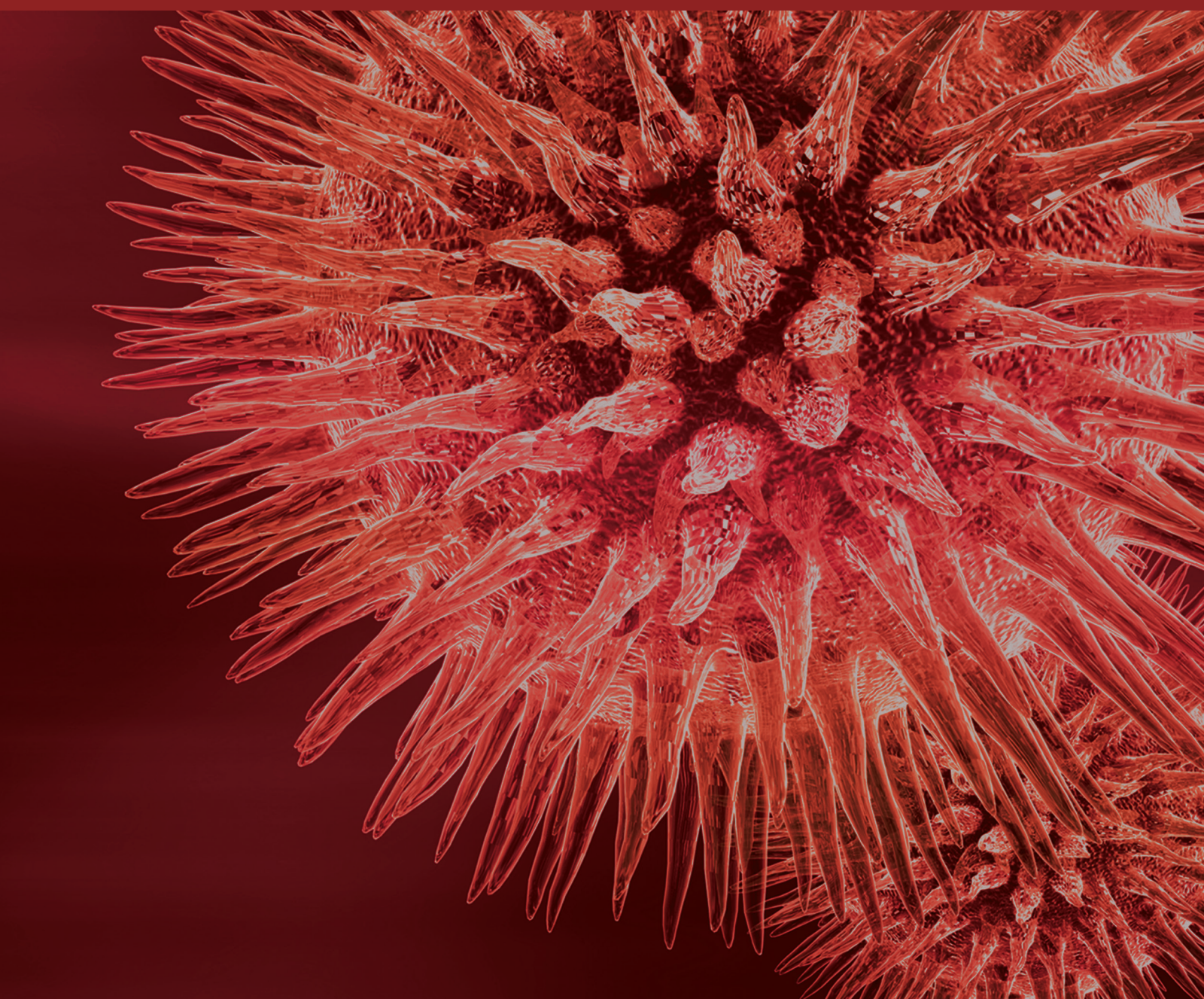
BioMed Research International

Cancer Diagnostic and Predictive Biomarkers 2016

Lead Guest Editor: Franco M. Buonaguro
Guest Editors: David C. Pauza, Maria L. Tornesello, Pierre Hainaut, Renato Franco, and Massimo Tommasino



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Editorial

Cancer Diagnostic and Predictive Biomarkers 2016

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Early diagnosis and prediction of therapeutic responses are crucial in cancer patients to tailor and optimize the treatment, increase the likelihood of cure, reduce side-effects, and avoid overtreatment. This special issue compiles relevant articles focused on the development of innovative cancer biomarkers and their validation.

New molecular biology tools, including genome-wide analysis, deep-sequencing, and RNA-seq, are currently available for the identification of novel unique biomarkers with great potential for developing more sensitive and specific diagnostic tools as well as discovering new candidate therapeutic targets for personalized medicine.

In this special issue the relevance of systemic biomarkers for early diagnosis and/or cancer characterization/monitoring has been emphasized by several articles focused on blood tumor markers with an overview by S. Holdenrieder et al. The great variety of blood tumor markers is shown by the wide range of biomarkers spanning from the basic parameter changes of blood count in renal cell carcinoma (reported by G. Prokopowicz et al.) to the detection of circulating tumor cells in gastric cancer (observed by K. Zou et al.), the level of antibodies to the hidden IgG antibodies to the tumor-associated Thomsen-Friedenreich antigen (described in gastric cancer patients by O. Kurtenkov and K. Klaamas), and the levels of plasma circulating tumor DNA, observed in melanoma patients by B. Busser et al. Moreover, the diagnostic relevance of systemic miRNAs (in particular miRNA 21) in biliary tract cancer seems even greater than current markers (i.e., CA19-9) and has been reported by C. Mayr et al.

Most articles, however, have been focused on tissue biomarkers in the early discovery and/or characterization stage, with a great potentiality of becoming relevant cancer biomarkers for molecular characterization/diagnosis, prognostic evaluation, and therapeutic responsivity. The articles on the predictive positive clinical significance of novel long noncoding RNA in HCC (by L. Zhang et al.) whose expression levels are directly related to overall survival, the low proliferation level of the human lung cancer cell lines A549 in presence of high expression of circular RNA-ITCH (by L. Wan et al.), and limited disease in SCLC of patients with miRNA-related polymorphisms in P13K/Akt/mTOR pathway genes (by W. Jiang et al.) are establishing a new paradigm.

Moreover, high EF24 levels have been associated with suppression of invasion and migration of HCC cells (by R. Zhao et al.), while high expression levels of Cadherin 17 have been associated with high frequency of bone marrow metastasis in a murine breast cancer model (by T. Okada et al.) and high expression of meiotic recombination 11 homolog A (MRE11) oncoprotein with clinical breast cancer progression (by C.-H. Yang et al.). The overall characterization of the primary tumor has been described for renal cell carcinoma

by S. H. Kim et al., glioblastoma by W. Szopa et al., and nasopharyngeal carcinoma by A. Lu et al. The latter study includes the primary tumor immune-evaluation showing that in patients with elevated neutrophil to lymphocyte ratio (NLR) the balance is tipped in favor of tumor-promoting inflammation resulting in tumor cell proliferation and cancer metastasis.

Finally specific gene signatures have been proposed to optimize personalized cancer treatment; in particular the overall higher responsiveness to PARPi of patients carrying BRCA2 mutations has been reviewed by S. Murata et al. and specifically for pancreatic cancer by J. Martinez-Useros and J. Garcia-Foncillas.

We hope that our current paper collection will enrich our readers and researchers, giving them an overview of the current broad range of cancer biomarkers being pursued by research teams around the world.

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Review Article

Plasma Circulating Tumor DNA Levels for the Monitoring of Melanoma Patients: Landscape of Available Technologies and Clinical Applications

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Melanoma is a cutaneous cancer with an increasing worldwide prevalence and high mortality due to unresectable or metastatic stages. Mutations in *BRAF*, *NRAS*, or *KIT* are present in more than 60% of melanoma cases, but a useful blood-based biomarker for the clinical monitoring of melanoma patients is still lacking. Thus, the analysis of circulating tumor cells (CTCs) and/or cell-free circulating tumor DNA (ctDNA) analysis from blood (liquid biopsies) appears to be a promising noninvasive, repeatable, and systemic sampling tool for detecting and monitoring melanoma. Here, we review the molecular biology-based strategies used for ctDNA quantification in melanoma patients, as well as their main clinical applications. Droplet digital PCR (ddPCR) and next generation sequencing (NGS) technologies appear to be two versatile and complementary strategies to study rare variant mutations for the detection and monitoring of melanoma progression. Among the different clinical uses of ctDNA, we highlight the assessment of molecular heterogeneity and the identification of genetic determinants for targeted therapy as well as the analysis of acquired resistance. Importantly, ctDNA quantification might also be a novel biomarker with a prognostic value for melanoma patients.

1. Introduction

It is well established that circulating tumor DNA (ctDNA) is a valid surrogate tumor biomarker for monitoring tumor burden and responses to anticancer therapies [1, 2]. This minimally invasive method to access cancer-derived DNA is also potentially useful for monitoring solid tumors and would avoid the need to perform repeated invasive biopsy procedures [3].

It was recently shown in a prospective proof-of-concept study that the monitoring of metastatic breast cancer via ctDNA was a both highly specific and sensitive strategy [4]. These results even suggest that ctDNA has better sensitivity than the well-established breast tumor biomarker (TM) carbohydrate antigen (CA 15-3). Similarly, serum levels of carcinoembryonic antigen (CEA), CA 19-9, and prostate specific antigen (PSA) can be used as surrogate markers of tumor burden changes in colon, pancreas, and prostate carcinomas. However, there is no valid and specific blood biomarker that is currently used either for the assessment of melanoma burden/recurrence or for clinical monitoring of the disease. The only TM accepted as a standard prognostic

factor of survival for melanoma is lactate dehydrogenase (LDH), which is a nonspecific enzyme that can be elevated in various benign or malignant diseases [5].

A relevant blood biomarker for routine clinical melanoma monitoring is thus highly and urgently needed. Melanomas are among the cancers that harbor the highest number of mutations per tumor [6] and ctDNA is detectable in about 80% of cases, with more than 1000 mutant fragments per 5 mL of plasma [7]. Mutations, deletions, or amplifications in *BRAF*, *NRAS*, *TP53*, or *KIT* are generally present in approximately 85% of melanomas [8]. These high-frequency genetic alterations can be found in the blood of melanoma patients and make it possible to distinguish ctDNA from circulating normal DNA. They could collectively serve as specific molecular biomarkers to track ctDNA levels and as personalized biomarkers of melanoma disease.

BRAF is the most frequently mutated gene in melanoma [8]. From 43 to 66% of cutaneous melanomas carry *BRAF* mutations, among which the *BRAF* V600E transversion is the most common (80%), followed by V600K (12%), V600R (5%), V600M (4%), and V600D (<5%) [9]. These activating *BRAF* mutations induce the constitutive downstream activation of the MEK-ERK signaling pathway, leading to tumor proliferation and survival [10].

NRAS encodes a small GTPase, which was the first protooncogene discovered in melanoma [11], and is found to be mutated in approximately 20% of cases [12]. Both *BRAF* and *NRAS* mutations are predictors of poorer outcome and lower overall survival (OS) of patients than those with nonmutated melanoma [13].

KIT is a gene which is found mutated in approximately 3% of melanomas [8]. The positive detection of *KIT* has also been successfully performed in the peripheral blood of patients with gastrointestinal stromal tumors [14, 15]. Patients with melanomas harboring *KIT* mutations are eligible for imatinib therapy [16].

Hence, there is a reason to believe that ctDNA will play the unhoped-for role of the currently missing gold-standard blood-based biomarker for the monitoring of melanoma in the near future.

Here, we review the existing molecular biology approaches that have been used for ctDNA quantification for melanoma patients and describe the main clinical applications and associated results that were obtained.

2. Technical Strategies for ctDNA Detection and Quantification

Patients with solid malignancies have higher levels of normal (wild-type) circulating cell-free DNA than healthy individuals [37]. Most conventional PCR-based methods, such as classical Sanger sequencing or pyrosequencing, can detect mutant alleles but are limited by the presence of disproportionate amounts of wild-type alleles in the blood. These two methods can only fulfill the requirements for ctDNA quantification for patients with very high levels of mutant ctDNA, which is rare in plasma [38].

Detecting somatic genetic alterations in the circulation is challenging, but novel approaches have facilitated sensitive and specific detection at low levels. Several recently developed methodologies, such as allele-specific amplification refractory mutation system PCR (ARMS), bead emulsification amplification and magnetics (BEAMing) technology, allele-specific PCR (AS-PCR), droplet digital PCR (ddPCR), and next generation sequencing (NGS), have been used to detect and quantify rare variants in the blood of melanoma patients, with analytical sensitivity ranging from 0.005 to 5% (Table 1).

One of the first studies dedicated to the evaluation of both an innovative ctDNA quantification method and its clinical utility in melanoma showed that quantitative real-time clamp reverse transcription PCR by peptide locked nucleic acid (PNA) and locked nucleic acid hybrid probes (LNAs) detected serum *BRAF* V600E with a sensitivity of 0.001%. The use of allele-specific blockers to suppress the amplification of the wild-type allele was also chosen for the screening of *BRAF* mutant alleles in plasma via competitive allele-specific PCR (CastPCR) [25]. However, the analytical sensitivity was lower at 0.5%.

The detection of *BRAF* mutations in isolated CTCs from *BRAF*-mutated melanoma patients has been performed after an obligatory step of whole genome amplification (WGA) [24]. The authors compared the performance of ddPCR with CastPCR and concluded that ddPCR was more robust and more sensitive than CastPCR. In this study, the detection of *BRAF* mutations with ddPCR reached a sensitivity of 0.0005%, which is 200 times greater than that obtained using CastPCR [24].

AS-PCR methods have recently reached sensitivities below 0.01%, which allow their use for rare variant detection [31].

BEAMing technology is sufficiently sensitive (0.01%, Table 1) to reliably detect mutant ctDNA in the plasma of melanoma patients. For example, *BRAF* mutations were detectable in 76% and 81% of patients with *BRAF* V600E and V600K mutations among 732 patients enrolled in four recent clinical trials evaluating targeted therapies [34].

The sensitivity of AS-PCR is generally not below 0.1% [3, 9, 19, 36]. However, the recent development of a novel, rapid, and inexpensive AS-PCR assay reached a sensitivity of 0.005% due to the use of a PNA designed to inhibit the amplification of the wild-type allele [18].

The technique of ddPCR is a robust method that can detect and quantify very small amounts of ctDNA without the need of a calibration curve [28]. It is indeed more precise for the detection of rare genetic variants and is less sensitive to inhibitors than quantitative RT-PCR [39, 40]. In a recent study, the ddPCR approach reliably distinguished mutant from wild-type alleles with no false positives [29]. *BRAF* ctDNA was never detected in a healthy patient cohort, yielding a clinical specificity of 100%. Using the same approach, the *NRAS* ctDNA test showed a specificity higher than 75% [30].

Most of the technologies presented in this review are generally used to detect and quantify known alterations. Despite being useful for longitudinal monitoring, they do not allow the discovery of other mutations, either the other innumerable mutations contained in the tumor or the de

Table 1: Overview of techniques used for detection and quantification of plasma ctDNA for melanoma patients. PCR: polymerase chain reaction; AS-PCR: allele-specific PCR; ARMS: amplification refractory mutation system allele-specific PCR; MS-PCR: mutant-specific PCR with fluorescent detection; CastPCR: competitive allele-specific PCR; CTCs: circulating tumor cells; Bi-PAP: mutation-specific bidirectional pyrophosphorolysis-activated polymerization; ddPCR: droplet digital PCR; BEAMing: beads, emulsification, amplification, and magnetics; NGS: next generation sequencing; WES: whole exome sequencing.

Method	Gene (mutation)	Analytical sensitivity (% of mutated copies)	References
Quantitative real-time clamp reverse transcription PCR	*BRAF* (p.V600E)	0.001%	[17]
AS-PCR or ARMS	*BRAF* (p.V600E/K/D)	0.1%	[3]
AS-PCR or ARMS	*BRAF* (p.V600E)	0.3%	[18]
AS-PCR or ARMS	*BRAF* (p.V600E)	0.25%	[9]
AS-PCR or ARMS	*BRAF* (p.V600E)	2.0%	[19]
ARMS	*BRAF* (p.V600E) *BRAF* (p.V600D) *BRAF* (p.V600K) *BRAF* (p.V600R)	1.82% 3.19% 4.34% 4.85%	[20]
MS-PCR	*BRAF* (p.V600E)	0.01%	[21]
RT-PCR + restriction enzyme digestion	*BRAF* (p.V600E)	0.1%	[22, 23]
ddPCR on DNA from CTC	*BRAF* (p.V600E) *BRAF* (p.V600K)	0.0005% after WGA enrichment	[24]
CastPCR on DNA from CTC	*BRAF* (p.V600E) *BRAF* (p.V600K)	0.1% after WGA enrichment	[24]
CastPCR	*BRAF* (p.V600E)	0.5%	[25]
Bi-PAP	*GNAQ* (c.626A>T) *GNAQ* (c.626A>C) *GNA11* (c.626A>T)	~0.05%	[26, 27]
ddPCR	*BRAF* (p.V600E)	0.005%	[28]
ddPCR	*BRAF* (p.V600E) *BRAF* (p.V600K) *NRAS* (p.Q61H)	0.01%	[29]
ddPCR	*BRAF* (p.V600E) *BRAF* (p.V600K) *NRAS* (p.Q61K) *NRAS* (p.Q61R)	0.01%	[30]
AS-PCR or ARMS	*BRAF* (p.V600E)	0.005%	[18]
AS-PCR	*BRAF* (p.V600E/E2/D/K/R/M)	0.01%	[31]
BEAMing technology	*BRAF* (p.V600E) *BRAF* (p.V600K)		[32]
BEAMing technology	*BRAF* (p.V600E) *NRAS* (p.Q61K) *NRAS* (p.Q61R)	<0.01%	[33]
BEAMing technology	*BRAF* (p.V600E) *BRAF* (p.V600K)	0.01%	[34]
PCR+NGS	*TERT* promoter	<0.1%	[33]
NGS (WES)	Exome		[35]

novo mutations occurring during the acquisition of resistance mechanisms. From this perspective, sequencing of ctDNA via NGS-based technologies is a reliable strategy that has already succeeded in identifying novel alterations at a frequency as low as one mutant copy in several thousand wild-type copies [41, 42].

Accordingly, digital PCR and NGS technologies are considered to be relevant and complementary diagnostic tools for quantifying rare variants in the blood of cancer patients [40, 43].

Plasma is a better source of ctDNA than serum [19], especially because of the large amounts of wild-type DNA

released by white cells lysis during clotting. However, discordant studies have shown that higher levels of ctDNA could be recovered from paired serum samples [9]. There is general agreement concerning the use of either dedicated blood collection tubes that prevent plasma cell-free DNA contamination by cellular DNA [44, 45] or conventional EDTA-containing tubes coupled with a stringent protocol for the separation of the plasma from blood cells by two consecutive centrifugations within 2-3 hours after the blood draw [46].

Analytical sensitivity (Table 1) refers to the estimated fraction of mutated copies that can be detected within the high number of wild-type alleles present in the plasma. The sensitivity of a given method is a percentage that can vary depending on the allele of interest, the technology used, or the pathophysiological state of the patient. For plasma ctDNA quantitation, the use of very sensitive methods is particularly necessary for quantifying the presence of extremely small fractions of mutant alleles (<1.0%). We believe that comparing the absolute sensitivities obtained from various studies aiming to quantify ctDNA is not informative. The diverse experimental protocols used in these studies introduce bias, preventing their comparison. The analysis of ctDNA coming from different sources (plasma, serum, or CTCs), with or without rapid processing after blood collection, obtained using different extraction methods, followed by whole genome amplification or not, and sequenced/quantified using different technologies, impedes any attempt to select the best approach for ctDNA quantification based on claimed (or measured) analytical sensitivities [47]. Importantly, most plasma or serum ctDNA is not derived from CTCs [7].

3. Clinical Applications for ctDNA in Melanoma

The recent advances resulting in improved sensitivity and specificity of ctDNA analysis have been shown to be useful in a wide variety of malignancies for several clinical applications, including early cancer detection, assessment of the molecular heterogeneity of overall disease, the monitoring of tumor dynamics, identification of genetic determinants for targeted therapy, evaluation of early treatment responses, monitoring of minimal residual disease, and assessment of the evolution of resistance in real time [38]. Several of these applications have been tested on melanoma patients and the results all support the serious need for routine serial ctDNA monitoring to improve the clinical management of these patients (Table 2).

3.1. Assessment of Molecular Heterogeneity. The assessment of genetic heterogeneity is a current technical challenge of major clinical importance. As an example, the mutational status of *BRAF* was found to be different between different sites of the primary tumor (intratumor heterogeneity), between the primary tumor and metastases, and between several metastases from the same patient [21, 48]. Biopsy and subsequent sequencing can miss detecting a mutation because of the site from which it was taken. A potential strategy to overcome such a bias in routine sequencing is to sequence plasma ctDNA. Indeed, ctDNA is a valid source for theoretically finding all mutations present in all tumor sites of a given patient [38].

3.2. Prognostic Value. The highest levels of circulating *BRAF* V600E were found in more advanced stages of disease [17]. This finding was in accordance with results of another seminal study, dedicated to quantifying circulating *BRAF* mutations in the blood of melanoma patients, which showed that detectable *BRAF* levels were only found in advanced stage III/IV disease [9]. The authors of this study postulated that this blood biomarker would not represent an interesting early-stage marker for melanoma patients, despite the fact that *BRAF* mutations occur early in melanomagenesis [49].

The prognostic significance of ctDNA quantification in melanoma has more recently been clearly demonstrated. Patients with OS of more than two years had significantly fewer copies of mutant *BRAF* V600E per mL of plasma than patients with OS of less than two years [28]. The prognostic value of ctDNA levels was also demonstrated in a phase II cohort of melanoma patients which included histologically confirmed patients with *BRAF* mutant stage IV melanoma [32]. Increasing amounts of circulating *BRAF* V600E were associated with a reduced overall response rate and shorter progression-free survival (PFS). Elevated ctDNA levels were also significantly associated with both shorter PFS and lower OS in uveal melanoma [26]. In addition, the absence of detectable ctDNA prior to treatment (baseline) within a large cohort of melanoma patients with *BRAF* mutations was an independent predictor of better disease outcome. Patients negative for *BRAF* ctDNA had higher response rates to dabrafenib and trametinib, longer PFS, and higher OS than patients for whom *BRAF* mutations were detected in the plasma [34]. Longer PFS also correlated with undetectable ctDNA levels after treatment initiation in an independent 36-patient cohort receiving dabrafenib and trametinib [31]. In a 48-patient cohort treated by MAPK inhibitors or immunotherapies, low baseline ctDNA was a good predictor of response to treatment and longer PFS [30]. More recently, the presence of detectable levels of ctDNA at baseline strongly correlated with longer OS [20]. All these studies suggest that rising or elevated ctDNA levels may predict poorer clinical outcome.

3.3. Identification of Genetic Determinants for Targeted Therapy. A phase II study of a large cohort of *BRAF* mutant melanoma patients failed to demonstrate the prognostic value of ctDNA detection in advanced melanoma. However, the authors stressed that there was a strong rationale for using blood testing to identify whether there are any genetic determinants for associated targeted therapy [3]. The definitive evidence of the benefit of using plasma rather than serum for ctDNA studies was provided in a large cohort of melanoma patients [19]. In this study, the authors proposed that *BRAF* V600 mutation testing from ctDNA should be considered as a first screening test for positively selecting patients with *BRAF* mutations eligible for targeted therapy. This blood-based mutation screening approach could significantly shorten the

Table 2: Applications of ctDNA quantification and monitoring for melanoma patients. PBL: peripheral blood lymphocytes.

Gene	Sample	Application	References
BRAF	Serum	(i) Tumor response monitoring (ii) Prognostic value	[17]
BRAF	Serum/plasma	Advanced stage IV monitoring	[9]
BRAF	Serum	Identification of genetic determinants for targeted therapy	[3]
BRAF	Plasma	Identification of genetic determinants for targeted therapy	[21]
BRAF	Serum/plasma	Identification of genetic determinants for targeted therapy	[19]
BRAF	Plasma	Identification of genetic determinants for targeted therapy	[36]
BRAF	PBL	(i) Identification of genetic determinants for targeted therapy (ii) Evaluation of early treatment response (iii) Monitoring of minimal residual disease	[22]
GNAQ *GNA11*	Plasma	Prognostic value	[26]
BRAF	Plasma	(i) Identification of genetic determinants for targeted therapy (ii) Monitoring of tumor dynamics (iii) Prognostic value	[28]
BRAF *NRAS* *TERT* Promoter	Plasma	Evaluation of early treatment response to immunotherapy	[33]
BRAF	Plasma/serum	(i) Prognostic value (ii) Monitoring of tumor dynamics (iii) Evaluation of early treatment response (iv) Monitoring of minimal residual disease	[18]
BRAF	Plasma	Prognostic value	[32]
BRAF *NRAS*	Plasma	(i) Monitoring of tumor dynamics (ii) Monitoring of minimal residual disease (iii) Real-time assessment of resistance	[30]
BRAF	Plasma	Prognostic value	[34]
BRAF	Plasma	(i) Prognostic value (ii) Monitoring of tumor dynamics (iii) Evaluation of early treatment response (iv) Real-time assessment of resistance	[34]
BRAF *NRAS*	Plasma	(i) Prognostic value (ii) Evaluation of early treatment response (iii) Real-time assessment of resistance	[30]
BRAF	Plasma	(i) Identification of genetic determinants for targeted therapy (ii) Prognostic value	[20]
Exome	Plasma	(i) Evaluation of early treatment response (ii) Real-time assessment of resistance	[35]

turnaround time relative to that required for mutation testing from archived FFPE tumors [19].

3.4. Monitoring of the Disease. The first attempt to monitor ctDNA levels in melanoma patients before and after treatment resulted in a significant positive correlation between the level of remaining detectable serum *BRAF* V600E and the absence of response to bio/chemotherapy [17]. Another study reported a rapid and marked decrease in *BRAF* V600E blood levels following the initiation of treatment, whether it was surgical or based on targeted anti-BRAF and/or anti-MEK therapies. The decrease correlated with the response to treatment, shown by imaging [22].

The longitudinal variations of ctDNA levels during the treatment of melanoma are logarithmic [28], reinforcing its potential role as a relevant surrogate marker for monitoring

efficacy (decrease and/or minimal residual disease) and recurrence (increase after nadir) of melanoma therapy. The only blood TM routinely incorporated in the management of melanoma is LDH, but it is neither sensitive nor specific and is considered to be an unreliable marker for monitoring treatment response [29]. A small case series demonstrated that ddPCR-based ctDNA quantification allowed the monitoring of treatment responses or the emergence of tumor resistance more consistently and informatively than LDH [29]. The superiority of measuring ctDNA over that of LDH levels was demonstrated in three other patients for whom ctDNA levels more accurately reflected the evolution of disease than those of LDH, as the latter tends to increase following immunotherapy [35]. In contrast, two other studies reported a positive correlation between ctDNA and LDH levels [20, 30].

Changes in ctDNA levels that occurred in patients receiving immune checkpoint blocking agents were predictive of treatment efficacy [33]. In this study, the authors also postulated that (1) decreases in ctDNA levels could help to identify patients who are responding to treatment, (2) increases in ctDNA levels might indicate tumor progression or tumor lysis preceding regression, (3) rising ctDNA levels after a long period off treatment might reflect tumor recurrence, and (4) ctDNA could be used as a marker of minimal residual disease after surgical resection. The monitoring of metastatic melanoma patients receiving targeted therapies also suggested that ctDNA levels dramatically decline during the first weeks of therapy, sometimes decreasing to undetectable levels, which is also associated with response to therapy [30]. An increase of ctDNA levels was never found in patients with an ongoing response but was found in 19/27 patients with progressive disease under targeted therapy, thus resulting in a sensitivity of 70% and a specificity of 100% [31].

The interest of such a longitudinal follow-up is that the recurrence of tumor burden was apparent by ctDNA analysis when radiological analysis still classified the tumor as stable [30]. That biological recurrence precedes radiological signs of recurrence by several weeks is well established [50, 51].

3.5. Resistance to Treatment. The assessment of tumor resistance to a given treatment can be determined by ctDNA studies in a dual manner. The first option consists of detecting increased levels of ctDNA carrying the original mutation after a prolonged period of undetectable or low values. *BRAF* mutations are usually conserved when recurrence/resistance to targeted therapies occurs and concentrations of plasma ctDNA generally decrease after treatment initiation [30]. Therefore, if progressive disease occurs after a period of tumor response, it is likely that an increase of ctDNA levels with the same primary *BRAF* mutation as that of the relapsing tumor cells will be detected. The second option consists of detecting resistance mutations. For melanoma treated with BRAF inhibitors, resistance generally occurs within the first year and usually involves reactivation of the MAPK pathway by the acquisition of secondary *NRAS* or *MEK* mutations or alternative splicing or amplification of the *BRAF* gene itself [52]. Tracking such secondary mutations is an approach that has already demonstrated its utility in the routine clinical monitoring for the early recurrence of lung cancers [53]. For melanoma patients, ctDNA monitoring of seven patients with progressive disease after a prior response to anti-BRAF treatments allowed the identification of secondary resistance mutations of the *NRAS* gene for three of them [30]. Similarly, a retrospective analysis of ctDNA from patients with tumor resistance was also performed by whole exome sequencing (WES) [35] and identified secondary *NRAS* mutations. A specific 10-gene panel was further developed and successfully used for the assessment in plasma of secondary mutations known to mediate resistance to BRAF and MEK inhibitors [35].

4. General Conclusion

Liquid biopsies are a promising area of investigation for improving the clinical monitoring of patients with solid malignancies [7] and ctDNA has been demonstrated for several applications to be a diagnostic, prognostic, and predictive biomarker for several malignancies [47]. The recent progress in the analytical sensitivity of digital genomic technologies allows the investigation of very rare mutant variants of ctDNA, representing an indisputable advantage over most strategies requiring CTC isolation [7]. The routine sequencing of *BRAF* (exon 15), *NRAS* (exon 2), and *KIT* (exons 9, 11, 13, and 17) for clinical purposes, such as molecular-based targeted therapy, suggests that tailored monitoring of the kinetics of plasma ctDNA could be used in the near future for melanoma patients for whom a mutation is detected.

Indeed, ctDNA can also harbor epigenetic modifications, such as methylation that has already been identified as a relevant prognostic factor for melanoma patients [54, 55]. Digital PCR and NGS strategies appear to be two of the most versatile technologies to study rare variants, such as mutations for cancer or SNPs for pharmacogenetics, as well as methylation [56].

Given the heterogeneity of tumor mutations between primary and metastatic sites, or between several metastatic sites, ctDNA might provide a more relevant representation of the mutational status of a patient than a biopsy from a single lesion or site [3, 20, 28]. Liquid biopsies, especially plasma ctDNA, have made the analysis of acquired resistance to anticancer treatments possible [50]. Recent studies showed that the monitoring of plasma ctDNA accurately reflected real-time sampling of multifocal tumor evolution [57]. These findings are fully transposable to melanoma patients, and such monitoring will help clinicians to better understand the molecular evolution of the individual primary and metastatic tumors of their patients. Further studies on large cohorts will be needed to prospectively evaluate the benefit of using serial ctDNA investigations in the monitoring of melanoma patients.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Research Article

Prognostic Significance of Neutrophil to Lymphocyte Ratio, Lymphocyte to Monocyte Ratio, and Platelet to Lymphocyte Ratio in Patients with Nasopharyngeal Carcinoma

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The peripheral blood neutrophil to lymphocyte ratio (NLR), lymphocyte to monocyte ratio (LMR), and platelet to lymphocyte ratio (PLR) have been reported to correlate with the prognosis of many malignancies. This study evaluated the prognostic value of pretreatment NLR, LMR, and PLR in nasopharyngeal carcinoma (NPC). A retrospective analysis of clinical and pathological data of 140 NPC patients without distant metastasis during initial treatment was conducted to identify correlations between NLR, LMR, and PLR and clinicopathological features, overall survival, and progression-free survival. Cox proportional hazard regression analysis was used to reveal the independent factors affecting the prognosis of NPC patients. NLR was associated with T staging, N staging, and overall clinical stage grouping of the NPC patients ($P < 0.05$). NLR ≥ 2.28, LMR < 2.26, and PLR ≥ 174 were significantly associated with a relatively short overall survival ($P < 0.05$). In addition, NLR ≥ 2.28 was significantly associated with a relatively short progression-free survival ($P < 0.05$). Cox proportional hazard regression analysis showed that NLR was an independent prognostic factor in NPC. Pretreatment NLR, LMR, and PLR might be a useful complement to TNM staging in the prognostic assessment of NPC patients.

1. Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common head and neck cancers in Southeast Asia, with a particularly high incidence in the provinces of Southern China [1–3]. Due to its anatomical location and radiosensitivity, radiotherapy or combined chemotherapy is a standard treatment for NPC. As for other solid tumors, the prognosis of NPC mainly depends on the TNM staging system [4]. However, TNM staging alone cannot predict NPC treatment efficacy. NPC patients with the same clinical staging often have different clinical courses. The possible explanation is that TNM staging is mainly based on the anatomical information and does not reflect the biological heterogeneity of the tumor. Hence, identification of prognosis-related biological markers may be an effective complement to TNM staging for the prognostic assessment of NPC patients.

Recent studies have shown that systemic inflammation promotes tumor progression and metastasis via the inhibition of apoptosis, promotion of angiogenesis, and damaging of DNA [5]. Hematological indices for these systemic inflammatory conditions, such as leukocyte count, monocyte count, platelet count, neutrophil to lymphocyte ratio (NLR), lymphocyte to monocyte ratio (LMR), and platelet to lymphocyte ratio (PLR), have been found to be independent prognostic factors for patients with non-small cell lung cancer [6], gastric cancer [7], and breast cancer [8]. This study evaluated the correlations between NLR, LMR, and PLR and clinicopathological features of NPC for the evaluation of their prognostic value in NPC patients.

2. Materials and Methods

2.1. Patients. In this study, 140 NPC patients admitted to Wuzhou Red Cross Hospital (Guangxi, China) from Feb 2009 to May 2010 were recruited, and the study protocol was approved by the Ethics Committee of Wuzhou Red Cross Hospital. The patients comprised 101 males and 39 females with a median age of 47 (range 10–76 years). The following criteria were applied for the inclusion of research subjects in this study: (1) pathologically diagnosed with NPC; (2) no prior malignancy; (3) no distant metastasis; (4) no current antitumor therapy; and (5) no infection or symptoms of inflammation. All patients were clinically staged in accordance with Chinese 2008 staging system [9] and received radical radiotherapy. Stages at III and IVa NPC patients received a combination of radiotherapy and chemotherapy.

2.2. Blood Tests. A blood sample was collected from each patient in an EDTA anticoagulant-treated tube and analyzed for routine peripheral blood cells (e.g., lymphocytes, neutrophils, monocytes, eosinophils, basophils, and platelets) using a Sysmex XE-2100 automated hematology system and its reagent kits (Sysmex, Japan).

2.3. Statistical Analysis. SPSS 13.0 (SPSS, Chicago, IL) software was used for statistical analysis in this study. Hematological indices were presented as medians (minimum to maximum value). In each patient, NLR was calculated by dividing the neutrophil number by the lymphocyte number; LMR was calculated by dividing the lymphocyte number by the mononuclear cell number; and PLR was calculated by dividing the platelet count by the lymphocyte number. A Chi-square (χ^2) test was used to determine the correlations between NLR, LMR, and PLR and clinicopathological features of the NPC patients. ROC curve analyses were used to determine the best cut-off values of the hematological indices for patients' survival. A Kaplan-Meier analysis was used to calculate patients' survival and prepare survival curves. The log-rank test was used to compare the survival rate in each group. A Cox proportional hazard regression analysis was used to evaluate multiple prognostic factors. Overall survival (OS) was defined as the duration from diagnosis to death or last follow-up. Progression-free survival (PFS) was the duration from diagnosis to local recurrence/metastasis or last follow-up. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Patients' Survival. One hundred and forty NPC patients had completed treatment, including 34 cases (24.3%) of radiotherapy alone and 106 cases (75.7%) of combined radiotherapy and chemotherapy. Median follow-up of the patients was 68 months (range 5–77 months). Eleven patients were lost to follow-up; 17 patients had local recurrence; 21 patients had distant metastasis; and 29 patients died. Among the NPC patients, the 5-year OS was 78.8%, and the 5-year PFS was 76.2%.

3.2. Association between Pretreatment NLR, LMR, and PLR, and Clinicopathological Features of the NPC Patients. Of the 140 NPC patients, the medians of pretreatment peripheral blood lymphocytes, neutrophil number, monocyte number, platelet count, NLR, LMR, and PLR were 1.76×10^9/L (0.52–3.61), 3.96×10^9/L (1.47–10.86), 0.52×10^9/L (0.01–2.00), 234×10^9/L (61–370), 2.34 (0.70–6.60), 3.31 (0.89–320), and 136 (43–361), respectively.

ROC curves are constructed between death events and censors. The cut-off values of pretreatment NLR, LMR, and PLR were 2.28, 2.26, and 174, respectively, and were used to divide the NPC patients into high- and low-level groups. NLR was significantly correlated to the T staging, N staging, and overall clinical stage grouping of the NPC patients ($P < 0.05$) but did not correlate with the age, gender, and pathological type ($P > 0.05$). LMR and PLR were not significantly correlated to the age, gender, pathological type, T staging, N staging, or overall clinical stage grouping of the NPC patients ($P > 0.05$, Table 1).

3.3. Correlations between Pretreatment NLR, LMR, and PLR and Prognosis of the NPC Patients. Five-year OS and 5-year PFS of the patients with pretreatment NLR ≥ 2.28 were significantly lower than the patients with pretreatment NLR < 2.28 (OS: 70.3% versus 87.8%, $P = 0.010$; PFS: 66.8% versus 86.2%, $P = 0.005$) (Figure 1).

As shown in Figure 2, 5-year OS of the patients with pretreatment LMR < 2.26 was significantly lower than the patients with pretreatment LMR ≥ 2.26 (53.9% versus 84.1%, $P = 0.003$). Five-year PFS of the patients with pretreatment LMR < 2.26 and ≥ 2.26 were 71.1% and 77.4%, respectively, which was not a significant difference ($P = 0.579$).

In Figure 3, 5-year OS of the patients with pretreatment PLR ≥ 174 was significantly lower than the patients with pretreatment PLR < 174 (66.5% versus 83.2%, $P = 0.040$). Five-year PFS of the patients with pretreatment PLR ≥ 174 and < 174 were 70.6% and 78.1%, respectively, which was not a significant difference ($P = 0.481$).

The associations of pretreatment NLR, LMR, and PLR with OS and PFS were examined further with Cox proportional hazards regression modeling, with adjustment for age, gender, WHO pathological classification, clinical staging, and chemotherapy. The results showed that NLR was an independent prognostic factor of OS and PFS in NPC patients; in addition, T staging was also an independent prognostic factor of OS (Table 2).

4. Discussion

Inflammation is commonly recognized to play a key role in cancer development and possibly affects the survival of cancer patients [10]. In this study, we found that patients with high T staging, N staging, and locally advanced NPC had relatively high pretreatment NLR. Increased NLR was significantly associated with poor OS and PFS of the patients, suggesting that NLR was independent prognostic factors for NPC. These results were consistent with the findings of An et al. [11]. The mechanism behind poor tumor prognosis due to

Table 1: Association of pretreatment NLR, LMR, and PLR with clinicopathologic characteristics in patients with nasopharyngeal carcinoma.

Characteristics	Case	NLR			LMR			PLR		
		<2.28	≥2.28	*P*	<2.26	≥2.26	*P*	<174	≥174	*P*
Age (year)										
<45	55	28	27	0.561	10	45	0.924	41	14	0.833
≥45	85	39	46		16	69		62	23	
Sex										
Male	101	47	54	0.614	21	80	0.277	76	25	0.469
Female	39	20	19		5	34		27	12	
Pathology (WHO)										
Type I/II	23	11	12	0.997	5	18	0.669	16	7	0.634
Type III	117	56	61		21	96		87	30	
T classification										
T_1-T_2	63	36	27	0.047	8	55	0.106	49	14	0.307
T_3-T_4	77	31	46		18	59		54	23	
N classification										
N_0-N_1	43	26	17	0.047	8	35	0.995	33	10	0.571
N_2-N_3	97	41	56		18	79		70	27	
Overall stage										
I-II	19	14	5	0.015	2	17	0.332	15	4	0.568
III-IVa	121	53	68		24	97		88	33	

NLR: neutrophil to lymphocyte ratio; LMR: lymphocyte to monocyte ratio; PLR: platelet to lymphocyte ratio; Type I: keratinizing squamous cell carcinoma; Type II: differentiated nonkeratinizing carcinoma; Type III: undifferentiated nonkeratinizing carcinoma.

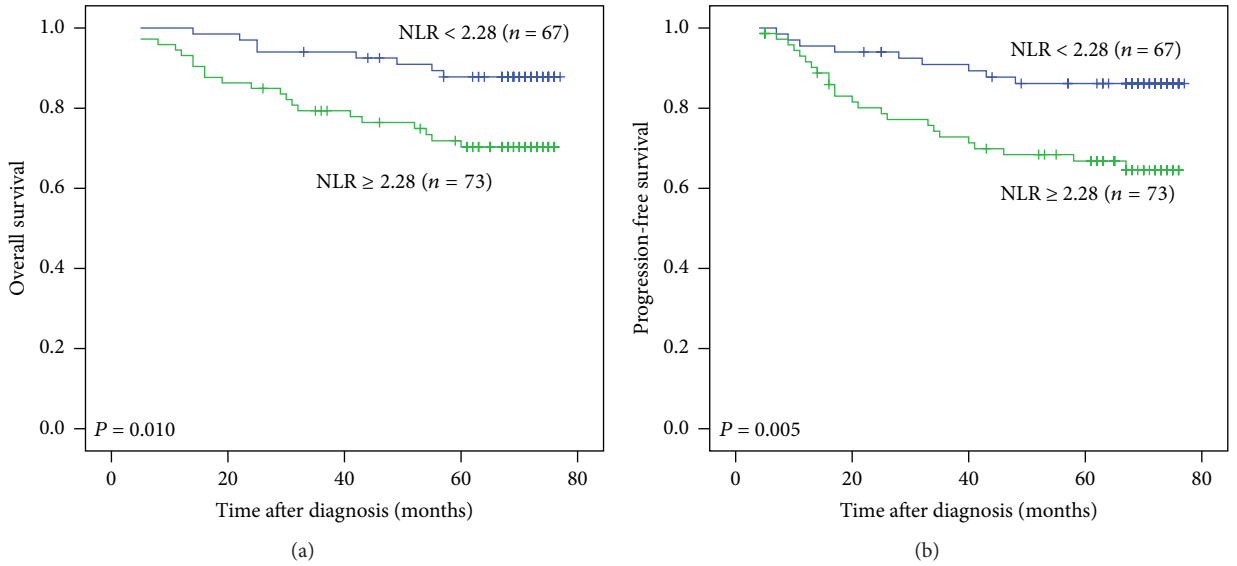

Figure 1: Kaplan-Meier curves for overall survival (OS) and progression-free survival (PFS) of patients with nasopharyngeal carcinoma according to pretreatment neutrophil to lymphocyte ratio (NLR). (a) OS stratified by NLR. (b) PFS stratified by NLR.

an increase in NLR remains unclear. Neutrophils, as a type of inflammatory cells, are considered to be involved in different steps of tumor development through the production of a variety of cytokines, such as oncostatin M, hepatocyte growth factor, and transforming growth factor- (TGF-) β [12]. In addition, neutrophils promote tumor angiogenesis through the release of angiogenic factors, such as vascular endothelial growth factor, angiopoietin-1, and fibroblast growth factor-2 [13, 14]. On the other hand, lymphocytes are also responsible for immune surveillance to remove tumor cells. The involvement of lymphocytes, such as T cells, in tumor infiltration is associated with better prognosis of cancer patients and has

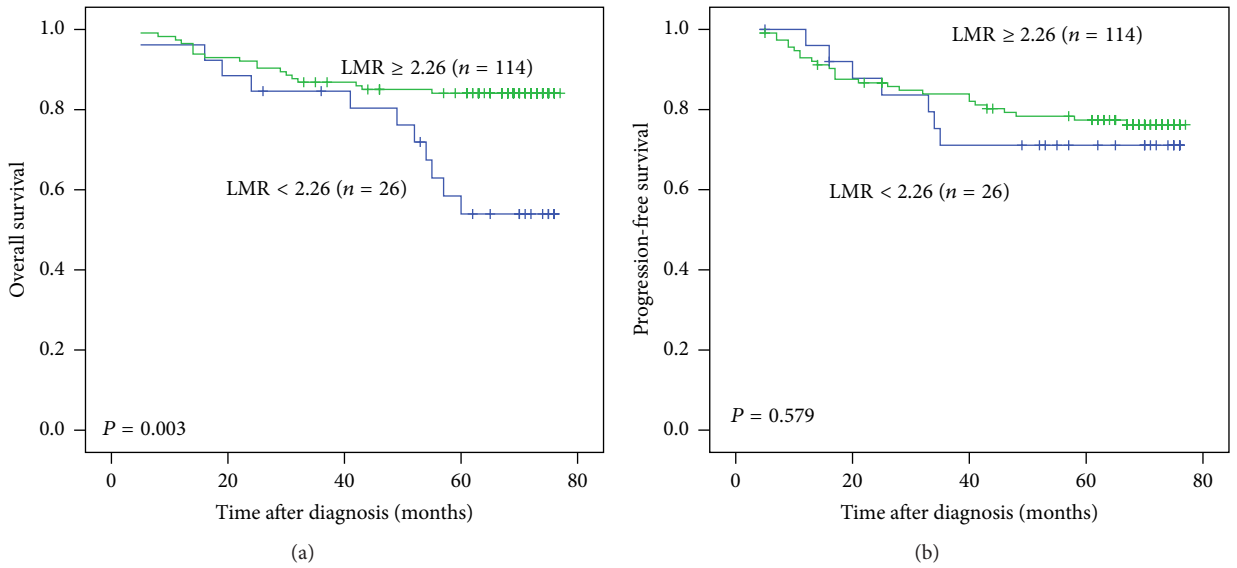

Figure 2: Kaplan-Meier curves for overall survival (OS) and progression-free survival (PFS) of patients with nasopharyngeal carcinoma according to pretreatment lymphocyte to monocyte ratio (LMR). (a) OS stratified by LMR. (b) PFS stratified by LMR.

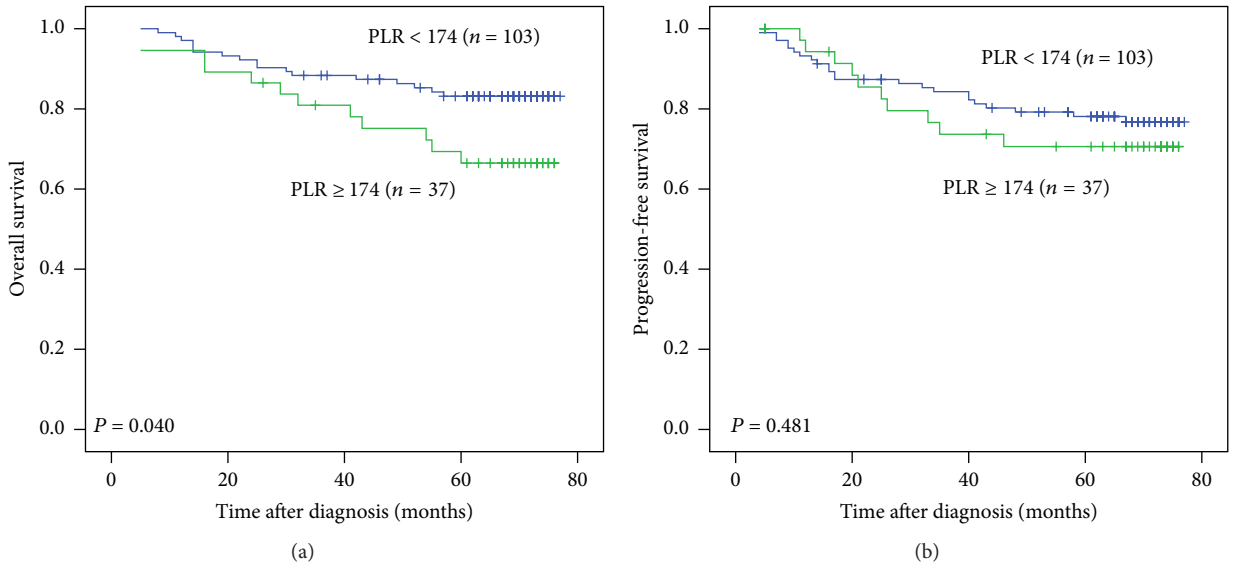

Figure 3: Kaplan-Meier curves for overall survival (OS) and progression-free survival (PFS) of patients with nasopharyngeal carcinoma according to pretreatment platelet to lymphocyte ratio (PLR). (a) OS stratified by PLR. (b) PFS stratified by PLR.

been used for tumor-targeted therapy [15, 16]. Therefore, NLR may affect the tumor microenvironment and immune system to influence the survival of NPC patients.

Tumor-infiltrating mononuclear cells were found to promote tumor invasion and cancer cell growth in lymphoma [17]. In addition, an increased number of monocytes before treatment has been associated with poor prognosis of lymphoma and other solid tumors [18, 19]. The results of this study also showed that a relatively high LMR was associated with better survival in NPC. Monocytes release monocyte chemoattractant protein- (MCP-) 1 to stimulate and mediate tumor-associated monocyte infiltration in solid tumors and then produce various chemokines, such as TGF-α, tumor necrosis factor- (TNF-) α, interleukin- (IL-) 1, and IL-6 which promote tumorigenesis, angiogenesis, and distant metastasis of malignant tumors [20]. NPC is usually infiltrated with lymphocytes such as Th17 cells. Th17 cells are partly regulated by macrophage migration inhibitory factor (MIF) and could generate a high level of cytokines including TNF and interferon- (IFN-) γ, which mediate the antitumor effects [21]. In NPC, infiltrating lymphocytes, including Th17 cells, can express MIF, and a high level of MIF was associated with

Table 2: Multivariate analysis of prognostic factors for survival of NPC patients using Cox proportional hazards regression model.

Endpoint	Hazard ratio (95% CI)	*P*
Overall survival		
T classification: T_{3-4} versus T_{1-2}	3.500 (1.387–8.830)	0.008
NLR (≥2.28 versus <2.28)	2.383 (1.041–5.457)	0.040
Progression-free survival		
NLR (≥2.28 versus <2.28)	2.615 (1.206–5.672)	0.015

NPC: nasopharyngeal carcinoma; NLR: neutrophil to lymphocyte ratio; CI: confidence interval.

a better treatment outcome of the disease [22]. Monocytes are likely to play an opposite role of lymphocytes and promote tumor development in NPC.

Platelets not only are involved in blood coagulate functions but also secrete a variety of cytokines to directly or indirectly participate in the inflammatory responses of the body. Chen et al. [23] reported that pretreatment increased platelet count was an unfavorable prognostic factor for NPC patients. In this study, a relatively high PLR was associated with short survival in NPC patients. The impact of the interaction between tumor progression and platelets remains unclear. Sharma et al. [24] have reported that platelets could mediate tumor growth, proliferation, and angiogenesis. Activated platelets promote tumor cell growth and survival through paracrine signaling or direct contact and interaction with tumor cells.

NLR, LMR, and PLR, as the ratio of absolute counts between 2 types of cells, have relative stability. Elevations of NLR, LMR, and PLR are often due to an imbalance between the 2 types of cells, and it can be considered that the balance between tumor-promoting inflammatory and antitumor immune status is violated. Patients with elevated NLR or PLR and decreased LMR denote that the balance is tipped in favor of tumor-promoting inflammatory with promoting tumor cell proliferation and cancer metastasis while weakening anti-tumor protection and is associated with poor oncologic outcome. NLR, LMR, and PLR measurements are easily obtained in clinical practice from routine blood tests. Therefore, they may be useful complements to the overall assessment of the clinicopathology of NPC patients.

5. Conclusion

In the current study, our results indicate that the pretreatment NLR was an independent prognostic factor in NPC, and NLR, LMR, and PLR might be a useful complement to TNM staging in the prognostic assessment of NPC patients. Limitations of this study include its small sample size and single-centered retrospective design. Multicenter, large scale prospective studies will be necessary to define the precise cut-off values of NLR, LMR, and PLR as prognostic markers for NPC.

Competing Interests

The authors declare that there is no conflict of interests.

Authors' Contributions

Aiying Lu and Haifeng Li contributed equally to this study.

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Research Article

Hidden IgG Antibodies to the Tumor-Associated Thomsen-Friedenreich Antigen in Gastric Cancer Patients: Lectin Reactivity, Avidity, and Clinical Relevance

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Natural antibodies to the tumor-associated Thomsen-Friedenreich antigen (TF) are related to tumor immunosurveillance and cancer patients' survival. Hidden IgG antibodies (HAbs) to TF, their lectin reactivity, avidity, and clinical relevance were studied. HAbs were present in cancer patients and controls. A decreased level of IgG HAbs was detected in cancer. The HAbs level positively correlated with the sialospecific SNA lectin binding in purified total IgG (tIgG) in donors and cancer patients, indicating that HAbs are higher sialylated. The avidity of anti-TF IgG in tIgG samples was lower in cancer patients ($P = 0.025$) while no difference in the avidity of free anti-TF IgG was established. A negative correlation between the avidity of anti-TF IgG in tIgG and SNA binding in both groups was observed ($P < 0.0001$). The HAbs level negatively correlated with the anti-TF IgG avidity in tIgG only in donors ($P = 0.003$). Changes in the level of HAbs and Abs avidity showed a rather good stage- and gender-dependent diagnostic accuracy. Cancer patients with a lower anti-TF IgG avidity in tIgG showed a benefit in survival. Thus the TF-specific HAbs represent a particular subset of anti-TF IgG that differ from free serum anti-TF IgG in SNA reactivity, avidity, diagnostic potential, and relation to survival.

1. Introduction

The expression of posttranscriptionally modified carboepitopes is a common feature of malignant cells. The Thomsen-Friedenreich disaccharide Galβ1-3GalNAcα/β-O-Ser/Thr (TF, CD 176) is overexpressed in a majority of adenocarcinomas [1–4] including gastric cancer which is considered one of the most deadly tumors worldwide. TF is also expressed on cancer stem cells [5] and is a marker of cancer-initiating cells [6]. Since the 1980s it has been demonstrated that TF- and αGal-glycotope- (Galα1,3-Galβ-) specific Abs appear early after birth and seem to be induced by intestinal microflora [7, 8].

The presence of naturally occurring TF-specific autoantibodies of different subclasses in cancer is a well-documented fact [9–12]. Moreover, the level of anti-TF antibodies (Abs) in the circulation is usually decreased in cancer, which is associated with tumor progression and patient survival [4, 11, 13] suggesting the important role of anti-TF Abs in antitumor immunity. The TF and endothelium-expressed galectin-3 have been identified as important molecular mechanisms initiating tumor/endothelial cell adhesion and metastasis [14, 15]. It has been shown that treatment of mice with a monoclonal anti-TF IgG Abs JAA-F11 inhibited lung metastasis and improved prognosis in a mouse breast cancer model [16] indicating that anti-TF humoral immune response has a therapeutic potential.

There is evidence that an appreciable amount of Abs is present in the circulation in a bound form, which phenomenon is called "hidden antibodies" (HAbs), and remains undetectable by conventional serologic methods [17, 18]. No special studies have been performed so far on the role of hidden IgG Abs to tumor-associated antigens in cancer. Recently we have demonstrated that anti-TF Abs in the serum (a pool of all Ig isotypes) of patients with gastric cancer show a significantly higher reactivity to sialic acid-specific *Sambucus nigra* lectin (SNA) than in controls [19].

Table 1: The characteristics of groups under investigation.

Group	n	Males	Females	Median age (range)
Donors	28	13	15	62 (24–72)
Gastric cancer patients	41	20	21	68 (28–83)

Moreover, the SNA-reactive anti-TF IgG Abs demonstrated a higher avidity in cancer [20]. These findings suggest that an altered glycosylation of anti-TF natural Abs may be used as a serologic biomarker for cancer.

In the present study we show that, in contrast to xenogeneic naturally occurring anti-αGal IgG, an appreciable amount of natural anti-TF IgG Abs is present in the circulation in a hidden form in both cancer patients and controls. HAbs showed a decreased level in cancer and exhibited a distinct SNA lectin reactivity, avidity, and relation to survival of gastric cancer patients indicating that TF-specific HAbs represent a particular subset of anti-TF IgG Abs which deserves further study to specify their clinical importance.

2. Material and Methods

2.1. Subjects and Samples. Serum samples were obtained from healthy blood donors ($n = 28$) and patients with histologically verified gastric carcinoma ($n = 41$) (Table 1). The investigation was carried out in accordance with the ICH GCP Standards and approved by the Tallinn Medical Research Ethics Committee, Estonia. A written informed consent was obtained from each subject. Tumor staging was based on the histopathological (pTNM) classification of malignant tumors. Serum samples were stored in aliquots at −20°C until use.

2.2. The Purification of Serum Total IgG. The purification of serum total IgG (tIgG) was performed on the Protein G HP Spin Trap column as described by the manufacturer (GE Healthcare, USA). The tIgG samples were eluted at pH 2.5, immediately neutralized, dialyzed against phosphate buffered solution (PBS, 0.1% NaN_3), and stored at +4°C until being tested. About 8.5 mg of IgG was obtained from 1 mL of serum applied onto the Protein G Sepharose column. To obtain the IgG-depleted serum we used the same method on the Protein G HP Spin Trap column, except the serum volume applied to the Protein G column was three times lower, and the complete depletion of IgG was controlled using the Easy-Titer IgG Assay Kit (Thermo Scientific, USA).

2.3. The TF-Specific Antibody Assay. The anti-TF and anti-αGal IgG antibody levels were determined by enzyme-linked immunosorbent assay (ELISA) as described elsewhere [19, 21] with minor modifications. Briefly, the plates (Maxisorp, Nunc, Denmark) were coated with a synthetic TFα- or αGal-polyacrylamide conjugate (Lectinity, Russia) in carbonate buffer, pH 9.6, 5 μg per well. After overnight incubation at +4°C, triple washing, and blocking with Superblock solution (Pierce, USA) for 15 min at 25°C, the serum or purified IgG samples diluted to 1 : 25 in PBS-0.05% Tween (Tw) were applied for 1.5 hr at 25°C. The concentration of IgG in serum and tIgG samples was measured by the Easy-Titer IgG Assay Kit (Thermo Scientific, USA) and the IgG concentration in the tIgG probe adjusted to that in serum. After subsequent washing with PBS-Tw, the bound anti-TF or anti-αGal IgG was detected with alkaline phosphatase conjugated goat anti-human IgG (Dako, Denmark) and p-nitrophenylphosphate disodium hexahydrate (Sigma, USA). The absorbance values were read at 405 nm (Tecan Reader, Austria).

2.4. The Hidden Anti-TF IgG Antibody Level and the Impact of IgG-Depleted Serum. The IgG concentration in tIgG samples was adjusted to that in serum as described above and the level of anti-TF IgG Ab in serum and tIgG samples was measured by ELISA. The level of hidden IgG antibodies (HAbs) was calculated as the difference between the levels of anti-TF IgG (OD values) in tIgG and serum.

To evaluate the impact of IgG-depleted serum the purified tIgG samples of cancer patients ($n = 5$) and controls ($n = 5$) were incubated with an equal volume of the autologic IgG-depleted serum diluted from 1 : 10 to 1 : 100, or bovine serum albumin (BSA, 0.5–2,0 mg/mL) for 15 min at 25°C, and the HAb levels (mean OD value) after incubation with PBS-BSA or after addition of IgG-depleted serum dilutions were determined as described above and presented in Figure 3.

2.5. The Reactivity of Anti-TF and Anti-αGal Antibodies to Sambucus nigra agglutinin (SNA) and Concanavalin A (ConA) Lectin. The lectin reactivity of the TF-specific IgG was measured by ELISA in a similar way, except that the binding of neuraminic acid- (sialic acid-) specific *Sambucus nigra* agglutinin (SNA) and mannose-specific concanavalin A (ConA) to the absorbed serum anti-TF Abs (all isotypes) or anti-TF IgG from tIgG samples was measured as described elsewhere [19, 21]. Biotinylated SNA (Vector Laboratories Inc., USA) in 10 mmol/L HEPES, 0.15 mol/L NaCl, and 0.1 mmol/L $CaCl_2$, pH 7.5, and biotinylated ConA (Sigma, USA) in the ConA binding buffer (0.05 mol/L Tris-HCl buffer, pH 7.2, containing 0.2 mol/L NaCl and 3 mmol/L $CaCl_2$, $MgCl_2$, and $MnCl_2$) were both applied at a concentration of 5 μg/mL each, for 1.5 h at 25°C. The bound lectins were detected with a streptavidin-alkaline phosphatase conjugate (Dako, Denmark) and p-nitrophenylphosphate (Sigma, USA). The optical density value of control wells (no sample) was subtracted from that of Ab-coated wells to determine the lectin binding. Each sample was analyzed in duplicate.

2.6. The Avidity of TF-Specific Antibodies. The assay is based on the dissociation of Ab-Ag complexes by 1.25 M ammonium thiocyanate (NH_4SCN). This concentration of the chaotrope was selected in the preliminary titration experiments. Under the conditions used, about 60% of serum IgG antibodies of controls were detached after treatment with 1.25 M thiocyanate. From each sample, a series with and without thiocyanate treatment were made.

Purified total IgG samples with an adjusted IgG concentration and serum samples diluted to 1 : 25 were added to the wells coated with TF-PAA or αGal–PAA glycoconjugate and blocked with the Superblock solution (Pierce, USA). After incubation for 1.5 hr at 25°C the wells were exposed to either

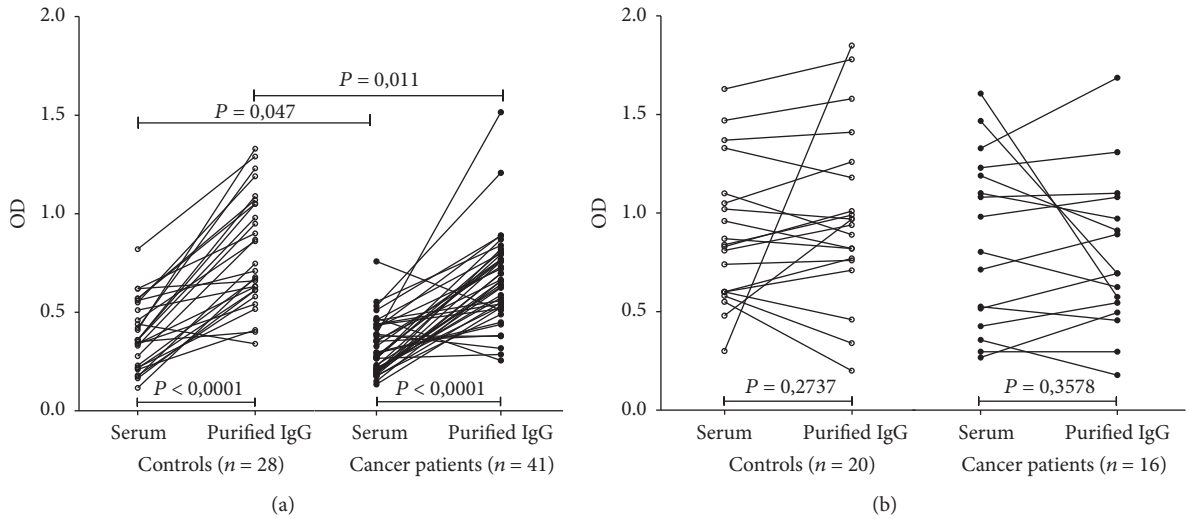

Figure 1: The level of anti-TF and anti-αGal IgG antibodies in serum and total purified IgG. (a) The TF-specific antibody level (OD) in cancer patients and controls. Each dot represents one individual. The values in serum and purified tIgG for each individual are connected with lines. (b) Anti-αGal IgG antibody levels. P values were calculated by the Mann–Whitney U test and are shown for significant differences.

PBS-0.05% Tw or NH_4SCN solution (1.25 mol/L) for 15 min at +25°C. After triple washing, the wells were incubated with alkaline phosphatase-labeled anti-human IgG for 60 min at 37°C. The bound antibodies were detected with alkaline phosphatase conjugated goat anti-human IgG (Sigma, USA) and p-nitrophenylphosphate (Sigma, USA). The results were expressed as a relative avidity index (AI) representing the percentage of reactivity remaining in the thiocyanate treated well.

2.7. Statistical Analysis. Comparisons between the groups were performed using the nonparametric Mann–Whitney U test for unpaired data or Student's t-test, where appropriate, and the Pearson two-tailed correlation. A $P \leq 0.05$ value was considered statistically significant. Survival analysis was carried out by the Kaplan-Meier method using the Estonian Cancer Registry database. The group median was used as a cut-off limit. The differences between cancer patients and controls in Ab levels and the avidity were evaluated for the diagnostic accuracy for cancer by the receiver operator characteristic (ROC) curve analysis. All calculations were performed using the GraphPad Prism 5 and SPSS 15.0 software.

3. Results

3.1. Anti-TF IgG, Anti-αGal IgG, and Hidden Antibody Levels in Cancer Patients and Controls. Decreased anti-TF IgG levels were found in both the serum and purified tIgG samples ($P = 0.047$ and 0.011, resp.) in cancer patients compared to controls. The OD values were significantly higher in purified tIgG than in serum samples in donors ($P < 0.0001$) and cancer patients ($P < 0.0001$) (Figure 1(a)). Only one subject of 28 controls and three of 41 cancer patients (all stage 3 patients) showed a slightly lower level of anti-TF IgG in tIgG than in serum. No significant difference in anti-αGal IgG level in the serum and purified IgG samples between patients and controls was observed (Figure 1(b)).

The level of hidden antibodies (HAb) defined as the difference between the levels in tIgG and serum was lower in cancer patients ($P = 0.04$). This decrease was mostly associated with stage 3 patients unlike controls ($P = 0.005$) (Figure 2) and related mainly to females (mean ± SEM: 0.19 ± 0.074; $P = 0.01$) compared with males (0.37 ± 0.058, $P = 0.74$). In contrast to anti-TF IgG, the level of hidden anti-αGal IgG Abs was very low and showed no difference between controls and patients (mean OD ± SEM: 0.01 ± 0.09 and 0.09 ± 0.08 for donors and cancer patients, resp., $P = 0.36$). In eight of 20 controls and seven of 16 patients the level of anti-αGal IgG in tIgG was even lower than in serum samples.

It is notable that after incubation of purified tIgG with IgG-depleted autologic serum the level of anti-TF IgG HAbs decreased dramatically and dose-dependently nearly down to the level in the serum already at the 1 : 10 dilution of the IgG-depleted serum (Figure 3). The impact of BSA addition (0.5–4 mg/mL) instead of IgG-depleted serum on the HAbs level was negligible being always below 10%. In addition, no such effect was observed with anti-αGal Abs where the HAbs level was very low.

Thus, rather a high level of hidden anti-TF IgG Abs but not anti-αGal hidden IgG was demonstrated in both gastric cancer patients and controls. A decreased level of anti-TF IgG HAb was observed in gastric cancer patients, especially in stage 3 females. It appears that the autologic IgG-depleted serum contains ligands that react with anti-TF IgG Abs, making them undetectable in the serum.

3.2. Anti-TF Antibody SNA and ConA Lectin Reactivity. The levels of SNA and ConA binding to anti-TF Abs were much higher in serum than in total IgG samples ($P < 0.0001$) in patients and controls (Figures 4(a) and 4(b)), suggesting a higher sialylation of anti-TF IgM and/or IgA compared to

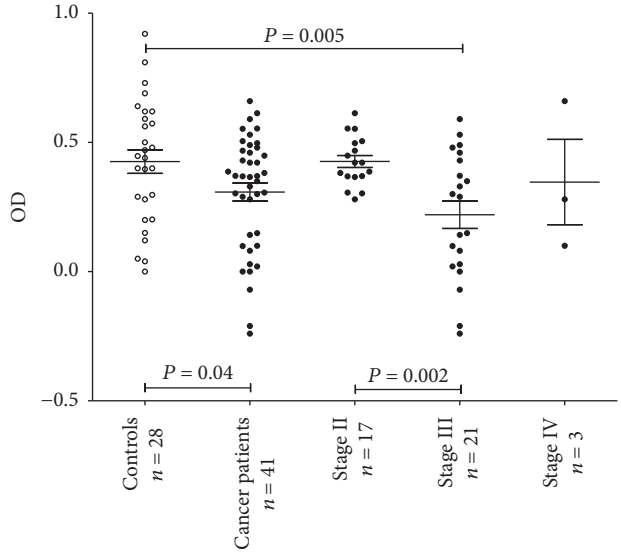

Figure 2: The level of TF-specific hidden IgG antibodies (HAbs) in controls and cancer patients by stage. Each dot represents one individual and group mean ± SEM is indicated by horizontal lines. The HAb level was calculated as the difference between the levels (OD values) of anti-TF-IgG in tIgG and serum. *P* values are shown for significant differences.

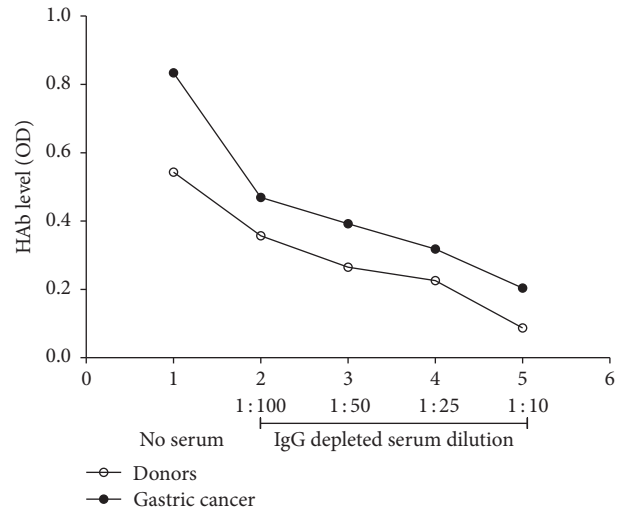

Figure 3: The effect of IgG-depleted serum on the level of anti-TF HAb in cancer patients and controls. The purified tIgG of cancer patients ($n = 5$) and controls ($n = 5$) was incubated with autologic IgG-depleted serum diluted from 1 : 10 to 1 : 100 for 15 min at 25°C. The level of anti-TF hidden antibodies was determined as described in Material and Methods; the mean value of HAb levels (OD) before and after addition of IgG-depleted serum dilutions is presented.

IgG. A significantly higher SNA binding to serum anti-TF Abs in the cancer patients group was observed (mean OD ± SEM: 1.61 ± 0.08 and 1.37 ± 0.11; in patients and controls, resp., $P = 0.03$, Mann–Whitney test), whereas no difference between patients and controls was found for tIgG samples (mean OD ± SEM: 0.40 ± 0.053 and 0.426 ± 0.038, resp., $P = 0.70$).

The ConA lectin binding to anti-TF Abs in serum and tIgG samples did not show a significant difference between patients and controls. The SNA binding to anti-TF IgG in purified tIgG samples positively correlated with the level of HAbs in donors ($r = 0.572$, $P = 0.0015$) and, to a lesser extent, in cancer patients ($r = 0.347$, $P = 0.027$) (Figures 5(a) and 5(b)), indicating that anti-TF hidden antibodies in purified tIgG are higher sialylated, especially in controls. No correlation between the SNA or ConA binding and anti-αGal or anti-αGal HAb levels was found.

3.3. The Avidity of Anti-TF IgG in Serum and Purified tIgG. In donors, the avidity of anti-TF IgG Abs in purified tIgG was found to be higher than that in serum ($P = 0.007$), while no difference in cancer patients was observed ($P = 0.80$) (Figure 6). Moreover, the avidity of anti-TF IgG Abs in purified tIgG in donors was also higher than that in cancer patients ($P = 0.025$). The avidity of free serum anti-TF IgG was similar in patients and controls ($P = 0.47$).

The avidity of anti-TF IgG in tIgG negatively correlated with the SNA binding in both groups: $r = -0.75$ and $r = -0.63$ in controls and cancer patients, respectively ($P < 0.0001$) (Figures 5(c) and 5(d)). The avidity was lower in controls with a high level of HAbs ($r = -0.54$, $P = 0.003$), while no correlation between the HAbs level and IgG avidity was found in patients with cancer ($r = -0.03$, $P = 0.86$) (Figures 5(e) and 5(f)).

The data show that cancer patients differ from controls in HAbs avidity. Cancer patients reveal some deficiency of high-avidity TF-specific IgG in tIgG samples where HAbs are present.

3.4. Diagnostic Potential and Survival Analysis. Changes in the anti-TF IgG HAb level in the whole cancer group showed rather low accuracy (area under the ROC curve (AUC) = 0.61, 95% CI 0.47–0.75) as analyzed by ROC curve analysis. The sensitivity increased in stage 3 patients: AUC = 0.69 (95% CI 0.54–0.84) (Figure 7(a)). Interestingly, the HAb level showed higher diagnostic potential in females especially in stage 3 patients: area under the ROC curve 0.78 (95% CI 0.61–0.94, $P = 0.0094$) (Figure 7(b)), compared to males (AUC = 0.55, 95% CI 0.26–0.83).

The avidity of anti-TF IgG in purified tIgG samples also showed a moderate diagnostic value (AUC = 0.638, 95% CI 0.51–0.77, $P = 0.052$). A better discrimination of patients and controls was obtained by using the ratio: the avidity of anti-TF IgG in tIgG samples/the avidity of IgG in serum samples (AUC = 0.68, 95% CI 0.55–0.80, $P = 0.010$) especially in stage 3 males (AUC = 0.784, 95% CI 0.53–1.0, $P = 0.04$) (Figures 7(c) and 7(d)).

The higher level of anti-TF IgG in serum was associated with better survival in stage 3 patients (HR = 3.23 (0.69–15.0), $P = 0.13$), whereas no relation of this level in purified tIgG samples to survival was found (Figures 8(a) and 8(b)). Survival benefit was demonstrated by patients with a higher level of SNA binding to serum anti-TF Abs (a pool of all Ig isotypes): HR = 2.94 (1.2–7.2), $P = 0.019$. In contrast, the SNA reactivity of anti-TF IgG in tIgG samples showed no association with survival (HR = 1.01 (0.4–2.5), $P = 0.98$,

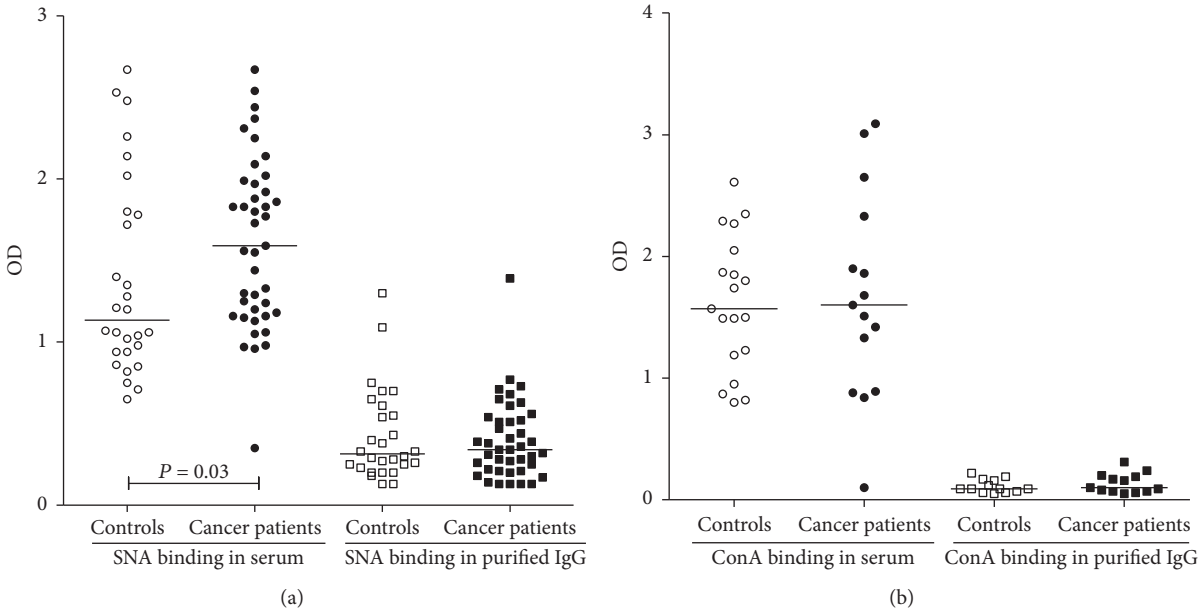

Figure 4: The binding of SNA (a) and ConA lectin (b) to anti-TF antibodies in serum and purified tIgG samples of cancer patients and controls. Each dot represents one individual and group median is indicated by horizontal lines. *P* values are shown for significant differences.

Figures 8(c) and 8(d)). The level of HAbs had no association with survival. However, the higher avidity of anti-TF IgG in purified tIgG samples where HAbs were present (Figure 8(f)) was related to worse survival (HR = 0.439 (0.179–1.074), $P = 0.07$). This was mostly on account of stage 3 patients (HR = 0.34 (0.09–1.26), $P = 0.10$). No association of the avidity of serum anti-TF IgG Abs with survival (HR = 1.02 (0.43–2.41), $P = 0.95$) was established (Figure 8(e)).

In summary, the level of HAbs and the avidity of anti-TF IgG showed a moderate stage- and gender-dependent diagnostic accuracy. A better outcome was observed in patients with a high level of TF-specific IgG in serum (stage 3), in those with a high level of SNA binding to serum anti-TF Abs (a pool of all Ig isotypes), and in patients with a low avidity of anti-TF IgG antibodies in purified tIgG.

4. Discussion

In this study, we focused on the anti-TF IgG HAb for several reasons. First, since the Fab fragment is not involved in the binding of IgG to PtG, the tIgG preparations are enriched in immune complexes, while the use of glycan- (TF-) specific sorbents for anti-TF Ab purification from the serum will exclude such a possibility due to the involvement of Fab fragments in the purification process. Second, the higher avidity of IgG allows the preferential binding of TF-positive ligands despite the possible interference of the other Ig isotypes. Third, using the Protein G it is much easier to purify IgG without the other Ig isotypes admixture. Fourth, the changes in the anti-TF IgG level showed a significant association with cancer progression and patients survival [11, 13] thus demonstrating a promising clinical potential. The possible role of the other isotypes of anti-TF Abs in the formation of HAbs needs a special study.

The results obtained in this work show that anti-TF IgG HAbs are present in the blood of both cancer patients and healthy controls. In contrast, no anti-αGal HAbs or a very low amount of them was found in the same samples. Differently from controls a decreased level of anti-TF IgG HAbs was found in cancer patients ($P = 0.005$ for stage 3 patients).

It has been shown that the in vitro exposure of human IgG to protein-destabilizing chemical or physical factors results in the exposure of IgG "hidden" reactivity [17]. However, it remains unclear whether this exposure results from the dissociation of preexisting immune complexes of Abs with some ligands, the presence of IgG dimers, anti-idiotypic Abs, or the exposure of the polyreactivity/polyspecificity of hidden Abs [22–25]. The absence of αGal-specific HAbs in the same purified tIgG samples strongly suggests that the purification of IgG per se is not a sufficient reason to explain the appearance of IgG HAbs. Moreover, the inhibition of the reactivity of hidden Abs by addition of IgG-depleted serum implies the role of serum-derived factors such as TF-positive or cross-reactive ligands that remain in the IgG-depleted serum and react with anti-TF IgG Abs again, making them HAbs. This restoration phenomenon (Figure 3) was absent in anti-αGal IgG where a very low level of HAbs was detected, which indicates that no ligands for αGal Abs are present in the IgG-depleted serum. We used anti-αGal IgG as a distinctive control to show that the purification of IgG at acid pH is not the main reason behind changes in anti-glycan IgG reactivity. We suggest that the ligands for TF-specific Abs should be more informative and more specific for cancer than anti-TF Abs themselves or their subsets (glycoforms).

Immunoglobulins (Igs) are glycosylated molecules which display a set of glycoforms that differ in number, type, and site of oligosaccharide attachment [26]. The N-glycans of

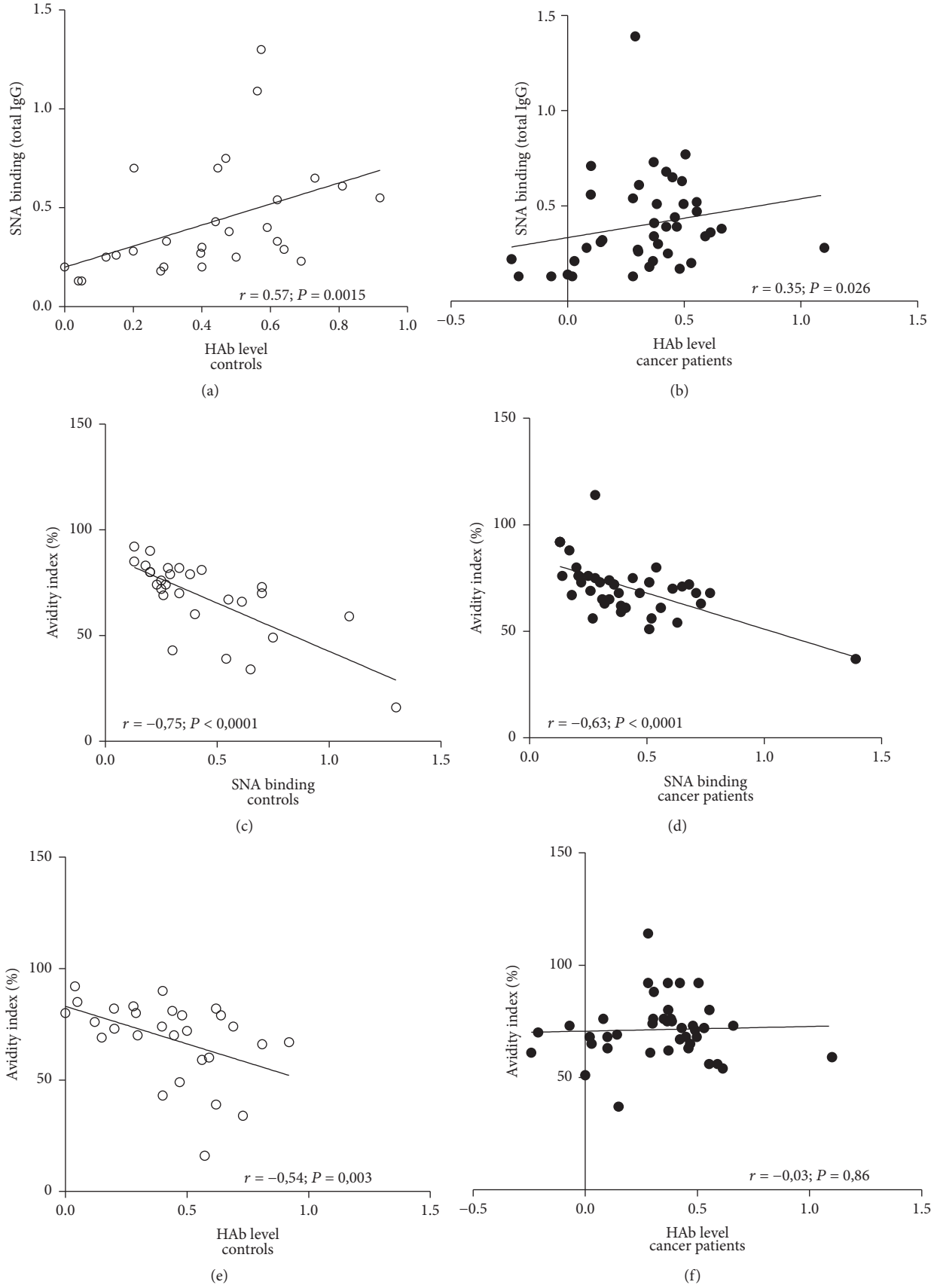

Figure 5: Correlation between the level of hidden TF-specific IgG antibodies, SNA binding, and the avidity of anti-TF IgG antibodies in gastric cancer patients and controls. (a, b) Correlation between the SNA binding to TF-specific IgG in purified tIgG and the HAb level; (c, d) correlation between the SNA binding and the avidity of TF-specific IgG in purified tIgG; (e, f) correlation between the HAb level and the avidity of TF-specific IgG in purified tIgG.

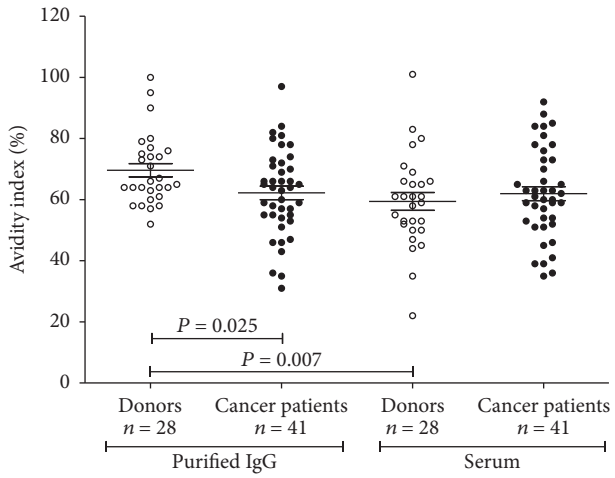

Figure 6: The avidity of TF-specific IgG antibodies in cancer patients and controls. The avidity indexes of anti-TF antibodies in serum and purified tIgG are compared. Each dot represents one individual and group mean ± SEM is indicated. P values were calculated by the unpaired Student's t-test and are shown for significant differences.

the Fc-fragment strongly influence IgG-Fcγ receptor interactions and thus the Fc-mediated effector mechanisms [27, 28]. Agalactosylated (asialylated) IgGs show an increased inflammatory activity that may promote tumor growth, while the Fc glycan sialylation may determine anti-inflammatory properties [29–31] though, besides IgG Fc sialylation, multiple mechanisms are involved in the anti-inflammatory effect of intravenous immunoglobulins [32]. It has also been shown that the anti-inflammatory potential of the sialylated IgG is mostly related to the SNA lectin-reactive sialic acids in the Fab fragment [33]. It is notable that the glycosylation of antigen-specific IgG can differ from total serum IgG glycosylation indicating that different B cell subsets produce differently glycosylated IgG [34, 35].

There is evidence that the aberrantly glycosylated serum IgG may be either of tumor origin, or accumulated in tumor tissue [36]. The higher levels of agalactosylated IgG oligosaccharides, which increase with tumor progression, were reported for patients with gastric, prostate, and ovarian cancers [36–38]. Up to now there are little data available on the glycosylation changes of Abs directed against antigens which are intrinsically involved in the pathogenesis of a specific disease, including cancer [21, 30, 36, 39–41].

The comparison of lectin binding patterns of anti-TF Abs in serum and tIgG shows that the main targets for binding SNA and ConA lectin in serum are TF-specific IgM and/or IgA. The SNA binding to free anti-TF Abs in serum was significantly higher in cancer patients ($P = 0.03$) (Figure 4(a)) whereas anti-TF IgG Abs in purified tIgG showed a similar SNA and ConA lectin binding in patients and controls. Since the SNA binding to TF-specific IgG positively correlated with the level of HAbs only in tIgG samples where both free and hidden anti-TF IgG were present, we conclude that HAbs are higher sialylated.

It is known that the total IgG purified on PtG sorbents is enriched by immune complexes and contains a lot of so-called "hitchhiker" ligands [42, 43]. We believe that this phenomenon is akin to the HAbs formation or is closely related to it, and many hitchhikers unidentified and untested as yet may be present in HAbs, including TF-positive ligands of tumor origin such as MUC1 which is overexpressed and aberrantly glycosylated in cancer, and identified as an antigenic component in IgG immune complexes in cancer patients [44, 45]. The modified self-components such as those derived from aged red blood cells may contain complexes of natural antibodies with TF-positive asialoglycophorin A or other senescent-associated epitopes connected to the clearance of aged erythrocytes [46, 47]. Thus the elimination of such complexes with natural Abs appears to be a normal physiological mechanism.

Contrary to blood donors, cancer patients demonstrated a lower avidity of anti-TF IgG in tIgG samples while no differences in the avidity of free serum TF-specific Abs between the two groups were observed. Since the tIgG contains both free Abs and HAbs, this decrease is obviously related to the HAbs that display a lower avidity in cancer.

The lower level of HAbs showed a rather good diagnostic accuracy for cancer in stage 3 females (AUC = 0.78) compared to males (AUC = 0.55). In contrast, the ratio of anti-TF IgG avidity in tIgG to the avidity of IgG in serum revealed a better diagnostic value in males (AUC = 0.784) than in females (AUC = 0.629) (Figure 7). Since the avidity of anti-TF IgG in serum showed no changes in cancer, the decrease of high-avidity anti-TF IgG HAbs in cancer patients, especially in males, may be of diagnostic value. At present we can give no explanation for these gender-related differences and further study is needed to evaluate the clinical utility of the above changes.

The SNA binding to anti-TF IgG in tIgG positively correlated with the HAbs levels in both groups (Figures 5(a) and 5(b)). At the same time, a negative correlation between the anti-TF Abs avidity in tIgG and the HAbs level was observed in controls ($r = -0.54$, $P = 0.003$) but not in cancer patients ($r = -0.03$, $P = 0.86$) (Figures 5(e) and 5(f)). These findings indicate that IgG HAbs are higher sialylated in cancer and display a lower avidity, which suggests that they represent a particular subset of TF- specific Abs.

It has been reported that the Fc glycan of adaptive IgG Abs is lower sialylated [48], though the vaccination in humans induced increased levels of antigen-specific IgG galactosylation and sialylation [35]. However, there are still no data about the sialylation of IgG-Fab fragments of tumor-related Abs in cancer. Notable is that the higher SNA reactivity of TF-specific Abs (a pool of all isotypes) in serum was associated with better survival (HR = 2.94 (1.2–7.2), $P = 0.019$), whereas no connection with the SNA reactivity of anti-TF IgG in tIgG (HR = 1.01 (0.4–2.5), $P = 0.98$) was demonstrated. We have shown recently that the higher avidity of SNA-reactive TF-specific Abs in the serum of cancer patients was not related to the IgG isotype [20]. In the present study, the higher avidity of anti-TF IgG in purified tIgG preparations containing HAbs was associated with worse survival (Figure 8(f)). In other words, a deficiency of high-avidity anti-TF IgG HAbs

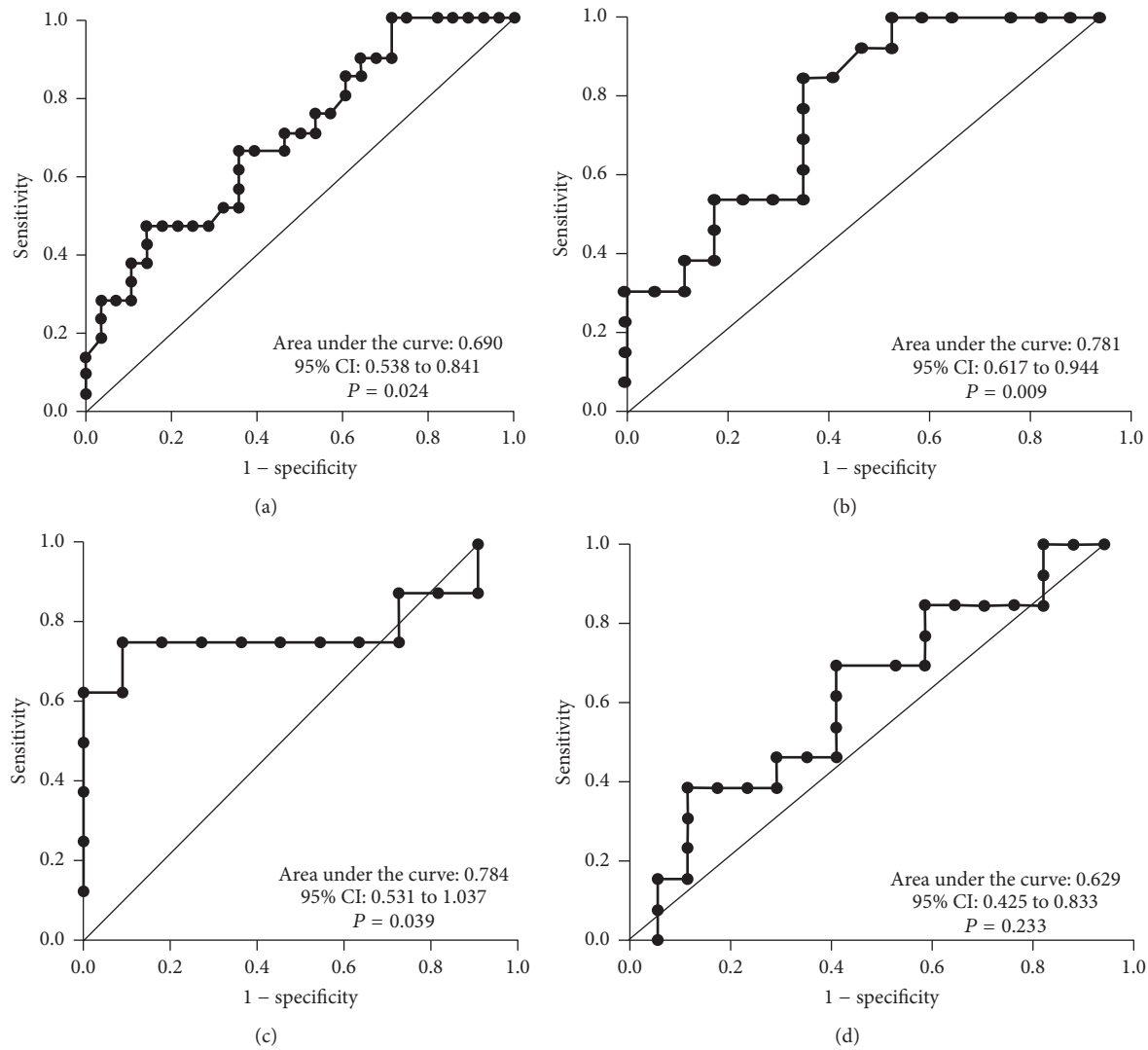

Figure 7: A receiver operator characteristic (ROC) curve analysis for the HAb level in stage 3 patients (a); in stage 3 females (b); and for the ratio anti-TF IgG avidity in tIgG/anti-TF IgG avidity in the serum of stage 3 males (c) and stage 3 females (d). The area under the ROC curve represents the diagnostic accuracy of the changes for gastric cancer.

in cancer tIgG samples is associated with survival benefit, whereas the avidity of free serum TF-specific IgG was not related to survival.

It is to be noted that despite the TF expression on tumor cells there is no evidence that any adaptive humoral immune response to TF occurs in the tumor host. Instead, a decrease in the level of serum TF-specific Abs is usually observed [9–11, 13]. However, no data still exist on the possible involvement of any particular subset of anti-TF IgG Abs in this decrease. We found that healthy individuals demonstrated a significantly higher avidity of anti-TF IgG in tIgG samples, unlike gastric cancer patients whose avidity values in tIgG were low and similar to those in serum samples. We suggest that this may occur, at least in part, due to the elimination of high-avidity anti-TF Ab subset from the circulation of patients with cancer. This decrease of the avidity of naturally occurring anti-TF IgG Abs in the purified tIgG of patients with gastric cancer is, to our knowledge, described for the first time. We speculate that such a decrease may be a result of the interaction of these Abs with tumor-derived TF-positive ligands in situ or their increased clearance with circulating tumor cells. Which ligands are involved in the HAbs formation in cancer patients and healthy individuals remains to be elucidated.

In conclusion, TF-specific HAbs represent a particular subset of anti-TF IgG Abs that differ from free anti-TF IgG Abs in SNA reactivity, avidity, diagnostic potential, and relation to survival. Thus it appears that free serum anti-TF natural Abs in the circulation are only the "tip of the iceberg." The hidden anti-TF antibody reactivity emerging in purified IgG suggests that serological testing with whole serum does not reflect the whole picture, and the HAbs analysis could tell us more about the other players and targets involved. Given that there are still no reliable biomarkers for gastric cancer, the changes in the level of anti-TF hidden IgG antibodies

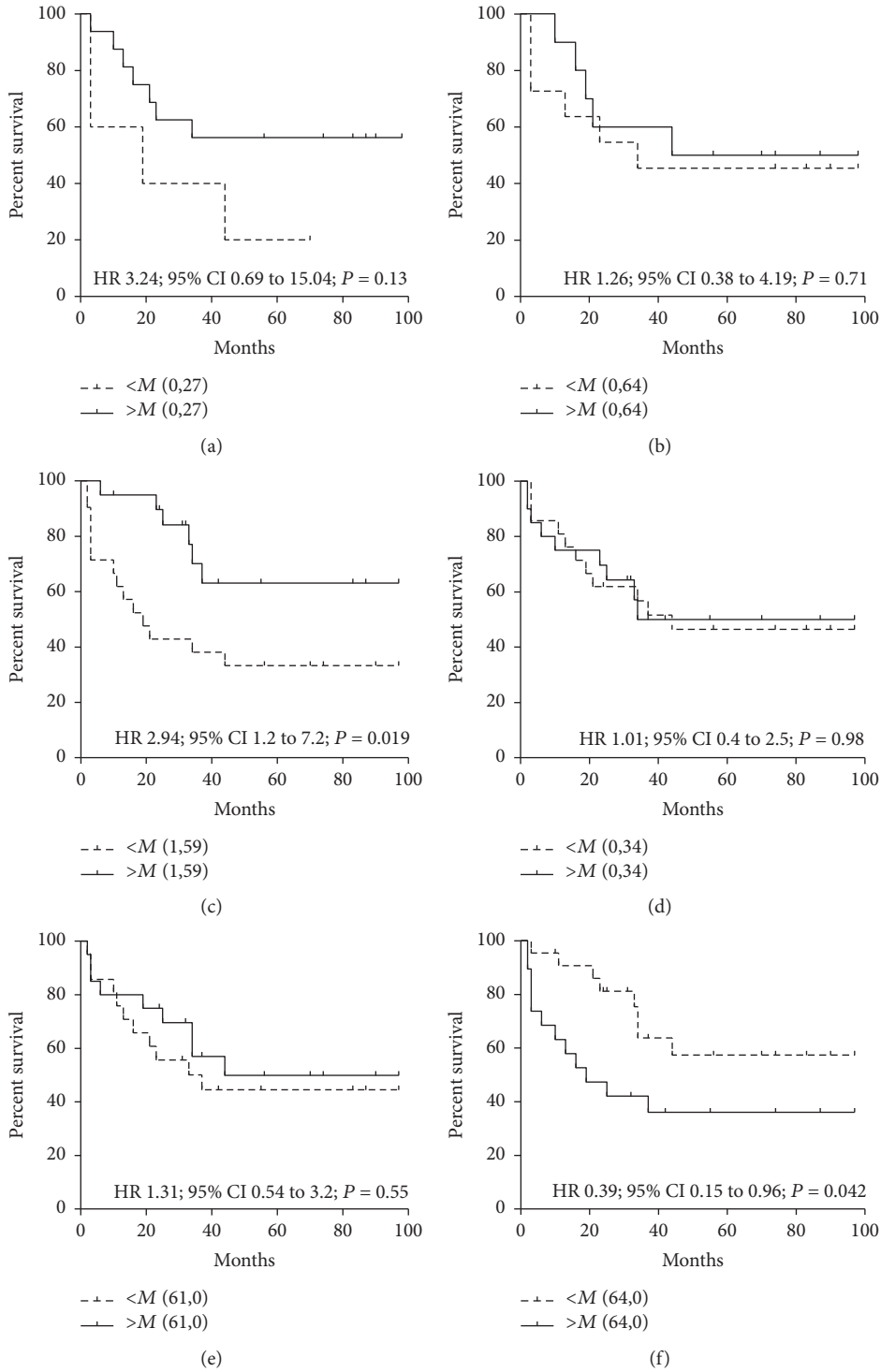

Figure 8: The probability of cancer patients survival in relation to anti-TF IgG level, SNA reactivity, and the avidity of TF-specific antibodies in serum and purified total IgG samples. Patients with either lower/equal (a dashed line) or higher values than median (a solid line) are compared using the Kaplan-Meier method. HR: hazard ratio with 95% confidence interval and P values are shown. (a) Anti-TF IgG level in serum (stage 3); (b) anti-TF IgG level in tIgG (stage 3); (c) the SNA binding to serum TF-specific antibodies; (d) the SNA binding to anti-TF IgG in tIgG; (e) the avidity of anti-TF IgG in serum; (f) the anti-TF IgG antibody avidity in tIgG and survival.

and their avidity found in this study merit further study. Whether the tumor-specific ligands are involved in the HAbs formation in patients with cancer remains to be clarified. The functional activity and clinically important effects of anti-TF Abs may apply to only a particular subset/glycoform of TF-specific Abs which contributes to cancer and its progression. Further characterization of TF-specific antibody subsets and potential ligands involved in the HAbs formation in health and disease may extend the concept of natural autoantibody signatures and improve the clinical utility of antibody-based diagnostic and predictive biomarkers.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Review Article

Diagnostic and Therapeutic Biomarkers in Glioblastoma: Current Status and Future Perspectives

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Glioblastoma (GBM) is a primary neuroepithelial tumor of the central nervous system, characterized by an extremely aggressive clinical phenotype. Patients with GBM have a poor prognosis and only 3–5% of them survive for more than 5 years. The current GBM treatment standards include maximal resection followed by radiotherapy with concomitant and adjuvant therapies. Despite these aggressive therapeutic regimens, the majority of patients suffer recurrence due to molecular heterogeneity of GBM. Consequently, a number of potential diagnostic, prognostic, and predictive biomarkers have been investigated. Some of them, such as IDH mutations, 1p19q deletion, MGMT promoter methylation, and EGFRvIII amplification are frequently tested in routine clinical practice. With the development of sequencing technology, detailed characterization of GBM molecular signatures has facilitated a more personalized therapeutic approach and contributed to the development of a new generation of anti-GBM therapies such as molecular inhibitors targeting growth factor receptors, vaccines, antibody-based drug conjugates, and more recently inhibitors blocking the immune checkpoints. In this article, we review the exciting progress towards elucidating the potential of current and novel GBM biomarkers and discuss their implications for clinical practice.

1. Introduction

Glioblastoma (GBM) is a primary neuroepithelial tumor of the central nervous system, characterized by an extremely aggressive clinical phenotype that has arisen from inter- and intrapatient genomic and histopathological diversity (Figure 1; Table 1). GBM is the most common of malignant primary brain tumors in adults. It accounts for 12% to 15% of all intracranial tumors and about 50% of astrocytic tumors.

Patients with GBM have a poor prognosis of just 12–15 months following standard therapy, with only 3–5% of patients surviving up to 5 years after diagnosis [1, 2]. The most favorable prognostic factors include younger age at diagnosis (<50 years), a Karnofsky Performance Status of at least 70 points, and the tumor being located in a noneloquent area of the brain [3]. The current GBM treatment standards include maximal resection (complete resection is achieved extremely rarely due to the diffusely infiltrative nature of these tumors) followed by radiotherapy with concomitant and adjuvant therapies, for example, temozolamide (TMZ). If there is progression, bevacizumab against circulating vascular endothelial growth factor (VEGF) is widely used, more recently also in combination with lomustine (CCNU) [4]. Despite these aggressive therapeutic regimens the majority of patients suffer recurrence due to the molecular heterogeneity of GBM tumors and penetration of therapeutic agents through the blood-brain barrier (BBB). Both of these factors affect treatment response and prognosis leading to acquired tumor resistance in GBM patients. However, recent developments in next-generation sequencing methods have led to identification of specific molecular signatures of GBM that allow for better understanding of the molecular pathogenesis of this disease [5]. Consequently, a number of potential diagnostic, prognostic, and predictive biomarkers have been proposed. Diagnostic biomarkers enable more accurate tumor classification; prognostic biomarkers inform about a likely cancer outcome (e.g., disease recurrence, disease progression, and overall survival) and predictive biomarkers facilitate patient

Table 1: Molecular biomarkers in GBM.

p53 signalling altered in 87%	MDM2 (amplification in 14%)
RTK/RAS/PI3 signalling altered in 88%	PI3K (mutation in 15%)
RB signaling altered in 78%	CDK4 (amplification in 18%)

management by helping to tailor the treatment strategy to patient-specific biology. There are some molecular markers still under evaluation, but several are commonly tested as part of the routine clinical interrogation of GBM patients including O^6-methylguanine DNA methyltransferase (MGMT), isocitrate dehydrogenase (IDH), epidermal growth factor receptor (EGFR), VEGF, tumor suppressor protein TP53, phosphatase and tensin homolog (PTEN), p16INK4a gene, phospholipid metabolites, cancer stem cells, and recently also imaging biomarkers (Table 2). Importantly, detailed characterization of these molecular signatures has facilitated a more personalized therapeutic approach and contributed to the development of a new generation of anti-GBM therapies such as small molecular inhibitors targeting growth factor receptors, vaccines, antibody-based drug conjugates, and more recently inhibitors blocking the immune checkpoints [6].

The aim of this article is to review the exciting progress towards elucidating the potential of diagnostic, prognostic, and predictive biomarkers of GBM and discuss their implications for clinical practice.

2. Glioblastoma Multiforme: A New Look

Gliomas have historically been classified and treated according to the World Health Organization (WHO) criteria, which are determined by histopathological examination, for example, nuclear atypia, cellular pleomorphism, mitotic activity, vascular thrombosis, microvascular proliferation, and necrosis [7]. In the official reclassification of Tumor Types of the Central Nervous System, published by WHO on May 9, 2016, the GBMs are listed in the group of diffuse astrocytic and oligodendroglial tumors which reflect their highly malignant behavior [8].

Clinically, most patients present de novo grade IV lesions (primary GBMs), whereas only a small fraction of patients (5–10%) show progression from less aggressive WHO grade II diffuse astrocytomas and WHO grade III anaplastic astrocytomas (secondary GBMs) [9].

Medical onset and progression of primary GBMs vary from those seen in secondary GBMs, with the latter being typically diagnosed at a younger age (45 versus 62 years), having longer clinical history (16.8 versus 6.3 months) and, although they are histologically largely indistinguishable, having a better prognosis in terms of survival (7.8 versus 4.7 months) [10].

Importantly, these two clinical presentations have distinct molecular signatures. For example, primary GBMs frequently present amplification/mutations of the EGFR gene (36–60% of primary and 8% of secondary tumors), PTEN mutation (25% of primary versus 4% secondary tumors), and CDKN2A-$p16^{INK4a}$ deletion (31–78% of primary versus 8% secondary tumors) [10]. Additionally, there are genetic aberrations that are expressed more frequently in secondary GBMs including TP53 mutations (28% of primary versus 65% of secondary tumors) [10], MGMT promoter methylation (36% of primary versus 75% of secondary tumors) [11], and IDH1 mutations (5% of primary versus 75% of secondary tumors) [12]. While histopathological analysis of gliomas has formed the basis of diagnosis and treatment up to this point, the widespread implementation of sequencing and profiling technologies has resulted in more comprehensive analysis of the molecular aberrations underlying gliomagenesis, as well as providing insights into their biological heterogeneity.

The more recent analysis of GBMs by The Cancer Genome Atlas Research Network (TCGA) highlighted the most frequent alterations in GBM genes, including amplification of EGFR and platelet-derived growth factor receptor alpha (PDGFRA); mutation of TP53, phosphatidylinositol-4,5-bisphosphate 3-kinase A (PIK3CA), PTEN, IDH1, RB1, and TERT promoter; and deletions of PTEN, CDKN2A/B, and MGMT, as well as alterations in chromatin remodeling genes. Based on these dominant gene expression patterns, four transcriptional subclasses of GBMs have been identified: classical, proneural, mesenchymal, and neural [13, 14]. Each of these subtypes is associated with specific genetic and epigenetic alterations. The classical subtype is characterized by the loss of chromosome 10 and amplification of chromosome 7 with coexisting EGFR amplification/mutation, impaired level of proapoptotic proteins, mitogen-activated protein kinase (MAPK), and Notch1 and Notch3 proteins [14, 15]. The proneural variant is associated with PDGFRA, CDK6, CDK4 MET, and frequent IDH1 mutations, activation of the phosphatidylinositol 3-kinase (PI3K), and inhibition of the translation repressor 4EBP1 [14].

Mesenchymal GBMs frequently show deletions and silencing mutations of NF1 on chromosome 17q11.2 and point mutations in PTEN, activation of MAPK pathway, and downregulation of mammalian target of rapamycin (mTOR) signaling.

Less is known about the neural tumors, which are characterized by the expression of neurofilament light polypeptide, synaptotagmin I, and overexpression of EGFR [16]. In terms of prognosis, no difference was found between the classical, mesenchymal, and neural subtypes. However, the proneural subtype was associated with onset at a younger age and prolonged survival time [14]. This has been attributed to mutations in the IDH1 gene, which are exclusively linked with the proneural phenotype and grade II/III of astrocytic and oligodendroglial tumors (72–100%) along with secondary glioblastomas (85%). While primary GBMs could be of any subtype and mutations in IDH1 are rarely found in these tumors (5%) [17].

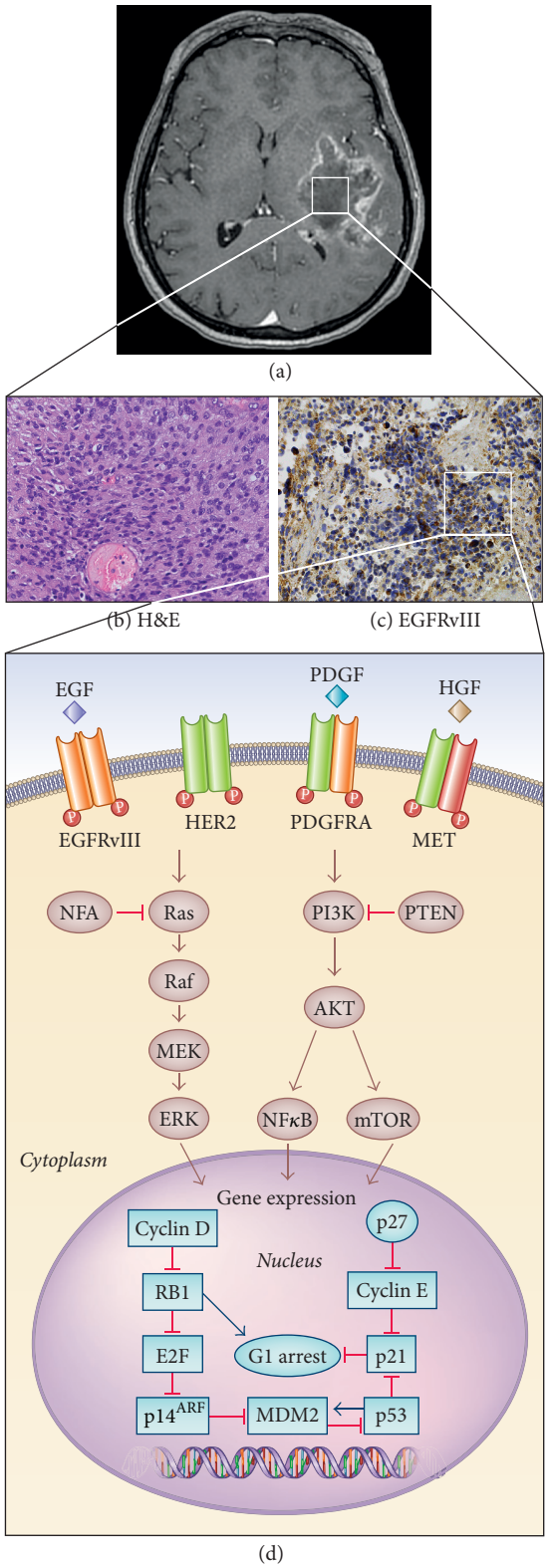

Figure 1: Glioblastoma characterization. (a) Axial contrast-enhanced T1-weighted MR image shows a large enhancing mass in the left temporal lobe in a 54-year-old woman diagnosed with GBM. (b, c) GBM formalin-fixed paraffin-embedded sections stained for H&E and EGFRvIII. (d) Frequent alterations in critical signaling pathways found in GBM.

Classification and subclassification of GBMs is not always easy. Recent studies analyzing expression signatures of single cells within GBM samples showed substantial intratumoral heterogeneity of expression subclasses within each tumor. Sattoriva et al. examined genome-wide somatic copy number levels in 38 fragments derived from 11 patients with GBM. Although the fragments from the same patient shared a common gene profile, they displayed a significant variety of copy number alterations that were present in only a subset of fragments. Moreover, using gene expression arrays, they found that in 6 out of 10 cases the fragments from the same tumor mass were classified into at least 2 different GBM subgroups, which indicated that tumor clones with different phenotypic profiles are present within the same malignancy [18]. This may explain the difficulties associated with oncologic biomarker validation and contribute to an incorrect selection of patients for targeted therapies, treatment failure, or drug resistance [19].

Additionally, GBM exhibit heterogeneity at the cellular level, with a small subpopulation of tumor cells harboring stem-like properties. These GBM stem cells (GBSC) are capable of self-renewal and differentiation into neuronal, macroglial, and mixed neuronal/astroglial phenotypes [20]. Recent genome-wide transcriptional analysis identified two phenotypically different subtypes of GBSC, namely, proneural and mesenchymal, which also correlate well with corresponding proneural and mesenchymal signatures in GBMs [21, 22]. Mesenchymal GBSC (35–40% of cases) similarly to mesenchymal GBMs, display a more aggressive phenotype and are more resistant to radiation as compared to proneural GBSC. Moreover, GBSC possess unique surface markers (e.g., CD133, CD15, and ALD1A3) and modulate characteristic signaling pathways to promote tumorigenesis (e.g., hedgehog and Notch) [23]. Interestingly, these GBSC have the ability to shift phenotypic features from one subtype to another when put under increased cellular stress (e.g., radiation treatment) [22–24] and transdifferentiate into tumor microenvironment cells such as endothelial cells and pericytes, providing more favorable conditions for GBM growth [25].

3. A Highway to Hell: Molecular Pathways and Genetic Aberrations Found in GBM

Tumor growth in GBM cells is facilitated by high expression of cell surface membrane receptors that control the intracellular signal transduction pathways regulating proliferation and cell cycle abnormalities including an increase in DNA repair proteins and abnormal cell death pathways [26, 27]. An integrated analysis of the genetic alterations, performed by the TCGA research network, confirmed that the most commonly disrupted signaling cascades in GBM include changes in pathways related to receptor tyrosine kinase (RTK) signaling through the RAS/MAPK (mitogen-activated protein kinase) and PI3K/AKT/mTOR, along with the cell cycle-regulating retinoblastoma (RB) tumor suppressor and p53 pathways.

Table 2: Major biomarkers relevant to the management of patients with glioblastoma.

Type of biomarker	EGFR mutation/ amplification	MGMT promotor methylation	IDH1/IDH2 mutation	Imaging	Reference
Diagnostic	EGFRvIII highly correlates with glioma subtypes. Real-time monitoring via typing of microvesicles with EGFR specific RNA.	Help to distinguish true progression and pseudoprogression in patients with newly diagnosed GBM treated with surgery followed by radiochemotherapy.	Differentiate between primary and secondary GBM. IDH-mutant diffuse gliomas and nonanaplastic reactive gliosis distinction.	Detection of specific molecular abnormalities. For example, EGFRvIII, MGMT promotor methylation, and 2-HG which correlates with IDH mutation.	[9, 76, 112–115, 118–120, 131–134]
Development status	+/under evaluation	Under evaluation	+	+	
Prognostic	Better prognosis with (i) EGFRvIII + Ki64 20% or less, (ii) EGFRvIII + normal PTEN, (iii) EGFRvIII + methylated MGMT promotor.	Better OS and PFS (probably with IDH mutations) in malignant gliomas treated with radio- and/or chemotherapy.	Better OS and PFS	MRI: extent of tumor edema and necrosis has negative correlation with OS. PET: ^{11}C-MET uptake is associated with poorer patient survival.	[32–36, 43, 44, 76, 78, 83, 85, 93, 126, 135–140]
Development status	+/under evaluation	+	+	Under evaluation	
Predictive	Possible biomarker for vaccine-based treatment.	Predicts response to chemotherapy with alkylation agents and radiotherapy. Correlate with better response to TMZ in (i) newly diagnosed GBM with TMZ as a first-line treatment, (ii) recurrent GBM, (iii) elderly patients.	IDH1 mutation is independently associated with complete resection in patients with malignant gliomas treated with surgery. Complete surgical resection is associated with improved survival in patients with IDH1 mutation. Absence of mutation suggests predictive role of MGMT promotor methylation for PFS in patients treated with chemotherapy.	Functional Diffusion Maps (fDMs) predicts PFS and OS in patients treated with radiochemotherapy. ADC predicts better response to bevacizumab combined with chemotherapy. Association between hypoxia level (measured by ^{18}F-FIMSO and radiotherapy response.	[77, 86–91, 93, 122, 123, 136, 140, 141]
Development status	Under evaluation	+	Under evaluation	Under evaluation	

OS, overall survival; PFS, progression-free survival.

4. RTK Signaling in GBM

Mutations or amplifications of RTK including EGFR, PDGFRA, basic fibroblast growth factor receptor 1 (FGFR-1), and insulin-like growth factor receptor (IGFR-1) are present in more than 80% of primary GBM [28]. These structurally related proteins coordinate a complex signaling network that drives and regulates many cellular processes. In gliomas the two main signaling pathways utilized by RTK are the RAS/RAF/MAPK pathway that leads to cellular proliferation, differentiation, and migration and the PI3K/AKT/mTOR pathway that primarily serves to promote cell proliferation and survival through progression of the cell cycle and inhibition of apoptosis [28]. The activity of PI3K is regulated by the tumor suppressor gene PTEN that is a negative regulator of this pathway [29]. Loss of PTEN, found in approximately 36% of gliomas, may result in dramatic upregulation of this pathway and be a major source of resistance to EGFR therapies [30].

EGFR mutations, rearrangements, alternative splicing, and focal amplifications are the most frequent genetic alterations, occurring in nearly 57% of GBM tumors [31]. EGFR amplification can be found, nearly exclusively, in patients with a classical subtype of GBM and is very rare in secondary GBMs [16]. However, the role of EGFR amplification as a prognostic biomarker has yielded conflicting results. There

are reports showing no association with overall survival (OS) in patients, others showing a negative impact, and some even indicating a favorable impact on patient survival [32–36]. Unfortunately, despite the high frequency of EGFR gene amplification, EGFR inhibitors (e.g., gefitinib and erlotinib) have not been successfully brought into clinical trials for patients with GBMs [37–39].

The lack of a meaningful response may be due to the relatively poor penetration of these drugs through BBB, acquired resistance promoting mutations in the targeted RTKs, and intratumoral heterogeneity in GBM tumors [40, 41]. In addition, around 50% of patients with EGFR amplification harbor EGFRvIII mutation, which results from an in-frame deletion of exons 2–7 and leads to constitutive and ligand independent receptor activity [42]. However, EGFRvIII prognostic relevance is still controversial, for instance, Shinojima et al. have shown that EGFRvIII expression in the presence of EGFR amplification is a strong indicator of poor survival and prognosis [43]. On the other hand, Montano et al. prospectively analyzed the relationship between EGFRvIII expression and OS in patients with newly diagnosed GBM treated with gross total resection and standard radiochemotherapy (TMZ). Notably in this case, EGFRvIII identified that patients had significantly longer OS. Furthermore, association of EGFRvIII/Ki67 of 20% or less, EGFRvIII/normal PTEN, and EGFRvIII/methylated MGMT allowed identification of subgroups in GBM patients with better prognosis [44].

Although EGFRvIII seems to be a well-defined drug target the clinical trials with tumor vaccine rindopepimut have demonstrated immunologic effect and apparent clinical benefit only in early phase trials. Tests of this vaccine in randomized, placebo-controlled phase III studies failed to show survival benefit [45]. Nevertheless, EGFR still remains an attractive molecular target and current clinical trials are focusing on testing new inhibitors that are more potent and specific to the GBM mutations and introducing mechanism-based combination therapies [40, 46, 47].

When it comes to other RTK alterations, in a smaller proportion of secondary GBMs (13%), high-level amplification of the PDGFRA has been detected, and nearly half of these tumors also contained amplification and/or mutation of EGFR [31, 48]. Despite PDGFRA being strongly associated with GBM, an anti-PDGFRA therapy using glivec resulted in only a limited clinical response [49].

Although much less frequent in GBMs, alterations such as mesenchymal-epithelial transition factor (c-Met) amplification and FGFR mutations have been reported in 2% of the analyzed GBMs [50, 51].

All these activating genetic aberrations can occur simultaneously in multiple RTKs within individual GBM and concurrently express mutations in downstream components of growth factor receptor pathways. The PI3K/AKT/mTOR, the most powerful oncogenic pathway in GBM, can be activated by mutations in either the catalytic (PIK3CA) or regulatory (PIK3R1) domains of PI3K [52]. The TCGA study found that almost 10% of the GBMs had mutations in the PIK3R1, which has not been found to be frequently expressed in any other cancer [53]. Furthermore, it has been reported that AKT classification can be a predictive marker that identifies a subset of GBM patients responding to carmustine (BCNU)/CCNU and PI3K/AKT/mTOR pathway inhibitors [54]. More recent studies have revealed that the tumor suppressor gene NF1, that encodes neurofibromin (RAS negative regulator), is mutated/deleted in 15% to 18% of primary GBMs (mesenchymal subclass) [53, 55].

5. TP53/MDM2/p14^{ARF} Pathway

The TP53 tumor suppressor gene, at chromosome 17q13.1, encodes a p53 protein that regulates target genes involved in (i) cell cycle arrest in the G1 and/or G2 phase of cell cycle, (ii) cell death and differentiation, (iii) DNA repair, and (iv) neovascularization [56–58]. TP53 may be inactivated indirectly, as a result of mutation or deletion, or directly due to damage of cooperating genes [59]. MDM2 oncoprotein negatively regulates p53 activity through the ubiquitination and proteasomal degradation of p53. In turn, the p14^{ARF} protein functionally antagonizes MDM2 and, thus, prevents the silencing of p53 [60]. Initially, TP53 mutations have been associated with secondary GBMs rather than with primary (65% versus 28%) [10], but recent TCGA data has reported TP53 mutations in many primary GBMs. The overall frequency of genetic alterations in the TP53/MDM2/p14^{ARF} pathway was found in 87% of GBMs, in 35% through TP53 mutations or homozygous deletion, in 14% due to MDM2 amplification, and in 49% as a result of p14^{ARF} homozygous deletion or mutation [53].

6. p16^{INK4a}/CDK4/RB1 Pathway

The RB1 protein controls progression through G1 into the S-phase of the cell cycle. The CDKN2A (p16^{INK4a}) protein binds to CDK4 cyclin and inhibits the CDK4/cyclin D1 complex that prevents cell cycle transition from G1 to S-phase [61]. Therefore, loss of normal RB1 function may result from altered expression of any of the p16INK4a, CDK4, or RB1 genes.

Inactivation of this pathway is commonly observed in both primary and secondary GBMs. While mutations in RB1 are not common (11%), genes encoding its upstream regulators are frequently altered, in particular mutations and deletions of CDKN2A/p16 (52%) and amplification of CDK4 (18%) [62]. Despite frequent occurrence of these defects none of them have been identified as a useful prognostic biomarker in GBMs [63].

7. IDH Mutations

The IDH1 and IDH2 genes encode two critical metabolic enzymes: isocitrate dehydrogenase 1 (present in the peroxisomes and cytosol) and isocitrate dehydrogenase 2 (present in the mitochondria). These proteins catalyze the oxidative carboxylation of isocitrate to alpha-ketoglutarate, which results in the production of NADPH in the citric acid (Krebs) cycle [64, 65]. Mutations of these IDH genes promote reactions that generate the oncometabolite 2-hydroxyglutarate (2-HG) [66, 67]. In gliomas the most frequent missense mutations in

IDH genes are present at the 132 residue in IDH1 (85%) and at 172 in IDH2 (3%) [12, 68]. They have been mainly found in secondary GBMs (73%–85%), along with grades II and III astrocytic and oligodendroglial tumors (72–100%) but appear to be rare or absent in primary GBMs (5%) [12, 69]. Several studies have reported that the presence of IDH mutations in diffuse glioma is associated with younger age (mean 32–47) [12, 70, 71]. Although, IDH-wild-type and IDH-mutant gliomas are histologically similar, numerous groups have reported that gliomas harboring IDH mutations represent a distinct disease entity that arises from a different cell type and occur in the presence of other genomic abnormalities, such as TP53 mutation or 1p/19q chromosome deletion, and happen mutually exclusively in gliomas with EGFR amplification and chromosome 10 loss [17]. Moreover, IDH-mutant tumors have also been linked with substantial epigenetic changes, such as DNA methylation disorders, which harbor a striking pattern of hypermethylation of certain DNA promoter regions termed as glioma-CpG island methylator phenotype (G-CIMP) [72]. It has been shown that 78% of G-CIMP+ tumors carry IDH1 mutations [73], and 98% of these malignancies are positive for IDH2 [74].

The wild-type IDH gliomas including pilocytic astrocytomas and primary GBMs are independent of the IDH pathway (G-CIMP−). Conversely, most grades II and III gliomas and secondary GBMs share IDH mutations (G-CIMP+). Up to 87.5% of G-CIMP+ tumors represent a proneural gene expression subtype and are usually found in younger patients (mean age at diagnosis: 36 years versus 59 years) [73, 75]. Moreover, they also carry a better prognosis than the IDH-wild-type gliomas of the same histological grade [76]. In addition, Beiko et al. demonstrated that IDH1 mutation status is associated with the benefit of surgical resection in malignant astrocytic gliomas (WHO grades III and IV). More aggressive resection involving the whole tumor (both enhancing and nonenhancing part) correlated with better prognosis in patients with IDH1 mutant GBMs rather than with wild-type IDH1. In the case of the latter, no prognostic benefit was observed after the resection of nonenhancing part [77].

Importantly, since the presence of IDH mutations has been shown to correlate with better OS and progression-free survival (PFS) in GBM patients, this aberration became the only molecular marker included in the updated 2016 WHO classification of astroglial brain tumors [8, 78]. Furthermore, it prompted efforts to develop inhibitors of the mutated IDH protein for therapeutic purposes. So far, these new drugs have been reported to induce differentiation in preclinical models, and clinical proof of concept has been achieved in early phase I trials (NCT02074839 and NCT01915498) using AG-120 and AG-221 in adults with relapsed or refractory acute myelogenous leukemia [79–82]. These promising early results are now driving expansion of these trials into solid tumors. Phase I dose escalation studies of AG-120 in patients with IDH-mutated gliomas and other solid tumors (NCT02073994) and of AG-221 also in patients with IDH-mutated gliomas (NCT02273739) are now open. Further work is urgently needed to determine the most appropriate IDH mutation detection technique to facilitate early identification of patients who may benefit from these novel therapies. At the moment IDH mutations are evaluated by immunohistochemistry, standard sequencing, or genotyping methods. Furthermore, 2-HG level has been noninvasively assessed in patients with glioma using magnetic resonance spectroscopy and proved to be a useful biomarker in monitoring treatment response [82].

8. MGMT Methylation

Promotor of MGMT encoding O_6-methylguanine-DNA methyltransferase is a DNA repair enzyme which can effectively protect cells against alkylating agents (e.g., TMZ and CCNU) through preventing G:C→A:T gene mutations [83]. Disorders of MGMT promoter methylation are associated with transcriptional silencing of the MGMT gene and loss of MGMT expression that results in decreased DNA repair and retention of alkyl groups, thereby allowing alkylating agents to be more effective in patients with MGMT promoter hypermethylation.

MGMT promoter methylation is more often found in secondary GBMs than in the lesions they derive from, such as low-grade gliomas and primary GBMs (75% versus 48% versus 36%) [11, 84]. Recently, a number of clinical trials have shown that MGMT methylation corresponds to greater PFS and OS in patients who are treated with alkylating agents [83, 85–87]. Therefore, MGMT promoter methylation status represents one of the most relevant prognostic factors in GBMs and has been considered as a potent predictor of response to alkylating agents.

Furthermore, recent prospective randomized trials (NOA-08), the Nordic trial, and RTOG 0525 have shown MGMT promoter methylation can also be a useful predictive biomarker to stratify elderly GBM patients for RT versus chemotherapy with alkylating agents. Several studies have demonstrated that patients with tumors with methylated MGMT promoter had a survival benefit when treated with TMZ and radiotherapy, compared with those who received RT only, whereas patients with MGMT promoter-unmethylated tumors had no survival benefit from chemotherapy, regardless of whether it was given at diagnosis together with RT or as a salvage treatment [88–92]. Consequently, it has been suggested that elderly GBM patients eligible for either RT or TMZ should undergo MGMT promoter methylation testing prior to the clinical decision being made. In addition, MGMT promoter methylation was associated with greater PFS and improved of OS in patients with recurrent GBMs (Director trial) [93]. These findings highlight the necessity for different therapeutic approaches in patients with GBMs depending on their MGMT status and introducing MGMT biomarker assessment into routine clinical practice.

9. Immune Checkpoints

In addition to mutations in cell signaling and growth proteins, part of the aggressive nature of the GBM is related to its ability to escape immune system surveillance. GBM has established

a mechanism of dampening the immune response by expressing immunosuppressive cytokines (e.g., prostaglandin E2 and transforming growth factor-β) and increasing activation of T-regulatory cells [94]. This suppressive immune microenvironment is manipulated by two important checkpoint proteins, cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1) [95]. CTLA-4 is exclusively upregulated on T cells and negatively regulates the early stages of T-lymphocyte activation by competing with the costimulatory molecule CD28 at binding the B7 ligands. In contrast, PD-1 is expressed by B, natural killer and dendritic cells as well as activated monocytes and tumor-infiltrative macrophages in addition to T cells [96, 97]. Moreover, PD-1 regulates immunity at multiple phases of the immune response effecting T-lymphocyte activity in the peripheral tissues [98]. TCGA analysis has reported high mRNA expression level of PD-L1, a PD-1 ligand, and CTLA-4 in mesenchymal GBMs suggesting a correlation between these immune checkpoint proteins and severity of GBM [99]. Yet, the prognostic value of these immune checkpoints in GBM is still controversial. Berghoff et al. performed a study on 117 GBM patient samples and found no correlation between PD-L1 and survival [100]. Liu et al. have revealed that PD-L1 can have both a positive and negative effect on GBM patient survival depending on the glioma subclass, on expression levels of PD-L1 regulatory molecules, and most importantly on the cell type that expresses PD-L1 in the tumor microenvironment [101]. But most current clinical studies have demonstrated that PD-1 and/or PD-L1 are immunohistochemically detectable in the majority of GBM samples and PD-L1 gene expression significantly correlates with molecular GBM subtypes (mainly mesenchymal) [100]. Additionally, Nduom et al. have shown that PD-L1 in GBM patients is overexpressed in a small subpopulation, where higher expression of PD-L1 is correlated with worse outcome [102].

With the dramatic success of checkpoint inhibitors (e.g., nivolumab and ipilimumab targeting PD-1 and CTLA-4, resp.) in melanoma, brain metastases, and lung and kidney cancers, hope has increased regarding the potential activity of these drugs in glioma [103–106]. Multiple clinical trials of these checkpoint inhibitors in GBMs and recurrent lower-grade gliomas are currently in progress (e.g., NCT02017717). These studies will determine whether this approach has any beneficial effects for these patients. So far, data from a phase III trial (NCT00045968) using a dendritic cell vaccine (DCVax-L) has indicated that monitoring CTLA-4 expression may predict survival in GBM patients, indicating there may be a role for CTLA-4 as a novel biomarker for treatment response [107, 108]. Furthermore, the RTOG is also planning a randomized phase II/III trial to test ipilimumab in combination with TMZ in patients with newly diagnosed glioblastoma [107]. Taken together, current findings indicate that the complexity of tumor microenvironment poses a major challenge to the development of immunotherapy approaches for GBMs and proper stratification of CTLA-4/PD-1/PD-L1-positive and negative patients will be important criterion for high-quality clinical trials in GBMs.

10. Imaging Biomarkers

The role of histopathology, proteomics, and next-generation sequencing methods as a standard reference for assessment of GBM progression is increasingly being challenged. In addition to invasiveness and sampling bias they do not address inter- and intratumor heterogeneity. Therefore, in contrast to conventional evaluation of ex vivo tissue specimens, development of imaging biomarkers for monitoring tumor response following therapeutic interventions could greatly improve individual patient management.

Currently there are no clinically approved imaging biomarkers for GBM. However, advanced functional imaging techniques including diffusion-weighted magnetic resonance imaging (DW-MRI) with apparent diffusion coefficient (ADC) mapping, dynamic susceptibility-weighted contrast-enhanced perfusion imaging, MR spectroscopy (MRS), and positron emission tomography (PET) have recently demonstrated a great potential for identifying distinct phenotypes of GBM tumors. While these results are promising, there is a large variation in sensitivity and specificity reported, which likely was a result of small sample size in some of these studies, and differences in acquisition protocols, as well as reference standards that have been used [109–111].

Nevertheless, several reports have highlighted that utilizing genomic and imaging data may improve the selection and implementation of the appropriate treatment for targeting the unique biology of GBM tumors and the detection of early treatment failure. For example, evaluation of 2-hydroxyglutarate (2-HG) by proton MRS has been reported to correlate with the IDH1 or IDH2 mutations in the tumor [112] indicating that upregulated levels of 2-HG in IDH-mutated gliomas have the potential in the future to provide important diagnostic and prognostic information. Additionally, numerous MRI parameters such as a high ratio of contrast enhancing tissue to necrotic tissue ($\geq$1), lower ADC values, increased T2 to contrast enhancing volume, deceased T2 border sharpness, and elevated relative cerebral blood volume (rCBV) have been reported as being predictive of EGFR amplification [113–115]. rCBV measurements have also shown to be a good predictive factor for the malignant degeneration-free survival, PFS, and OS as well as discrimination of tumor recurrence and nonneoplastic contrast enhancing tissue after radiotherapy in low-grade gliomas [116, 117].

Furthermore, an increase in tumor blood volume has been associated with EGFR amplification, PTEN deletion, and normal unmethylated MGMT [118–120].

Many of these MRI features are also essential in monitoring the clinical effectiveness of treatment regimens. Larsen et al. reported nearly 100% sensitivity and specificity using calculated CBV, which is comparable to those achieved by ^{18}F-fluorodeoxyglucose (^{18}F-FDG) on the same patients [121]. Besides, tumor ADC value has been shown to be a useful indicator for predicting response to bevacizumab. For example, in a large cohort of 97 bevacizumab-treated patients with recurrent GBM, low ADC was associated with a poor outcome in post hoc analysis from the multicenter randomized, phase II BRAIN study [122]. However, larger studies are needed for this imaging biomarker to become universally

accepted. Also, functional diffusion mapping based on ADC values determined prior to and after radiochemotherapy were shown to correlate with both survival benefit and longer PFS in GBM patients [123].

Aside from MRI, a number of PET radiotracers have been evaluated as potential imaging biomarkers which may offer additional insight into brain tumor pathophysiology. Currently, ^{18}F-FDG is the most frequently used PET radioligand, but it has limited capabilities for GBM imaging due to elevated glucose uptake in the brain compared to other tissues, which results in low-grade tumors, small tumors, and tumors with early recurrence remaining undetected. Therefore, for the past few years other PET ligands have been assessed including radiolabeled amino acids and their aromatic analogues (e.g., ^{11}C-methionine (^{11}C-MET), ^{18}F-flouroethyltyrosine (^{18}F-FET), and 3,4-dihydroxy-6-^{18}F-fluoro-L-phenylalanine (^{18}F-FDOPA)), and hypoxia agents (e.g., ^{18}F-fluoromisonidazole (^{18}F-FMISO)) since they overcome the limitations of ^{18}F-FDG providing much higher tumor/background contrast. The amino acid PET tracers have attracted most of the attention due to the fact they enter the brain via amino acid transporters allowing visualization of both low- and high-grade gliomas regardless of integrity of the BBB [124, 125]. Kim et al., have demonstrated that among several clinical and metabolic factors, ^{11}C-MET uptake is associated with poorer patient survival indicating a prognostic value of this tracer in glioma patients [126]. Pauleit et al. found that there is increased ^{18}F-FET uptake in nonenhancing tumor areas which are difficult to delineate using MRI [127]. In addition, Fueger et al., have shown that imaging using ^{18}F-FDOPA could differentiate between low- and high-grade gliomas and that tracer uptake correlated with tumor proliferation in newly diagnosed gliomas, but not in previously treated recurrent tumors [128]. A study of 22 participants with GBM has shown association between preradiation volume and degree of tumor hypoxia as measured by ^{18}F-FIMSO and a shorter time to tumor progression and decreased survival. These promising results indicate that hypoxia imaging may also serve in the future as an early biomarker of radiation resistant tumor regions and provide insight into radiotherapy planning for patients with GBM [129]. While current data highlights the potential of molecular imaging biomarkers for the evaluation of treatment response and survival, further prospective studies are needed to evaluate their clinical impact. What is needed is an integration of comprehensive genomic information together with imaging data that will not only strengthen our understanding of heterogeneity in GBM's genetics, metabolomics, or epigenetics, but also provide an opportunity to identify robust predictive biomarkers that could improve therapeutic outcome and minimize drug resistance.

11. Conclusions

Although numerous challenges remain, recently substantial progress has been made in the molecular characterization of diffuse gliomas, providing useful insights into the development of more effective targeted therapeutics. Even though some of these therapies, including IDH or RTK pathway inhibitors, have so far produced limited or no therapeutic efficacy in phase III trials, our improved understanding of their mechanisms of action has helped to determine how to better incorporate their use in existing treatment paradigms. Importantly, based on the challenges these drugs have initially presented, innovative clinical trials have been designed evaluating different therapeutic strategies. The detailed description of these regimens was beyond the scope of this review but, as briefly mentioned in each paragraph, recent clinical reports are very promising. For example, given the potential to manipulate or enhance the immune system machinery to attack and kill tumor cells, immunotherapy has shed new light on and generated a lot of excitement in the treatment of GBM, especially with clinical trials that are currently underway. Phase I/II trials testing DCVax-L in patients with newly diagnosed GBM showed significant increase in the median life expectancy [6]. Furthermore, clinical trials based on either retroviral or adenoviral vectors have demonstrated that the herpes simplex virus-1 thymidine kinase (HSV1-TK)/ganciclovir (GCV) system HSV1-TK/GCV is well tolerated. However, due to immune suppression mechanisms present in GBM microenvironment, the study has not shown as expected significant therapeutic benefit [130].

Therefore, against this background, there is an urgent need to incorporate the status of known biomarkers into the routine clinical practice which may assist not only in patient selection, but also in the adjustment of treatment schedule based on the patient-specific biology. The biggest challenge lies in better understanding of GBM heterogeneity and the ability to successfully translate the vast amounts of data generated by large-scale, next-generation sequencing, and single tumor cell sequencing, as well as genomic and molecular imaging analyses into a clinically applicable format. Furthermore, appropriate combination of novel targeted and immunotherapeutic approaches that are biomarker driven will hopefully improve the management and lead to more durable responses in GBM patients.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

MicroRNA-Related Polymorphisms in PI3K/Akt/mTOR Pathway Genes Are Predictive of Limited-Disease Small Cell Lung Cancer Treatment Outcomes

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The phosphoinositide-3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway plays an important role in cancer progression and treatment, including that of small cell lung cancer (SCLC), a disease with traditionally poor prognosis. Given the regulatory role of microRNA (miRNA) in gene expression, we examined the association of single nucleotide polymorphisms (SNPs) at miRNA-binding sites of genes in the mTOR pathway with the prognosis of patients with limited-disease SCLC. A retrospective study was conducted of 146 patients with limited-disease SCLC treated with chemoradiotherapy. Nine SNPs of six mTOR pathway genes were genotyped using blood samples. Cox proportional hazard regression modeling and recursive partitioning analysis were performed to identify SNPs significantly associated with overall survival. Three SNPs, *MTOR*: rs2536 (T>C), *PIK3R1*: rs3756668 (A>G), and *PIK3R1*: rs12755 (A>C), were associated with longer overall survival. Recursive partitioning analysis based on unfavorable genotype combinations of the rs2536 and rs3756668 SNPs classified patients into three risk subgroups and was internally validated with 1000 bootstrap samples. These findings suggest that miRNA-related polymorphisms in the PI3K/Akt/mTOR pathway may be valuable biomarkers to complement clinicopathological variables in predicting prognosis of limited-disease SCLC and to facilitate selection of patients likely to benefit from chemoradiotherapy.

1. Introduction

Small cell lung cancer (SCLC), which accounts for approximately 13% of lung cancer cases [1], is a common neuroendocrine malignancy characterized by aggressive growth and early metastasis. Chemotherapy with agents of etoposide or topoisomerase 1 inhibitors (irinotecan or topotecan), along with cisplatin and radiotherapy, is the recommended treatment for SCLC with limited disease (LD SCLC) [2, 3]. Unfortunately, there is only a 15% to 20% cure rate and most cases recur rapidly, leading to a very poor overall prognosis [4]. Genetic variations may play an important role in treatment sensitivity; therefore, identification of novel genetic predictors could be helpful for individualized treatment options.

The phosphoinositide-3 kinase (PI3K)/Akt/mechanistic target of rapamycin (mTOR) signaling pathway has been a hot therapeutic target for SCLC in recent research [5]. This pathway is activated in various cancer types, including SCLC [6], and is associated with radiation and chemotherapy resistance [7]. Genetic variations in this pathway are reported to modulate clinical outcomes in patients with esophageal cancer and non-small-cell lung cancer who have undergone chemoradiotherapy [8, 9]. MicroRNAs (miRNAs), a class of small, noncoding RNAs, are key regulators of gene expression in many biological processes [10], including the mTOR

pathway [11]. Single nucleotide polymorphisms (SNPs) in miRNA-binding sites may affect the regulatory effect of miRNAs on oncogenes and tumor suppressor genes. However, little published research has considered the impact of miRNA-related genetic polymorphisms in the PI3K/Akt/mTOR pathway and their relationship to SCLC outcome. We therefore analyzed the effect of these genetic variations on the prognosis of patients with LD SCLC receiving curative chemoradiotherapy and selected valuable biomarkers for decision making.

2. Materials and Methods

2.1. Ethics Statement. This investigation was conducted in accordance with the ethical standards outlined in the Declaration of Helsinki and national and international guidelines. Our institutional review board (Cancer Hospital, Chinese Academy of Medical Sciences) approved this retrospective study, and informed consent was waived.

2.2. Study Population. Patients in this study were retrospectively recruited from the Cancer Hospital, Chinese Academy of Medical Sciences, between January 2007 and June 2014. All SCLC were histologically confirmed, staged as LD SCLC based on the International Association for the Study of Lung Cancer (IASLC) classification [12], and initially treated with curative-intent platin-based chemotherapy combined with intensity-modulated radiation therapy (IMRT). Patients who had any other malignancy within five years of enrollment were excluded to avoid a potential confounding effect. Prophylactic cranial irradiation (PCI) was offered to patients who had achieved a complete remission (CR) or partial response (PR), depending on the treating physician's discretion and patient's preference. Clinical information was collected from medical records. Long-term archived serum samples from recruited patients were analyzed [13].

2.3. SNP Selection and Genotyping. Candidate miRNA-related SNPs of PI3K/Akt/mTOR pathway genes were selected in two ways: SNPs located at the 3′-UTR of miRNA target genes with a minor allele frequency greater than 0.1 in the Chinese Han population in the PolymiRTS Database 3.0 (http://compbio.uthsc.edu/miRSNP/) and Ensemble Asia database (release 79, http://asia.ensembl.org/in-dex.html?redirect=no) as well as those previously reported in the literature to be associated with cancer were included. Tagging SNPs in linkage disequilibrium were identified using HaploReg version 3 (http://www.broadinstitute.org/mammals/haploreg/haploreg_v3.php) with a cut-off value of $r^2 > 0.8$.

Genomic DNA was extracted using a TIANamp Blood DNA Kit (TIANGEN Biotech, Beijing, China). Genotyping was conducted using the MALDI-TOF mass spectrometry-based iPLEX Gold assay on the Sequenom MassARRAY Platform (San Diego, CA, USA) and was analyzed using MassARRAY TyperAnalyzer v4.0 software (Sequenom). SNPs with a more than 95% success rate and samples with a call rate exceeding 96% were included. Ultimately, a total of nine tagSNPs in six genes were selected.

2.4. Statistical Analysis. Response rates were evaluated according to RECIST 1.0 criteria. The objective response rate (ORR) included CR and PR to treatment. The association of genetic features with ORR was estimated using the Pearson χ^2 test with odds ratios (ORs) and 95% confidence interval (CI). Overall survival (OS) was calculated from pathologic diagnosis to the date of death or last follow-up. The Kaplan-Meier method and log-rank test were used to assess survival for each genotype and clinical characteristics. Hazard ratios (HRs) and 95% CI were estimated by Cox proportional hazards regression models. The adjustment factors included age, gender, smoking history, Karnofsky performance score (KPS), and Charlson comorbidity index (CCI). Genotype analyses were conducted in three genetic models (dominant, recessive, and additive) for each SNP and the model with the smallest P value was used. Models with rare genotypes (<5% of patients) were excluded. Recursive partitioning analysis (RPA) was conducted to evaluate the cumulative effects of the genetic variants in the pathway. Concordance probability estimate (CPE) was used to assess the predictive ability of RPA classification [14] from 1000 bootstrap samples.

A two-sided P value < 0.05 was considered significant for all statistical analyses. Multiple comparison was performed by Benjamini-Hochberg False Discovery Rate (FDR) correction based on tests for 3 models with a q-value of 0.05 [15]. Statistical power of the RPA classification was calculated with an α of 0.1. All statistical analyses were carried out using IBM SPSS Statistics 21.0 software (IBM Corp., Armonk, NY) and R version 3.2.3 (http://www.r-project.org.com/).

3. Results

3.1. Clinical Characteristics. The clinical characteristics of 146 patients with LD SCLC recruited to this study are shown in Table 1. Patients' median age at diagnosis was 56.8 years (range 29–80 years). Of these patients, 91.8% were diagnosed as stage III. Chemotherapy was delivered based on an EP (etoposide + cisplatin) or EC (etoposide + carboplatin) regimen, usually for 2–4 cycles; concurrent treatment was given to 54.8% patients with no difference in overall survival compared to those receiving sequential treatment. A total of 37 patients achieved CR and 84 had a PR. PCI was administered to 43.2% patients, 90.5% of whom had a CR or PR in the primary lesion. The overall median survival time (MST) and 5-year OS rate were 35.1 months and 38.9%, respectively, at a median follow-up time of 42.2 months. Age, KPS, CCI, and PCI were prognostic factors for OS ($P < 0.05$, log-rank test).

3.2. Associations of Individual SNPs with Outcome. The survival analysis by genotype for each SNP is shown in Table 2. Two SNPs were significantly associated with OS (*MTOR*: rs2536 and *PIK3R1*: rs3756668), rising to three (*PIK3R1*: rs12755) after adjustment for age, gender, KPS, smoking history, and CCI. Patients carrying heterozygous TC of *MTOR*: rs2536 (T>C) had a significantly increased risk of reduced OS compared to those with TT genotype (adjusted HR = 1.948, 95% CI: 1.090–3.482). In addition, variant homozygous genotypes of rs12755 (A>C) and rs3756668 (A>G) in PI3K

Table 1: Clinical characteristics of 146 patients with limited disease-small cell lung cancer.

Variables	*N* (%)	5yOS	*P*
Gender			
Male	104 (71.2%)	33.0%	0.065
Female	42 (28.8%)	47.2%	
Age			
≤60	95 (65.1%)	43.0%	0.015
>60	51 (34.9%)	30.8%	
KPS			
≥90	61 (41.8%)	47.5%	0.009
<90	85 (58.2%)	33.1%	
Location			
Left lobe	69 (47.3%)	39.3%	0.987
Right lobe	77 (52.7%)	37.8%	
Smoking			
Yes	96 (65.8%)	35.7%	0.088
No	50 (34.2%)	40.1%	
Charlson comorbidity index			
≤3	130 (89.0%)	41.7%	
4-5	13 (8.9%)	19.5%	0.030
6-7	3 (2.1%)	0%	
Weight loss			
With	27 (18.5%)	51.3%	0.393
Without	119 (81.5%)	35.2%	
AJCC stage			
IA	1 (0.7%)	100.0%	
IB	2 (1.4%)	0%	
IIA	5 (3.4%)	NA	0.573
IIB	4 (2.7%)	NA	
IIIA	68 (46.6%)	41.7%	
IIIB	66 (45.2%)	34.9%	
Treatment modality			
Concurrent	80 (54.8%)	46.8%	0.401
Sequential	66 (45.2%)	31.2%	
Chemotherapy cycles			
<4	9 (6.2%)	NA	
4–6	126 (86.3%)	40.2%	0.382
>6	11 (7.5%)	38.6%	
Radiotherapy dose			
<60	46 (30.7%)	39.3%	0.525
≥60	100 (69.3%)	39.9%	
PCI			
With	63 (43.2%)	62.1%	$1.54E-4$
Without	83 (56.8%)	23.1%	

OS: overall survival; AJCC: American Joint Committee on Cancer; PCI: prophylactic cranial irradiation.

regulatory subunit 1 (alpha) (*PIK3R1*) were associated with 77.5% (adjusted HR = 0.225, 95% CI: 0.054–0.931) and 61.2% (adjusted HR = 0.388, 95% CI: 0.176–0.856) decrease in the risk of death, respectively (Figures 1(a)–1(c)). However, after multiple comparison correction, the associations with OS of these SNPs were not statistically significant.

Of the three prognostic SNPs, rs12755 and rs3756668 were associated with ORR of chemoradiotherapy in patients with LD SCLC. Individuals carrying the rs12755 C allele showed a significantly higher ORR than those with AA genotype (ORs = 0.908, 95% CI: 0.858–0.961, $P = 0.037$). For rs3756668, carriers of the G allele had a higher ORR compared with the AA genotype (OR = 0.790, 95% CI = 0.720–0.867, $P = 0.012$).

3.3. Associations of Individual SNPs with OS in Stage III Patients. Assessing only the 134 patients with stage III SCLC, the overall MST and 5-year OS rate were 35.1 months and 38.2%, respectively. *MTOR*: rs2536 (T>C) and *PIK3R1*: rs3756668 (A>G) remained significantly associated with survival in stage III patients (Table 3). *PIK3R1*: rs12755 (A>C) was a borderline prognostic factor after adjusting for clinical covariates (HR adjusted = 0.242, 95% CI: 0.058–1.010). However, after correcting for multiple comparison, the discriminations of these genetic variants for survival were not statistically significant (Table 3). Other polymorphisms in the PI3K/Akt/mTOR pathway continued not to show significant association with OS.

3.4. Recursive Partitioning Analysis (RPA). To explore the combined effect of unfavorable genotypes, three SNPs significantly associated with OS (rs2536 (T>C) in an additive model, as well as rs12755 and rs3756668 in recessive models), were included in a recursive partitioning analysis. The RPA model was developed based on *PIK3R1*: rs12755 (A>C) and *PIK3R1*: rs3756668 (A>G), and the data set was split into three risk classifications (Table 4). The MST and 5-year OS rate were 48.6, 35.1, and 18.4 months and 47.7%, 40.8%, and 17.6% in the low, intermediate, and high-risk classes, respectively, after adjustment for age, gender, KPS, smoking history, and CCI (Figure 1(d)). The ORs of ORR in the intermediate and high-risk classes were 0.757 (95% CI: 0.679–0.845) and 0.359 (95% CI: 0.236–0.546) using the low risk group as a reference. The statistical power of this classification was 0.74.

3.5. Validation of the RPA Classification Using Bootstrap Analysis. Bootstrap analysis was conducted and confirmed a good performance of the RPA predictive model based on PI3K/Akt/mTOR pathway SNPs in survival discrimination by 1000 resampling internal validation datasets (Table 4). The CPE of the RPA classification was 0.69 from 1000 bootstrap samples, supporting the satisfactory predictive efficacy.

4. Discussion

SCLC is second only to melanoma in terms of malignancies with a high degree of genomic alternations [16, 17]. As one of the most promising therapeutic targets in SCLC, the PI3K/Akt/mTOR pathway was shown to have a high prevalence of genetic alternations in a comprehensive genomic analysis of SCLC in Asian populations [18]. The PI3K/Akt/mTOR pathway plays a critical role in cancer progression by regulating cell growth, proliferation, and survival [19]. This pathway was also reported to be involved in

Table 2: Survival analysis of miRSNPs in mTOR pathway in 146 patients with limited-disease small cell lung cancer.

Gene SNP		Model	5yOS	HR (95% CI)	Log-rank P	HR (95% CI) (adjusted)*	P adjusted*	q
VEGFA rs10434	DOM	GG (100) GA + AA (46)	39.0% 39.7%	0.768 (0.449–1.314)	0.334	0.864 (0.498–1.497)	0.601	0.651
DDIT4 rs1053639	DOM	AA (87) AT + TT (59)	34.0% 44.8%	0.949 (0.577–1.560)	0.835	0.910 (0.553–1.498)	0.712	0.688
MAPK1 rs1063311	DOM	CC (107) CT + TT (36)	36.6% 45.8%	0.637 (0.338–1.199)	0.158	0.546 (0.288–1.037)	0.064	0.165
PTEN rs11202607	ADD	CC (122) CT (23)	37.2% 55.1%	0.714 (0.325–1.570)	0.400	0.596 (0.267–1.331)	0.207	0.391
PIK3R1 rs12755	REC	CA + CC (134) AA (11)	28.7% 55.7%	0.508 (0.284–0.909)	0.107	0.225 (0.054–0.931)	0.040	0.129
MTOR rs2536	ADD	TT (117) TC (25)	43.4% 22.9%	1.838 (1.033–3.272)	0.036	1.948 (1.090–3.482)	0.024	0.103
VEGFA rs3025039	DOM	CC (89) CT + TT (55)	42.3% 35.1%	1.025 (0.617–1.705)	0.923	1.083 (0.639–1.837)	0.767	0.704
PIK3R1 rs3756668	REC	GA + GG (119) AA (25)	35.5% 47.7%	0.417 (0.190–0.916)	0.025	0.388 (0.176–0.856)	0.019	0.103
PTEN rs701848	DOM	TT (52) TC + CC (92)	30.2% 41.9%	0.802 (0.472–1.363)	0.413	0.725 (0.420–1.252)	0.249	0.435

*Adjusted for age, gender, Karnofsky performance score (KPS), smoking history, and Charlson comorbidity index (CCI).
miRSNPs: miRNA-related single nucleotide polymorphisms; OS: overall survival; HR: hazard ratio; CI: confidence interval.
Reference sequences: *VEGFA* GenBank NG_008732; DDIT4 GenBank NM_019058; *MAPK1* GenBank NG_023054; *PTEN* GenBank NG_007466; *PIK3R1* GenBank NG_012849; *MTOR* GenBank NG_033239.

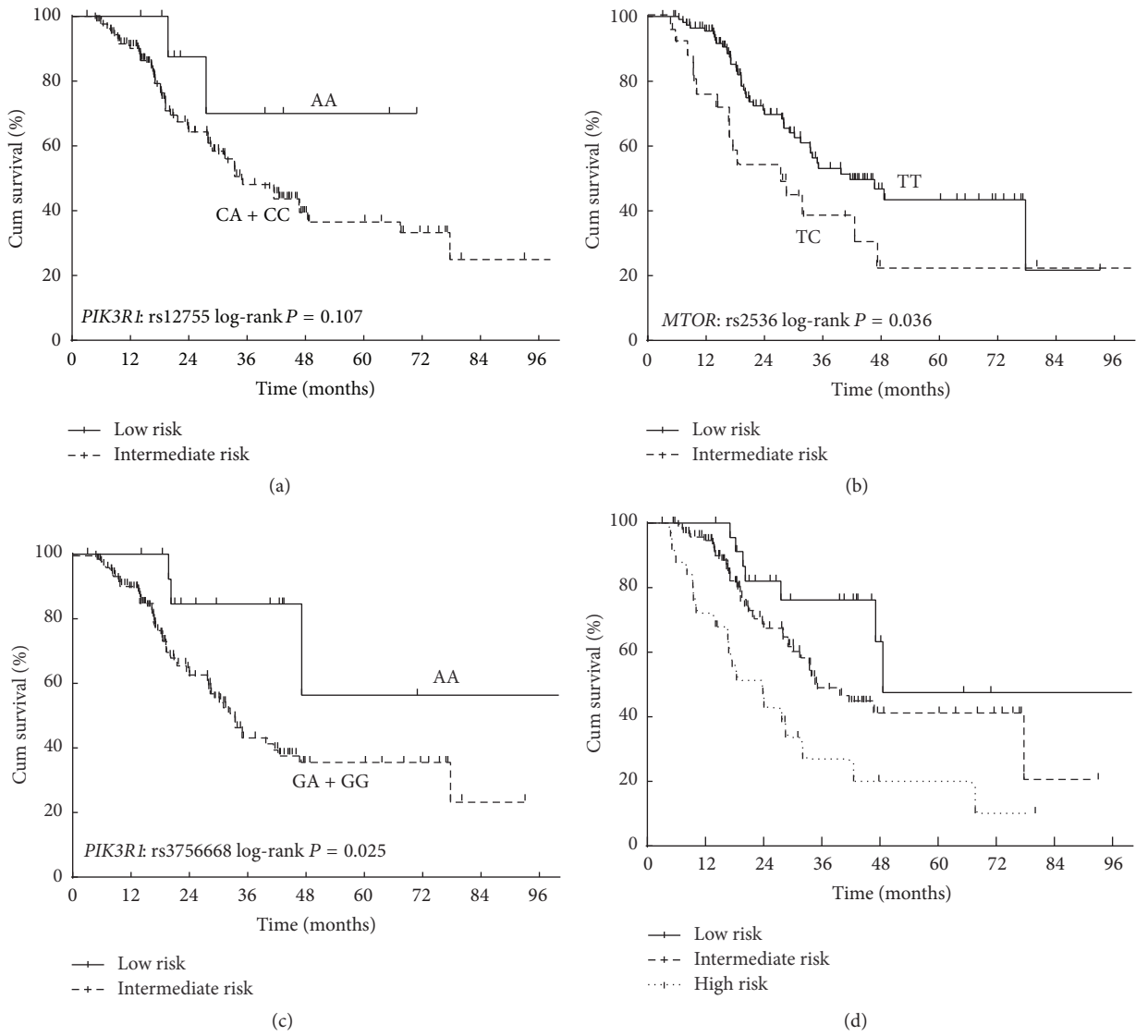

Figure 1: The Kaplan-Meier survival curve of selected SNPs and the RPA classification in patients with limited-disease small cell lung cancer treated with curative chemoradiotherapy. (a) *PIK3R1*: rs12755 (A>C); (b) *MTOR*: rs2536 (T>C); (c) *PIK3R1*: rs3756668 (A>G); (d) RPA classification.

the development of resistance to radiation and chemotherapy [20–22]. Genetic variants in this pathway were demonstrated to be associated with platinum-based chemotherapy response and prognosis in patients with advanced NSCLC [23]. In this study, we performed a pathway-specific analysis to determine whether polymorphisms in the PI3K/Akt/mTOR pathway may predict outcomes of patients with LD SCLC treated with curative chemoradiotherapy. Three SNPs in miRNA-binding genes in this pathway were identified to be associated with overall survival of these patients, and the unfavorable genotypes of *MTOR*: rs2536 (T>C) and *PIK3R1*: rs3756668 (A>G) were combined by RPA to optimize the prognostic value. To our knowledge, this is the first study to evaluate the pathway-based effect of polymorphisms in the PI3K/Akt/mTOR pathway on clinical outcome of SCLC. The combination of multigenic variants classified these patients into three risk groups and provided a basis to identify patients with LD SCLC that will benefit from chemoradiotherapy.

The *MTOR* gene, located in exon 59 of chromosome 1p36.2, encodes a serine/threonine kinase and emerges as a key downstream effector of the PI3K/Akt/mTOR signaling pathway. Moreover, *MTOR* has been identified as a potential target in SCLC [22]. rs2536 is the mostly frequently studied polymorphism to be associated with cancer susceptibility in the 3′UTR of this gene. However, reports of the association vary in the literature. *MTOR* rs2536 TC/CC genotypes were reported to have an association with decreased risk in acute lymphoblastic leukemia [24], but increased risk in prostate cancer [25], and no association in gastric and esophageal cancer [26, 27]. Therefore, it is possible that the effect of this polymorphism on *MTOR* expression may be cancer type-specific. Until now, there has been no

Table 3: Survival analysis of miRSNPs in mTOR pathway in 134 patients with stage III small cell lung cancer.

Gene SNP		Model	5yOS	HR (95% CI)	Log-rank P	HR (95% CI) (adjusted)*	P adjusted*	q
VEGFA rs10434	DOM	GG (90) GA + AA (44)	37.9% 39.6%	0.739 (0.424–1.288)	0.284	0.830 (0.468–1.472)	0.523	0.584
DDIT4 rs1053639	DOM	AA (80) AT + TT (54)	35.1% 42.1%	1.042 (0.622–1.744)	0.877	0.970 (0.573–1.639)	0.908	0.709
MAPK1 rs1063311	DOM	CC (97) CT + TT (34)	36.8% 41.7%	0.694 (0.366–1.315)	0.260	0.542 (0.308–0.953)	0.101	0.219
PTEN rs11202607	ADD	CC (111) CT (22)	35.9% 58.5%	0.607 (0.260–1.416)	0.243	0.493 (0.208–1.169)	0.108	0.225
PIK3R1 rs12755	REC	CA + CC (123) AA (10)	36.2% 70.0%	0.360 (0.088–1.475)	0.155	0.242 (0.058–1.010)	0.052	0.161
MTOR rs2536	ADD	TT (108) TC (22)	43.1% 21.2%	1.978 (1.087–3.600)	0.026	2.039 (1.117–3.720)	0.020	0.103
VEGFA rs3025039	DOM	CC (80) CT + TT (52)	42.9% 33.4%	1.127 (0.666–1.908)	0.655	1.185 (0.687–2.044)	0.541	0.592
PIK3R1 rs3756668	REC	GA + GG (109) AA (23)	34.0% 49.5%	0.362 (0.155–0.845)	0.014	0.341 (0.146–0.797)	0.013	0.103
PTEN rs701848	DOM	TT (47) TC + CC (85)	23.5% 40.8%	0.885 (0.504–1.556)	0.672	0.759 (0.423–1.362)	0.356	0.488

*Adjusted for age, gender, Karnofsky performance score (KPS), smoking history, and Charlson comorbidity index (CCI).

miRSNPs: miRNA-related single nucleotide polymorphisms; OS: overall survival; HR: hazard ratio; CI: confidence interval.

Reference sequences: *VEGFA* GenBank NG_008732; DDIT4 GenBank NM_019058; *MAPK1* GenBank NG_023054; *PTEN* GenBank NG_007466; *PIK3R1* GenBank NG_012849; *MTOR* GenBank NG_033239.

Table 4: RPA classification based on unfavorable genotype combinations.

	rs3756668	rs2536	*n*	5y-OS	HR (95% CI) adjusted*	*P* adjusted*	HR (95% CI) bootstrap	*P* bootstrap
Low risk	AA	Any	25	47.7%	Ref	0.004	Ref	
Intermediate risk	GA + GG	TT	96	40.8%	2.163 (0.963–4.866)	0.062	2.210 (2.154–2.268)	$2E-16$
High risk	GA + GG	TC	21	17.6%	4.535 (1.813–11.345)	0.001	4.539 (4.409–4.673)	$2E-16$

*Adjusted for age, gender, Karnofsky performance score (KPS), smoking history, and Charlson comorbidity index (CCI).
RPA: recursive partitioning analysis; OS: overall survival; HR: hazard ratio; CI: confidence interval.

published work on the association between *MTOR*: rs2536 and cancer outcomes. In the present study, patients with rs2536 TC genotype showed a poorer survival than those with a TT genotype. As proposed previously, the binding miRNA-576 at the T allele was substituted for miRNA-767 at the C allele predicted by the bioinformatics web server (https://snpinfo.niehs.nih.gov/snpinfo/snpfunc.php) [25]. Therefore, rs2536 TC genotype was speculated to affect *MTOR* expression, probably because of its effect on affinity for miRNA; however, *MTOR* expression is very complicated. It was found that phosphorylated *MTOR* (*p-MTOR*) was more highly expressed in limited-stage than extended-stage SCLCs, and higher *p-MTOR* expression was associated with better prognosis [28], in contrast to other cancers [29, 30]. Therefore, elucidation of the exact mechanisms requires additional functional studies.

Two SNPs of *PIK3R1*, rs12755 (A>C) and rs3756668 (A>G), were also found to be associated with survival in SCLC. Class IA *PI3K*, composed of a p110 catalytic unit and a regulatory unit, is activated by growth factors and subsequently induces a kinase cascade downstream [31]. *PIK3R1* encodes the 85-kD regulatory subunit, which is indispensable for stabilization of the catalytic subunit and downregulating PI3K signaling [32]. A handful of studies showed that polymorphisms of *PIK3R1* were associated with survival in bladder cancer and endometrial cancer [33, 34]. Neither *PIK3R1* SNP discussed in this study has been reported to date. These *PIK3R1* SNPs were found to be associated with favorable survival under a recessive model. Moreover, Ueki et al. [35] demonstrated that heterozygous disruption of *PIK3R1* reduced p85α by 50% and enhanced PI3K signaling, while complete depletion of p85α in homozygous generated knockout cells significantly decreased PI3-kinase activity. This may be indicative of the functional effect of these loci on *PIK3R1* regulation and activity.

mTOR inhibitors displayed limited antitumor activity in SCLC on account of feedback reactivation of this pathway [36]. Dual inhibition of PI3K and mTOR can block the loop and enhance therapeutic effectiveness [18], and inhibition has been shown to improve tumor radiosensitivity by impairing DNA damage repair and normalizing tumor vasculature [37, 38]. Tumor activity depends on complex signaling networks. Thus, recent studies of cancer-related genetic alternations have also taken a pathway-based approach. In the present work, the combination of two SNPs in *PIK3R1* and *MTOR* provided a more precise discrimination than individual SNPs. Consequently, it is biologically plausible for these potentially functional polymorphisms to have a synergistic effect.

To summarize, this study found three miRNA-related SNPs (*MTOR*: rs2536 (T>C), *PIK3R1*: rs3756668 (A>C) and *PIK3R1*: rs12755 (A>G)) in the PI3K/Akt/mTOR pathway and pathway-based RPA that were associated with survival in patients with LD SCLC treated with chemoradiotherapy. The identification of these predictive factors may be help in selecting the optimal therapeutic strategy for individual patients. However, only nine functional SNPs were selected, based on prior knowledge, for the present study, which means that some important variations in this pathway may have been missed. In addition, multiple correction for these SNPs by 1000 bootstrap samples showed significant associations with OS, suggesting that the negative results after multiple correction were due to the limited number of subjects, although this is the largest study on miRNA-SNPs associated with prognosis in SCLC. Moreover, despite internal validation by bootstrap, there is a lack of independent external validation to confirm our findings. Therefore, additional larger, well-designed studies, including molecular mechanism investigation, are imperative to confirm these findings.

5. Conclusions

In conclusion, the current study identified 3 miRNA-related SNPs in the PI3K/Akt/mTOR pathway as prognostic biomarkers for patients with limited-disease SCLC treated with chemoradiotherapy. A risk classification incorporating *MTOR*: rs2536 (T>C) and *PIK3R1*: rs3756668 (A>C) was developed to identify patients likely to benefit from treatment. Replication in a large independent cohort and assessment of biological function is imperative to confirm these findings.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Primary Tumor Characteristics Are Important Prognostic Factors for Sorafenib-Treated Patients with Metastatic Renal Cell Carcinoma: A Retrospective Multicenter Study

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We aimed to identify prognostic factors associated with progression-free survival (PFS) and overall survival (OS) in metastatic renal cell carcinoma (mRCC) patients treated with sorafenib. We investigated 177 patients, including 116 who received sorafenib as first-line therapy, using the Cox regression model. During a median follow-up period of 19.2 months, the PFS and OS were 6.4 and 32.6 months among all patients and 7.4 months and undetermined for first-line sorafenib-treated patients, respectively. Clinical T3-4 stage (hazard ratio [HR] 2.56) and a primary tumor size >7 cm (HR 0.34) were significant prognostic factors for PFS among all patients, as were tumor size >7 cm (HR 0.12), collecting system invasion (HR 5.67), and tumor necrosis (HR 4.11) for OS ($p < 0.05$). In first-line sorafenib-treated patients, ≥4 metastatic lesions (HR 28.57), clinical T3-4 stage (HR 4.34), collecting system invasion (univariate analysis HR 2.11; multivariate analysis HR 0.07), lymphovascular invasion (HR 13.35), and tumor necrosis (HR 6.69) were significant prognosticators of PFS, as were bone metastasis (HR 5.49) and clinical T3-4 stages (HR 4.1) for OS ($p < 0.05$). Our study thus identified a number of primary tumor-related characteristics as important prognostic factors in sorafenib-treated mRCC patients.

1. Introduction

Up to one-third of patients with renal cell carcinoma (RCC) present with advanced disease globally, and 20–40% of those who undergo nephrectomy for localized RCC subsequently develop metastases [1]. In the previous era of immunotherapy, the prognosis of patients with unresectable and/or metastatic RCC (mRCC) was dismal, and the average survival was approximately 12 months; only a fraction of patients (10–20%) benefit from cytokine treatment because of limited therapeutic options and the resistance of RCC to conventional chemotherapy [2, 3].

Since 2005, a number of novel targeted therapy (TT) agents that show better efficacy for the treatment of advanced RCC, compared to previous immunochemotherapy agents, have been introduced, and the prognoses of advanced diseases such as mRCC have greatly improved. However, prognoses vary widely, causing clinicians to question the predictive prognostic models of TTs in mRCCs. Various studies have attempted to stratify patients into poor, intermediate, and favorable prognosis groups by investigating multiple risk factors. The Memorial Sloan Kettering Cancer Center (MSKCC) [4] and Heng [5] risk criteria form the bases of prognostic classifications. Several predictive prognostic factors for TTs include laboratory findings, performance and immune status, physical condition, and tumor burden [6–9].

Among the various TTs, sorafenib was one of the first available and globally used tyrosine kinase inhibitors for mRCC patients, with good tolerability and safety. The 2015 guidelines of the National Comprehensive Cancer Network (NCCN) recommended sorafenib as a first-line treatment for patients with relapsed or medically unresectable, predominantly clear cell, stage IV RCC (category 2A) [10]. Many large-scale multicenter studies are currently ongoing in different countries to assess sorafenib's efficacy and safety profile in mRCC patients of different ethnicities using progression-free survival (PFS) and overall survival (OS) as endpoints; these studies seek to identify important prognostic factors in their populations [11–13]. However, few studies have investigated Asian patients with mRCC. Therefore, a representative group of mRCC patients treated with sorafenib was selected from the databases of 11 academic institutions to identify the relevant prognostic factors, including primary tumor-related factors, for PFS and OS. In addition, sorafenib-treated patient survival curves were plotted to compare the prognoses of different risk groups according to the MSKCC [4] and Heng [5] risk criteria.

2. Patients and Methods

2.1. Patient Selection. A retrospective analysis of 184 clinically diagnosed mRCC patients from 11 Korean academic institutions was performed and included those who had been treated with sorafenib with or without prior systemic therapies between 2006 and 2012. After excluding patients aged <18 years and those with unavailable medical follow-up records, 177 patients were ultimately enrolled after pathological confirmation of RCC in their primary or metastatic site(s) by nephrectomy, metastasectomy, or tumor biopsy. Tumors were stage IV according to the 2009 American Joint Committee on Cancer staging classification.

2.2. Treatment and Diagnostic Modalities. Sorafenib treatment was commenced at 400 mg orally twice daily on a continuous basis until disease progression was noted in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0 or the development of intolerance was noted. Tumor response was measured starting at 4–12 weeks after initiation of treatment using RECIST. The primary tumor size was calculated using the contrast phase of baseline computed tomography (CT) images, as was the longest horizontal diameter of the primary unresectable mRCC-affected kidney in situ. For resectable mRCCs, measurements were performed on the pathologic specimen after nephrectomy but before formalin fixation.

Pre- and posttreatment evaluation consisted of a complete history and physical examination, complete blood count, liver and renal function tests, CT of the chest, CT or magnetic resonance imaging of the abdomen and pelvis, and total body bone scintigraphy. Fluorodeoxyglucose positron emission tomography-CT scanning was optional and was performed at each clinician's discretion to evaluate small suspicious multiple metastatic lesions that were not identifiable on CT images. During follow-up, all patients were required to undergo examination by their attending urologists, with work-ups performed according to their respective institutional protocols. Follow-up continued after the termination of treatment until death.

2.3. Statistical Methods. PFS was determined from the date of the initiation of sorafenib treatment until documentation of radiologically confirmed disease progression or death from any cause. OS was calculated from the date of initiating sorafenib treatment until death from any cause. All baseline clinicopathological parameters were analyzed as discrete variables with the Chi-square and Wilcoxon rank sum tests as appropriate. The Kaplan-Meier method was used to estimate time-to-event distributions of PFS and OS according to the MSKCC (2002 version) [4] and Heng [5] risk criteria. Univariate and multivariate Cox regression models were employed to identify potential baseline prognostic variables for PFS and OS in all patients as well as in the first-line sorafenib-treated patients separately. Cox regression analysis with Firth's penalized likelihood was used for rare events. Clinically important variables, such as primary tumor-related factors, were subjected to multivariate analysis even if not found to be significant on univariate analyses. All statistical analyses were performed using the STATA statistical software (release 13.1, STATA Inc., TX, USA).

2.4. Ethics Statement. This retrospective study was approved by the Institutional Review Board (IRB) of the National Cancer Center (IRB number NCCNCS-11-439) and other participating hospitals. The informed consent requirement was waived by the IRB.

3. Results

During a median follow-up of 19.2 months (range, 0.2–62.3 months), the median PFS and OS were 6.4 (range, 5.2–8.9) and 32.6 (range, 27.3–63.8) months for all 177 patients, respectively; for the 116 first-line sorafenib-treated patients, the median PFS and OS were 7.4 (range, 5.5–10.0) months and unattained at 57.8 months, respectively (Table 1). With respect to the best response to sorafenib, the objective response and disease control rates among all 177 patients were 22% and 53.1%, respectively, while the rates among the 116 first-line sorafenib-treated patients were 23.2% and 56%, respectively. All the other baseline demographics, including clinicopathological data and imaging parameters, are described in Table 1.

Significant prognostic factors for PFS and OS were found on multivariate analysis. Clinical T3-4 stage (hazard ratio [HR] 2.56, 95% confidence interval [CI] 1.08–6.09) was a negative predictor of PFS (Table 2), while collecting system invasion (HR 5.67, 95% CI 1.59–22.56) and tumor necrosis (HR 4.11, 95% CI 1.06–21.78) were negative predictors of OS (Table 3) ($p < 0.05$). Conversely, a primary tumor size >7 cm was indicative of significantly better PFS (HR 0.34, 95% CI 0.12–0.98) and OS (HR 0.12, 95% CI 0.02–0.7) ($p < 0.05$; Tables 2 and 3, resp.).

Further multivariate subanalysis of the 116 first-line sorafenib-treated patients revealed that ≥4 lesions at the metastatic sites (HR 28.57, 95% CI 1.74–468.69), clinical T3-4 stage (HR 4.34, 95% CI 1.20–15.71), lymphovascular invasion (HR 13.35, 95% CI 1.91–93.37), and necrosis within the primary kidney tumor (HR 6.69, 95% CI 2.06–21.73) were significantly poor prognostic indicators of PFS, whereas collecting system invasion (HR 0.07, 95% CI 0.01–0.55) was the sole favorable parameter ($p < 0.05$; Table 4). Furthermore, bone metastasis (HR 5.49, 95% CI 1.62–18.65) and clinical T3-4 stage (HR 4.1, 95% CI 1.08–15.51) were significantly negative prognostic parameters of OS ($p < 0.05$; Table 5).

Kaplan-Meier analyses revealed that patients categorized according to MSKCC versus Heng criteria had different PFS and OS. The MSKCC intermediate group (i.e., those with 1-2 risk factors) had the worst PFS (4.8 months; versus 6.6 months for the poor group and 12.8 months for the favorable group; $p < 0.001$) and OS (23.2 months; versus both the poor and favorable groups where OS was yet undetermined, $p < 0.002$) when considering all 177 sorafenib-treated patients (Figure 1). When categorized according to the Heng risk criteria, the intermediate group also had worse PFS (4.5 months) than the favorable and poor groups (12.8 and 5.2 months, resp.); however, the OS in the intermediate group (23.2 months) was similar to that of the poor group (17.9 months) when compared to the reference group (i.e., the favorable group; OS yet undetermined) ($p < 0.001$) (Figure 1). Additional subanalyses of the first-line sorafenib-treated patients with the MSKCC and Heng risk criteria were performed using the Kaplan-Meier and log-rank tests, revealing that similar patterns of OS and PFS were observed according to the MSKCC criteria, but not according to the Heng criteria (Figure 2). Furthermore, we compared the OS of the first-line sorafenib groups according to the presence of bone metastasis and/or T3-4 stage, which we had found to be poor prognostic factors; significant differences in OS were observed ($p = 0.0003$; Figure 3).

4. Discussion

Globally, sorafenib has proven to be tolerable, safe, and effective for treating mRCC patients. It is essential that the prognostic factors of PFS and OS be clarified in order to guide patient care and yield the best therapeutic response. Several previous studies suggested various parameters and models for classifying mRCC patients into favorable, intermediate, and poor risk groups according to the number of survival risk factors [2, 5, 8, 9, 14, 15]. Using a nationwide Korean kidney cancer database encompassing 11 Korean academic institutions, this study clarified the prognostic importance of several independent primary tumor characteristics, as well as of the extent of disease, in sorafenib-treated mRCC patients with or without prior systemic therapies. Specifically, clinical T3-4 stage was a negative prognosticator of PFS, as were collecting system invasion and tumor necrosis for OS. Conversely, a primary tumor size >7 cm was a favorable prognostic factor for both PFS and OS.

Previous studies suggested that the intermediate risk group, as defined by the MSKCC and Heng criteria, had multiple pitfalls because of the uneven distribution of a large number of diverse patients; this produced heterogeneous outcomes that necessitated further stratification [16, 17]. We encountered similar findings as well, with the intermediate group having disproportionately worse PFS (4.8 months) and OS (23.2 months) than the poor risk groups; this was also the case when using the Heng risk model. In addition, significantly different survival outcomes were observed for groups stratified according to the MSKCC and Heng risk criteria when analyzing all patients as well as only first-line sorafenib-treated patients ($p < 0.05$, Figures 1 and 2). The Heng criteria appeared to be slightly more correlative with OS than the MSKCC criteria, which is consistent with a previous Korean study with sunitinib that showed the Heng risk model to have slightly better discriminatory ability than the MSKCC model [17].

Among the significant prognostic factors for PFS and OS in all patients, primary tumor size had an HR <1.0 on both univariate and multivariate analyses, indicating that mRCC patients with greater sized primary tumors, especially those >7 cm, responded better to sorafenib and had more favorable prognoses in our study. This implies that smaller sized renal tumors with metastatic lesions might be more aggressive than larger tumors. Subgroup analysis that stratified tumor size into four groups (<4 cm, 4–7 cm, 7–10 cm, and >10 cm) was performed on 140 patients with previous nephrectomies; these 4 subgroups were statistically correlated with low (1-2) and high (3-4) Fuhrman nuclear grades. A greater proportion of small tumors were of higher tumor grades, albeit without statistical significance owing to a small sample size ($p = 0.068$; odds ratio 0.675; data not shown).

In addition to a primary tumor size >7 cm, other significant factors determining survival among the 177 patients were clinical T3-4 stage (for PFS and OS), collecting system invasion (for OS), and tumor necrosis (for OS) within the

Table 1: Baseline demographics.

Parameter	Overall ($N = 177$) N (percentage) or median (range)	First-line ($N = 116$) N (percentage) or median (range)
Gender (male/female)	136/41 (76.8/23.2)	91/25 (78.4/21.6)
Age (years)	62.0 ± 10.9	63.8 ± 10.5
Follow-up duration (months)	19.2 (0.2–63.8)	18 (0.2–57.8)
Treatment duration (weeks)	20.1 (1–216)	23.9 (4.4–176)
Body mass index (kg/cm^2)	23.3 (14.5–37.2)	23.1 (14.5–37.2)
Comorbidity		
Diabetes	42 (23.7)	29 (25.0)
Hypertension	75 (42.4)	49 (42.2)
Cerebrovascular accident (CVA)	5 (2.8)	2 (1.7)
Cardiovascular disease	6 (3.4)	6 (5.2)
Liver disease	3 (1.7)	2 (1.7)
Renal disease	8 (4.5)	5 (4.3)
Deep vein thrombosis (DVT)	1 (0.6)	1 (0.9)
Hypercholesterolemia	3 (1.7)	3 (2.6)
Presenting symptom	101 (57.1)	62 (53.4)
Incidental renal mass	11 (6.2)	9 (7.8)
Symptom developed	83 (46.9)	52 (44.8)
Other	7 (4.0)	1 (0.9)
Body surface area (m^2)		
≤1.7	74 (41.8)	52 (44.8)
>1.7	87 (49.2)	60 (51.7)
Unknown	16 (9.0)	4 (3.4)
ECOG		
0	110 (62.1)	59 (50.9)
1	54 (30.5)	46 (39.7)
2	9 (5.1)	8 (6.9)
3	1 (0.6)	1 (0.9)
Unknown	3 (1.7)	2 (1.7)
Karnofsky performance score		
>80	107 (60.5)	63 (54.3)
50–80	22 (12.4)	22 (19.0)
<50	7 (4.0)	6 (5.2)
Unknown	41 (23.2)	25 (21.6)
MSKCC risk criteria		
Favorable	49 (27.7)	33 (28.4)
Intermediate	82 (46.3)	48 (41.4)
Poor	9 (5.1)	7 (6.0)
Unknown	37 (20.9)	28 (24.1)
Heng risk criteria		
Favorable	39 (22.0)	29 (25.0)
Intermediate	78 (44.1)	46 (39.7)
Poor	19 (10.7)	15 (12.9)
Unknown	41 (23.2)	26 (22.4)
Prior surgical therapy		
Nephrectomy (radical/partial/embolization)	150 (135/5/10) [84.7 (76.3/2.8/5.6)]	99 (87/5/7) [85.3 (75/4.3/6.0)]
Metastasectomy	44 (24.9)	26 (22.4)
Prior systemic therapy	56 (31.6)	0
Immuno/chemo/sunitinib therapy	33 (18.6)/4 (2.3)/19 (10.7)	—

Table 1: Continued.

Parameter	Overall (N = 177) N (percentage) or median (range)	First-line (N = 116) N (percentage) or median (range)
Number of metastatic sites (18)		
1 organ	94 (53.1)	65 (56.0)
2 organs	44 (24.9)	27 (23.3)
3 organs	19 (10.7)	10 (8.6)
≥4 organs	8 (4.5)	4 (3.4)
Unknown	12 (6.8)	10 (8.6)
Metastatic sites	3 (1–5)	3 (1–5)
Brain	42 (23.7)	33 (28.4)
Bone	38 (21.5)	23 (19.8)
Liver	17 (9.6)	9 (7.8)
Lung	124 (70.1)	81 (69.8)
Lymph node	34 (19.2)	23 (19.8)
Pancreas	8 (4.5)	7 (6.0)
Kidney, contralateral	7 (4.0)	5 (4.3)
Other	30 (16.9)	17 (14.7)
Primary kidney tumor-related parameter		
Size of primary tumor (cm)	8 (1–117)	8 (1–117)
Collecting system invasion	28 (15.8)	16 (13.8)
Capsule invasion	36 (20.3)	24 (20.7)
Lymphovascular invasion	34 (19.2)	29 (25.0)
Tumor necrosis	46 (26.0)	31 (26.7)
TNM stage		
T1	25 (14.1)	16 (13.8)
T2	35 (19.8)	25 (21.6)
T3	74 (41.8)	54 (46.6)
T4	8 (4.5)	8 (6.9)
Tx	35 (19.8)	13 (11.2)
N1	27 (15.3)	22 (19.0)
M1	131 (74.0)	85 (73.3)
Fuhrman grade		
1	5 (2.8)	2 (1.7)
2	39 (22.0)	26 (22.4)
3	69 (39.0)	47 (40.5)
4	36 (20.3)	23 (19.8)
Unknown	28 (15.8)	18 (15.5)
Histology		
Clear cell, pure	159 (89.8)	110 (94.8)
Non-clear cell	3 (1.7)	1 (0.9)
Unknown	15 (8.5)	5 (4.3)
Best overall response (CR + PR + SD)	94 (53.1)	65 (56.0)
Complete remission	6 (3.4)	4 (3.4)
Partial response	33 (18.6)	23 (19.8)
Stable disease	55 (31.1)	38 (32.8)
Progressive disease*	83 (46.9)	51 (44.0)
Progression-free survival (median months)	6.4 (5.2–8.9)	7.4 (5.5–10.0)
Overall survival (median months)	32.6 (27.3–63.8)	NR
Survival	114 (64.4)	85 (73.3)
Cancer-specific death	51 (28.8)	24 (20.7)

*Progressive disease = progressive disease + not evaluated disease.

CR, complete response; ECOG, Eastern Cooperative Oncology Group; MSKCC, Memorial Sloan Kettering Cancer Center; NR, not yet reached; PR: partial response; SD, stable disease.

Table 2: Multivariate analyses of prognostic factors for progression-free survival in all sorafenib-treated patients ($N = 177$).

Category	Univariate analysis of PFS			Multivariate analysis of PFS		
	Hazard ratio	p value	Confidence interval	Hazard ratio	p value	Confidence interval
Gender, female	0.76	0.228	0.48–1.19			
Age ≥65 years	1.10	0.620	0.76–1.59			
KPS <80%	1.15	0.654	0.63–2.09			
LDH >1.5x ULN	1.32	0.511	0.57–3.04			
Hemoglobin <LLN	1.62	0.012	1.11–2.37	0.86	0.724	0.36–2.02
cCa >10 mg/dL	0.88	0.769	0.39–2.02			
Time from diagnosis to treatment <1 year	1.99	<0.001	1.37–2.90	1.32	0.531	0.56–3.12
Leukocytosis	1.82	0.114	0.87–3.80			
Thrombocytosis	3.24	0.002	1.54–6.82	0.70	0.677	0.13–3.75
Hypoalbuminemia	1.23	0.541	0.64–2.36			
Prior nephrectomy	0.83	0.751	0.26–2.63			
Prior metastasectomy	0.93	0.723	0.61–1.41			
Brain metastasis	0.97	0.881	0.64–1.47			
Bone metastasis	1.38	0.117	0.92–2.07			
Liver metastasis	2.01	0.025	1.09–3.71	1.66	0.377	0.54–5.08
Lung metastasis	0.77	0.192	0.52–1.14			
Lymph node metastasis	1.24	0.340	0.80–1.93			
Pancreas	1.17	0.673	0.57–2.40			
Contralateral kidney	0.22	0.036	0.05–0.91	0.29	0.286	0.03–2.80
Metastatic sites						
1	1.00					
2-3	1.04	0.845	0.71–1.52			
≥4	1.29	0.532	0.58–2.85			
T stage						
T1-2	1.00			1.00		
T3-4	1.55	0.028	1.05–1.52	2.56	0.034	1.08–6.09
N1	2.26	0.001	1.38–2.85	1.31	0.714	0.31–5.54
M1	1.98	0.033	1.06–2.30	0.84	0.721	0.31–2.22
Fuhrman grade						
1-2	1.00					
3-4	0.96	0.857	0.63–3.68			
Tumor-related factor						
Primary tumor size						
<4 cm	1.00			1.00		
4–7 cm	0.92	0.811	0.45–1.87	0.54	0.281	0.18–1.66
>7 cm	0.78	0.447	0.40–1.49	0.34	0.045	0.12–0.98
Collecting system invasion	1.67	0.045	1.01–2.74	0.73	0.582	0.24–2.23
Capsule invasion	1.01	0.981	0.62–1.64			
Lymphovascular invasion	2.13	0.004	1.27–3.57	2.01	0.324	0.5–8.09
Tumor necrosis	2.13	0.003	1.30–3.47	2.36	0.055	0.98–5.65
Immunotherapy	1.00					
Other therapy	0.96	0.858	0.62–1.5			

KPS, Karnofsky performance status; LDH, lactate dehydrogenase; ULN, upper limit normal; LLN, lower limit normal; cCa, corrected calcium.

primary renal tumor. These data corresponded to previous studies of invasive and advanced RCC, where clinical T3-4 stage, aggressive characteristics such as collecting system invasion [18, 19], and faster growth rates with tumor necrosis [20–22] reflected advanced disease states with a negative impact on survival. However, none of these previous reports included TT agent-treated mRCC patients; this is the first study to show that certain primary tumor-related factors were significant indicators of prognosis in TT agent-treated mRCC patients, particularly those receiving sorafenib.

Table 3: Multivariate analyses of prognostic factors for overall survival in all sorafenib-treated patients ($N = 177$).

Parameter	Univariate analysis of OS			Multivariate analysis of OS		
	Hazard ratio	p value	Confidence interval	Hazard ratio	p value	Confidence interval
Gender, female	0.81	0.507	0.44–1.5			
Age ≥65 years	1.05	0.863	0.62–1.77			
KPS <80%	1.14	0.756	0.49–2.67			
LDH >1.5x ULN	1.62	0.311	0.64–4.09			
Hemoglobin <LLN	2.72	<0.001	1.61–4.61	1.54	0.527	0.39–5.77
cCa >10 mg/dL	0.91	0.861	0.33–2.53			
Time from diagnosis to treatment <1 year	1.63	0.072	0.96–2.78			
Leukocytosis	1.03	0.963	0.25–4.30			
Thrombocytosis	7.52	<0.001	3.04–18.63	1.58	0.787	0.01–21.63
Hypoalbuminemia	1.56	0.270	0.71–3.45			
Prior nephrectomy	0.54	0.396	0.13–2.24			
Prior metastasectomy	0.71	0.305	0.37–1.36			
Brain metastasis	1.07	0.813	0.60–1.92			
Bone metastasis	2.16	0.004	1.28–3.66	2.71	0.104	0.81–8.61
Liver metastasis	2.79	0.005	1.36–5.72	2.39	0.318	0.42–14.09
Lung metastasis	1.22	0.505	0.68–2.18			
Lymph node metastasis	1.43	0.225	0.80–2.57			
Pancreas	0.77	0.719	0.19–3.16			
Contralateral kidney	1.02	0.978	0.25–4.19			
Metastatic sites						
1	1.00					
2-3	1.83	0.024	1.08–4.65	1.12	0.885	0.24–4.74
≥4	4.20	0.004	1.59–14.11	1.63	0.663	0.13–12.07
T stage						
T1-2	1.00					
T3-4	1.76	0.068	0.96–3.22			
N1	1.68	0.193	0.77–3.69			
M1	5.70	0.016	1.38–23.45	0.87	0.888	0.15–9.29
Fuhrman grade						
1-2	1.00					
3-4	1.19	0.594	0.63–2.26			
Primary tumor size						
<4 cm	1.00					
4–7 cm	1.07	0.897	0.38–3.04	0.47	0.358	0.09–2.45
> 7cm	0.83	0.705	0.31–1.62	0.12	0.020	0.02–0.7
Collecting system invasion	2.51	0.012	1.22–3.62	5.67	0.008	1.59–22.56
Capsule invasion	0.79	0.555	0.36–3.05			
Lymphovascular invasion	1.37	0.448	0.61–2.22			
Tumor necrosis	4.32	<0.001	1.83–5.16	4.11	0.041	1.06–21.78
Immunotherapy	1.0					
Other therapy	0.71	0.232	0.41–1.72			

KPS, Karnofsky performance status; LDH, lactate dehydrogenase; ULN, upper limit normal; LLN, lower limit normal; cCa, corrected calcium.

Separate analysis of prognostic factors for PFS and OS in 116 patients with first-line sorafenib treatment showed that clinical T3-4 stages (HR 4.34), collecting system invasion (HR 2.11 on univariate analysis and HR 0.07 on multivariate analysis), tumor necrosis (HR 6.69), lymphovascular invasion (HR 13.35), and metastatic sites ≥4 lesions (HR 28.57) were significant predictors of PFS; this is also the first study to show that collecting system invasion, tumor necrosis, and lymphovascular invasion are significant prognostic factors in naive sorafenib-treated mRCC patients. However, the HRs for collecting system invasion were not correlated on univariate versus multivariate analyses because of multicollinearity

Table 4: Multivariate analyses of prognostic factors for progression-free survival in first-line sorafenib-treated patients ($N = 116$).

Parameter	Univariate analysis of PFS			Multivariate analysis of PFS		
	Hazard ratio	p value	Confidence interval	Hazard ratio	p value	Confidence interval
Gender, female	0.56	0.066	0.30–1.04			
Age ≥65 years	1.04	0.859	0.66–1.66			
KPS <80%	1.87	0.062	0.97–3.61			
LDH >1.5x ULN	0.70	0.625	0.17–2.90			
Hemoglobin <LLN	1.78	0.019	1.10–2.87	0.41	0.220	0.10–1.70
cCa >10 mg/dL	0.83	0.684	0.33–2.06			
Time from diagnosis to treatment <1 year	2.88	0.000	1.72–4.81	1.90	0.242	0.65–5.59
Leukocytosis	2.35	0.032	1.08–5.13	2.41	0.274	0.50–11.61
Thrombocytosis	4.06	0.001	1.78–9.30	0.83	0.875	0.09–8.02
Hypoalbuminemia	1.93	0.089	0.91–4.10			
Prior nephrectomy	1.98	0.499	0.27–14.33			
Prior metastasectomy	0.89	0.683	0.52–1.53			
Brain metastasis	1.04	0.867	0.63–1.73			
Bone metastasis	1.65	0.066	0.97–2.81			
Liver metastasis	2.16	0.058	0.98–4.80			
Lung metastasis	0.72	0.187	0.44–1.17			
Lymph node metastasis	1.82	0.040	1.03–3.23	1.98	0.365	0.45–8.72
Pancreas	1.16	0.708	0.53–2.56			
Contralateral kidney	0.23	0.149	0.03–1.69			
Metastatic sites						
1	1.00			1.00		
2-3	1.28	0.320	0.78–2.10	0.80	0.783	0.17–3.80
4	3.96	0.012	1.35–11.56	28.57	0.019	1.74–468.69
T stage						
T1-2	1.00			1.00		
T3-4	1.97	0.008	1.19–3.26	4.34	0.025	1.20–15.71
N1	2.66	0.001	1.51–4.68	0.75	0.837	0.05–11.95
M1	2.08	0.044	1.02–4.22	0.75	0.674	0.20–2.86
Fuhrman grade						
1-2	1.00					
3-4	0.98	0.955	0.57–1.71			
Primary tumor size						
<4 cm	1.00					
4–7 cm	0.87	0.737	0.37–2.00	0.37	0.288	0.06–2.30
>7 cm	0.85	0.690	0.39–1.86	0.31	0.186	0.05–1.76
Collecting system invasion	2.11	0.026	1.09–4.07	0.07	0.012	0.01–0.55
Capsule invasion	1.05	0.869	0.59–1.88			
Lymphovascular invasion	2.41	0.004	1.33–4.37	13.35	0.009	1.91–93.37
Tumor necrosis	2.01	0.022	1.10–3.65	6.69	0.002	2.06–21.73

KPS, Karnofsky performance status; LDH, lactate dehydrogenase; ULN, upper limit normal; LLN, lower limit normal; cCa, corrected calcium.

between collecting system invasion, lymphovascular invasion, and tumor necrosis. When excluding both lymphovascular invasion and tumor necrosis, collecting system invasion was found to be a poor prognostic factor for PFS in the first-line sorafenib group (HR: 2.24; data not shown).

Bone metastasis and clinical T3-4 stages were the only significant predictors of OS in first-line sorafenib-treated patients. The OS curves were compared among patients with poor prognostic factors (i.e., those with bone metastasis, T3-4 stage, or both); there were significant differences in OS rates among each of these groups. The presence of bone metastases in mRCC patients has been shown to be a poor prognostic factor despite sorafenib treatment [23]; therefore, mRCC patients with newly diagnosed bone metastases should be considered for active treatment of the metastatic bone lesion with either surgery or radiation therapy in combination with

Table 5: Multivariate analyses of prognostic factors for overall survival in first-line sorafenib-treated patients ($N = 116$).

Category	Univariate analysis of OS			Multivariate analysis of OS		
	Hazard ratio	p value	95% confidence interval	Hazard ratio	p value	95% confidence interval
Gender, female	0.34	0.076	0.10–1.12			
Age ≥65 years	1.13	0.736	0.55–2.36			
KPS <80%	1.55	0.374	0.59–4.07			
LDH >1.5x ULN	0.64	0.659	0.09–4.74			
Hemoglobin <LLN	2.10	0.044	1.02–4.30	1.14	0.824	0.36–3.67
cCa >10 mg/dL	0.53	0.390	0.13–2.24			
Time from diagnosis to treatment <1 year	2.16	0.048	1.01–4.63	1.36	0.584	0.45–4.07
Leukocytosis	1.36	0.685	0.31–5.88			
Thrombocytosis	8.95	0.000	3.08–26.05	4.92	0.068	0.89–27.30
Hypoalbuminemia	2.66	0.035	1.07–6.62	1.94	0.337	0.50–7.46
Prior nephrectomy	0.69	0.714	0.09–5.11			
Prior metastasectomy	0.86	0.748	0.34–2.17			
Brain metastasis	1.18	0.674	0.55–2.50			
Bone metastasis	3.25	0.001	1.57–6.73	5.28	0.015	1.38–20.19
Liver metastasis	2.55	0.087	0.87–7.44			
Lung metastasis	0.83	0.627	0.39–1.76			
Lymph node metastasis	1.71	0.192	0.76–3.86			
Pancreas	0.56	0.563	0.08–4.08			
Contralateral kidney	0.79	0.814	0.11–5.78			
Metastatic sites						
1	1.00					
2-3	1.74	0.150	0.82–3.70			
≥4	2.89	0.160	0.66–12.66			
T stage						
T1-2	1.00			1.00		
T3-4	2.45	0.050	1.00–6.01	4.10	0.038	1.08–15.51
N1	2.14	0.120	0.82–5.56			
M1	7.15	0.054	0.97–52.98			
Fuhrman grade						
1-2	1.00					
3-4	1.85	0.264	0.63–5.45			
Tumor-related factor						
Primary tumor size						
<4 cm	1.00			1.00		
4–7 cm	1.08	0.909	0.29–4.00	0.70	0.633	0.16–3.02
>7 cm	0.81	0.751	0.22–2.95	0.31	0.142	0.07–1.48
Collecting system invasion	2.31	0.130	0.78–6.85			
Capsule invasion	0.69	0.483	0.25–1.92			
Lymphovascular invasion	2.45	0.068	0.94–6.39			
Tumor necrosis	2.75	0.061	0.95–7.93			

KPS, Karnofsky performance status; LDH, lactate dehydrogenase; ULN, upper limit normal; LLN, lower limit normal; cCa, corrected calcium.

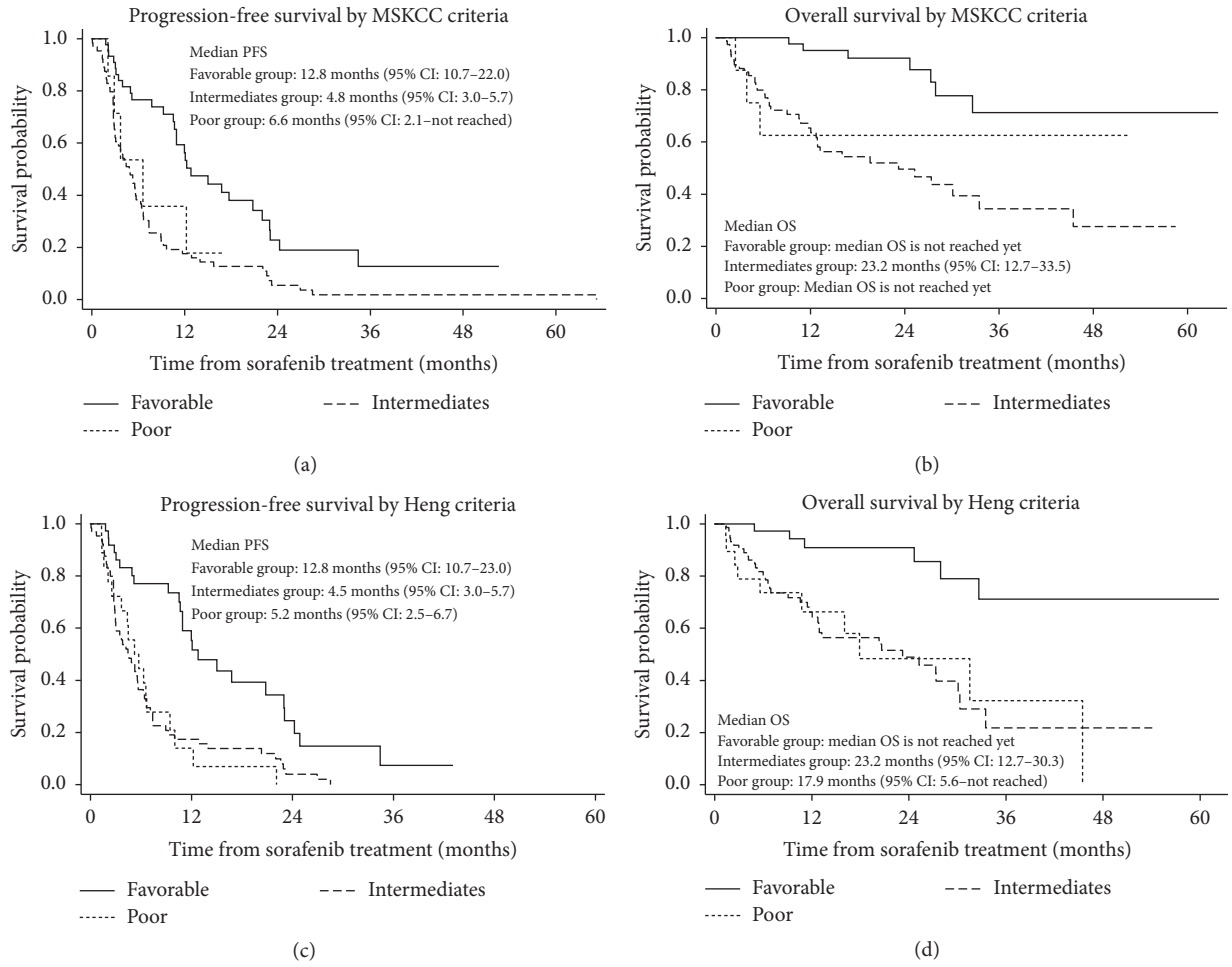

Figure 1: Kaplan-Meier analysis of progression-free survival (a, c) and overall survival (b, d) with log-rank tests for all sorafenib-treated patients ($N = 177$) according to the Memorial Sloan Kettering Cancer Center (MSKCC) (a, b) and Heng (c, d) risk groups. PFS, progression-free survival; OS, overall survival; CI, confidence interval.

systemic medical therapy. This can improve both the OS and quality of life for mRCC patients treated with sorafenib.

First-line sorafenib-treated patients were separately subjected to subanalyses to identify additional significant prognostic factors of OS, since multianalyses with numerous interrelated parameters, or those conducted with missing values and multicollinearities from retrospectively collected data, may have decreased statistical power and significance. Multivariate Cox statistics with backward selection were performed in 2 independent analyses while dividing the variables into (1) laboratory parameters and risk criteria and (2) primary pathological tumor-related factors (including TNM staging). Our results were consistent with those of previous studies [23–26]; we found that bone metastasis (HR 4.6) and clinical T3-4 stage (HR 3.8) were significant negative prognostic factors for survival and that female sex (HR 0.16), thrombocytosis (HR 0.23), lymphovascular invasion (HR 0.20), and primary tumor size >7 cm (HR 0.15) were significant positive prognostic factors ($p < 0.05$, data not shown).

The prognostic factors identified in this study collectively indicated that the therapeutic response to sorafenib could be more dependent on the characteristics of the primary renal tumor and the overall extent of the disease than on general health status. A greater primary tumor size with smaller necrotic areas, the absence of lymphovascular and collecting system invasion within the primary tumor, and less metastatic lesions without bone involvement are expected to reflect better prognosis with sorafenib treatment, whether before or after nephrectomy or metastasectomy [15, 27, 28]. Additionally, Araki et al. emphasized the importance of primary tumor characteristics, especially tumor growth patterns, and also found that the Fuhrman nuclear grade, presence of a sarcomatoid component, lymphovascular invasion, tumor necrosis, growth pattern, and other pathological parameters of the primary tumor were potentially useful prognostic indicators, as they could be assessed easily at the time of nephrectomy [22]. The importance of primary tumor characteristics and disease extent in this study was noteworthy because sorafenib is considered systemically less potent than other similarly acting tyrosine kinase inhibitors, although with more tolerability, fewer dose reductions, and/or less severe adverse events [12]. Furthermore, the

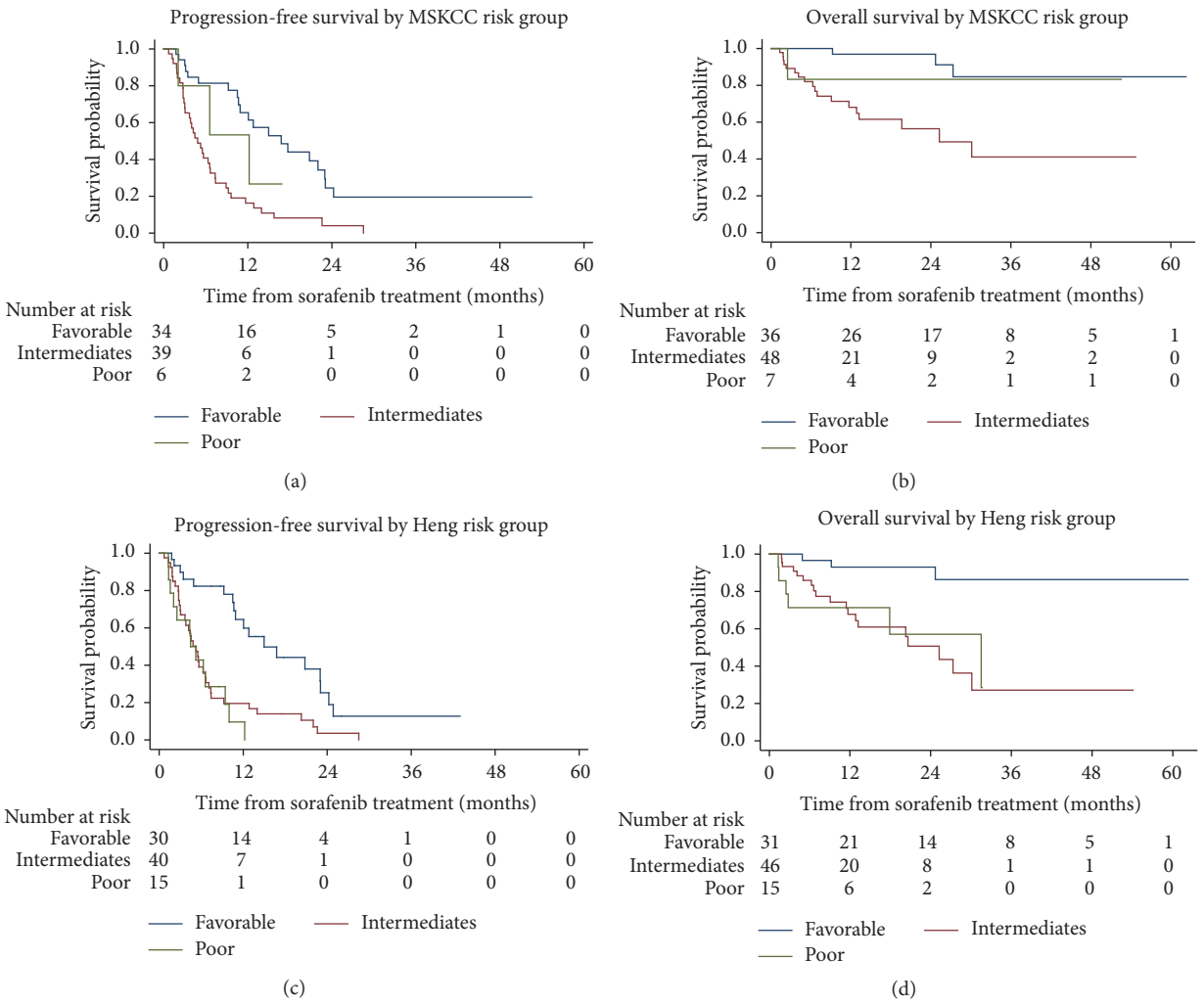

Figure 2: Kaplan-Meier analysis of progression-free survival and overall survival with log-rank tests for first-line sorafenib-treated patients ($N = 116$) according to the Memorial Sloan Kettering Cancer Center (MSKCC) (a) and Heng (b) risk groups. PFS, progression-free survival; OS, overall survival; CI, confidence interval.

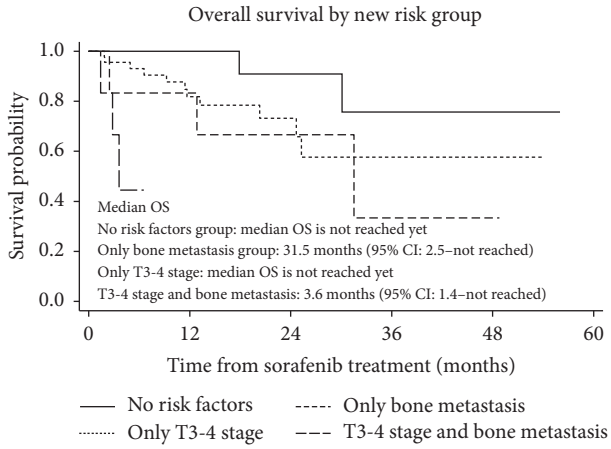

Figure 3: Comparison of overall survival (OS) according to the presence of bone metastasis and/or T3-4 stage, which were poor prognostic factors of OS in first-line sorafenib-treated patients.

prognostic significance of histology (clear and non-clear cell pathology) was generally not found to be of statistical significance; the small number of non-clear cell RCC patients in this study may not have been sufficient to influence the statistical analyses, and sorafenib has multiple inhibitory mechanisms involving multiple pathways [13].

This study harbored potential selection bias because of its retrospective nature as well as incomplete data collection, no further treatment information on other second-line and/or salvage therapy following treatment failure with sorafenib, and heterogeneity of clinicians' use of sorafenib due to this being a multicenter study. Further large-scale, prospectively designed studies are necessary to confirm our findings. These would include comparing the therapeutic effectiveness of sorafenib to other targeted therapies in patients with primary mRCC tumors sized >7 cm, as patients whose primary tumors were T2 stage were good candidates for sorafenib therapy in contrast to patients with T3-4 stage mRCCs.

Moreover, this study showed that primary tumor size and T3-4 stage were significant prognostic factors in terms of PFS and OS, not only for sorafenib-treated patients but also for mRCC patients in general.

Despite these limitations, the current study is the first large-scale multicenter study to reveal the prognostic factors of primary tumor-related characteristics associated with first-line sorafenib treatment in terms of PFS and OS in Korean mRCC patients. Our results might be of relevance to poor risk patients without aggressive primary tumor characteristics (such as clinical T3-4 stage and bone metastasis) who may be candidates for sorafenib therapy, especially as the NCCN guideline does not currently specify the patients for whom sorafenib is indicated [10]. Older patients and those with high underlying comorbidities, hepatic dysfunction, and major toxic adverse effects with sunitinib and pazopanib might be indicated for sorafenib therapy [11, 12, 29, 30]. Although the use of sorafenib as a first-line treatment is limited, and resulting PFS and OS rates are inferior to those observed with other TTs such as sunitinib, pazopanib, and combination of bevacizumab and interferon alpha, proper selection of patients as recommend by category 2A of the NCCN guideline may result in more positive prognoses following sorafenib therapy [4, 5, 10–12].

5. Conclusions

In this study of the long-term efficacy of sorafenib as an overall or first-line therapy, we showed that primary renal tumor-related characteristics, as well as the extent of disease, were significant prognostic indicators in sorafenib-treated Korean patients with mRCC.

Competing Interests

The authors would like to disclose the following: Choung-Soo Kim was a consultant for Pfizer, Bristol-Myers Squibb, and Taiho and received advisory and/or speaker fees from these companies. Jinsoo Chung was a consultant for Pfizer and Novartis and received advisory and/or speaker fees from Pfizer, Novartis, and Bayer. However, the authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Jinsoo Chung, Sang Eun Lee, Choung-Soo Kim, Ill Young Seo, Tae Nam Kim, Sung-Hoo Hong, Tae Gyun Kwon, Seong Il Seo, Kwan Joong Joo, Kanghyon Song, and Cheol Kwak contributed to manuscript conception. Sung Han Kim and Jinsoo Chung contributed to manuscript preparation. Sohee Kim and Byung-Ho Nam contributed to statistical analysis. Jinsoo Chung and Choung-Soo Kim contributed to funding source. Sung Han Kim and Jinsoo Chung contributed to internal review for draft. All authors approved the manuscript.

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Research Article

Expression of Cadherin-17 Promotes Metastasis in a Highly Bone Marrow Metastatic Murine Breast Cancer Model

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We previously established 4T1E/M3 highly bone marrow metastatic mouse breast cancer cells through *in vivo* selection of 4T1 cells. But while the incidence of bone marrow metastasis of 4T1E/M3 cells was high (~80%) when injected intravenously to mice, it was rather low (~20%) when injected subcutaneously. Therefore, using 4T1E/M3 cells, we carried out further *in vitro* and *in vivo* selection steps to establish FP10SC2 cells, which show a very high incidence of metastasis to lungs (100%) and spines (85%) after subcutaneous injection into mice. qRT-PCR and western bolt analysis revealed that cadherin-17 gene and protein expression were higher in FP10SC2 cells than in parental 4T1E/M3 cells. In addition, immunostaining revealed the presence of cadherin-17 at sites of bone marrow and lung metastasis after subcutaneous injection of FP10SC2 cells into mice. Suppressing cadherin-17 expression in FP10SC2 cells using RNAi dramatically decreased the cells' anchorage-independent growth and migration *in vitro* and their metastasis to lung and bone marrow *in vivo*. These findings suggest that cadherin-17 plays a crucial role in mediating breast cancer metastasis to bone marrow.

1. Introduction

Bone is a main target of breast cancer metastasis such that nearly 80% of patients with advanced breast cancer suffer from bone metastasis [1, 2]. And because once bone metastasis has occurred, the quality of life of the affected breast cancer patient is seriously diminished, new approaches to preventing and reducing bone metastasis are urgently required. We previously established the 4T1E/M3 highly bone marrow metastatic breast cancer cell line [3] and showed that the chemokine CCL2/MCP-1 negatively regulates the cells' growth, migration, and metastasis [4], while BMP7 signaling increases the metastatic potential of these cells [5]. However, although the metastatic potential of 4T1E/M3 cells is high (about 80%) when injected intravenously into mice, it is only 20% to 30% when injected subcutaneously [3]. In the present study, we established more highly metastatic breast cancer cell line, FP10SC2, which shows 85% to 100% metastasis to spine when injected subcutaneously into mice.

Cadherin-17, also known as liver-intestine cadherin or human peptide transporter-1, is a structurally unique member of the cadherin superfamilies [6, 7]. Whereas classical cadherins have five cadherin repeats, cadherin-17 contains seven cadherin repeats and is closely associated with cadherin-16 in the seven cadherin domain subfamily. In addition, cadherin-17 has only about 20 amino acids in its cytoplasmic domain, while classical cadherins contain a highly conserved cytoplasmic domain of 150–160 amino acids. Cadherin-17 is expressed in mice and humans almost exclusively in epithelial cells of both embryonic and adult small intestine and colon, fetal liver, and B lymphocytes [8], where it plays an important role during embryonic gastrointestinal development and also functions as a peptide transporter. In cancer, cadherin-17 expression reportedly correlates with clinical association with tumor metastasis and advanced tumor stages in hepatocellular carcinoma [9], knockdown of cadherin-17 inhibited cell proliferation, adhesion, migration, and invasion in gastric cancer [10],

and an anti-cadherin-17 antibody suppresses subcutaneous hepatocellular carcinoma growth and lung metastasis [11]. In the present study, we found that expression of cadherin-17 in our highly metastatic FP10SC2 cells was higher than that in the parental 4T1E/M3 cells and that suppressing cadherin-17 expression reduced the metastatic potential of FP10SC2 cells. These results suggest that the expression of cadherin-17 promotes breast cancer metastasis to bone marrow.

2. Materials and Methods

2.1. Animals. Seven- to eight-week-old female BALB/c mice were purchased from Japan Clea (Tokyo, Japan).

2.2. Establishment of the FP10SC1 and FP10SC2 Cell Lines. The 4T1E/M3 highly bone metastatic mouse breast cancer cell line was established as described previously [3]. Briefly, 4T1 murine breast cancer cells were initially transfected with a plasmid harboring the neomycin resistance gene and selected in medium (RPMI 1640 supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% fetal bovine serum: GIBCO, Invitrogen, Carlsbad, CA, USA) containing 120 μg/mL G418 (Invitrogen). The selected cells were injected into the tail vein of BALB/c mice (1×10^6 cells/mouse), and 12 days later bone marrow cells were recovered from the femurs and tibias by flushing. These cells were cultured in the medium containing 120 μg/mL G418 and intravenously injected into a new set of mice, and this cycle was repeated three times to establish the 4T1E/M3 cells. When 4T1E/M3 cells were intravenously injected into mice, the incidence of metastasis to the spine was about 80%, but it was only 20% when the cells were injected subcutaneously [3].

To establish cells with greater metastatic potential after subcutaneous injection, 4T1E/M3 cells (2×10^5/mL, 200 μL/well) were seeded onto polycarbonate membranes (8 μm pores) within transwell chambers (Krabo, Osaka, Japan) in 24-well plastic tissue culture plates. After incubation for 2 days, the cells that migrated through the pores in the membrane were harvested and seeded onto a new membrane. After repeating this process 10 times, the cells were subcutaneously injected into BALB/c mice (1×10^6 cells/mouse), and 25 days later mice were sacrificed and bone marrow cells were collected from the spines by flushing the bones using a 25G needle and syringe (Terumo Co. Ltd., Tokyo, Japan). The collected cells were cultured in medium containing 120 μg/mL G418, and FP10SC1 cells were established. FP10SC1 cells (1×10^6 cells/mouse) were then subcutaneously injected into a new set of mice, and this cycle was repeated to establish FP10SC2 cells.

2.3. Cell Proliferation Assay. Cells (1×10^4/mL, 200 μL/well) were seeded into 96-well plates and cultured for 0 to 4 days, and 10 μL of Cell Counting Kit-8 reagent (Dojin Laboratories, Tokyo, Japan) was added to each well. After the additional 4 h incubation at 37°C under 5% CO_2, the absorbance at 450 nm was measured using a microplate reader (Model 680, Bio-Rad Laboratories, Inc., Hercules, CA).

2.4. Anchorage-Independent Proliferation Assay. Anchorage-independent colony formation in soft agar was assayed as described previously [5]. First, culture medium containing 0.5% agarose (Difco noble agar: BD Diagnostic Systems, Sparks, MD, USA) was added to 6 cm dishes (5 mL/dish). Thereafter, cells (1×10^4/mL, 2 mL/dish) suspended in culture medium containing 0.3% agarose (Difco noble agar) were layered onto the 0.5% agarose in the dishes. The cells were then cultured for 12 days at 37°C under 5% CO_2, after which the total numbers of colonies per dish were counted under a microscope.

2.5. In Vitro Invasion Assay. The *in vitro* invasion assay system used was described previously [12]. Briefly, cells (2×10^5/mL, 200 μL/well) were seeded onto polycarbonate membranes (8 μm pores) in transwell chambers (Krabo) and placed in 24-well plates. For transendothelial invasion assays, a bone marrow derived endothelial cell (BMEC) monolayer [13] was formed on fibronectin-coated membranes, and the cells (2×10^5/mL, 200 μL/well) were seeded onto the monolayer. After incubation for 18 h, the cells that migrated through the pores to the underside of the membrane were counted under a microscope.

2.6. In Vivo Tumor Experiments. Cells (1×10^6/mouse) were subcutaneously injected into the backs or mammary fad pad of BALB/c mice. Twenty-five days later, the mice were sacrificed and their lungs and spines were collected and subjected to histological examination. Each experiment was repeated by three times. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of National Institute of Advanced Industrial Science and Technology (AIST).

2.7. Histology. Lungs and spines from mice were fixed in 10% neutral buffered formalin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), processed, embedded in paraffin, sectioned at 5-6 μm, stained with H&E, and examined and photographed under a microscope. The spines were decalcified using decalcifying solution A (Plank Rychlo Method, Wako Pure Chemical Industries) following the manufacturer's protocol before embedding in paraffin. The percent metastatic area per tissue in the histological sections was calculated using Image J software. For immunohistochemical analysis, specimens were stained using rabbit anti-cadherin-17 IgG (sc-25628; Santa Cruz Biotech Inc., Santa Cruz, CA, USA) and HRP-conjugated goat anti-rabbit IgG (R&D).

2.8. RNA Isolation and Quantitative RT-PCR. Total RNA isolation and cDNA synthesis were performed as described previously [3]. The reverse transcription protocol entailed incubation at 25°C for 10 min, 55°C for 30 min, 85°C for 5 min, and 0°C for 5 min. Specific forward and reverse primers were designed using Primer 3 software (Genetyx Co., Tokyo, Japan) and synthesized by Fasmac Co. Ltd. (Kanagawa, Japan). The primer sequences (5′-3′) used were as follows: for GAPDH (glyceraldehyde-3-phosphate dehydrogenase, product size, 209 bp), CCCCTTCATTGACCTCAACTAC (forward) and TGGTGGTGAAGACACCAGTAGA (reverse);

for cadherin-17 (product size, 180 bp), ACTGAAGTAGGTGGGTCCTCT (forward) and CCGAAGTGACTGCTGGTCAT (reverse). The quantitative RT-PCR (qRT-PCR) reactions were set up according to the Light Cycler manual (Roche Diagnostics GmbH, Mannheim, Germany) using a Light Cycler Fast Start DNA Master SYBR Green I Kit (Roche Diagnostics). The PCR protocol entailed denaturation at 95°C for 10 min, 45 cycles of denaturation at 95°C for 10 s, annealing at 59°C for 10 s, and extension at 72°C for 11 s, and a final cooling step at 40°C for 30 s. For each reaction, the crossing point (defined as the cycle number at which the noise band intersected the fluorescence curves) was determined using the "Second Derivative Maximum Method" in the Light Cycler software (ver. 3.52, Roche Diagnostics). Relative mRNA levels were calculated using the software with a standard curve constructed using various concentrations of a 1 : 1 mixture of the RT product and then normalized to GAPDH mRNA.

2.9. RNA Interference. For vector-based RNAi analysis, we synthesized miRNA oligo DNA (CTGTACAAGTAAGCTAAGCACTTCGTGGCCGTCGATCGTTTAAAGGGAGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGCTGTATGCTGAACACAGGCACTTCATTCACAGTTTTGGCCACTGACTGACTGTGAATGGTGCCTGTGTTCAGGACACAAGGCCTGTTACTAGCACTCACATGGAACAAATGGCCCAGATCTGGCCGCACTCGAGATATCTAGACCCAGCTTTCTTGTACAAAGTGGTTG) using Cadherin17 BLOCK-iT™ miR RNAi Select (Invitrogen), Mmi 506198_top_Cadherin17, and Mmi 506198_bot_Cadherin17 (Invitrogen) to generate knockdown clones numbers 1 and 2. The sequence was inserted into an expression vector to obtain pcDNA6.2-GW/EmGFP-miR-mCDH17, the sequence of which was verified by Life Technologies Inc. (Tokyo, Japan). The plasmid pcDNA6.2-GW/EmGFP-miR-neg (Invitrogen), which carried a scrambled sequence and could not target any known vertebrate gene, was served as a control nontargeting vector. To knock down the targeted genes, FP10SC2 cells were transfected with the vector using FuGene HD (Roche Diagnostics) following the manufacturer's protocol. Cells were then cultured in medium containing 2 μg/mL blasticidin (Wako, Tokyo, Japan) to yield stable control/FP10SC2 and Cadherin-17(−)/FP10SC2 clones numbers 1 and 2 after limiting dilution.

2.10. Western Blotting. Cells were seeded into 10 cm dishes and treated with Complete Lysis buffer (Roche Diagnostics) following the manufacturer's protocol, after which the protein obtained was measured using a BCA Protein Assay Kit (Pierce. Rochford IL, USA). The proteins were then resolved by SDS-PAGE and transferred to i-Blot Gel Transfer Stacks PVDF membranes (Invitrogen) using an i-Blot Dry Blotting system (Invitrogen). After blocking the membranes with 5% skim milk, they were incubated overnight at room temperature with anti-cadherin-17 (1/200, rabbit polyclonal IgG, sc-25628; Santa Cruz Biotech Inc., Santa Cruz, CA, USA) or anti-β-actin (1/2000, rabbit polyclonal IgG, sc-7210; Santa Cruz Biotech Inc.) diluted in Can Get signal immunoreaction Enhancer Solution 1 (Toyobo, Tokyo, Japan). The membranes were then washed three times with TBS-T and incubated for 1 h at room temperature with HRP-conjugated anti-rabbit IgG (1/5000, GE Healthcare) diluted in Can Get signal immunoreaction Enhancer Solution 2 (Toyobo, Tokyo, Japan). Finally, the membranes were treated with ECL Prime detection reagent (GE Healthcare) according to manufacturer's protocol, and the chemiluminescence was detected using Chemi Doc XRS (Bio-Rad Laboratories Inc.).

Table 1: Incidences of metastasis by 4T1E/M3 and FP10SC2.

Cell	Lung	Spine
4T1E/M3	100% (6/6)	33% (2/6)
FP10SC2	100% (13/13)	85% (11/13)

Note. 4T1E/M3 ($n = 6$) or FP10SC2 ($n = 13$) cells (1×10^6/mouse) were subcutaneously injected to mice and 25 days later tissue metastasis was evaluated in histological sections.

3. Results

3.1. Newly Established FP10SC1 and FP10SC2 Cells Showed Enhanced Spontaneous Bone Marrow Metastasis. We established FP10SC1 cells from parental 4T1E/M3 cells through 10 *in vitro* selection steps and 1 *in vivo* selection step. From the FP10SC1 cells, we then established FP10SC2 cells through another *in vivo* selection steps. Histological sections of lungs and spines collected from mice subcutaneously injected with FP10SC2 or 4T1E/M3 cells after 25 days revealed the presence of metastasis in all lungs from 4T1E/M3- and FP10SC2-injected mice. In addition, metastasis was detected in the spines of 20% to 33% (2/10 or 2/6) of 4T1E/M3-injected mice and 85% (11/13) of FP10SC2-injected mice (Table 1). It means that the metastatic potential was strongly augmented in FP10SC2 cells. The photomicrographs in Figure 1 show metastasis in spines from mice subcutaneously injected with 4T1E/M3 and FP10SC2 cells.

3.2. Proliferation Rates of FP10SC1 and FP10SC2 Was Not Changed. To investigate the mechanism underlying the enhanced metastatic activity of FP10SC1 and FP10SC2, we carried out modified MTT assays to examine their *in vitro* proliferation rates. As shown in Figure 2(a), there was no difference in the proliferation rates of 4T1E/M3, FP10SC1, and FP10SC2 cells.

3.3. Anchorage-Independent Growth of FP10SC2 Cells Was Markedly Accelerated. Anchorage-independent proliferation is a hallmark of tumor cell malignancy. We therefore assessed the anchorage-independent proliferation of FP10SC1, FP10SC2, and 4T1E/M3 cells in soft agar colony formation assays. Twelve days after plating, colonies were evident in medium containing 0.3% agar. FP10SC1 cells formed nearly twice as many colonies as did 4T1E/M3 cells, while FP10SC2 cells formed more than three times as many colonies as 4T1E/M3 cells (Figure 2(b)). The diameters of individual colonies did not differ among the three cell types (Figure 2(c)).

3.4. Migration of FP10SC1 and FP10SC2 Cells Was Significantly Enhanced. The ability to migrate is another important feature

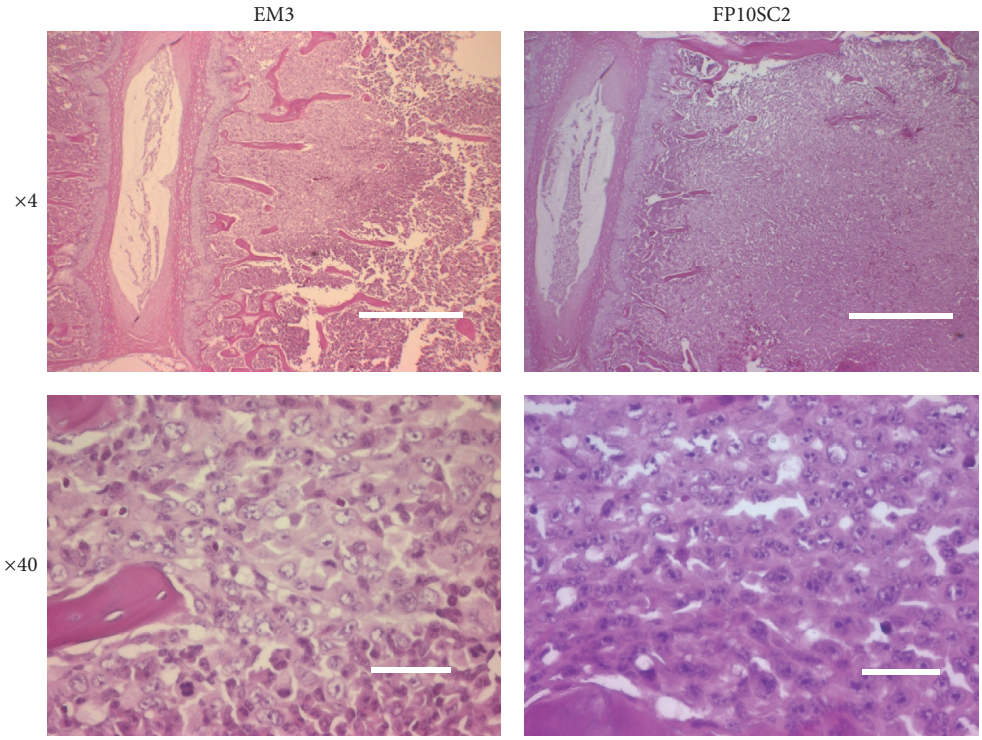

Figure 1: Histological examination of the spines of mice subcutaneously injected with 4T1E/M3 or FP10SC2 cells. Shown are representative histological sections of spines. Cancer metastasis in spine samples collected 25 days after injection of cells (1×10^6 cells/mouse) was detected using H&E staining. Images are shown at objective lens magnifications of 4x and 40x; the bars indicate 500 and 25 μm, respectively.

of metastatic tumor cells. We therefore assessed the migration activity of FP10SC1 and FP10SC2 cells using transwell chambers in which the insert bottom was a polycarbonate membrane with 8 μm pores. To measure transendothelial invasion activity, we grew a BMEC monolayer [14] on fibronectin-coated polycarbonate membranes. After seeding FP10SC1, FP10SC2, or 4T1E/M3 cells onto polycarbonate membranes, with or without BMEC monolayers, and incubating the cells for 18 h, the numbers of cells that migrated through the membrane pores were counted. As shown in Figures 3(a) and 3(b), the migration activity of FP10SC1 and FP10SC2 cells was more than 3-4 times higher than that of 4T1E/M3 cells.

3.5. Cadherin-17 Expression Was Dramatically Increased in FP10SC2 Cells. Cadherin-17 is reportedly overexpressed in liver, stomach, intestinal, and pancreatic cancers, and the increased expression is associated with the occurrence of metastasis [9]. We therefore used qRT-PCR analysis to compare the levels of cadherin-17 gene expression in FP10SC1 and FP10SC2 cells with that in the parental 4T1E/M3 cells. We found that, as compared to 4T1E/M3 cells, expression of cadherin-17 mRNA was markedly (about 10 times) increased in both FP10SC1 and FP10SC2 cells (Figure 4(a)). In addition, as shown in Figure 4(b), cadherin-17 protein expression in FP10SC1 and SC2 was augmented measured by western blot analysis. We also examined the gene expression of some molecules including cadherin and integrin family and found that the cadherin-17 gene expression was significantly augmented among other adhesion molecules in FP10SC1 and FP10SC2.

Moreover, immunohistochemical analysis of sections of lungs and spines collected 28 days after subcutaneous injection of FP10SC2 cells into mice revealed the clear presence of cadherin-17-positive metastatic cancer cells in both the lungs (Figures 5(a) and 5(b)) and spines (Figures 5(c) and 5(d)). This suggests that cadherin-17 is highly expressed *in vivo* at sites of metastasis.

3.6. Knocking down Cadherin-17 Gene Expression Did Not Affect In Vitro Proliferation. To assess the effects of the augmented cadherin-17 expression on the behavior of FP10SC2 cells, we established two stable transfectant clones, cadherin-17(−)/FP10SC2#1 and #2, in which cadherin-17 was knocked down by miR RNAi. Using qRT-PCR, we confirmed that levels of cadherin-17 mRNA were significantly lower in cadherin-17(−)/FP10SC2#1 and #2 cells than in the mock transfectant (control/FP10SC2) (Figure 6(a)). Consistent with that finding, western blotting showed that cadherin-17 protein was also expressed at a lower level in cadherin-17(−)/FP10SC2#1 and #2 cells than in control/FP10SC2 cells (Figure 6(b)). By contrast, there was no difference in the proliferation rates of cadherin-17(−)/FP10SC2#1 and #2 cells and those of FP10SC2 and control/FP10SC2 cells (Figure 7(a)).

3.7. Cadherin-17 Knockdown Reduced Anchorage-Independent Growth and Cell Migration. High potentials for anchorage-independent cell growth and cell migration are well-known *in*

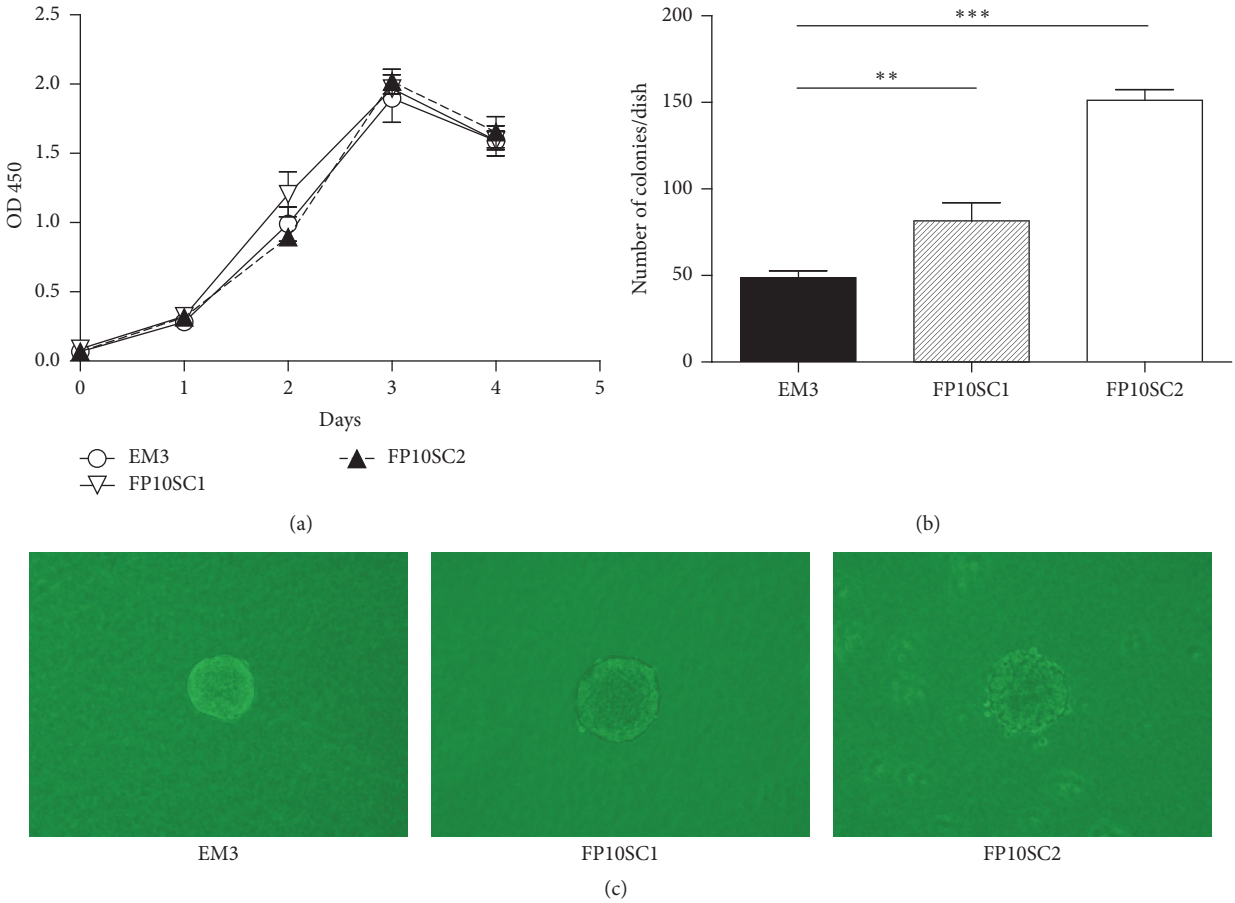

Figure 2: Growth curves and anchorage-independent growth of 4T1E/M3, FP10SC1, and FP10SC2 cells. (a) Growth curves for the 4T1E/M3, FP10SC1 and FP10SC2 cells measured in MTT assays. Data are means ± SD. (b) Anchorage-independent growth of 4T1E/M3, FP10SC1 and FP10SC2 cells measured in colony formation assays. Cells (1×10^4/dish, 3 dishes/cell type) were cultured for 12 days in medium containing 0.3% agarose layered on 0.5% agarose. Data are means ± SD; $^{**}p < 0.01$; $^{***}p < 0.001$. (c) Representative images of colonies.

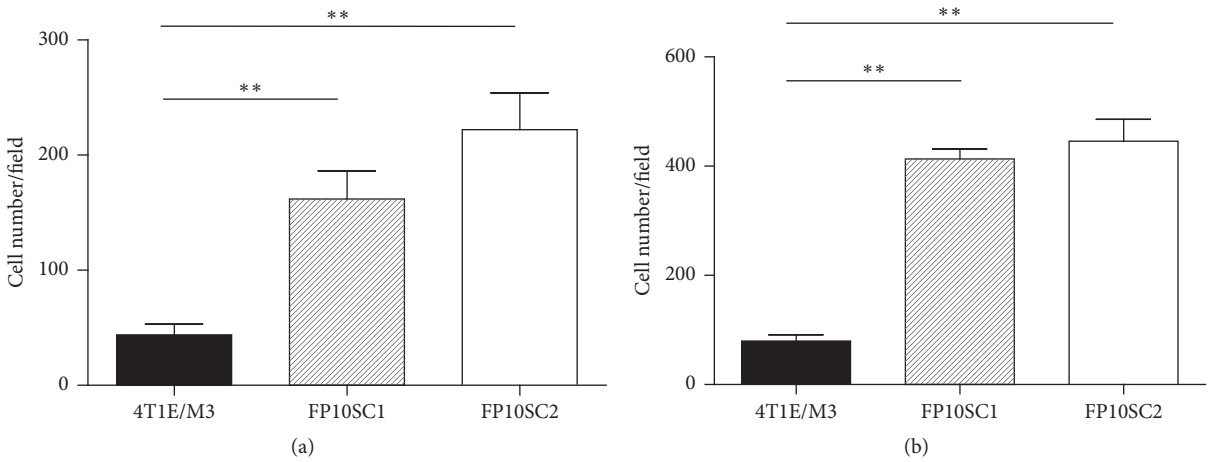

Figure 3: Migration of 4T1E/M3, FP10SC1, and FP10SC2 cells. (a) Cells (4×10^4 cells/well) were seeded onto BMEC monolayers grown on polycarbonate porous membranes, and the numbers of cells migrating through the pores were counted after 18 h. (b) Cells (4×10^4 cells/well) were seeded directly onto polycarbonate porous membranes, and the numbers of the cells migrating through the pores were counted after 18 h. In both panels, data are means ± SD; $^{**}p < 0.01$.

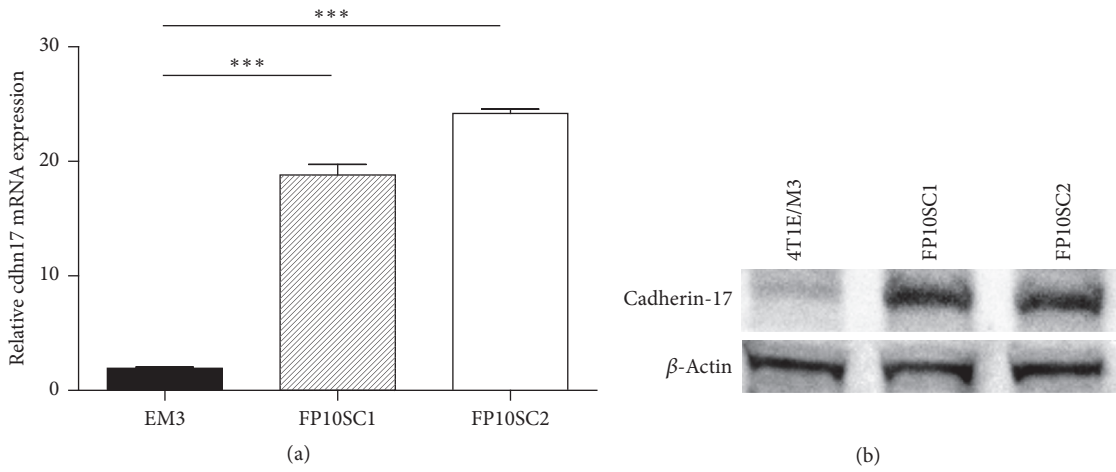

Figure 4: Expression of cadherin-17 in 4T1E/M3, FP10SC1, and FP10SC2 cells. (a) Quantitative real-time RT-PCR (qRT-PCR) analysis of cadherin-17 expression in 4T1E/M3, FP10SC1 and FP10SC2 cells. Levels of cadherin-17 mRNA are shown relative to that of GAPDH mRNA. Values are means ± SD; $^{***}p < 0.001$. (b) Western blot analysis of cadherin-17 in lysates of 4T1E/M3, FP10SC1, and FP10SC2 cells.

vitro properties related to cancer metastasis and malignancy. We therefore used colony formation and migration assays to evaluate the effect of cadherin-17 suppression on anchorage-independent cell growth and cell migration, respectively. We found that cadherin-17(−)/FP10SC2 cells showed dramatically less anchorage-independent growth (Figure 7(b)) than control/FP10SC2 cells such that cadherin-17(−)/FP10SC2#1 and #2 cells formed almost no colonies (Figure 7(b)). Photographs of the colonies of FP10SC2 and control/FP10SC2 are shown in Figure 7(c). In addition, migration was significantly diminished in cadherin-17(−)/FP10SC2#1 and #2 cells as compared to control/FP10SC2, with (Figure 8(a)) and without a BMEC monolayer (Figure 8(b)). The mean numbers of cadherin-17(−)/FP10SC2#1 and #2 cells showing transendothelial migration were 40.8 and 61.0 respectively, while those of the FP10SC2 and control/FP10SC2 cells were 201.5 and 171.7, respectively (Figure 8(a)). In the absence of a BMEC monolayer, the mean numbers of migrated cadherin-17(−)/FP10SC2#1 and #2 cells was 78.5 and 89.5, respectively, while those of FP10SC2 and control/FP10SC2 cells were 460 and 403, respectively (Figure 8(b)).

3.8. Cadherin-17(−)/FP10SC2 Cells Showed Reduced Metastasis to Bone Marrow. When cadherin-17(−)/FP10SC2 cells were subcutaneously injected to BALB/c mice, the incidence of metastasis to spine was 25% for cadherin-17(−)/FP10SC2#1 cells and 47% for cadherin-17(−)/FP10SC2#2 cell (Table 2). By contrast the incidence of spinal metastasis for control/FP10SC2 cells was 85%. Moreover, the incidence of metastasis to lung was 75% for cadherin-17(−)/FP10SC2#1 cells but was 100% for control/FP10SC2 cells. Thus expression of cadherin-17 clearly enhances the metastatic activity of breast cancer cells in our model.

We further analyzed percent metastatic area per lung or spine in the histological sections using Image J software. As a result, the percent metastatic area in the lung of the mice injected by CDH17(−)/FP#1 and #2 was almost the same as that by control/FP10SC2 (approximately 60%). On the other hand, percent metastatic area in the spine of the mice injected by CDH17(−)/FP#1 and #2 was significantly decreased (12.4 and 9.7%, resp.) compared to that in the spine of the mice injected by control/FP10SC2 (42.6%). Although the incidence of metastasis of CDH17(−)/FP#1 (25%) was lower than that of CDH17(−)/FP#2 (47%), the percent metastatic area in the spine of CDH17(−)/FP#1 injected mice (12.4%) was rather higher than that of CDH17(−)/FP#2 injected mice (9.7%). As a result, we estimate that there is not so much difference in the metastatic activity of these two subclones. When the sections of metastatic tumors developed by CDH17(−)/FP#1 and #2 were stained with anti-cadherin-17 antibody, the expression of cadherin-17 was not detected.

Table 2: Incidences of metastasis and percent metastatic area per tissue by control/FP10SC2, CDH17(−)/FP#1, and CDH17(−)/FP#2.

Cell	Lung	Spine
Control/FP10SC2	100% (13/13)	85% (11/13)
	60.5 ± 3.35	42.6 ± 2.88
CDH17(−)/FP#1	75% (9/12)	25% (3/12)
	58.3 ± 4.23	12.4 ± 3.57
CDH17(−)FP#2	100% (15/15)	47% (7/15)
	56.8 ± 3.68	9.7 ± 2.48

Note. Control/FP10SC2 ($n = 13$), CDH17(−)/FP#1 ($n = 12$), or CDH17(−)/FP#2 ($n = 15$) cells (1×10^6/mouse) were subcutaneously injected to mice and 25 days later tissue metastasis was evaluated in histological sections. Percent metastatic area per lung or spine was calculated using Image J.

4. Discussion

Cadherin-17 reportedly mediates significant increases in the adhesion and proliferation of highly metastatic human colorectal cancer cells, and cadherin-17 silencing in these cells suppresses tumor growth and liver metastasis [15]. In addition, the prognosis of gastric and ovarian cancer patients with tumors expressing cadherin-17 is significantly worse

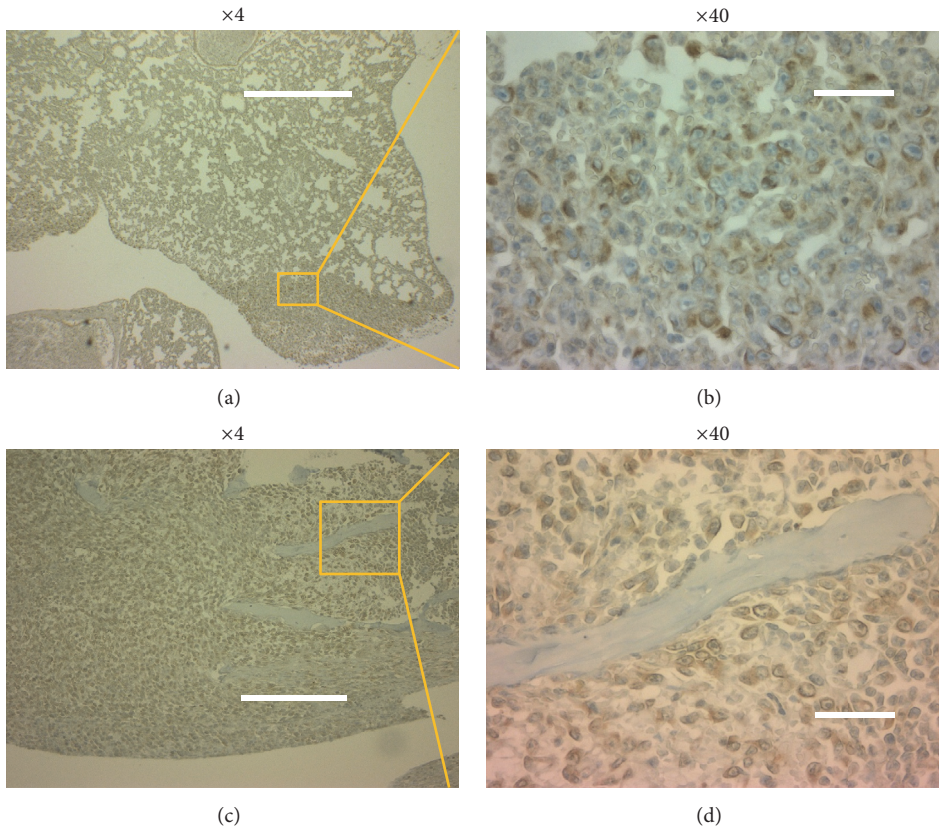

Figure 5: Immunohistochemical examination of the spines and lungs of BALB/c mice subcutaneously injected with FP10SC2 cells. (a)–(d) Immunostaining for cadherin-17 in spine (a, b) and lung (c, d) samples collected 28 days after subcutaneous injection of FP10SC2 cells into the backs of BALB/c mice (1×10^6 cells/mouse). Objective lens magnification, (a) 4x; (b) 40x; (c) 4x; (d) 40x; the bars indicate 500 (a, c) and 25 μm (b, d), respectively.

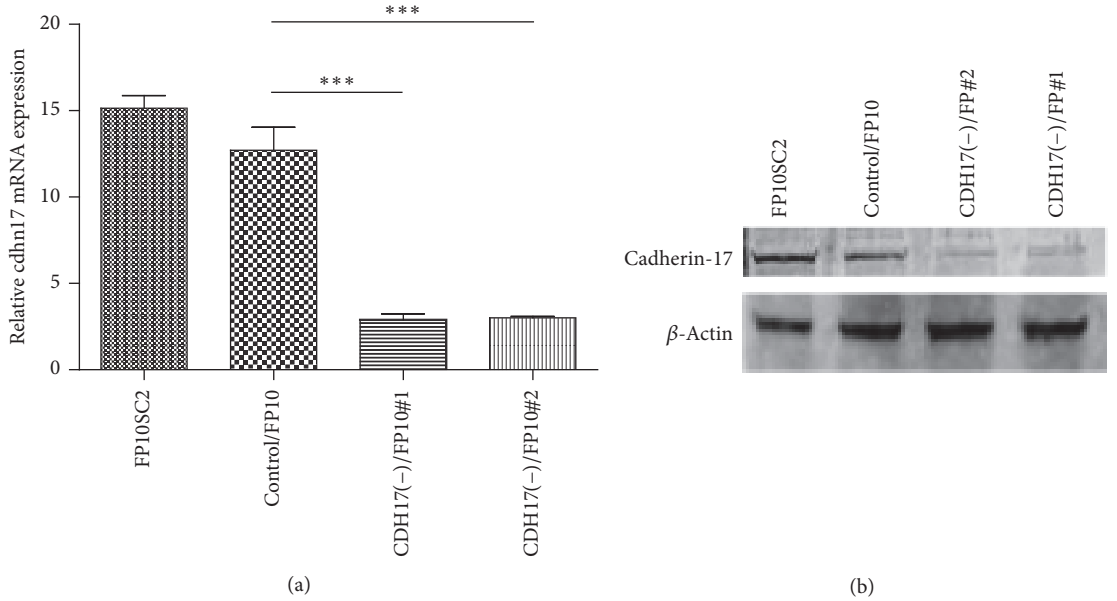

Figure 6: Establishment of cadherin-17 knockdown FP10SC2 clones and expression of cadherin-17. (a) qRT-PCR analysis of cadherin-17 expression. Levels of the indicated mRNAs in FP10SC2, control/FP10SC2, CDH17(−)/FP10SC2 clone #1, and CDH17(−)/FP10SC2 clone #2 are shown relative to the level of GAPDH mRNA. Values are means ± SD; $^{***}p < 0.001$. (b) Western blot analysis of cadherin-17 in lysates of FP10SC2 cells, control/FP10SC2, CDH17(−)/FP10SC2 clone #1, and CDH17(−)/FP10SC2 clone #2.

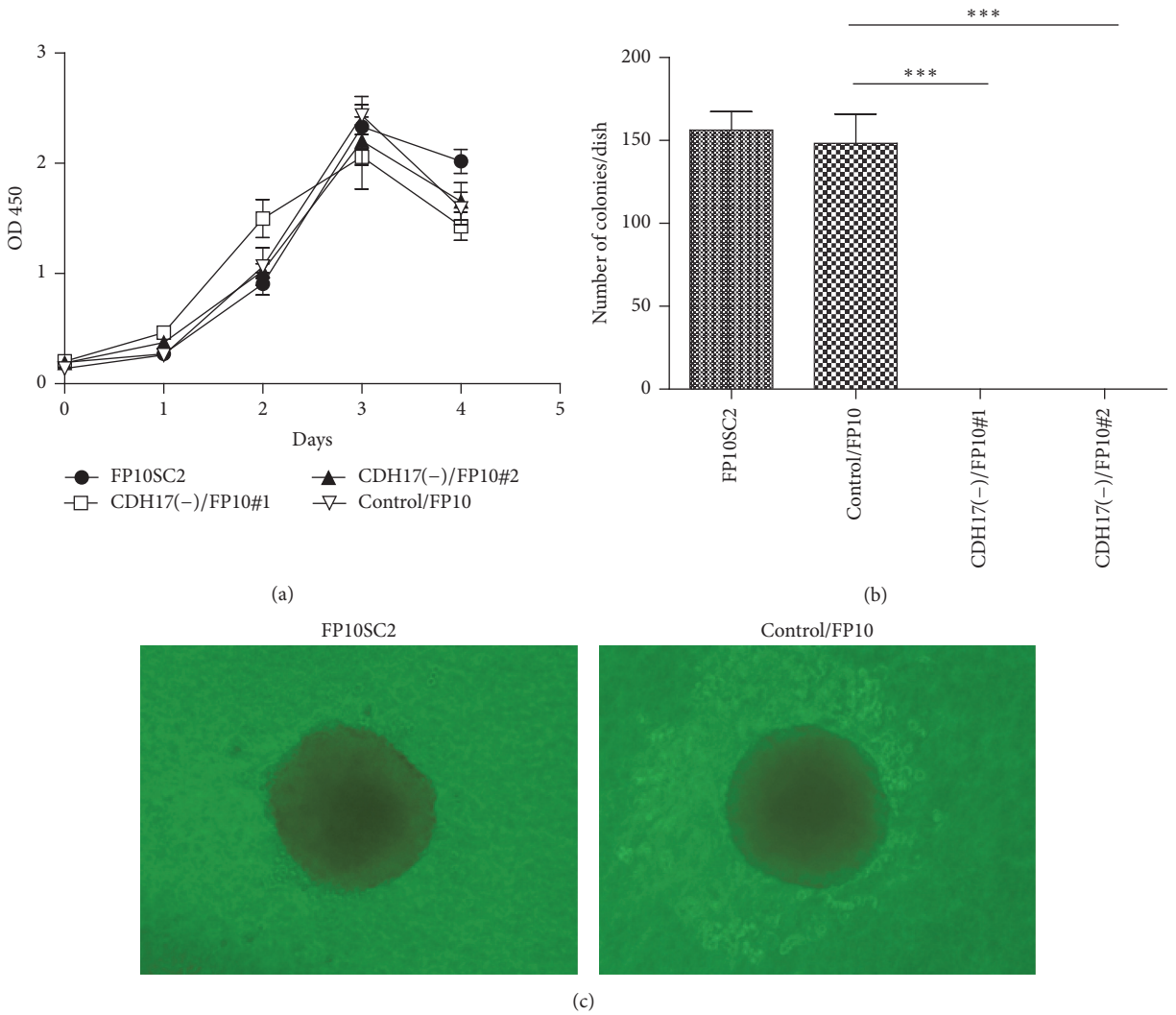

Figure 7: Growth curves and anchorage-independent growth of FP10SC2 cells, control/FP10SC2, CDH17(−)/FP10SC2 clone #1, and CDH17(−)/FP10SC2 clone #2. (a) Growth curves for the indicated cells measured in MTT assays. Data are means ± SD. (b) Anchorage-independent growth of the indicated cells measured in colony formation assays. Cells (1×10^4/dish, 3 dishes/cell type) were cultured for 12 days in medium containing 0.3% agarose layered on 0.5% agarose. Data are means ± SD; $^{***}p < 0.001$. (c) Representative images of colonies.

than that of patients whose tumors do not express cadherin-17 [16–18]. However, little is known about the actions of cadherin-17 in breast cancer metastasis.

We previously established 4T1E/M3 highly bone marrow metastatic breast cancer cells. The metastatic potential of 4T1E/M3 cells to lung (100%) and spine (85% to 100%) was high when intravenously injected into mice but was rather low (about 20%) when injected subcutaneously [3]. However, by passing 4T1E/M3 cells through a number of *in vitro* and *in vivo* selection steps (see Materials and Methods for details), we established FP10SC2 cells, which exhibited higher metastatic activity when subcutaneously injected into mice (Table 1). A notable difference between FP10SC2 and the parental 4T1E/M3 cells is the higher expression of cadherin-17 in FP10SC2 cells (Figures 4(a) and 4(b)). Furthermore, although suppressing cadherin-17 in FP10SC2 cells had no effect on their proliferation (Figure 7(a)), the *in vitro* anchorage-independent growth and migration activity of FP10SC2 cells were markedly decreased (Figures 7(b), 7(c), 8(a), and 8(b)), as was *in vivo* pulmonary and spinal metastasis (Table 2). Because 4T1E/M3 breast cancer cells originally have very strong metastatic activity to lung (almost 100%), it seems to be difficult to suppress the metastasis to lung strongly. As a result, the knockdown of cadherin-17 may look more effective on the suppression of spine metastasis (Table 2).

It was reported that downregulation of cadherin-17 inactivates WNT signaling and inhibits tumor growth in hepatocellular carcinoma and gastric cancer [9–11, 19]. The involvement of WNT signaling was also shown in human breast cancer cell line, MDA-MB-231 [20]. It was demonstrated that downregulation of cadherin-17 induced inactivation of NFkB signaling pathway with the reductions of downstream proteins including VEGF-C and MMP-9 and suppressed

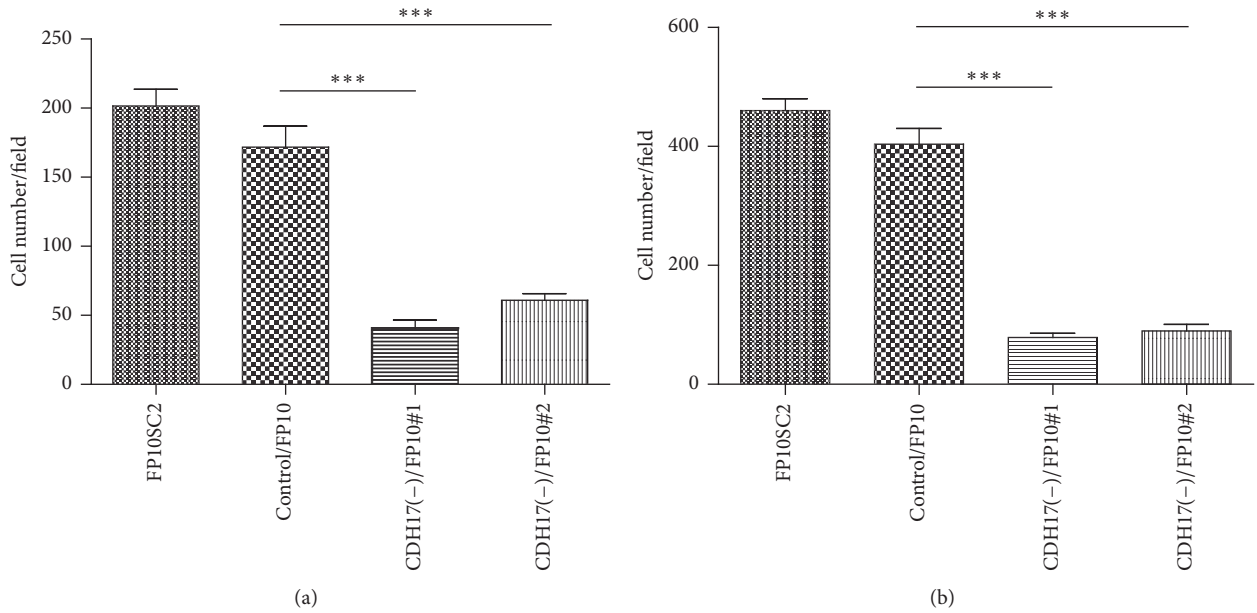

Figure 8: Migration of FP10SC2 cells, control/FP10SC2, CDH17(−)/FP10SC2 clone #1, and CDH17(−)/FP10SC2 clone #2. (a) Cells (4×10^4 cells/well) were seeded on BMEC monolayers formed on polycarbonate porous membranes, and the numbers of the cells migrating through the were counted after 18 h. (b) Cells (4×10^4 cells/well) were seeded directly on polycarbonate porous membranes, and the numbers of the cells migrating through the pores were counted after 18 h. In both panels, data are means ± SD; $^{***}p < 0.001$.

proliferation, adherence, and invasion in gastric cancer [21]. It was further shown that targeting cadherin-17 inactivated Ras/Raf/MEK/ERK signaling and inhibited cell proliferation in gastric cancer [22]. Moreover, cadherin-17 was shown to modulate $\alpha 1\beta 2$ integrin signaling to induce specific focal adhesion kinase and Ras activation and led to the increase in cell adhesion and proliferation in colon cancer cells and the RGD motif in cadherin-17 was important in this process [14, 23]. Although the underling mechanism of the effect of cadherin-17 suppression in our model is still unclear, the same mechanism above demonstrated may be involved in the decrease of metastasis in the lung and spine. We consider that analysis of the potential interaction of cadherin-17 with the molecules in signaling pathways will be an important future study in our model.

In the studies on many tumors, for example, gastric, ovarian, hepatocellular, and colorectal cancer, expression of cadherin-17 in human tissues was examined and estimated as an important candidate of prognosis marker [16, 24]. Examination of cadherin 17 expression in human breast cancer tissues may be useful to detect a therapeutic target for future research.

Taken together, these findings suggest that expression of cadherin-17 promotes the metastatic activity of the highly bone marrow metastatic breast cancer cells in our model, and that cadherin-17 expression may be a useful marker of bone marrow metastasis in breast cancer.

5. Conclusion

In this study, we concluded that the expression of cadherin-17 promotes the metastatic activity of bone marrow metastatic breast cancer cells and that cadherin-17 may be a useful marker of bone marrow metastasis in breast cancer.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Interaction of MRE11 and Clinicopathologic Characteristics in Recurrence of Breast Cancer: Individual and Cumulated Receiver Operating Characteristic Analyses

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The interaction between the meiotic recombination 11 homolog A (MRE11) oncoprotein and breast cancer recurrence status remains unclear. The aim of this study was to assess the interaction between MRE11 and clinicopathologic variables in breast cancer. A dataset for 254 subjects with breast cancer (220 nonrecurrent and 34 recurrent) was used in individual and cumulated receiver operating characteristic (ROC) analyses of MRE11 and 12 clinicopathologic variables for predicting breast cancer recurrence. In individual ROC analysis, the area under curve (AUC) for each predictor of breast cancer recurrence was smaller than 0.7. In cumulated ROC analysis, however, the AUC value for each predictor improved. Ten relevant variables in breast cancer recurrence were used to find the optimal prognostic indicators. The presence of any six of the following ten variables had a high (79%) sensitivity and a high (70%) specificity for predicting breast cancer recurrence: tumor size ≥ 2.4 cm, tumor stage II/III, therapy other than hormone therapy, age ≥ 52 years, MRE11 positive cells > 50%, body mass index ≥ 24, lymph node metastasis, positivity for progesterone receptor, positivity for epidermal growth factor receptor, and negativity for estrogen receptor. In conclusion, this study revealed that these 10 clinicopathologic variables are the minimum discriminators needed for optimal discriminant effectiveness in predicting breast cancer recurrence.

1. Introduction

Breast cancer is the most common cancer in women worldwide and is diagnosed in one in three of all women with cancer. Reported risk factors for breast cancer include age, family history, genetic specificity, and lifestyle [1–4]. Local and/or systematic treatments for breast cancer now enable a high survival rate, especially when breast cancer is diagnosed at an early stage [5]. However, breast cancer recurrence or metastasis (i.e., the spread of tumor cells from the original site) can reduce survival time [6].

Prognostic indicators of breast cancer complication and recurrence can be used to predict survival after diagnosis of breast cancer [7]. Estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) are reportedly accurate and independent prognostic

indicators of breast cancer recurrence risk [6], and combining these independent indicators can improve accuracy in predicting recurrence. Representative prognostic indicators can also be used to evaluate the effectiveness of adjuvant therapy and to estimate the risk of tumor recurrence [8].

Our previous study [9] reported the important role of meiotic recombination 11 homolog A (MRE11) in cell proliferation, tumor invasion, and DNA repair in patients with breast cancer. The MRE11 is considered an oncoprotein because it is overexpressed in colorectal cancer [10] and in highly malignant breast cancer [9]. However, no studies have evaluated the use of breast cancer tumor marker MRE11 as a diagnostic or prognostic indicator in breast cancer. Specifically, no studies have evaluated whether MRE11 interacts with clinicopathologic variables associated with breast cancer recurrence.

Receiver operating characteristic (ROC) analysis is widely used method of evaluating the performance of a diagnostic test according to a continuous spectrum of results [11, 12]. By graphically depicting the quantitative analysis results, the ROC curve reveals the discriminant thresholds based on the probability of positive results (sensitivity against 1 − specificity) in individual subjects [13]. The area under the curve (AUC) is a measure of the overall accuracy of the dichotomous methods of the measurements. Recently, ROC has been used as a tool for comparing the accuracy of various models for predicting cancer diagnosis, prognosis, and survival [14–20].

This study developed a scoring system based on ROC analysis to identify patient characteristics associated with susceptibility to breast cancer recurrence. Thus, aims of this study were (i) to assess breast cancer recurrence based on MRE11 expression and clinicopathologic characteristics and (ii) to identify the patient characteristics that are risk factors for breast cancer recurrence.

2. Methods

2.1. Study Participants. After obtaining IRB approval, this study enrolled 254 female breast cancer patients who had received surgical treatment for pathology-confirmed invasive ductal carcinoma at the Department of Surgery, Kaohsiung Medical University Hospital, during 2006–2010. Informed consent was obtained from all patients. The ethics statement, laboratory procedures, and other study procedures were identical to those in our previous study [9]. The dataset for all clinicopathological variables is available online at https://wp.kmu.edu.tw/changhw/files/2015/10/ROC_MRE11_DATASET.xlsx.

2.2. Criteria for Breast Cancer Recurrence. Patients with and without breast cancer recurrence were classified into a recurrence group and a nonrecurrence group, respectively. In the recurrence group, breast cancer recurrence was defined as a local/regional recurrence with or without distant metastasis diagnosed according to symptoms observed in clinical examination, pathology study, or imaging study. Patients who remained disease-free for 60 months after diagnosis or who were disease-free at the end of the follow-up period were classified into the nonrecurrence group.

2.3. Dichotomous Results of ROC Analysis for Each Clinicopathologic Variable. The 13 clinicopathologic variables included in the ROC analysis included MRE11 positive cells (%), tumor stage, tumor grade, age, body mass index (BMI), tumor size, lymph node (LN) metastasis, estrogen receptor (ER) status, progesterone receptor (PR) status, human epidermal growth factor receptor 2 (HER2) status, radiotherapy (RT), chemotherapy (CT), and hormone therapy (HT). First, the clinicopathologic variables were dichotomized by ROC curve analysis. The ROC curve is a graphical plot of the true positive rate (sensitivity) against the false positive rate (1 − specificity) at various threshold settings. Each cut-off point estimated by ROC analysis indicates the distinguishing characteristic of each clinicopathologic variable used to classify participants into the recurrence group. The area under the ROC curve (AUC) is used to calculate the accuracy of dichotomous results.

2.4. AUC of Cumulated ROC Analysis. A cumulated ROC analysis was performed to detect the combined effects of the clinicopathologic variables used to predict recurrence. Variables that had a strong association with recurrence (i.e., values larger than the cut-off point in AUC from the individually dichotomous results) received a score of 1. The remaining variables received a score of 0 (i.e., values less than the cut-off point in AUC from the individually dichotomous results). The indicators were then ranked by cumulated AUC results for individually dichotomous results. Positive changes in the cumulated AUC values for these variables were tracked until the addition of other variables no longer increased the AUC values. Accordingly, clinicopathologic variables that contributed to positive changes in AUC were selected for further analysis.

2.5. Cut-Off Point for Cumulated Scoring System. The cumulated scoring system was then used to rank the clinicopathologic variables as breast cancer predictors. The cumulated score for each subject was obtained by adding the risk factors to the recurrence score (1 or 0). The correctly classified rate for each possible cut-off point within the range of cumulated scores was dependent on the number of clinicopathologic variables selected for ROC analysis. For example, if "n" variables were selected according to a positive change in AUC value in the previous step, cumulated scores ranging from 0 to "n" were generated. The last step was calculating the specificity, sensitivity, and correctly classified rate for each cut-off point in the cumulated score range.

2.6. Risk Relationship of the Selected Variables in the Cumulated Scoring System. For each subject, the cumulated score represented the total number of clinicopathologic variables that were breast cancer risk factors. For instance, a score of 3 was interpreted as the presence of three risk factors that were more relevant to breast cancer recurrence compared to the selected clinicopathologic variables.

2.7. Statistical Analyses. Differences in the distributions of clinicopathologic variables between the recurrence and the nonrecurrence groups were estimated by frequency tables and the χ^2 test. The AUC represents the accuracy of the dichotomous results for a single clinicopathologic variable for predicting breast cancer recurrence. The dichotomous results with high AUC values were considered better predictors of breast cancer recurrence.

In cumulated ROC analysis, the likelihood ratio was used to assess recurrence status in subjects with different cumulated scores. The likelihood ratio for a positive test result [LR+: sensitivity/(1 − specificity)] represents the ratio of the probability of a positive test in the recurrence subjects to the probability of a positive test in the nonrecurrence subjects. Comparatively, the likelihood ratio for a negative test [LR−: (1 − sensitivity)/specificity] result represents the ratio of the probability of a negative test in the recurrence subjects to the probability of a negative test in the nonrecurrence subjects. All statistical analyses were performed by STATA version 11.0.

3. Results

3.1. Clinicopathologic Characteristics and Recurrence of Breast Cancer. During the 5-year follow-up period, recurrence developed in 34 (13.39%) of the 254 breast cancer patients. Table 1 compares clinicopathological variables between the recurrence and the nonrecurrence groups. Compared to the nonrecurrence group, the recurrence group had significantly larger proportions of patients with MRE11 positive cells > 50% (85.29%), breast cancer stage II/III (94.12%), age ≥ 52 years (67.65%), tumor size ≥ 2.4 cm (67.65%), LN metastasis (58.82%), negative expression of ER (58.82%), negative expression of PR (64.71%), triple negative breast cancer (35.29%), RT (79.41%), and no-HT treatment (61.76%).

3.2. AUC for Clinicopathologic Variables for Breast Cancer Recurrence. Table 2 shows the AUCs obtained when these 13 variables were considered in estimates of recurrence risk. The clinical criteria for high and low risk of breast cancer recurrence were identical to those used in our previous study [9]. Notably, although the dichotomized tumor size yielded the highest AUC value (0.679 with sensitivity of 0.677 and specificity of 0.682), it did not meet the criterion of AUC ≥ 0.7 [21] for classification of recurrence.

3.3. Cumulated ROC Analysis of Breast Cancer Recurrence. No single dichotomized variable showed satisfactory performance in predicting breast cancer recurrence (defined as AUC < 0.700). Therefore, this study developed an improved scoring system that considered the combined effects of these variables. Table 3 shows the ROC analysis results obtained for the developed scoring system with cumulated top-ranked predictors. The scoring system obtained a good AUC value (0.806) when 6 dichotomized variables (tumor size, tumor stage, ER, HT, LN metastasis, and age) were used. The AUC values showed further positive changes (range, 0.806 to 0.821) when the scoring system consisted of the cumulated top tenth rank of dichotomized variables (tumor size, stage, ER, HT, LN metastasis, age, PR, MRE11 positive cells, BMI, and HER2). When the number of variables exceeded ten cumulated top-ranked variables, however, the AUC value slightly decreased. That is, the scoring system obtained the best AUC values when ten dichotomized variables were used.

3.4. Cut-Off Point for Cumulated Scoring System. Hence, the performance of possible cut-off points ranging from score 0 to 10 were compared in the scoring system. Table 4 compares the results. For predicting recurrence, a cut-off point of 0 had a sensitivity of 100% (all recurrence patients correctly classified) but had a specificity of 0% (no recurrence patients correctly classified). In contrast, a cut-off point of 100% had a specificity of 10 (all nonrecurrence subjects correctly classified) but a sensitivity of 0 (no recurrence patients correctly classified). The cut-off point for 6 dichotomized variables had the highest sensitivity and the highest specificity. This cut-off point correctly classified 71.3% with the best combination of sensitivity (0.794) and specificity (0.700; LR+ 2.647 and LR− 0.294).

3.5. Risk Relationships of the Selected Variables in the Cumulated Scoring System. Table 5 lists the clinicopathologic variables that contributed the five largest possible changes in AUC values (0.799 to 0.821) in Table 3. The scores for 10 dichotomized variables were then computed into these five clinicopathologic variables as indicated in Table 5. In the 254 patients analyzed, scores were ≤ 5 in most (63.39%; 161) patients. In the 161 patients with scores of ≤ 5, most (63.98%, 103) patients were HER2 negative. The score was 6 in 16.93% (43/254) of the patients. In patients with a score of 6, most (74.42%; 32/43) had BMI ≥ 24. The score was 7 in 9.45% (24/254) of the patients. In patients with a score of 7, all (100.00%) had BMI ≥ 24. The score was 8 in 6.69% (17/254) patients. In patients with a score of 8, 88.24% (15) had BMI ≥ 24. The score was 9 in 3.15% (8/254) patients. All patients who had a score of 9 were negative for PR expression and had a BMI ≥ 24. Only 0.39% (1/254) patients had a score of 10, that is, high values for all five clinicopathologic variables that were risk factors for breast cancer recurrence.

4. Discussion

Conventional statistical methods used to estimate probability of breast cancer recurrence include logistic regression, Cox-proportional hazard regression model, Kaplan-Meier estimator, and log-rank test [22]. Our previous work using similar statistical methods revealed that MRE11 is associated with breast cancer malignancy [9]. However, possible interactions between MRE11 and clinicopathologic variables for breast cancer recurrence have not been reported in the literature.

An ROC analysis is a simple and powerful approach to discriminant analysis. In this study, dichotomization of clinicopathologic variables by AUC enabled quick and easy differentiation of variables associated with high and low recurrence risk. However, variations in breast cancer recurrence are rarely affected by a single factor. Similarly, we found

Table 1: Clinicopathologic characteristics of breast cancer patients in recurrence status[*1].

Variable	No recurrence ($n = 220$)		Recurrence ($n = 34$)		P
	N	%	N	%	
MRE11 positive cells					0.030
≤50%	73	33.18	5	14.71	
>50%	147	66.82	29	85.29	
Stage					<0.001
I	88	40.00	2	5.88	
II, III	132	60.00	32	94.12	
Grade					0.543
1, 2	166	75.45	24	70.59	
3	54	24.55	10	29.41	
Age					0.007
<52 yrs	126	57.27	11	32.35	
≥52 yrs	94	42.73	23	67.65	
BMI (kg/m^2)					0.151
<24	126	57.27	15	44.12	
≥24	94	42.73	19	55.88	
Tumor size (cm)					<0.001
<2.4 cm	150	68.18	11	32.35	
≥2.4 cm	70	31.82	23	67.65	
LN metastasis					0.003
Negative	149	67.73	14	41.18	
Positive	71	32.27	20	58.82	
ER					<0.001
Negative	63	28.64	20	58.82	
Positive	157	71.36	14	41.18	
PR					0.009
Negative	90	40.91	22	64.71	
Positive	130	59.09	12	35.29	
HER2 status					0.430
Negative	140	63.64	24	70.59	
Positive	80	36.36	10	29.41	
Triple negative					0.002
No	190	86.36	22	64.71	
Yes	30	13.64	12	35.29	
RT					0.021
No	91	41.36	7	20.59	
Yes	129	58.64	27	79.41	
CT					0.866
No	30	13.64	5	14.71	
Yes	190	86.36	29	85.29	
HT					0.001
No	72	32.73	21	61.76	
Yes	148	67.27	13	38.24	

[*1]Dataset was retrieved from our previous study [9]. MRE11: meiotic recombination 11; BMI: body mass index; LN: lymph node; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; RT: radiotherapy; CT: chemotherapy; HT: hormone therapy.

that the contribution of each variable may be too weak (<0.7) in terms of AUC.

A recent study developed a cumulated ROC analysis strategy for assessing outcomes of orthodontic surgery [23] and for diagnosing metastasis in breast cancer [24] and other cancer types [25, 26]. In our study, a similar scoring system was applied in multivariate cumulated ROC analysis to evaluate diagnostic indicators of breast cancer recurrence. Table 3 shows that 10 variables were the minimum number of discriminators required to obtain the optimum discriminant

Table 2: AUC of clinicopathologic characteristics for recurrence status[*1].

Variable	AUC	High risk	Low risk	Sensitivity	Specificity
Tumor size (cm)	0.679	≥2.4 cm	<2.4 cm	0.677	0.682
Stage	0.671	II, III	I	0.941	0.400
ER	0.651	Negative	Positive	0.588	0.714
HT	0.645	No	Yes	0.618	0.673
LN metastasis	0.633	Positive	Negative	0.588	0.677
Age	0.625	≥52 yrs	<52 yrs	0.677	0.573
PR	0.619	negative	positive	0.647	0.591
MRE11 positive cells	0.592	>50%	≤50%	0.853	0.332
BMI	0.566	≥24	<24	0.559	0.573
HER2 status	0.535	Negative	Positive	0.706	0.364
Grade	0.524	3	1, 2	0.294	0.755
CT	0.505	No	Yes	0.147	0.864
RT	0.396	No	Yes	0.206	0.586

[*1]Data for high/low risks of breast cancer recurrence were retrieved from our previous study [9]. AUC: area under receiver operating characteristic; ER: estrogen receptor; HT: hormone therapy; LN: lymph node; PR: progesterone receptor; MRE11: meiotic recombination 11; BMI: body mass index; HER2: human epidermal growth factor receptor 2; CT: chemotherapy; RT: radiotherapy.

Table 3: Cumulated top-ranked prediction results using ROC analysis[*1].

Cumulated top-ranked variables	Variables	AUC
2	Tumor size and stage	0.724
3	Above variables plus ER	0.771
4	Above variables plus HT	0.765
5	Above variables plus LN metastasis	0.790
6	Above variables plus age	0.806
7	Above variables plus PR	0.800
8	Above variables plus MRE11 positive cells	0.799
9	Above variables plus BMI	0.810
10	Above variables plus HER2	0.821
11	Above variables plus grade	0.806
12	Above variables plus CT	0.799
13	Above variables plus RT	0.774

[*1]Dataset and high/low risks of breast cancer recurrence were retrieved from our previous study [9]. ER: estrogen receptor; HT: hormone therapy; LN: lymph node; PR: progesterone receptor; BMI: body mass index; CT: chemotherapy; RT: radiotherapy.

Table 4: Cut-off point identified by ROC analysis[*1].

Number of dichotomized variables[*2]	Sensitivity	Specificity	Sensitivity + specificity	Correctly classified	LR+	LR−
0	1.000	0.000	1.000	0.134	1.000	—
1	1.000	0.027	1.027	0.158	1.028	0.000
2	1.000	0.073	1.073	0.197	1.078	0.000
3	1.000	0.182	1.182	0.291	1.222	0.000
4	0.941	0.332	1.273	0.413	1.409	0.177
5	0.912	0.468	1.380	0.528	1.714	0.189
6	0.794	0.700	1.494	0.713	2.647	0.294
7	0.618	0.868	1.486	0.835	4.686	0.440
8	0.382	0.941	1.323	0.866	6.471	0.656
9	0.177	0.986	1.163	0.878	12.941	0.835
10	0.029	1.000	1.029	0.870	—	0.971

LR+: likelihood ratio for a positive test result; LR−: likelihood ratio for a negative test result.
[*1]Dataset was retrieved from our previous study [9].
[*2]The number of dichotomized variables was the cumulated effects of the various clinicopathologic variables from Table 3, including tumor size, stage, ER, HT, LN metastasis, age, PR, BMI, MRE11 positive cells, and HER2.

Table 5: Risk relationship of scores with selected variables[*1].

Score[*2]	Total	Age ≥ 52 yrs (n = 117)		PR negative (n = 112)		MRE11 positive cells > 50% (n = 113)		BMI ≥ 24 (n = 176)		HER2 negative (n = 164)	
	N	N	%	N	%	N	%	N	%	N	%
≤5	161	51	31.68	47	29.19	61	37.89	96	59.63	103	63.98
6	43	27	62.79	27	62.79	20	46.51	32	74.42	27	62.79
7	24	18	75.00	16	66.67	13	54.17	24	100.00	14	58.33
8	17	13	76.47	13	76.47	12	70.59	15	88.24	13	76.47
9	8	7	87.50	8	100.00	6	75.00	8	100.00	6	75.00
10	1	1	100.00	1	100.00	1	100.00	1	100.00	1	100.00

PR: progesterone receptor; MRE11: meiotic recombination 11; BMI: body mass index; HER2: human epidermal growth factor receptor 2.

[*1]Dataset was retrieved from our previous study (n = 254) [9].

[*2]Cumulated score representing the number of risk properties of the selected clinicopathologic variables in the subjects. The selected clinicopathologic variables included tumor size, tumor stage, ER, HT, LN metastasis, age, PR, BMI, MRE11 positive cells, and HER2.

effectiveness. Table 4 further shows that a cut-off point of 6 had the best combination of sensitivity and specificity for predicting breast cancer recurrence. These results suggest that the cumulated ROC analysis strategy also improves accuracy in predicting breast cancer recurrence.

Moreover, these data suggest that both gene-environment and environment-environment interactions have important roles in predicting recurrence when clinicopathologic variables are regarded as the environmental factors. Similar interactions between these variables have been reported. For example, ER and HER2 are reportedly both interdependent and independent prognostic indicators of breast cancer recurrence [27]. The Nottingham prognostic index considers tumor size, lymph node metastasis, and tumor grade to obtain an estimate of recurrence risk in patients with breast cancer [28, 29]. In contrast, the breast cancer severity score (BCSS) is a prognostic scoring system based on tumor size, number of metastatic lymph nodes, and HER2 status [30]. The BCSS may be the best predictor of both overall survival and disease-free survival. A common feature of these prognostic scoring systems is that all of the breast cancer characteristics mentioned in the current study are considered simultaneously.

Comparisons of these variables further showed that most subjects with a high risk of breast cancer recurrence had a high BMI (Table 5), which is consistent with reports that high BMI is associated with aggressive tumor characteristics in premenopausal [31] and postmenopausal women [32]. Another randomized trial showed that lymph node metastasis, ER-negativity, and HER2 negativity are associated with breast cancer risk and prognosis [33]. Breast cancer patients with the triple negative subtype (i.e., negativity for ER, PR, and HER2) have a high risk of disease progression [34]. Supplementary Table 1 (in Supplementary Material available online at https://doi.org/10.1155/2017/2563910) shows that MRE11 expression did not significantly differ among ER-positivity, ER-negativity, PR-positivity, or PR-negativity in the current study. Supplementary Table 2 further shows that BMI level did not significantly differ among ER-positivity, ER-negativity, PR-positivity, PR-negativity, HER2-positivity, or HER2-negativity.

Some limitations of this study should be noted. First, our previous work [9] suggested a survival analysis of the current dataset using Cox-proportional hazard model, Kaplan-Meier curve, and log-rank test. However, survival was not considered in the follow-up analyses of subjects in the current study. Another limitation is that, although the cumulated ROC analysis revealed potential joint effects of the selected clinicopathologic variables, the complex interactions of all possible combinations of clinicopathologic variables were not analyzed because the cumulated ROC was initially based on the highest ranked AUC for a single variable. In subsequent analyses, the variable with the lowest AUC was not considered. Moreover, this study did not validate the signature identified by ROC analysis in a testing dataset. Therefore, the use of a testing dataset and intelligent computational algorithms [35–44] is warranted in future studies of the complex high-order interactions between these clinicopathologic variables and breast cancer recurrence.

5. Conclusions

This study evaluated the contributions of 13 clinicopathologic variables in predicting breast cancer recurrence. In individual ROC analysis, each variable had a weak AUC for predicting breast cancer recurrence. In cumulative ROC analysis, however, each variable had an improved AUC. Finally, this study revealed that 10 clinicopathologic variables is the minimum number of discriminators needed for optimum accuracy in predicting breast cancer recurrence by discriminant analysis.

Competing Interests

The authors have no competing interests to declare.

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Research Article

Basic Parameters of Blood Count as Prognostic Factors for Renal Cell Carcinoma

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Background. Renal cell carcinoma is the most common type of kidney cancer. Taking account of morbidity and mortality increase, it is evident that searching for independent prognostic factors is needed. *Aim of the Study*. The aim of the study was to analyze routinely performed blood parameters as potential prognostic factors for kidney cancer. *Material and Methods*. We have retrospectively reviewed the records of 230 patients treated for renal cell carcinoma in the years 2000–2006. Preoperative blood parameters, postoperative histopathological results, and staging and grading were performed. To estimate the risk of tumor recurrence and cancer specific mortality (CSM) within five years of follow-up, uni- and multivariate Cox and regression analyses were used. To assess the quality of classifiers and to search for the optimal cut-off point, the ROC curve was used. *Results*. T stage of the tumor metastasis is the most important risk factor for early recurrence and cancer specific mortality ($p < 0.001$). The preoperative platelet count (PLT) above 351×10^3/uL (95.3%; 55.1%) and AUC of 77% are negative prognostic factors and correlate with increased cancer specific mortality (CSM) during the five-year follow-up ($p < 0.001$). Increased risk of local recurrence was observed for PLT above 243.5×10^3/ul (59%; 88%) and AUC of 80% ($p = 0.001$). The opposite was observed in the mean platelets volume (MPV) for cancer specific mortality (CSM). The cut-off point for the MPV was 10.1 fl (75.4%; 55.1%) and for the AUC is of 68.1% ($p = 0.047$). *Conclusions*. Many analyzed parameters in univariate regressions reached statistical significance and could be considered as potential prognostic factors for ccRCC. In multivariate analysis, only T stage, platelet count (PLT), and mean platelet volume (MPV) correlated with CSM or recurrent ccRCC.

1. Background

The incidence of kidney cancer has increased in recent years and has now reached about 2-3% of malignant tumors in adults. Renal cell carcinoma is the third most common cancer of the urinary tract [1–3]. Kidney cancer detected accidentally is found in approximately 1.5% of cases at autopsy [4]. Over 80% of all malignant kidney tumors are renal cell carcinoma (RCC). RCC clear cell type (otherwise known as conventional or classic) most commonly originates from the proximal nephron (in over 80% of cases).

The main treatment modality for renal cancer is surgery. Kidney tumors, in particular RCC tumors, are poorly sensitive to chemotherapy or radiotherapy; only the currently used targeted therapy achieves temporarily stabilizing effect in 30–40% of cases. Risk factors for kidney cancer include smoking, obesity, diuretics, phenacetin, exposure to cadmium and asbestos, high protein diet, and chronic dialysis.

The main prognostic factors are grade and stage of tumor and cellular anaplasia, tumor histology, the presence of vascular microinvasion, and the content of the genetic material in the cell analyzed by flow and static cytometry (DNA ploidy). Molecular prognostic factors are listed among the prognostic factors; however, their major drawbacks are cost and low availability [5–13].

2. Aim of the Study

The following study aims to answer whether preoperative determination of basic blood cell count can be used as a popular and inexpensive prognostic factor in the treatment of renal cell carcinoma limited to the organ.

3. Materials and Methods

The study was retrospective and used the medical records of 397 patients who underwent surgery for kidney tumors in our center in the years 2000–2006. During the five-year follow-up period of all 397 patients operated on, 230 patients with the postoperative diagnosis of nonmetastatic renal cell carcinoma were enrolled in the study. The median follow-up was 3,98 ± 0,99 years. According to the inclusion criteria of the study, patients with coexisting conditions that may have an impact on the blood morphology parameters were excluded. On the basis of the information gathered, routinely collected blood count parameters at the time of admission to the ward were analyzed. We analyzed the data on histopathological material, histology, TNM, size and location of the tumor removed, and the positive margin in the case of the nephron sparing surgery (NSS). Histopathologic tumor grade was rated according to the three-degree Fuhrman scale. In order to determine the clinical stage, given data were analyzed according to the TNM classification from 2010.

The studies were an attempt to determine whether any of the tested parameters can be used to assess the prognosis of kidney cancer, so that a group of patients that require special oncological surveillance can be selected. All the information gathered was analyzed in relation to the local recurrence, metastasis, and tumor-specific deaths. Statistical analysis was performed using the program R [R Core Team (2012)]. For determining the risk of metastasis, tumor recurrence, or death within five years, Cox proportional hazards model was used. To assess the quality of the classifier and the search for the optimal cut-off point, ROC (Receiver Operating Characteristic) curve was used as a tool to describe the overall sensitivity and specificity for the variability characteristics of the classifier.

The examination protocol was approved by the Bioethical Commission of the Medical University of Silesia, decision number KNW/0022/KB215/12.

4. Results

The study group included 123 men and 107 women. The average age was 60.9 ± 10.5 years. Tumor size was 6.8 ± 4.1 cm. 89 patients underwent nephron sparing surgery (NSS) (mean tumor size was 4.16 cm), and 141 patients underwent radical nephrectomy (mean tumor size was 7.9 cm). Local recurrence during follow-up occurred in 25 patients (10.8%) and distant metastases were found in 22 patients (9.5%). Tumor-specific death was reported in 6 cases after the NSS (2.6%) and in 43 (18.6%) after radical nephrectomy. There was an almost identical distribution of neoplastic changes, taking into account the side operated on, 113 in the right and 117 in the left kidney. We also evaluated the anatomical location of the tumor. The most frequently observed tumors were located on the dorsal side of the kidney, followed by the lower and upper pole. In the central part of the kidney, 7 tumors were detected (Figure 1).

The lowest clinical stage, T1, was observed in 112 patients (T1a-48, T1b-64), T2 in 60 patients (T2a-33, T2b-27), and T3 in 54 patients (T3a-50, T3b-4), and the most advanced type of cancer, T4, was found in 4 patients.

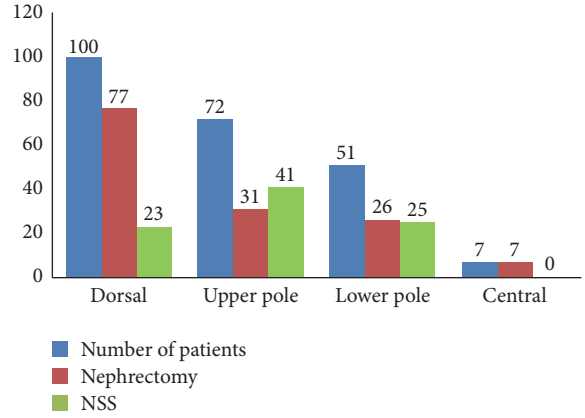

Figure 1: Anatomical location of the tumor according to the performed procedure.

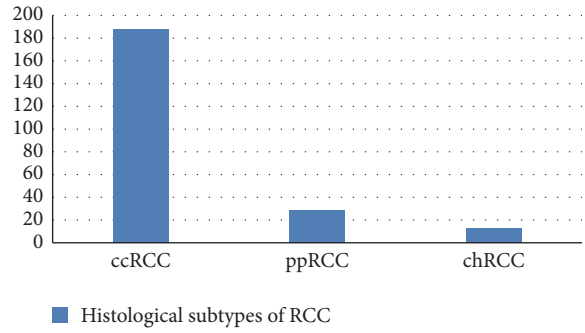

Figure 2: Histological subtypes of RCC in the examined group.

Histological subtypes of RCC in the examined group are presented in Figure 2.

Average values of the analyzed blood parameters are presented in Table 1.

For determining the risk of metastasis, tumor recurrence, or death, Cox proportional hazards model was used. In this statistical regression, complete and censored data were taken into account. Relatively, the highest number of medical, physical, chemical, and biological risk factors is assigned in this analysis to patients' death, followed by tumor recurrence and metastasis. Statistically significant results ($p < 0.05$) in the univariate model are summarized in Table 2.

In terms of the impact of a single risk factor, it can be said that the location of the tumor has a strong statistical relationship with the occurrence of metastasis. Cox regression results relating to the location of the tumor in the lower, upper, or dorsal pole compared to the central location are associated with a significant decrease in the risk of metastasis, respectively, $(1 - 0.096) * 100\% = 90\%$, $(1 - 0.084) * 100\% = 91.6\%$, and $(1 - 0.174) * 100\% = 82\%$. It was also observed that the decline in the value of the studied blood count parameters with the exception of platelet count (PLT) and MPV is an unfavorable prognostic factor ($p < 0.05$).

Table 1: Average values of the analyzed blood parameters.

RBC [$10^6/\mu$L]	HGB [g/dL]	HCT [%]	MCV [fL]	MCHC [g/dL]	MCH [pg]	RDW [%]	PLT [$10^3/\mu$L]	MPV [fL]
4,5 ± 0,5	13,4 ± 1,8	39,2 ± 4,5	87,08 ± 5,9	34,0 ± 2,0	30,6 ± 2,6	14.7 ± 1.9%	249,0 ± 87,5	11,0 ± 1,6

Table 2: Univariate Cox regression. Statistically significant parameters predictive of metastasis, recurrence, and death specifically due to renal cell carcinoma.

AD	AP	Regression coefficient	SE	HR	p value	N	Number of events
Metastasis	NSS/NF	1,550	0,744	4,710	0,0370	230	21
	Tumor location C (ref.)	0,000		1,000		230	21
	Location L	−2,340	0,764	0,096	0,0022		
	Location U	−2,470	0,817	0,084	0,0025		
	Location D	−1,750	0,652	0,174	0,0073		
	T	0,525	0,141	1,690	0,0002	230	21
	PLT [thousand/μL]	0,005	0,002	1,010	0,0030	230	21
Recurrence	Tumor size (cm)	0,088	0,030	1,090	0,0027	230	25
	G	0,626	0,281	1,87	0,0260	230	25
	T	0,426	0,123	1,530	0,0005	230	25
	HGB [g/dL]	−0,397	0,102	0,672	0,0001	230	25
	HCT [%]	−0,121	0,052	0,886	0,0200	230	13
	MCV [fL]	−0,113	0,026	0,893	0,0000	230	25
	MCH [pg]	−0,196	0,065	0,822	0,0025	230	25
	PLT [thousand/μL]	0,008	0,001	1,010	0,0000	230	25
Death	NSS/NF	1,280	0,436	3,600	0,0033	230	49
	Tumor size (cm)	0,101	0,020	1,110	0,0000	230	49
	G	0,704	0,202	2,020	0,0005	230	49
	T	0,510	0,089	1,670	0,0000	230	49
	HGB [g/dL]	−0,356	0,077	0,700	0,0000	230	49
	HCT [%]	−0,114	0,038	0,892	0,0024	230	27
	MCV [fL]	−0,105	0,019	0,900	0,0000	230	49
	MCHC [g/dL]	−0,148	0,057	0,863	0,0098	230	49
	MCH [pg]	−0,206	0,049	0,814	0,0000	230	49
	RDW [%]	0,155	0,056	1,170	0,0056	230	49
	PLT [thousand/μL]	0,009	0,001	1,010	0,0000	230	49
	MPV [fL]	−0,375	0,093	0,687	0,0001	230	49

AE: adverse event; AP: analyzed parameters; HR: hazard ratio; SE: standard error; location C:central location of the tumor; location L: tumor in the lower pole of the kidney; location U: tumor in the upper pole of the kidney; location D: tumor in the dorsal part of the kidney; T: tumor scale; G: Fuhrman scale; PLT: platelets count; HGB: hemoglobin level; HCT: hematocrit; MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration; MCH: mean corpuscular hemoglobin; RDW: red blood cell distribution width; MPV: mean platelet volume; NSS: nephron sparing surgery; NF: nephrectomy.

The results of the multivariate Cox regression are summarized in Table 3. Based on this analysis, factors correlated with metastasis have not been found. In this analysis, as in the logistic regression, low predictive value of single blood parameters was revealed. Charts of proportional survival rates of patients according to the number of platelets are presented in Figure 3.

In a Cox multivariate analysis, as in the logistic regression, low predictive value of individual morphology parameters was revealed. Graphs of proportional survival depending on the number and volume of the platelets are shown in Figures 3 and 4.

After analyzing the quality of classifiers (suspected risk factors) in the range of analyzed traits using the ROC curve, there was no statistically significant factor determining the increased risk of metastasis in patients. The number of statistically significant classifiers defining the occurrence of relapse and death is roughly the same. The most likely event is predicting the case of tumor recurrence using the platelet count (PLT). For the cut-off point of 243.5 platelet units (specificity 59%, sensitivity 88%), the likelihood of correctly predicting an event is up to 80% (AUC) (Figure 5). In the case of tumor size (cut-off point of 7.5 cm), HGB (cut-off point of 11.6 g/dL), MCV (cut-off point of 86.0 fL), and MCH (30.2 pg cut-off point), the likelihood of correctly predicting recurrence within five years of follow-up was 64%, 68%, 73%, and 67%, respectively. A similar analysis of potential predictive factors for death was carried out. In this case, we have a 68% likelihood of occurrence with a cut-off point at 7.5 cm (specificity 78.9%, sensitivity 49%). The best indicator

Table 3: Multivariate Cox regression. Statistically significant parameters predictive of recurrence and death specifically due to renal cell carcinoma.

AD	AP	Regression coefficient	SE	HR	p value	N	Number of events
Recurrence	T	0,288	0,129	1,334	0,0250	230	25
	PLT [thousand/μL]	0,006	0,002	1,006	0,0006		
Death	T	0,289	0,114	1,340	0,0110	230	49
	PLT [thousand/μL]	0,007	0,002	1,010	0,0000		
	MPV [fL]	−0,261	0,132	0,770	0,0470		

AE: adverse event; AP: analyzed parameters; HR: hazard ratio; SE: standard error; T: tumor scale; PLT: platelets count; MPV: mean platelet volume.

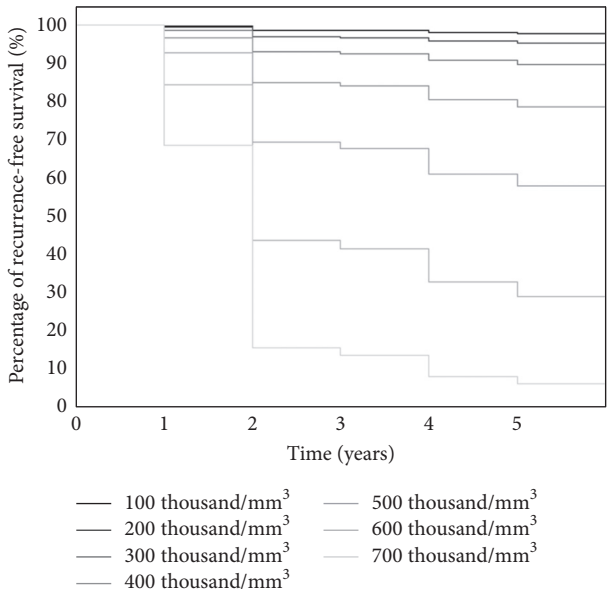

Figure 3: Estimated recurrence-free survival according to the number of platelets.

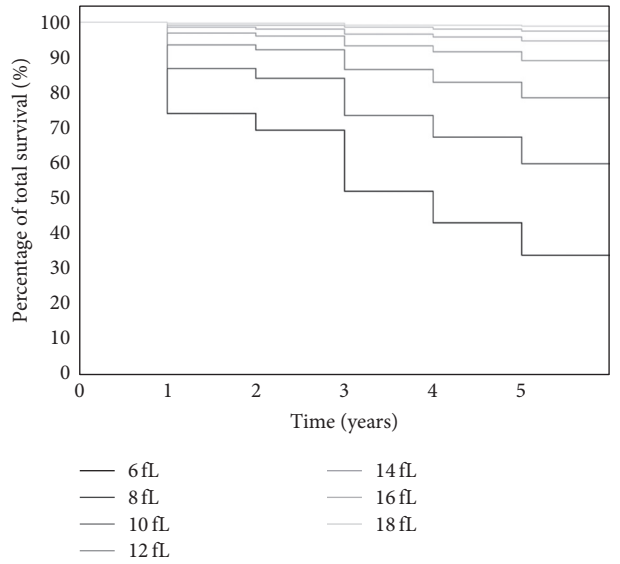

Figure 4: Expected total survival relative to the mean platelet volume.

in this case appears to be the platelet count. The probability of death within a specific five-year follow-up was 77.7% at a cut-off point at 351 thousand/μL. The specificity and sensitivity of this classifier are 95.3% and 55.1% (Figure 6). The worst indicator in terms of predicting mortality is MCH; the likelihood of predicting the correct event using this marker is less than 64% at a specificity and sensitivity of 77% and 53%.

5. Discussion

Despite advances in medicine, an increase in morbidity and mortality due to kidney cancer is still observed. The diversity of kidney cancers does not enable unambiguous determination of prognosis. Given that 30% of patients with disease confined to the organ will develop generalized disease after surgical treatment, it is important to search for prognostic factors. Numerous reports of treatment of erythropoietin (EPO) level elevation in patients with renal cancer prompted us to search the potential prognostic factors among blood counts.

An important factor in the tumor environment is hypoxia, which induces tumor cell defense mechanisms. One of these mechanisms is the increase in the transcription of hypoxia inducible factor (HIF) composed of two subunits (HIF-α and HIF-β). High expression of HIF-α subunit is determined mainly by low levels of intracellular oxygen. Under normal oxygenation, the HIF-α subunit is rapidly degraded by normally acting protein VHL (pVHL), forming ubiquitin ligase complex pVHL-E3. In the case of VHL gene mutation, the active inhibitory protein of subunit HIF-α is not produced. This leads to the stabilization and accumulation of HIF-α in cells and contributes to the formation of multiple hypoxia inducible factors that promote angiogenesis, drug resistance, and tumor progression [14, 15]. One such factor is erythropoietin (EPO). Under normal conditions, the cells of the proximal portion of the coil, from which the clear cell renal carcinoma originates, do not produce detectable amounts of EPO, even under hypoxic conditions. This may explain the importance of VHL gene mutation in the development of clear cell RCC [16, 17]. Erythropoietin is the primary regulator of erythropoiesis in tissues involved in the production of red blood cells. In other tissues, especially in cancer cells, it inhibits apoptosis, stimulates angiogenesis, and promotes cell proliferation. Many studies have confirmed the overexpression of EPO and its receptor (EPOR) in clear

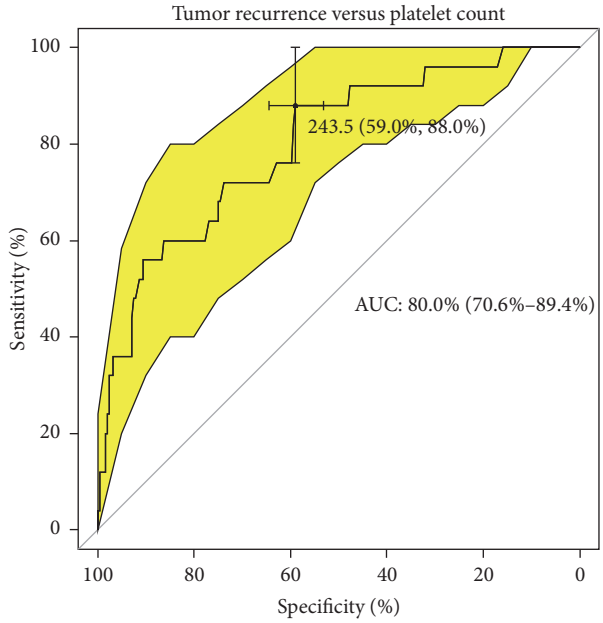

Figure 5: Upward sloping quality classifier of tumor recurrence versus platelet count.

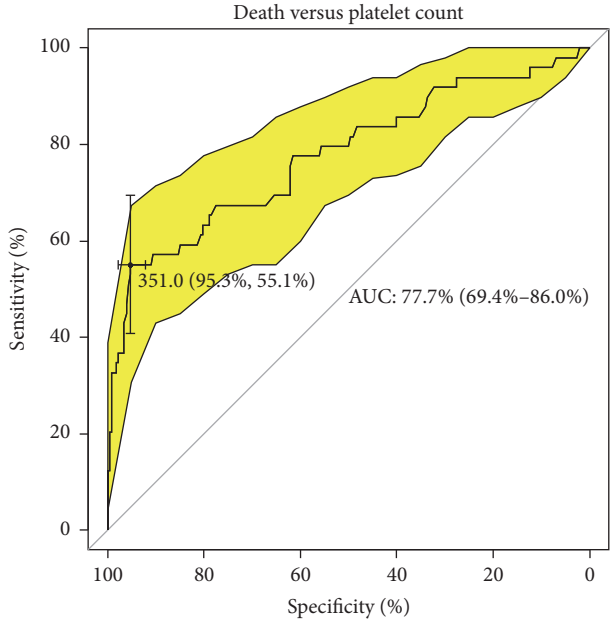

Figure 6: Upward sloping classifier of death versus platelet count.

cell renal carcinoma. The prognostic value of this finding merits further investigation [18–20].

If EPO level elevation is observed in patients with renal cell cancer, it would appear that we should watch for polycythemia in these patients. Clinical practice however demonstrates that this is not in fact the case. Despite the increase in the expression of EPO and EPOR, in up to 35% of patients with RCC, anemia is observed and only 1–5% of patients develop paraneoplastic polycythemia. Why this happens is not entirely clear. Attempts for explanation include the weak activity of EPO produced by the tumor and the low sensitivity to this factor of the tissues involved in the production of blood. The low level and abnormal iron metabolism may play an important role. The breakdown of tumor cells in the microvessels has also been described. Growth of inflammatory factors and antibodies that occurs during carcinogenesis may contribute to autoimmune hemolysis. Among the preoperative parameters associated with the red blood cells system, the erythrocyte count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, the rate of the average weight of corpuscular hemoglobin, and the coefficient of variation of volume distribution of erythrocytes (anisocytosis) were analyzed in the study. It has been proven that lowering the value of all the above-mentioned parameters except anisocytosis below a specified cut-off point results in an increased risk of tumor-specific death within the five years of follow-up. Reduced preoperative values of HGB, HCT, MCV, and MCH also predispose patients to early recurrence of cancer. Similar results were also obtained by other authors [21–24].

Our study failed to confirm the prognostic value of hemoglobin concentration in the univariate analyses. Multivariate logistic regression and multivariate Cox proportional hazard model failed to demonstrate a correlation between this factor and tumor recurrence or death or the occurrence of metastases. To date, there are no reports in the literature on the prognostic value of the parameters relating to the construction of red blood cells. Thrombocytosis as a paraneoplastic syndrome is widely described in the literature. Elevated blood platelets may be accompanied by kidney cancer, among other conditions. Thrombocytosis in this case is most likely associated with the neuroendocrine activity of tumor cells. Production of many types of cytokines, including IL-6, IL-11, thrombopoietin, and GM-CSF (granulocyte/macrophage colony stimulating factor), results in increased production of thrombocytes. The main role is played in this case by IL-6, which stimulates platelet production by stimulating the maturation of megakaryocytes [25]. There is evidence on the protective effect of platelets in relation to circulating tumor cells. They hinder their recognition by the immune system and facilitate their integration into the endothelium [26]. Platelets probably also affect tumor growth by producing growth factors such as VEGF, PDGF, hepatocyte growth factor, thrombospondin, or endostatin [27].

In research studies so far, many authors have confirmed that preoperative thrombocytosis is an unfavorable prognostic factor for renal cell carcinoma [28–33].

These reports based on a relatively small number of patients confirm the high value of the platelet count as an independent predictor for renal cell carcinoma. Less enthusiastic towards thrombocytosis and preoperative anemia are Karakiewicz and colleagues, who demonstrated in the single and multivariable analyses that these parameters correlate with the increase of cancer specific mortality. However, the predictive value of these parameters (0.3%) is lower than the factors associated with the anatomy of the tumor (TNM, G) or the patient's ECOG scale [34]. Similar conclusions have been shown by German authors [35].

Our analyses have shown that the increase in platelet count is a negative prognostic factor in patients undergoing surgery for renal cell carcinoma. Statistical significance was confirmed in the single and multidimensional analyses. In addition, the predictive value of the volume of platelets was confirmed to have statistical significance. The combination of high platelet counts with their small volume is characteristic for cancer diseases. To date, no reports on the predictive value of MPV in renal cancer have been published.

Previous studies looking for new, readily available prognostic factors do not constitute a breakthrough and only improve the predictive value of factors related to tumor stage and anatomy. It is estimated that the parameters of blood count and biochemical parameters may constitute independent prognostic factors for renal cell carcinoma, but they are not qualitatively superior to the TNM classification. It should also be remembered that all the evaluated morphological and biochemical parameters of blood are characterized by low repeatability and are dependent on many factors.

6. Conclusions

(1) Low preoperative hemoglobin concentration, hematocrit, average weight of hemoglobin, mean volume, and mean hemoglobin concentration may be considered in the category of risk factors for recurrence and progression of renal cell carcinoma after surgical treatment.

(2) The best predictor among the analyzed preoperative laboratory parameters is the platelet count and volume.

(3) Preoperative platelet count above 243.5 thousand/uL and 351 thousand/uL, respectively, is an independent predictor of recurrence and tumor-specific death during the five years of follow-up.

(4) Preoperative average size of platelets less than 10.1 fL is an independent predictor of tumor-specific mortality during the five-year follow-up.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Review Article

The Role of BRCA2 Mutation Status as Diagnostic, Predictive, and Prognosis Biomarker for Pancreatic Cancer

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Pancreatic cancer is one of the deadliest cancers worldwide, and life expectancy after diagnosis is often short. Most pancreatic tumours appear sporadically and have been highly related to habits such as cigarette smoking, high alcohol intake, high carbohydrate, and sugar consumption. Other observational studies have suggested the association between pancreatic cancer and exposure to arsenic, lead, or cadmium. Aside from these factors, chronic pancreatitis and diabetes have also come to be considered as risk factors for these kinds of tumours. Studies have found that 10% of pancreatic cancer cases arise from an inherited syndrome related to some genetic alterations. One of these alterations includes mutation in *BRCA2* gene. *BRCA2* mutations impair DNA damage response and homologous recombination by direct regulation of RAD51. In light of these findings that link genetic factors to tumour development, DNA damage agents have been proposed as target therapies for pancreatic cancer patients carrying *BRCA2* mutations. Some of these drugs include platinum-based agents and PARP inhibitors. However, the acquired resistance to PARP inhibitors has created a need for new chemotherapeutic strategies to target *BRCA2.* The present systematic review collects and analyses the role of *BRCA2* alterations to be used in early diagnosis of an inherited syndrome associated with familiar cancer and as a prognostic and predictive biomarker for the management of pancreatic cancer patients.

1. Introduction

In 1994, *BRCA2* (breast cancer gene 2) was located in chromosome 13ql2-13 by the group led by Wooster et al. [1]. Transmission of this gene follows an autosomal dominant pattern with incomplete penetrance [2]. Soon thereafter, *BRCA2* was reported as a tumour suppressor gene based on evidence of loss of heterozygosity in 7 out of 8 familial breast cancers [3]. Subsequently, *BRCA2* was associated with high-risk breast and ovarian cancer with a large component of heritability [4–7], although the risk for ovarian cancer due to *BRCA2* is much lower than the risk associated with *BRCA1* [8].

Only one year after this gene was discovered, the association between *BRCA2* and pancreatic cancer was assessed by Schutte et al. [9]. It was found that pancreatic cancer appeared in some individuals with a history of familial breast cancer associated with *BRCA2* alterations [10]; thus, it was estimated that 10% of cases of pancreatic cancer have an underlying inherited component [11, 12].

Worldwide pancreatic cancer incidence has increased from 185,000 in the 1980s [13] to 227,000 cases per year in 2014 [14]. In 2007, the highest incidence of pancreatic cancer was in the Baltic countries and central and eastern Europe. In northern European countries and the UK, this cancer has risen over most recent years and is rising in countries of southern, central, and eastern Europe [15]. It is hypothesised that this increase could be associated with increased consumption of high-sugar or carbohydrate-rich foods [16] or simply reflects the ageing of the population in recent decades.

Nowadays, the primary acquired risk factors for pancreatic cancer are cigarette smoking (HR = 1.74), high alcohol consumption (HR = 1.1–1.5), obesity (body mass index > 30; HR = 1.2–1.5), and some infectious diseases that include *Helicobacter pylori (HR = 1.5)*, *Hepatitis B virus,* or *Human Immunodeficiency virus* [17–19]. Interestingly, other studies suggested that heavy consumption of cooking and table salt appeared to be significantly associated with pancreatic cancer

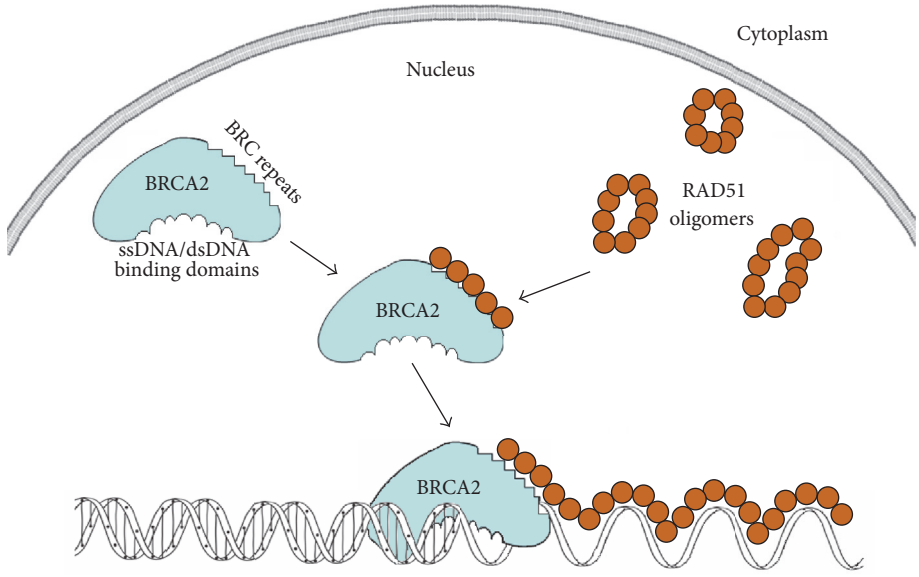

Figure 1: DNA damage response model performed by BRCA2 and RAD51. Dephosphorylation of BRCA2 at Ser3291 enables RAD51 binding to BRCA2 in BRC repeats and the subsequent binding to double-strand DNA. RAD51 oligomers bind to single-strand DNA at the site of damage that enables its repairs.

($P = 0.009$ and $P = 0.0001$, resp.), and a similar correlation was found with smoked food ($P < 0.01$) [20].

Interestingly, observational studies link pancreatic cancer incidence to cadmium, arsenic, and lead exposure [21]. The countries with the highest levels of arsenic (more than 10 μg/L, values recommended by the World Health Organization [22]) are those with highest incidence of pancreatic cancer. These countries include Baltic countries (especially Finland) and central and eastern European countries such as Austria, Czech Republic, Slovakia, and Hungary [23].

Pancreatic cancer has long been related to family history of pancreatic cancer (HR = 2.20, 95%; CI = 1.16–4.19) and melanoma (HR = 1.74, 95%; CI = 1.03–2.95), upon breast, ovarian, lung, gastrointestinal, or prostate cancer [24]. In addition, diabetes has also been associated with pancreatic cancer (HR = 1.4–2.2) [25].

Surgical resection is currently the best option so far to improve survival [26]. Mean life expectancy for pancreatic cancer is 1.4 years reaching 3.5 years for surgically resected patients versus 0.8 years for nonoperated patients ($P < 0.001$) [27]. However, cancers of the pancreas are usually asymptomatic, and the disease only becomes apparent after the tumour invades surrounding tissues or metastasises to distant organs [28]. As a result, there is a pressing need to find new approaches and strategies; of these, targeted therapies hold particular promise, and *BRCA2* is one such therapy that has great potential. *BRCA2* regulates sister chromatid cohesion and/or alignment [29] and plays a key role in response to DNA damage by direct regulation of RAD51 recombination (Figure 1).

2. *BRCA2* in DNA Damage Response

The first attempt to associate *BRCA2* with DNA damage response was as a cofactor associated with human RAD51-dependent DNA repair of double-strand breaks through 8 evolutionarily conserved BRC motifs encoded in exon 11 of *BRCA2* (Figure 1) [30]. The milestone of DNA strand exchange is RAD51 protein which is closely related to other tumour suppressor genes such as *TP53*, *ATM*, *BRCA1*, *BLM*, and *FANCD2*. Preclinical studies showed that *BRCA2* disruptions sensitize mice embryos to ionising radiation [30], which was previously observed in *RAD51* knockout mice embryos [31]. Furthermore, mice carrying truncations on *BRCA2* loci were one-third smaller than their wild-type littermates and had improper tissue differentiation, sterility, and a shorter overall survival [32, 33].

On the other hand, *BRCA2* is essential for repair of double-strand breaks by homologous recombination [34]. *BRCA2* alterations led to elevated P53 and P21 expression, spontaneous accumulation of chromosomal abnormalities, and aberrant chromatid exchanges, which suggests its role in pancreatic tumorigenesis (Figure 2). The aforementioned properties make *BRCA2* a crucial factor to maintain cell homeostasis.

3. *BRCA2* as a Prognostic Biomarker of Pancreatic Cancer

BRCA2 inactivation is due mainly to genomic mutations. The most common mutations of *BRCA2* found in pancreatic cancer patients are 6174delT frameshift mutation, 6158insT mutation, splice site mutation 16-2A > G, and the splice site mutation 15-1G > A [35, 36]. Another variant located in the 3′-untranslated region is significantly associated with lower expression of brca2 RNA and, consequently, with sporadic pancreatic cancer (HR = 1.3; $P < 0.0001$) [37]. *BRCA2* inactivation has been reported to be a late event in sporadic pancreatic tumorigenesis [38] preceded by *KRAS* mutation

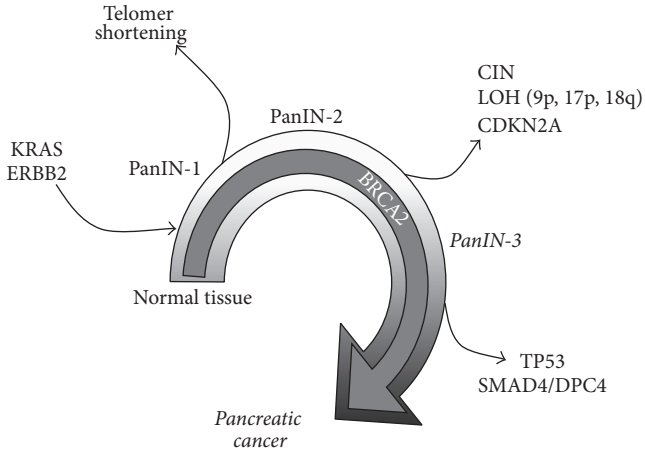

Figure 2: Transformation model of familiar pancreatic adenocarcinoma. Ingoing arrows concern oncogenic effectors that are acquired in the cancer progression. Outgoing arrows highlight tumour suppressor factors that become inactivated in pancreatic cancer. PanIN: pancreatic intraepithelial neoplasia; CIN: chromosome instability; LOH: loss of heterozygosity.

(G12D) or loss of *TP53* [39, 40]. Overall, *BRCA2* could be used to determine patient prognosis.

Ashkenazi Jews have been one of the most closely studied ethnic groups concerning the significance of *BRCA2* mutations and family involved pancreatic cancer. Struewing et al. found that >90% of Ashkenazi patients that carried *BRCA2* mutations detected in blood sample showed an association with increased risk of pancreatic cancer [41]. The 6174delT mutation of *BRCA2* was determined to be present in 1% (CI = 0.6–1.5) of 1.255 Jewish individuals [42]. Another study performed with 26 European families reported that 19% (CI = 7% to 39%) of the families with first-degree relatives with pancreatic cancer had either a mutation or a splice variant of *BRCA2* [43]. Murphy et al. reported 17% of *BRCA2* mutations in 31 samples from pancreatic cancer patients with at least two first-degree relatives affected by pancreatic cancer [35]. One study found 6% (10 of 180 families) with *BRCA2* mutation and moderate or high-risk pancreatic cancer predisposition and 6% (8 of 146) of families that presented two or more first-degree relatives affected with pancreatic cancer [44].

This kind of studies performed with high-risk pancreatic cancer families provides a true enlightenment of *BRCA2*-associated pancreatic cancer; however, *BRCA2* has not been directly related to patient outcome.

Pancreatic cancers with a high familial component are associated with mutations not only in *BRCA2* but also in *ATM, CDKN2, PALB2, PRSS1, STK11, or* mismatch repair (MMR) family genes [45]. MMR genes allow continuous point mutations in repeats regions of DNA known as microsatellites that become unstable. Alterations in microsatellites are found in oncogenes associated with initiation and progression of cancer [46]. Several MMR genes are involved in the acquisition of aggressive phenotype of cancer [47]. For example, alterations on EXO1, MLH1, MSH2, MSH3, MSH6, PMS2, PMS2L3, RECQL, TP73, and TREX1 were statistically significantly associated with overall survival of pancreatic cancer patients [48]. Nevertheless, the predisposition to pancreatic cancer by MMR family genes is due mainly to mutations in *MLH1* and *MSH2* and it is estimated to be <5% [49].

4. *BRCA2* as Predictive Biomarker in Pancreatic Cancer

BRCA2 mutations impair DNA repair; thus, they are considered biomarkers of genomic instability and DNA damage repair deficiency. Therefore, *BRCA2* mutations could be used as predictive biomarkers of response to some DNA damage agents. Some of these compounds include platinum-based agents and PARP inhibitors. They are considered targeted therapies indicated for BRCA2-positive tumours according to some good results achieved in clinical trials [50]. Platinum-based drugs are, despite their toxicity, one of the gold-standard chemotherapies administered to pancreatic cancer patients. Cisplatin, carboplatin, and oxaliplatin are some of the mostly used in clinical practice and allow cross-linking and forming DNA adducts which trigger apoptosis cascade [51].

Oliver et al. presented a cohort of pancreatic cancer patients; of them, those with family history of breast, ovarian, or pancreatic cancers showed significantly increased survival after platinum-based chemotherapy compared to other patients without family history (22.9 versus 6.3 months, $P < 0.01$) [52]. A case report of a 60-year-old pancreatic adenocarcinoma patient carrying the *BRCA2* mutation, 1153insertionT, presented recurrence after gemcitabine treatment but showed a complete response after cisplatin and gemcitabine as second-line therapy [53]. Subsequently, another study with pancreatic cancer patients with positive *BRCA1/2* mutations showed improved outcome after treatment with platinum-based chemotherapy. Here, patients with locally advanced disease were pathologically downstaged and those with metastatic disease had significant increase in their progression-free survival [54]. Golan et al. reported that stage III/IV patients treated with platinum-based chemotherapy carrying *BRCA1/2* mutations had improved overall survival *compared to* those patients treated with other drugs (22 versus 9 months, resp.; $P = 0.039$) [55]. One study reported that 5 out of 8 patients with pancreatic ductal adenocarcinomas that were treated with platinum-based chemotherapy presented *BRCA2* mutation. Of these 5 patients, 2 had complete radiological response and 2 had partial responses to platinum treatment [56]. All the above-mentioned studies suggest that *BRCA2* mutations predict not only platinum response but also better outcome and longer survival for pancreatic cancer patients with advanced disease.

Poly(ADP-ribose) polymerase inhibitors (PARPi) prevent the repair of double-strand DNA breaks, homologous recombination, and replication repair performed by the PARP family of proteins [57]. A preclinical study with CAPAN-1 cell line has suggested that 6174delT mutation of *BRCA2* is highly sensitive to PARPi [58]. However, another study also performed with pancreatic cancer cell lines reported how a PARPi increases sensitivity to chemoradiotherapy independently of *BRCA2* mutation status [59].

Table 1: Clinical trials for *BRCA2* mutated pancreatic cancer patients.

Clinical trial	Phase	Study type	Drugs	Sponsor	Inclusion criteria
NCT02309632	Screening	Nonrandomized	Screening of high-risk individuals	University of Arkansas	Peutz-Jegher's Syndrome BRCA1 mutation carrier BRCA2 mutation carrier Ataxia-telangiectasia Familial atypical malignant melanoma syndrome Colorectal neoplasms, hereditary nonpolyposis Hereditary pancreatitis
NCT02000089	Prospective observational	Cohort	Human synthetic secretin	Johns Hopkins University	Pancreas cancer Peutz-Jeghers Syndrome Gene mutation Germline mutation carrier Lynch Syndrome
NCT02775461	Prospective observational	Cohort	—	Icahn School of Medicine at Mount Sinai	Pancreas cancer Pancreatitis Chronic pancreatitis Pancreatic cyst Family history of pancreas cancer Genetic mutations
NCT01585805	Phase II	Randomized	Gemcitabine, cisplatin with or without veliparib or veliparib alone	National Cancer Institute	BRCA1 mutation carrier BRCA2 mutation carrier Metastatic pancreatic adenocarcinoma Pancreatic adenocarcinoma Recurrent pancreatic carcinoma Stage III pancreatic cancer Stage IV pancreatic cancer
NCT01102569	Prospective observational	Cohort	—	Columbia University	BRCA1 mutation carrier BRCA2 mutation carrier
NCT00438906	Prospective observational	Cohort	Human synthetic secretin	Johns Hopkins University	Pancreatic neoplasm Peutz-Jeghers Syndrome
NCT01233505	Phase I	Interventional	Veliparib, oxaliplatin, capecitabine	National Cancer Institute	Advanced solid tumors BRCA1 mutation carrier BRCA2 mutation carrier
NCT02703545	Prospective observational	Cohort	—	Johns Hopkins University	Peutz-Jeghers Syndrome Familial pancreas cancer BRCA1 mutation carrier BRCA2 mutation carrier Hereditary pancreatitis
NCT00714701	Prospective observational	Cohort	—	Sidney Kimmel Comprehensive Cancer Center	Early pancreatic neoplasia Familial pancreatic neoplasia
NCT00892736	Phase I	Interventional	Veliparib	National Cancer Institute	Advanced solid tumors BRCA1 mutation carrier BRCA2 mutation carrier Estrogen receptor negative HER2/Neu negative

Table 1: Continued.

Clinical trial	Phase	Study type	Drugs	Sponsor	Inclusion criteria
NCT01339650	Phase I	Interventional	ABT-767	AbbVie	Advanced solid tumors BRCA1 mutation carrier BRCA2 mutation carrier
NCT01078662	Phase II	Interventional	Olaparib	AstraZeneca	Advanced solid tumors BRCA1 mutation carrier BRCA2 mutation carrier

A different drug popularly used in pancreatic cancer treatment is gemcitabine and is able to induce DNA damage response and PARP degradation [60]. Gemcitabine in combination with PARPi showed promising antitumor activity compared to PBS, gemcitabine, or PARPi alone, in in vivo models of pancreatic cancer [61].

In clinical studies, *BRCA2*-positive status has been associated with better response to PARPi alone or in combination with other drugs. In one study, 3 out of 4 patients with a known *BRCA1* or *BRCA2* mutation showed partial response after receiving PARPi alone or in combination with platinum-based chemotherapy [62]. In a phase I/II trial of PARPi in combination with 5FU and oxaliplatin that included 2 patients with *BRCA2* mutation, one showed a partial response and the other achieved complete response [63]. Another phase IB trial of PARPi in combination with gemcitabine and platinum-based chemotherapy reported that *BRCA*-mutated patients achieved partial response in 56% and stable disease in 44% of cases. However, 62% of *BRCA* wild-type patients remained with stable disease and 25% with progression [64].

To date, personalised therapies in pancreatic cancer could improve patient survival if assisted by breakthrough techniques used in molecular diagnosis. Deep sequencing currently offers a high-throughput method of dissecting the underlying mechanisms of tumorigenicity, leading to new strategies for personalised medicine. However, pancreatic cancer genotype is highly heterogeneous, and this heterogeneity involves its therapeutic ineffectiveness [65]. The IMPaCT clinical trial was set out to improve patient survival using deep sequencing to guide treatment decisions. In the study, patients carrying BRCA2 mutations were eligible to receive targeted treatment based on 5FU and mitomycin versus gemcitabine alone [66]. Nevertheless, no consistent conclusions arose from this trial due to the low number of patients recruited with *BRCA2* mutations [67].

Nevertheless, patients could present acquired resistance to platinum-based chemotherapy by accumulation of secondary genomic alterations, such as *BCR-ABL* point mutations, in which case the *BRCA2* mutation effect is bypassed [68].

Table 1 summarises ongoing or recently completed clinical trials recruiting *BRCA2* mutated pancreatic cancer patients.

5. Conclusions

Pancreatic cancer is one of the most deadly cancers worldwide, and despite new methods of early diagnosis, surgery, and drug discovery, tumour cells tend to scatter and metastasise to vital organs, thereby reducing survival significantly. It is also highly resistant to treatments and responds poorly to chemoradiotherapy; indeed, chemoradiotherapy is used in most of cases as a palliative therapy. Therefore, patients are encouraged to participate in clinical trials regardless of disease stage.

Some studies attribute the increasing incidence of sporadic pancreatic cancer to the ageing of the population. However, several studies have reported different factors associated with this neoplasm. Obesity, cigarette smoking, high alcohol intake, and chronic pancreatitis are the most relevant factors [69].

On the other hand, it is estimated that 10% of pancreatic cancer cases are due to an inherited syndrome [11, 12] caused by mutations in the *BRCA1* or *BRCA2* genes [10]. Most of the clinical studies that relate pancreatic cancer to *BRCA2* mutations have been performed on Ashkenazim. Although this fact limits the findings' applicability to other populations, there is nonetheless great potential in the study of the heritability of *BRCA2* mutation and pancreatic cancer incidence [41, 42].

Several preclinical and clinical studies have suggested the potential use of *BRCA2* mutations as biomarkers for DNA damage agents' response like platinum-based chemotherapy and PARPi. Clinical trials have evaluated *BRCA2* as a predictive biomarker for use in platinum-based therapies but they were mainly retrospective and with a scarce cohort of patients. Thus, further multicenter prospective studies using larger cohorts are required to investigate multitarget therapies and their potential to minimize resistance to therapy.

Competing Interests

The authors declare no conflict of interests.

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Review Article

Clinically Meaningful Use of Blood Tumor Markers in Oncology

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Before the introduction of modern imaging techniques and the recent developments in molecular diagnosis, tumor markers (TMs) were among the few available diagnostic tools for the management of cancer patients. Easily obtained from serum or plasma samples, TMs are minimally invasive and convenient, and the associated costs are low. Single TMs were traditionally used but these have come under scrutiny due to their low sensitivity and specificity when used, for example, in a screening setting. However, recent research has shown superior performance using a combination of multiple TMs as a panel for assessment, or as part of validated algorithms that also incorporate other clinical factors. In addition, newer TMs have been discovered that have an increased sensitivity and specificity profile for defined malignancies. The aim of this review is to provide a concise overview of the appropriate uses of both traditional and newer TMs and their roles in diagnosis, prognosis, and the monitoring of patients in current clinical practice. We also look at the future direction of TMs and their integration with other diagnostic modalities and other emerging serum based biomarkers, such as circulating nucleic acids, to ultimately advance diagnostic performance and improve patient management.

1. Introduction

The term tumor marker (TM) traditionally has referred to substances, mainly proteins, that are either directly produced by malignant cells or are produced by other cells, in response to certain malignant or other nonmalignant conditions. TMs can be associated with malignancies of a specific organ (e.g., prostate surface antigen [PSA] in prostate cancer and thyroglobulin in thyroid cancer), but often a TM, such as cancer antigen 19-9 (CA 19-9), can be elevated in a variety of cancers (e.g., pancreatic cancer, hepatobiliary cancers, and gastric adenocarcinomas) [1]. In addition, TMs are not uniformly elevated in all patients diagnosed with a specific malignancy (e.g., carcinoembryonic antigen [CEA] in colorectal carcinoma [CRC]) [2]. Despite these limitations and prior to the advent of modern imaging techniques and advances in molecular diagnosis, TMs were among the few available diagnostic tools for management of oncologic patients. They are easily measured in bodily fluids, mainly in serum or plasma samples; the results are rapidly available, and the associated costs for TM testing are relatively low [3]. Thus, for many malignancies, TMs have become an established part of patient management and are also included in a number of clinical guidelines [4–11]. The lack of diagnostic alternatives and poor treatment options for patients with advanced cancers highlighted the need for early detection and led the medical community to conduct several studies that tested single TMs for the screening of several solid tumors. However, the various causes of their elevation in blood were associated with insufficient sensitivity and specificity in asymptomatic patients, thus making the use of a single TM for screening in the majority of solid tumors extremely challenging. Even in rare exceptions, such as prostate cancer, where a specific TM, namely PSA, was initially recommended for screening, the intended use of the marker has more recently come under scrutiny because PSA alone cannot distinguish the presence of clinically relevant forms of aggressive cancer from more indolent variants of the

disease and thus has led to overdiagnosis and overtreatment [12]. Nevertheless, in case of suspicious masses, studies have shown that newer TMs provide improved profiles of sensitivity and specificity for defined malignancies such as progastrin-releasing peptide (ProGRP) for small cell lung cancer [13] and human epididymis protein 4 (HE4) for ovarian cancer [14].

TMs were discovered in an era prior to the advent of evidence-based, guideline-driven medicine, and many studies examining the utility of TMs were either underpowered, were used to correlate TM levels with what are now outdated "gold standards" (such as plain X-rays to assess tumor response), or did not show the rigorous design required nowadays to conclusively demonstrate a clinically useful endpoint [15, 16].

Over the past decade, advances in molecular and cellular biology have led to the introduction of novel diagnostic tools in oncology which measure circulating tumor cells or elucidate the molecular events of tumors on a single patient level, leading to a paradigm shift in how antitumor therapies are developed and patients are selected for specific targeted therapies [17–19]. The intense focus on the characterization of tumor tissue over the past decade, using gene arrays, polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH), immunohistochemistry (IHC), and next generation sequencing (NGS), has transformed oncology and made precision medicine a reality for many patients [17]. Only very recently we have been able to measure total and mutated cell-free nucleic acid, specific to the patient's tumor, in peripheral blood, which will open up vast new opportunities for diagnosis and treatment decisions for the near future [20–27].

Given the exciting advances in molecular diagnostics, the question arises: will there be any future role for traditional TMs in oncology? While studies on molecular markers in tumor tissue and blood already show great progress in the establishment and validation of new technologies, many inherent biological limitations are still present, including the heterogeneity of the tumors, the prevalence of tumor-specific mutations only in subgroups of cancer patients, and the heterogeneous responses to targeted therapies in the same (e.g., mutation-positive) patient subgroups [28].

We therefore suggest that the optimal patient management flow of the future will integrate novel and established tools, including TMs, and it will be crucial to choose the right marker in the right setting, not only to optimize patient-level outcomes but also to contain associated health care costs to the society as a whole.

The primary goal of this concise review is to conceptually outline how "traditional" TMs can still be clinically valuable.

2. Role of TMs in Current Clinical Practice

Despite the diagnostic advances in oncology, the use of TM in the management of patients with solid tumors has several established indications as well as opportunities for broader use. In this section, we will briefly summarize how TMs can be used at each step along the entire spectrum of patient management. The focus here is not an in-depth discussion of novel biomarkers such as circulating tumor cells, circulating nucleic acids, or novel proteomics approaches, since this would go beyond the scope of this overview. However, the authors will define a continued role for TMs in the context of the growing importance of novel biomarkers.

2.1. Differential Diagnosis. In individuals with a suspected cancer, TMs can be very helpful to narrow down the potential differential diagnoses and to focus the further workup. For example, while carcinoma antigen 125 (CA125) alone has not been recommended for screening for ovarian carcinoma [29], Lokich et al. could show that the combination of CA125 with HE4, using the Risk of Ovarian Malignancy Algorithm (ROMA), might more accurately define the risk of an epithelial ovarian carcinoma, which would prompt a more aggressive diagnostic workup (e.g., laparoscopy) versus a more conservative management strategy of surveillance in low risk women [30]. Furthermore, the recent data on lung cancer screening in high risk individuals using low resolution computed tomography has shown a mortality benefit on a population basis [31]. However, many intermediate size pulmonary nodules evade a clear diagnosis based on imaging alone and might be too small for CT-guided or transbronchial biopsy. These patients would currently be followed up with short-term imaging, which is costly and exposes them to additional radiation [32]. A panel of six TMs (CEA, cancer Antigen 15-3 [CA15-3], squamous cell carcinoma antigen [SCCA], cytokeratin 19 fragment [CYFRA 21-1], neuron-specific enolase [NSE], and ProGRP) was recently shown to be more accurate in predicting the presence of lung cancer than either a single TM alone or clinical factors such as tumor size and smoking status [33]. Thus, these TMs could be used for appropriate triage of indeterminate lung nodules. All the more, the pattern of CEA, SCCA, CYFRA 21-1, NSE, and ProGRP has been shown to be very helpful to distinguish between small-cell and non-small-cell lung cancer subtypes (SCLC and NSCLC, resp.) [33]. This is quite relevant considering a significant portion of lung biopsies might be nondiagnostic [34]. Another example for improved diagnostic performance of combining TMs is in the realm of prostate cancer screening. In men with no palpable prostate nodule and a screening PSA value in the so-called grey zone, that is, a PSA between 2 and 10 ng/mL, the clinician cannot reliably distinguish between prostate cancer and benign prostate hyperplasia without an invasive prostate biopsy. In this setting, measuring, in addition to total PSA, the levels of free PSA and calculating their ratio (%fPSA) can be helpful to diagnose underlying prostate cancer [35]. The lower the %fPSA, the higher the probability of prostate cancer. In the seminal study by Catalona et al., testing of %fPSA reduced unnecessary biopsies by 20%, still maintaining a 95% detection rate for prostate cancer [36]. The Stockholm model, which incorporates free PSA with an array of other risk assessment data, was recently shown to be a more sensitive and specific screening test than PSA alone [37].

In patients with diagnosed cancer of unknown primary (CUP), the use of several TMs is part of the diagnostic flow as outlined in guidelines such as National Comprehensive Cancer Network (NCCN) [38]. Depending on which

TMs are elevated, a focused diagnostic workup can be directed to certain organs and thus the morbidity, time delay, and cost of further invasive diagnostic procedures (e.g., bronchoscopy) may be reduced. While a tissue diagnosis is almost uniformly required for treatment decisions in oncology, two notable exceptions heavily rely on the presence of TMs. In male patients with a testicular mass and elevated TMs (alpha-fetoprotein [AFP] and human chorionic gonadotropin [HCG], alone or in combination), a biopsy is not needed and orchiectomy is the next therapeutic and diagnostic step. Similarly, in patients with underlying liver cirrhosis, an elevated AFP level, together with characteristic findings on multiphase imaging, is sufficient to establish a diagnosis of hepatocellular carcinoma (HCC) and a biopsy is not recommended.

2.2. Staging/Prognosis. TMs are also useful after a diagnosis of a malignancy has been established. While multiple groups have investigated the prognostic role of genomic and immunologic biomarker signatures in early stage NSCLC, Muley et al. demonstrated a relatively straightforward combination of presurgery CEA and CYFRA-21 levels can be prognostic for relapse-free survival in this patient cohort of stage I-IIIA patients [39]. Another recent study demonstrated that, in patients with SCLC, both pretreatment levels and absolute levels of ProGRP at the end of the first chemotherapy cycle are prognostic for overall survival [40]. In primary breast cancers, CA15-3, alone or in combination with other TMs, has been shown to be prognostic in several papers [41–44]. Another example is CA19-9, which can be elevated in hepatobiliary carcinomas, and has long been recognized to be an independent prognostic factor in patients with advanced pancreatic adenocarcinoma and thus has been incorporated as a stratification factor in recent large clinical trials [45, 46].

In clinically organ-confined prostate cancer, PSA of 10–20 ng/mL defines intermediate risk and PSA > 20 ng/mL defines high risk, independent of T classification and Gleason score [47]. In metastatic testicular nonseminomatous germ cell tumors, TM are also used to define good, intermediate, and poor risk patients, independent of tumor sizes and locations [48, 49].

These examples illustrate that TMs can be a valuable tool for prognostication in defined patient cohorts, despite the availability of more elaborate and costly tests.

2.3. Treatment Monitoring. The arguably most common and best established use of TMs is disease monitoring during treatment. The association of many TMs with various solid tumors was recognized decades ago, and their use as monitoring markers has become an established component of patient management ever since. Table 1 shows a list of commonly used TMs with associated malignancies. A more detailed review of TMs with their recommended clinical use according to various guidelines can be found elsewhere [50]. The attractiveness of TMs in disease monitoring is rooted in the basic principle of having a tool which informs the oncologist about treatment success rapidly (at most centers within hours), at a relatively low cost (most TMs cost less than $40 [51]), with minimal inconvenience to the patient (i.e., blood draw versus invasive biopsy or imaging). Also, it is important to note that specificity is less of an issue in patients already diagnosed with a certain malignancy, which further increases the usefulness of TMs in this setting. Finally, a TM such as CA125 can indicate in gastrointestinal and other cancers of the abdominal cavity the presence of omental carcinomatosis, where imaging often fails to detect any measurable disease, and in some cases monitoring of CA125 levels remains the only means to assess treatment success [52].

However, for meaningful clinical interpretation of TM kinetics, the maintenance of the same methods for marker measurement is paramount. Further, additional laboratory parameters such as creatinine, transaminases, and C-reactive protein (CRP) levels are helpful to control potential influencing conditions such as renal and hepatic failure or inflammations. Therapy monitoring can be done most efficiently when blood draws are done at defined time points during treatment, for example, before every application of a new chemotherapeutic cycle, and when biochemical response or progression is done on the basis of defined TM changes in relation to the individual baseline values rather than on fixed cut-off levels like the reference value of any control group. Thereby relevant marker changes can vary considerably due to different half or doubling times of the markers. Although these facts seem to be logical, meaningful changes of TMs are poorly defined and clear schedules of marker determinations are rarely used for response estimation in clinical routine so far.

The next wave of diagnostic advances in therapy monitoring is focusing on detection of nucleic acids from an individual's tumor in peripheral blood, following the specific mutation in that particular patient [53]. Undoubtedly, this development is another step towards precision medicine and will be an important addition to concept of "treating the right patient with the right medicine." However, does that mean that TMs will become obsolete in this setting? The answer is very likely no. Molecular diagnostics will be highly valuable at decision points during patient management: at diagnosis to determine the right treatment and at progression to specify the particular resistance mutation (Figure 1). This will dictate the next line of therapy, but *between* decision points, during chemotherapy cycles, it will be very difficult to show a benefit of molecular diagnosis over traditional TMs in disease monitoring. Even today, an early rise in TM might herald future radiographic progression, but might not necessarily lead at the first rise to a change in treatment. Clinical studies comparing circulating tumor DNA (ctDNA) with TMs show ctDNA is best used in patients with nonelevated TM levels [54–57]. No additional benefit has been shown when combined with TMs. This would demonstrate that detecting "molecular progression" is associated with improved outcomes compared to progression based on TMs and thus would justify higher costs and turnaround time. The approach might be useful in selecting highly aggressive cancers with several lines of highly effective therapy, but in the majority of cases this "high sensitivity" method would have a hard time replacing the established TM. In addition, also for molecular markers, clinically meaningful changes have to

Table 1: Commonly used TMs and associated malignancies.

TM	Type of malignancy	Differential diagnosis	Prognosis/staging	Treatment monitoring/surveillance
Tg	Thyroid	x		x
Calcitonin	Thyroid (medullary)	x		x
β2M (beta-2-microglobulin)	Multiple myeloma, CLL		x	
CEA	CRC, pancreatic, gastric/gastroesophageal AC, esophageal AC, NSCLC AC, breast, endometrial, thyroid, c-cell			x
CA 125	Ovarian, breast, omental carcinomatosis	x		x
HE4	Ovarian, NSCLC, endometrial	x		x
Beta-HCG	GCT, choriocarcinoma, urothelial	x	x	x
AFP (alpha-feto protein)	HCC, GCT	x	x	x
CA 15-3	Breast, NSCLC AC		x	x
CA 19-9	Pancreatic, biliary tract, upper GI		x	x
CA 72-4	Upper GI, mucinous ovarian			x
CYFRA 21-1	NSCLC, esophageal, HNSCC, pancreatic, bladder	x		x
S100	Malignant melanoma			x
NSE	SCLC, NET, neuroblastoma	x		x
ProGRP	SCLC, thyroid medullary	x	x	x
Chromogranin A	SCLC, NET	x		x
PSA/free PSA	Prostate	x	x	x
SCCA	Cervix SCC, NSCLC SCC, esophageal SCC, HNSCC	x		
Ig (immunoglobulin)	Multiple myeloma			x
LC (light chains)	Multiple myeloma	x		x
Her-2-neu	Breast cancer			x
TK	Multiple myeloma, CLL	x	x	x

AC, adenocarcinoma; SCC, squamous cell carcinoma; CLL, chronic lymphocytic leukemia; HNSCC, head and neck squamous cell carcinoma; GCT, germ-cell tumor; GI, gastrointestinal; NET, neuroendocrine tumors; TK, thymidine kinase; Tg, thyroglobulin.

be defined on a single patient level in order to avoid false positive (or negative) results. Moreover, method continuity has to be considered and preanalytical and influencing factors have to be controlled. This is similar in principle to the introduction of imaging with positron emission tomography (PET) computerised tomography (CT) scan compared to conventional CT scans. For various reasons, including cost, PET scans were not able to routinely replace CT scans during regular disease monitoring intervals. So in the future, while molecular diagnostics will become increasingly important in triaging the patient at decision points, TM in-between those points will continue to be used as a trigger for further elaborate and more costly tests (e.g., imaging or molecular testing for new mutations).

One should not forget that despite fragmentation and subclassification of histologic diagnoses in oncology on a genotypic level to assign the best treatment (e.g., KRAS, NRAS, or BRAF, in mutated CRC [58]), the majority of these are captured under the umbrella of a few blood based TMs on a phenotypic level (e.g., CEA and CA19-9 in CRC). Simply put, that means one biomarker can be used for many molecularly distinct diseases to tell us whether tumor cells are being killed or not. This is clinically relevant as molecular patterns predict treatment response accurately only in a portion of patients while a considerable number (up to 50% in recurrent lung cancer patients) will not respond to targeted therapy approaches, despite positivity of epidermal growth factor receptor (EGFR) mutation analysis [59, 60]. In addition, many patients would not qualify for molecular monitoring as they do not have "drugable" mutations. As molecular profiling shows great interindividual differences, we do not need to create a specific primer set for each patient to monitor their treatment response but can use the same TM for most of them. Thus, disease monitoring during systemic treatment of advanced cancers will remain one of the main indication where TMs will still play an important role in the future.

2.4. Surveillance and Recurrence Monitoring. In nonmetastatic cancers which are treated with curative intent and advanced cancers with a good response to first line chemotherapy, surveillance of the patients for up to five years is recommended to detect early recurrence. In some cases, the surveillance time is even longer, for example, in germ-cell

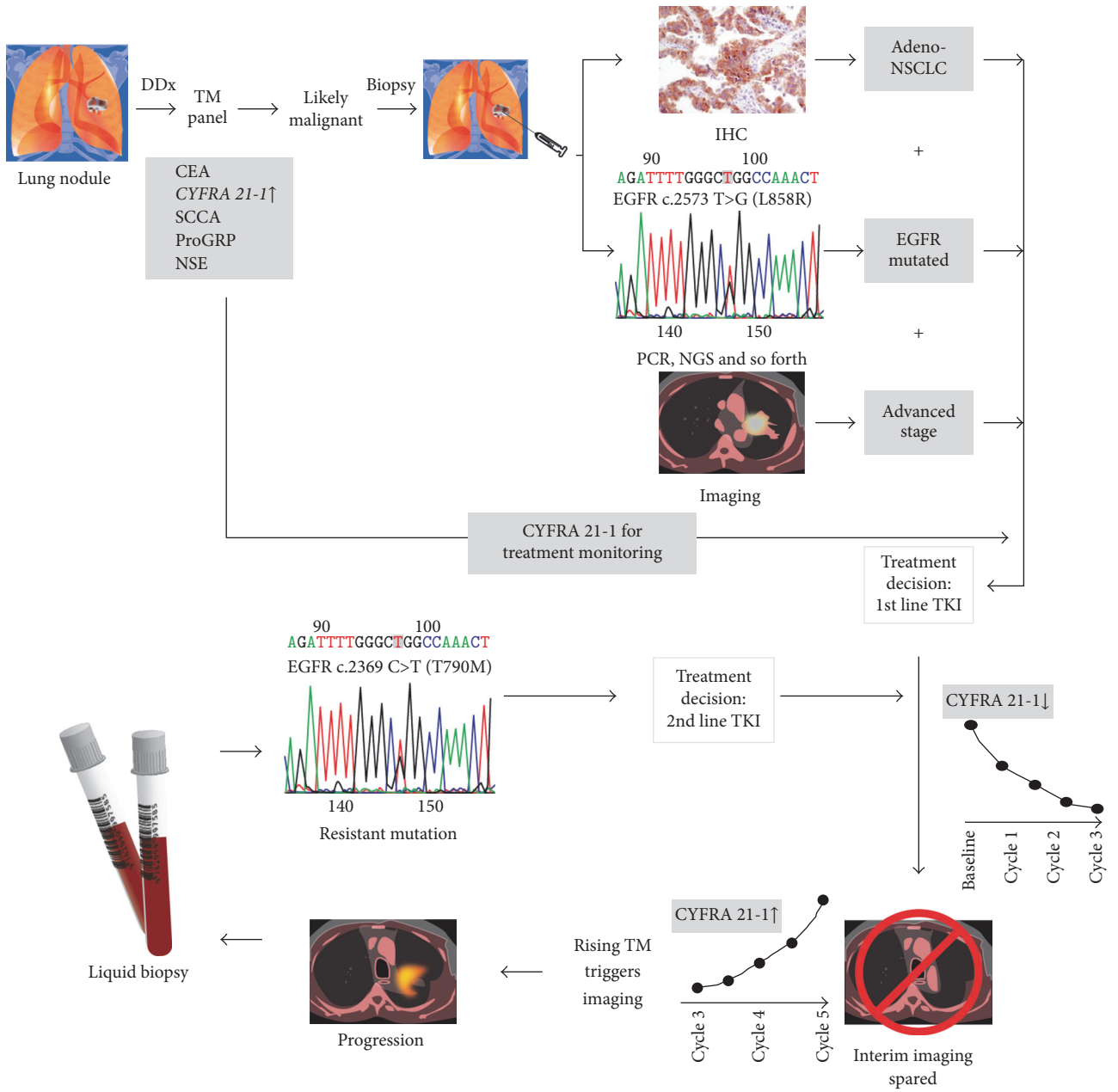

Figure 1: Integration of TMs with other diagnostic modalities, as exemplified in the management of lung cancer. Using a panel of different TMs will guide the decision to either observe a patient with an indeterminate lung nodule versus proceed with a biopsy. In this example, levels of CYFRA 21-1 are elevated. Tissue diagnosis with IHC establishes the diagnosis of adenocarcinoma NSCLC, and molecular testing shows an actionable EGFR mutation. On imaging, an advanced stage is confirmed, and a treatment decision is made based on the integrated information. During treatment (in this case with a tyrosine kinase inhibitor [TKI]), response can be monitored with serial CYFRA 21-1 measurements showing a decline, thus replacing interim staging imaging. Upon rise of the CYFRA 21-1 levels, repeat imaging is performed, which confirms progressive disease. A liquid biopsy avoids an invasive procedure and testing of cell-free DNA by PCR shows the development of a resistant mutation. Based on the result, a second line TKI is chosen. Treatment response is then again monitored using TMs. DDX, differential diagnosis.

tumors [61, 62]. The underlying hypothesis here is that early recurrence detection will increase the likelihood of having a limited disease volume and thus either (a) be able to treat the recurrence with definitive local therapy (surgery and/or radiation) or (b) have a better response to systemic treatment because of smaller tumor load. Hence, in many common cancers that are amenable to screening and thus are detected at nonmetastatic stage in the majority of patients (e.g., prostate, CRC, or breast), the use of TMs in posttreatment surveillance is either already included in the guidelines and/or part of common clinical practice [63–66]. From the above indications to monitor patients for relapse, one can see

that usually a salvage treatment, especially a local treatment modality (i.e., surgical or radiation), is based on anatomic localization of the recurrence. Therefore, the TM itself will not trigger the treatment, but rather it will be the next diagnostic test which will ultimately advise on the best treatment strategy. This approach has successfully been applied in breast cancer using CEA and CA 15-3 for after care surveillance [43, 67]. It has to be pointed out that the principles of marker monitoring (maintenance of the same methods, defined time schedules of TM determinations, interpretation according to marker changes in relation to individual baseline values and not according to fixed cut-offs) were the preconditions to develop the most efficient monitoring procedure [68]. Furthermore, triggering sensitive imaging diagnostics and therapeutic interventions were paramount to benefit from the time advantage by early recurrence detection.

Other randomized trials for recurrence monitoring with TMs, for example, in ovarian cancer using CA125, have not shown a survival benefit and were not included in recommendations [69, 70]. Several reasons for the disappointing results have been identified such as the unfavorable patient selection with poor prognosis, unsatisfactory surgical results with overly high numbers of tumor-positive margins, interpretation of CA125 levels on the basis of fixed high cut-offs that did not lead to an early recurrence detection, and insufficient second-line treatments [71, 72]. So if detection of recurrence with TMs in these instances is not recommended, then it is difficult to envision even more sophisticated blood markers, such as cell-free DNA, to be widely accepted as surveillance markers considering costs are higher and procedural schedules are not respected with the markers available nowadays.

However, survival might not be the best endpoint to evaluate the role of TMs in this setting. To fully investigate this, it would require large and long-term randomized trials. Given the fact that TMs have been around for decades and already incorporated in patient management, it would be very challenging to find resources to conduct those trials today. Alternative more immediate endpoints to investigate would be the number of surveillance CT scans saved by TM monitoring or the proportion of patients who undergo curative intent salvage treatment. Primrose et al. recently demonstrated in a large prospective trial that CRC recurrence monitoring with CEA alone was not worse than regular CT scans [73]. It is possible to imagine that blood-based molecular tests for tumor recurrence monitoring will compete with TMs in the future, given the probability that they will show a serologic recurrence without any radiographic correlation and hence no actual target for local salvage treatment may result in frequent, expensive repeat testing until a detectable lesion is identified.

3. Future Directions

It is clear that TMs can be very useful tools in patient management, if used appropriately. But it is also clear that more work is needed to optimize the clinical use of TMs in daily practice.

3.1. Improve Diagnostic Performance. Traditionally, TMs have been used as single markers, which have led to concerns about their low sensitivity or low specificity. This is especially true for tumors that do not overwhelmingly express a specific TM, for example, NSCLC [74]. In this patient population, no single TM is elevated in a large proportion of patients. However, a panel of several TMs will identify in the majority at least one elevated TM, which then could be followed for treatment monitoring. It is important to note that some TMs are correlated with histologic subtype, which can further guide the choice of TM. Other examples include CRC (CA19-9 in addition to CEA [75]) and ovarian cancer (HE4 in CA125 negative tumors [76]). The combination of two or more TMs can also increase the prognostic performance, for example, with CEA and CYFRA 21-1 in NSCLC [39].

Furthermore, in future trials assessing the diagnostic performance of TMs, investigators should not only focus on a single time point but assess the TM trend over a defined period of time, for example, the introduction of TM kinetics using the risk of ovarian cancer algorithm (ROCA) [77] or when assessing the PSA doubling time in patients with prostate cancer [78].

Finally, research is ongoing to find novel TM, which alone or in combination will improve the diagnostic performance in certain indications, for example, the combination of ProGRP, NSE, CYFRA 21-1, and CEA in lung cancer subtyping [33]. In addition, recent data shows that treatment monitoring in patients with SCLC might be optimized using ProGRP and NSE as TMs [40]. Based on the same concept, the addition of nucleic acids (e.g., ctDNA) might help in closing the diagnostic gap in tumors without known TMs (e.g., sarcomas) or in combination improve the monitoring of malignancies with less established TMs (e.g., S-100B in melanomas [79, 80]) Further ongoing novel marker research will hopefully add to our current armamentarium of available TM.

3.2. Generate More Robust Data and Educate. Many widely used TM, such as CEA, CA125, and AFP, were described more than 20 years ago, and their adoption into clinical practice preceded the rise of evidence-based medicine [81–83]. Thus, it would be very challenging, if not almost impossible, to find the resources to regenerate clinical data in separate clinical trials to show utility of these TMs. However, there are other opportunities to collect high-quality data to support the use of TMs. One might take already published data and analyze several papers together in form of a meta-analysis, which would provide more evidence for the use of TMs in certain indications. For disease monitoring purposes, it is possible to collect blood samples in a prospective fashion in consecutive patients who are treated for specific tumors with standard therapy outside of a therapeutic clinical trial and correlate the TM changes with documented tumor responses. While this option is certainly less expensive than performing dedicated prospective clinical TM trials, there are always issues with bias, ascertaining TM changes with response and outcome, as well as variable sample collection time points. Hence, we believe the way forward to generate more robust data for existing (and testing novel biomarkers)

Table 2: Incorporating TMs into clinical trials.

(a) Schematized prospective trial

	Baseline		Treatment		Surveillance	Progression (PFS)/recurrence (RFS)	Death (OS)
Imaging	T0		T2	T3	T4	T5	
TM	T0	T1	T2	T3	T4	T5	

(b) Uses of TMs at different clinical endpoints

Clinical endpoint	Prognostic
Use of clinical data	Correlate T0 levels with PFS/RFS and/or OS
Potential outcome	TM can be used in future trials as prognostic factor for risk stratification
Example for clinical implication	In "good risk patients": consider less intensive treatment, or shorter duration In "poor risk patients": consider maintenance after induction chemotherapy
Clinical endpoint	**Response**
Use of clinical data	Correlate change in T0 to T1 and T2 levels with response per RECIST on imaging at T2
Potential outcome	Early TM change at T1 predicts progression on first imaging at T2 Early TM change at T2 predicts progression on 2nd imaging at T3 (i.e., in patients with stable disease on 1st imaging at T2)
Example for clinical implication	Randomized trial of continuation of same chemotherapy versus early change to different regimen based on early TM stratification; primary outcome could be ORR, PFS/RFS, or OS
Clinical endpoint	**Treatment monitoring**
Use of clinical data	Correlate change in T0 to T3 levels with best response per RECIST on imaging at T3
Potential outcome	Decline in TM panel correlates with response on imaging
Example for clinical implication	Fewer interval scans for patients with declining markers
Clinical endpoint	**Detection of early relapse**
Use of clinical data	Correlate change from nadir of TM at T3 with posttreatment at T4 and T5
Potential outcome	Increase in levels of TM at T4 compared to T3 will predict progression at T5
Example for clinical implication	Tailor surveillance imaging based on TM levels

T0 to T5, various time points for blood draw and/or imaging; PFS, progression-free survival; RFS, recurrence-free survival; OS, overall survival.

would be retrospectively in available samples from large and well-annotated clinical trials. As a next step, prospective sample collections could be incorporated in future therapeutic clinical trials. A more rigorous assessment of novel markers in prospective clinical trials that are sufficiently powered and have clear endpoints (e.g., assessment of response by Response Evaluation Criteria In Solid Tumors [RECIST] [84]) will likely increase acceptance of these TMs into clinical practice. This is especially true for less widely used TMs (e.g., HE4), which should be incorporated into future therapeutic trials to demonstrate their utility as a secondary endpoint. These approaches would address many of the issues associated with samples collected from patients outside of trials, for example, the benefit of having a tight time correlation of sample collection with intervention, and clearly defined criteria for response. Finally, the appropriate endpoints to establish the role of TMs in clinical practice should not always be overall survival, as already outlined above. TMs can be used as an inexpensive and fast diagnostic modality to trigger downstream tests, and hence their utility should be tested in the context of health economic studies, that is, change in patient management and savings in treatment and advanced diagnostics-related costs, rather than purely clinical endpoints, such as overall survival.

The use of TMs outside of their already established uses (e.g., germ cell tumors, colorectal, prostate, ovarian, pancreatic, hepatocellular, and neuroendocrine tumors) will also depend on more education of clinicians. This will happen through publications and conference presentations but also should start early during the training of oncology fellows through their supervising senior physicians. Like many other aspects of clinical practice, the use of TMs is likely correlated with the level of exposure to these TMs during medical training.

Table 2 conceptually summarizes how TM collection could be incorporated into future prospective trials. TMs would be drawn at various time points indicated in Table 2(a), with routine imaging and patient followup as specified in the respective protocol. Various calculations using the TM levels at the time points indicated in Table 2(a) would then be carried out according to Table 2(b) to determine the diagnostic performance of the TM for different

clinical endpoints. These results would inform a more data driven and rational use of TMs based on the data provided.

4. Conclusion

TMs represent a convenient and cost-effective diagnostic tool for the management of various malignancies. Combining several TMs, serial measurements, and incorporation of novel TMs can all improve their diagnostic performance. In evaluating their usefulness, one should understand their role in certain indications (e.g., disease monitoring) as a first-line test to appropriately trigger further workup and more invasive diagnostics, not the TM as a stand-alone test that will directly affect outcomes of the patients. This fundamental conceptual framework will also result in their continued use despite, or as an important complementation to, novel diagnostic modalities such as cell-free DNA testing. Ongoing research and improved future discovery platforms will advance the field of TMs and add novel markers to the already available armamentarium.

Competing Interests

Farshid Dayyani was an employee of Roche Diagnostics International Ltd when this review was initiated. David Morgenstern is an employee of Roche Diagnostics International Ltd. Stefan Holdenrieder has received grants/research support from Roche Diagnostics. Lance Pagliaro has no conflict of interests to declare. Editorial support was provided by Kim Brown of Roche Diagnostics International Ltd.

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Research Article

EF24 Suppresses Invasion and Migration of Hepatocellular Carcinoma Cells *In Vitro* via Inhibiting the Phosphorylation of Src

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Diphenyl difluoroketone (EF24), a curcumin analog, is a promising anticancer compound that exerts its effects by inhibiting cell proliferation and inducing apoptosis. However, the efficacy of EF24 against cancer metastasis, particularly in hepatocellular carcinoma (HCC), remains elusive. In this study, the effect of EF24 on HCCLM-3 and HepG2 cell migration and invasion was detected by wound healing and transwell assay, respectively. The results revealed that EF24 suppressed the migration and invasion of both HCCLM-3 and HepG2 cells. Furthermore, EF24 treatment decreased the formation of filopodia on the cell surface and inhibited the phosphorylation of Src in both cell lines, which may help contribute towards understanding the mechanism underlying the suppressive effect of EF24 on HCC migration and invasion. Additionally, the expression of total- and phosphorylated-Src in primary HCC tissues and their paired lymph node metastatic tissues was detected, and phosphorylated-Src was found to be associated with HCC lymph node metastasis. The results of this study suggest that Src is a novel and promising therapeutic target in HCC and provide evidence to support the hypothesis that EF24 may be a useful therapeutic agent for the treatment of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most aggressive malignancies and the third leading cause of cancer-associated mortality worldwide [1]. The survival rate for the majority of patients is poor owing to the high incidence of postoperative recurrence and metastasis [2, 3]. Therefore, the development of an effective therapy to impede HCC metastasis remains a challenge.

Curcumin, a constituent of turmeric powder derived from the rhizome of *Curcuma longa*, is well known for its promising antiproliferative activity in many human cancers [4, 5]. It inhibits the enhancer of zeste homologue 2, signal transducer and activator of transcription 3, macrophage stimulating 1, and nuclear factor-κB signaling pathways that are critical in cancer development and progression [6–9]. However, natural curcumin has limited uses due to its poor absorption and low bioavailability [10].

Diphenyl difluoroketone (EF24), an artificially designed structural analog of curcumin, has been shown to be an effective and promising anticancer agent [11]. EF24 exerts anticancer effects via inhibition of cancer growth and induction of cancer cell apoptosis. It has been reported that EF24 induces G2/M arrest and apoptosis by increasing phosphatase and tensin homologue expression in ovarian cancer cells [12]. In addition, it has been reported to decrease lung cancer cell viability by increasing the rate of phosphorylation of extracellular signal-regulated kinase, c-Jun N-terminal kinase, and

p38 [13] and promoting apoptosis in HCC [14]. Recently, EF24 has been shown to suppress epithelial-to-mesenchymal transition (EMT) in melanoma cells by upregulating the expression of microRNA- (miR-) 33b [15], which implies that EF24 may inhibit cancer metastasis. However, limited information is available regarding the effect of EF24 on cancer metastasis, particularly in HCC.

In the present study, we examined the effect of EF24 on the migration and invasion of HCC cells. Additionally, we compared the expression of Src in HCC tissues with that in their paired lymph node metastasized tissues and evaluated the effect of EF24 on Src expression in HCC cells.

2. Materials and Methods

2.1. Reagents and Cell Culture. EF24 was purchased from Sigma (St. Louis, MO, USA). The HCCLM-3 cell line with high metastatic potential, derived from MHCC97 parental cells [16], was provided by the Liver Cancer Institute, Shanghai Medical College of Fudan University (Shanghai, China). HCCLM-3 and HepG2 cells were cultured in Dulbecco's modified Eagle's medium (HyClone; Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Carlsbad, CA, USA) and 1% antibiotic (100 IU/mL penicillin and 100 μg/mL streptomycin; Mediatech, Inc., Manassas, VA, USA). Cells were incubated in 5% CO_2 at 37°C.

2.2. Cell Viability and Cell Apoptosis Assays. Cell viability was detected by using an MTT assay, as previously described [17]. Briefly, HCCLM-3 and HepG2 cells were seeded into 96-well plates at 5×10^3 cells/well and incubated overnight at 37°C. After treatment with EF24 at different doses (0–8 μM) for 12 h, cells were incubated with 20 μL MTT (5 mg/mL) for 4 h. Then, the culture medium was removed and 150 μL dimethyl sulfoxide (DMSO) was added. The absorbance of each well was read at 490 nm using a microplate reader (model 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell viability was expressed as the percentage of absorbance of treated wells compared with that of untreated wells (DMSO control). Values (mean ± SD) are from five independent experiments.

A phycoerythrin- (PE-) labeled annexin V apoptosis detection kit (BD Biosciences) was used to detect apoptosis, according to the manufacturer's instructions. Briefly, HCCLM-3 and HepG2 cells (1×10^6 cells) were exposed to 1 μM EF24 for 12 h. Then, the cells were collected, washed, and resuspended in binding buffer at 1×10^6 cells/mL, and then 1×10^5 cells were incubated with 5 μL PE-annexin V and 5 μL 7-aminoactinomycin D for 15 min at room temperature in the dark. Finally, apoptotic cells were analyzed using a flow cytometer (BD Biosciences). Experiments were repeated twice.

2.3. Detection of Cellular Ultrastructure Alteration with Electron Microscopy. Electron microscopy was employed to detect cellular ultrastructure changes after treatment with EF24, as previously described [17]. Briefly, HCCLM-3 and HepG2 cells, with or without EF24 treatment, were collected and fixed in 2.5% glutaraldehyde overnight. Then, the cells were fixed with 1% osmium tetroxide for 1 h, dehydrated in a graded series of acetone, and embedded in Epon-812 (Nacalai Tesque, Inc., Osaka, Japan). Ultrathin sections were cut, double-stained with uranyl acetate and lead citrate, and examined under a JEM-1220 electron microscope (JEOL, Ltd., Tokyo, Japan).

2.4. Wound Healing Cell Migration Assay. Equal numbers of HCCLM-3 and HepG2 cells were seeded into 6-well plates one day before treatment with EF24. When the cell confluence reached ~90%, cells were treated with different doses of EF24 for 12 h. Then, an artificial wound was created by using a 200 μL pipette tip and the plates were washed with phosphate-buffered saline to remove the debris. A random field was chosen and photographed at 0 and 24 h. From this, the wound width was measured and the healing ability was represented as a ratio of the 24 h width to 0 h width from the same field. The experiments were performed in triplicate.

2.5. Transwell Cell Invasion Assay. After treatment with EF24 for 12 h, cells were trypsinized for the Matrigel invasion assay. Matrigel-coated (BD Biosciences) transwell chambers (Corning Costar, Corning, NY, USA) were used to detect the invasion of HCCLM-3 and HepG2 cells, as previously described [17]. Briefly, filters were precoated with 30 μL Matrigel for 3 h, and 4×10^4 cells in serum-free medium were added to the upper chambers. The lower chambers were supplemented with medium containing 10% FBS. After incubation at 37°C for 24 h, the invaded cells were fixed, stained with hematoxylin and eosin, counted, and photographed under a light microscope. Experiments were conducted in triplicate and cell numbers were expressed as mean ± SD.

2.6. Western Blotting. EF24-treated and EF24-untreated HCCLM-3 and HepG2 cells were collected for Western blotting analysis, as described in our previous report [18]. Total protein was extracted, and samples were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Merck Millipore). The membranes were incubated with primary antibodies against total- (t-) Src (cat. number 2109; Cell Signaling Technology, Inc., Danvers, MA, USA) and phosphorylated- (p-) Y416Src (cat. number 2101; Cell Signaling Technology, Inc.) at 1 : 1,000 dilution overnight at 4°C, washed in TBST, and then exposed to alkaline phosphatase-conjugated secondary antibody (1 : 800 dilution; Santa Cruz Biotechnology, Inc., Danvers, MA, USA) for 2 h at room temperature. Final detection was performed using Western blue (Promega, Madison, WI, USA). GAPDH was used as an internal control. The blots were imaged and the densitometric readings for the proteins were normalized to those of GAPDH (Quantity One software, version 4.4.0.36; Bio-Rad Laboratories, Inc.).

2.7. Immunocytochemistry. HCCLM-3 and HepG2 cells, with or without EF24 treatment, were fixed in 95% ethanol and permeabilized in 0.2% Triton X-100 (Sigma-Aldrich). Then, 3% H_2O_2 was used to arrest endogenous peroxidase activity. The standard indirect horseradish peroxidase method was

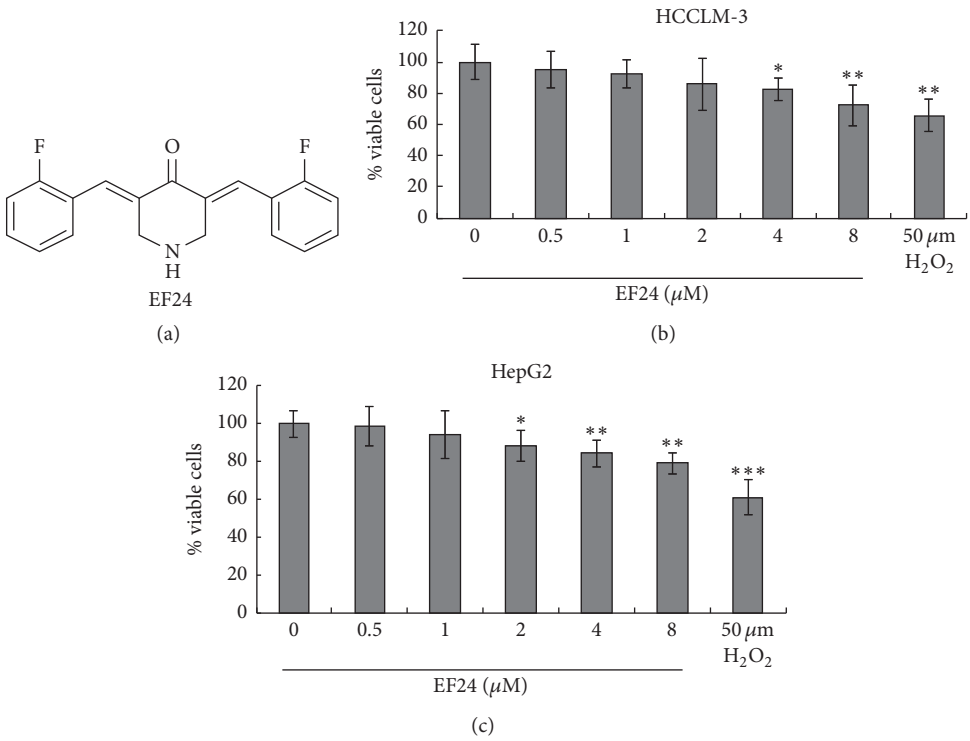

Figure 1: EF24 inhibits HCC cell viability in a dose-dependent manner. (a) The compound structure of EF24. (b) EF24 treatment inhibits the cell viability of HCCLM-3 cells. (c) EF24 treatment inhibits the cell viability of HepG2 cells. HCCLM-3 cells and HepG2 cells were treated with different doses (0–8 μM) of EF24 or 50 μM H_2O_2 (positive control) for 12 h; the cell viability was tested by MTT and expressed as the percentage of absorbance of treated wells compared with that of untreated wells. Data were shown as mean ± SD from five repeated experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

used for staining of the cells. Briefly, cell slides were incubated with antibodies against t-Src (1 : 400 dilution) and p-Y416Src (1 : 50 dilution) overnight at 4°C and then incubated with secondary antibody (PV-6001; ZSGB-Bio, Beijing, China) for 1 h at 37°C. After incubation with 3,3′-diaminobenzidine substrate (ZLI-9019; ZSGB-Bio) for 30 s, the cell slides were counterstained with hematoxylin, dehydrated, and mounted.

2.8. Immunohistochemistry. A total of six metastatic lymph node tissue samples and their six primary HCC tissue samples were obtained from Chinese patients diagnosed as having HCC. Sample collection was approved by the Harbin Medical University (Harbin, China) Institutional Ethics Committee. Formalin-fixed paraffin-embedded sections were incubated with t-Src and p-Y416Src antibodies, as described previously [19]. Briefly, the sections were heated for antigen retrieval at 95°C and blocked with 10% goat serum for 1 h. The slides were stained using the standard indirect horseradish peroxidase method, as described above for immunocytochemistry.

Src expression in HCC and lymph node tissues was assessed using the histoscore method, developed by Allred et al. [20]. In each specimen, the overall Src expression was calculated as a sum of the intensity (0, none; 1, weak; 2, moderate; and 3, strong) and proportion (0, none; 1, <5%; 2, 5–25%; 3, 26–50%; 4, 51–75%; and 5, >75%) scores to give a range of 0–8. Three investigators scored the slides independently and an agreement was reached for all samples.

2.9. Statistical Analysis. Statistical analyses were performed using SPSS software (version 10.0; SPSS, Inc., Chicago, IL, USA). All data were expressed as the mean ± SD. Comparisons between two groups were analyzed using Student's t-test. Mann–Whitney U test was used to analyze the differences in Src expression between groups. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. EF24 Inhibits HCC Cell Invasion and Migration without Affecting Cell Growth and Apoptosis. Before evaluating the effects of EF24 on HCC cell invasion and migration *in vitro*, we first determined the concentrations of EF24 that could be used for subsequent cell treatment. HCCLM-3 and HepG2 cells were treated with different concentrations of EF24 (0, 0.5, 1, 2, 4, and 8 μM) for 12 h, and then the viability and apoptosis of both cell lines were determined by MTT assay and flow cytometry, respectively. Our results showed that EF24 inhibited HCCLM-3 and HepG2 cell proliferation in a dose-dependent manner. EF24 at a concentration of <2 μM did not suppress HCCLM-3 cell viability ($P = 0.508$, $P = 0.293$, and $P = 0.167$; Figure 1) and, at concentration <1 μM, did not significantly decrease the viability of HepG2 cells ($P = 0.367$, $P = 0.407$; Figure 1). In addition, 1 μM EF24 treatment did not affect the apoptosis rate in both cell lines (Figure 2). Therefore, 0.5 and 1 μM EF24 were used to treat both cell

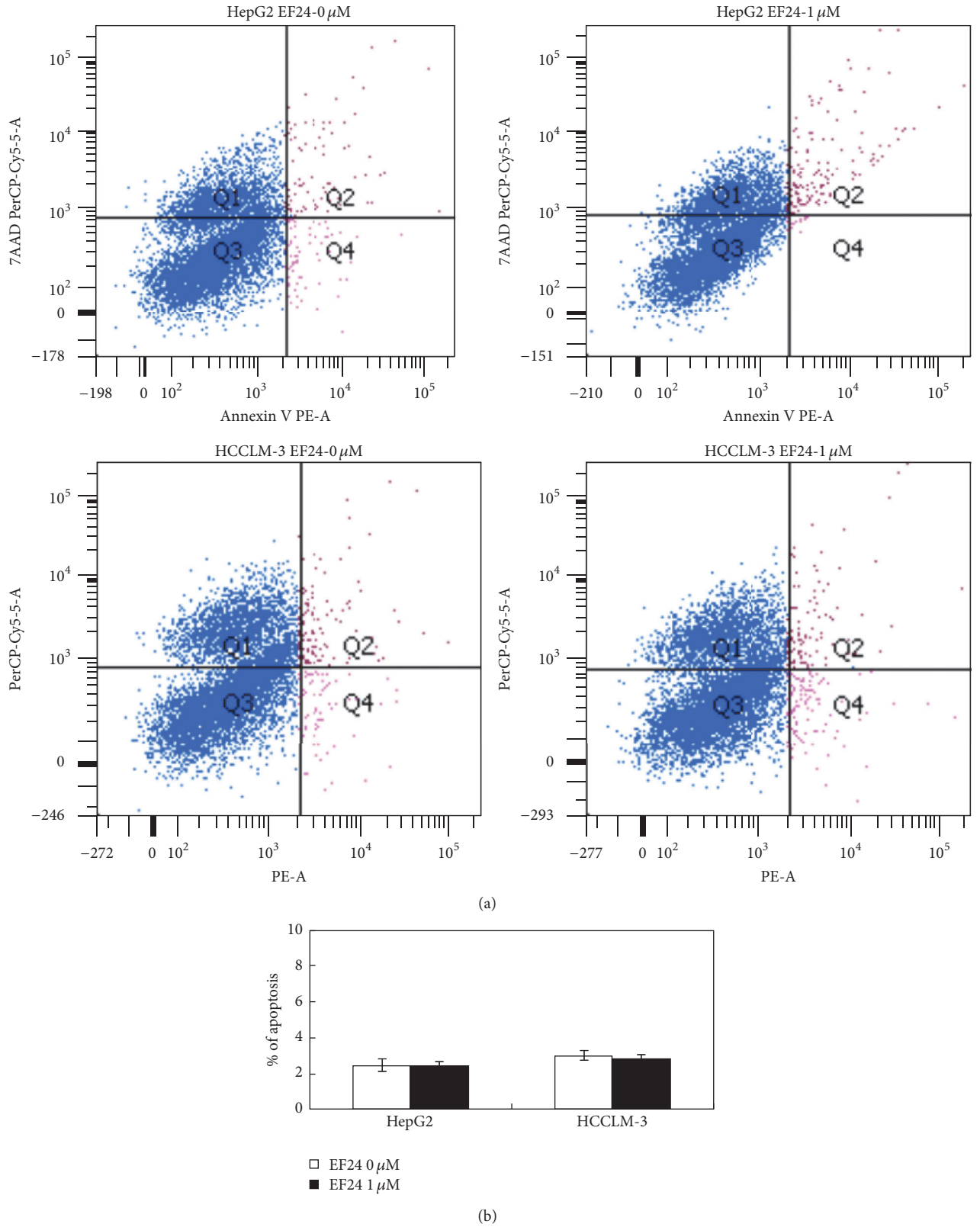

Figure 2: 1 μM EF24 treatment does not induce cell apoptosis in HCCLM-3 and HepG2 cell. (a) The representative flow cytometry result to show the cell apoptosis in two HCC cell lines treated with 0 μM and 1 μM EF24 for 12 h. (b) The statistical analysis about the apoptosis of HepG2 and HCCLM-3 cells. There is no significant difference for apoptosis in both cell lines with or without EF24 treatment.

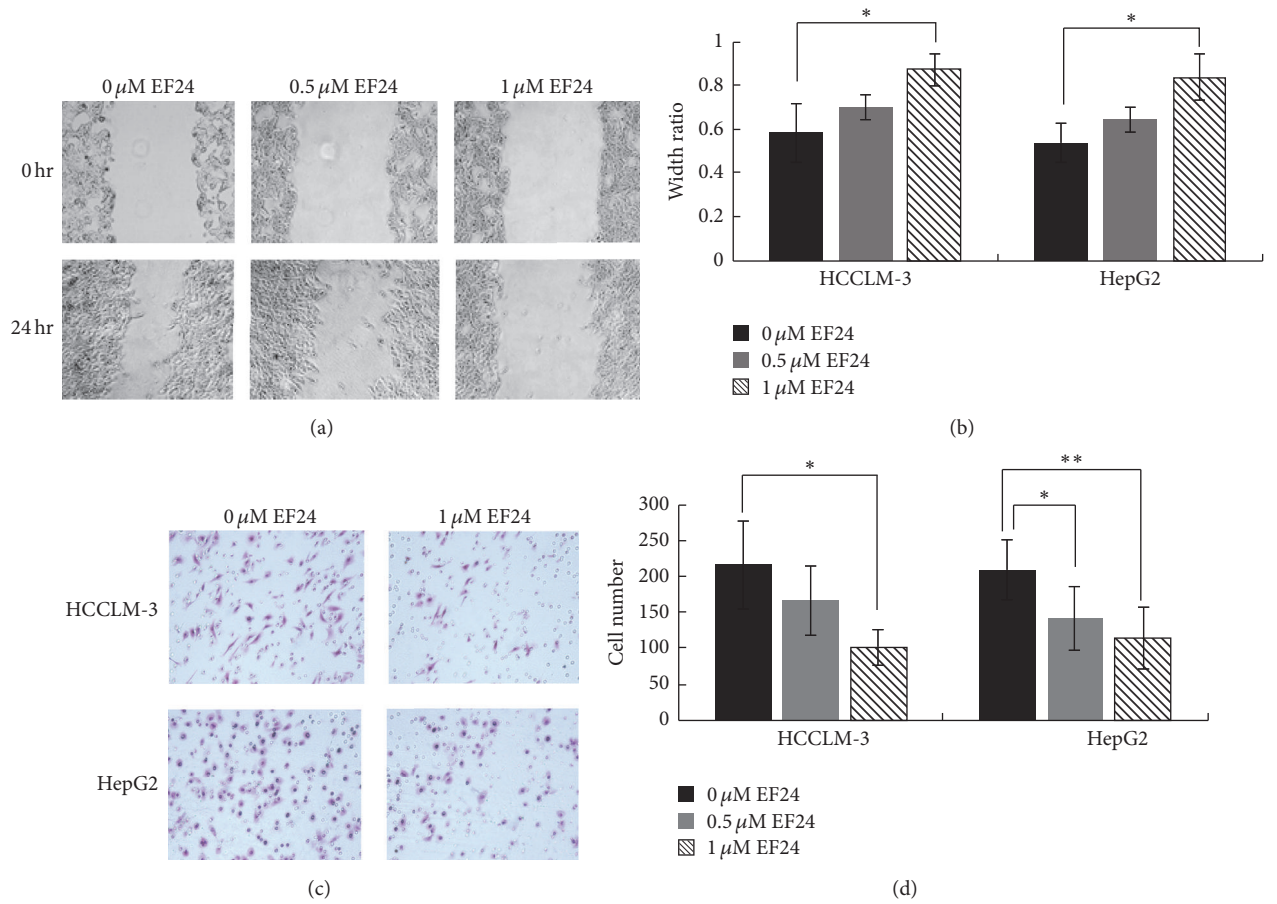

Figure 3: EF24 inhibits the migration and invasion of HCC. (a) EF24 treatment inhibits the migration of HCCLM-3 and HepG2 cells. The artificial wound was created by using a 200 μL pipette tip, and then a random field was chosen and photographed at 0 and 24 h, respectively. Representative images at 0 h and 24 h after wounding were shown at magnification of 100x. (b) Statistical analysis about the effect of EF24 on the migration of HCC. The wound width was measured and the healing ability was represented as a ratio of the 24 h width to 0 h width from the same field. (c) EF24 treatment inhibits the invasion of HCCLM-3 and HepG2 cells. After treatment with or without EF24 for 24 h, cell invasion ability was detected by transwell assay. The invaded cells were fixed, stained, and photographed under a light microscope. (d) Statistical analysis about the effect of EF24 on the invasion of HCC. Data are expressed as the average number of invaded cells under high power field from triplicate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

lines to evaluate the anti-invasion and anti-migration effect of EF24, since these doses have no significant effects on the proliferation and apoptosis of HCC cells.

Transwell and wound healing assays were performed to determine the effect of EF24 on HCC cell invasion and migration, respectively. HCCLM-3 and HepG2 cells showed a dose-dependent decrease in wound healing activities after EF24 treatment; EF24 at 1 μM significantly inhibited HCC migration ($P = 0.047$, $P = 0.022$; Figures 3(a) and 3(b)). Additionally, the transwell assay showed that the invasion ability of HCCLM-3 and HepG2 cells was reduced by EF24 treatment in a dose-dependent manner (Figures 3(c) and 3(d)). These results suggest that EF24 has a potent antimetastasis effect on HCCs and that this effect is independent of its antiproliferative and proapoptotic effects.

3.2. EF24 Treatment Decreases the Formation of Filopodia on the Surface of HCC Cells. To explore how EF24 affects cell migration and invasion, we detected the cellular ultrastructural changes in HCCLM-3 and HepG2 cells after EF24 treatment. HCCLM-3 and HepG2 cells that were not treated with EF24 exhibited abundant organelles, intact nuclei, and plentiful filopodia formation on the cell surface (Figures 4(a) and 4(c)). However, cells treated with 1 μM EF24 for 12 h showed markedly decreased filopodia formation (Figures 4(b) and 4(d)) and organelle degeneration. This result suggests that reduction of filopodia may contribute towards the effect of EF24 in suppressing the invasion and migration of HCC cells.

3.3. EF24 Inhibits the Phosphorylation of Src in HCC Cells. Recently, Src has been shown to serve important roles in promoting HCC [21, 22]. Therefore, we detected the effect of EF24 treatment on the expression of Src. The expression of t-Src and p-Y416Src was detected in HepG2 and HCCLM-3 cells following treatment with or without EF24.

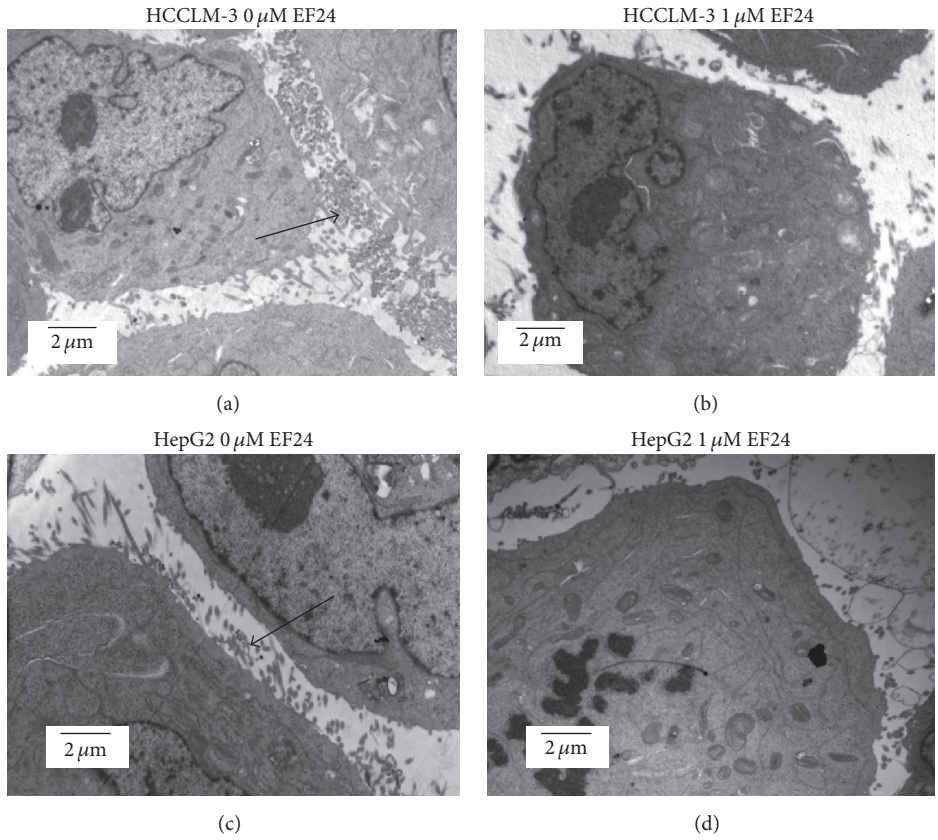

Figure 4: EF24 treatment decreases the formation of filopodia on the surface of HCC. HCCLM-3 and HepG2 cells were incubated with or without EF24 for 12 h and then harvested. Electron microscopy was employed to detect the impact of EF24 on the cellular ultrastructure. (a) and (c) showed that HCCLM-3 and HepG2 cells without EF24 treatment have plentiful filopodia (arrow) (×6000). (b) and (d) showed that the filopodia on the surface of HCCLM-3 and HepG2 cells treated with 1 μM EF24 were decreased (×6000).

Our result showed that EF24 treatment (1 μM) attenuated the phosphorylated-Src but did not affect the total-Src level (Figures 5(a) and 5(b)). Consistent with the Western blotting results, immunocytochemistry staining indicated that EF24 treatment decreased the phosphorylation of Src in both HCCLM-3 and HepG2 cells (Figures 5(c) and 5(d)). These results suggest that EF24 suppression on migration and invasion may be attributed to its inhibitory effect on the phosphorylation of Src in HCC cells.

3.4. Src Expression Increases in the Metastatic Lymph Node Tissue of HCC Patients. The expression of t-Src and p-Y416Src was evaluated by immunohistochemistry in human primary HCC samples and their paired lymph node metastasized tissues to detect their potential effects on HCC metastasis (Figure 6). The histoscore was analyzed using Mann–Whitney U test and the result revealed that the staining scores of t-Src (5.33 ± 1.21) and p-Y416Src (3.00 ± 1.10) were both significantly higher in metastatic lymph node tissue compared with those in the primary liver HCC tissue (P = 0.012 and P = 0.030, resp.; Table 1). These results suggest that Src may be a potential target for preventing and treating HCC metastasis.

Table 1: Src expression in metastatic lymph nodes and liver primary lesions of HCC (n = 6).

Variable	Metastatic lymph nodes	Liver primary lesions	P
t-Src	5.33 ± 1.21	3.17 ± 0.98	0.012
p-Y416Src	3.00 ± 1.10	1.33 ± 1.03	0.030

Mean histoscore values ± SD were calculated for t-Src and p-Y416Src expression in lymph node metastasis and liver primary lesions of HCC.

4. Discussion

Curcumin, as a promising anticancer agent, has attracted increasing attention for its antiproliferative and chemopreventive properties [4, 5]. An increasing amount of evidence showed that curcumin inhibits cancer metastasis via different mechanisms. For example, curcumin inhibits miR-21 transcription and suppresses invasion and metastasis in colorectal cancer [23]. In addition, curcumin inhibits breast cancer metastasis by decreasing the inflammatory cytokines, chemokine (C-X-C motif) ligand 1 (CXCL1) and CXCL2 [24]. Furthermore, curcumin was found to reduce the expression of SET8 to inhibit metastasis in pancreatic cancer [25]. EF24, a novel curcumin analog with greater biological activity and

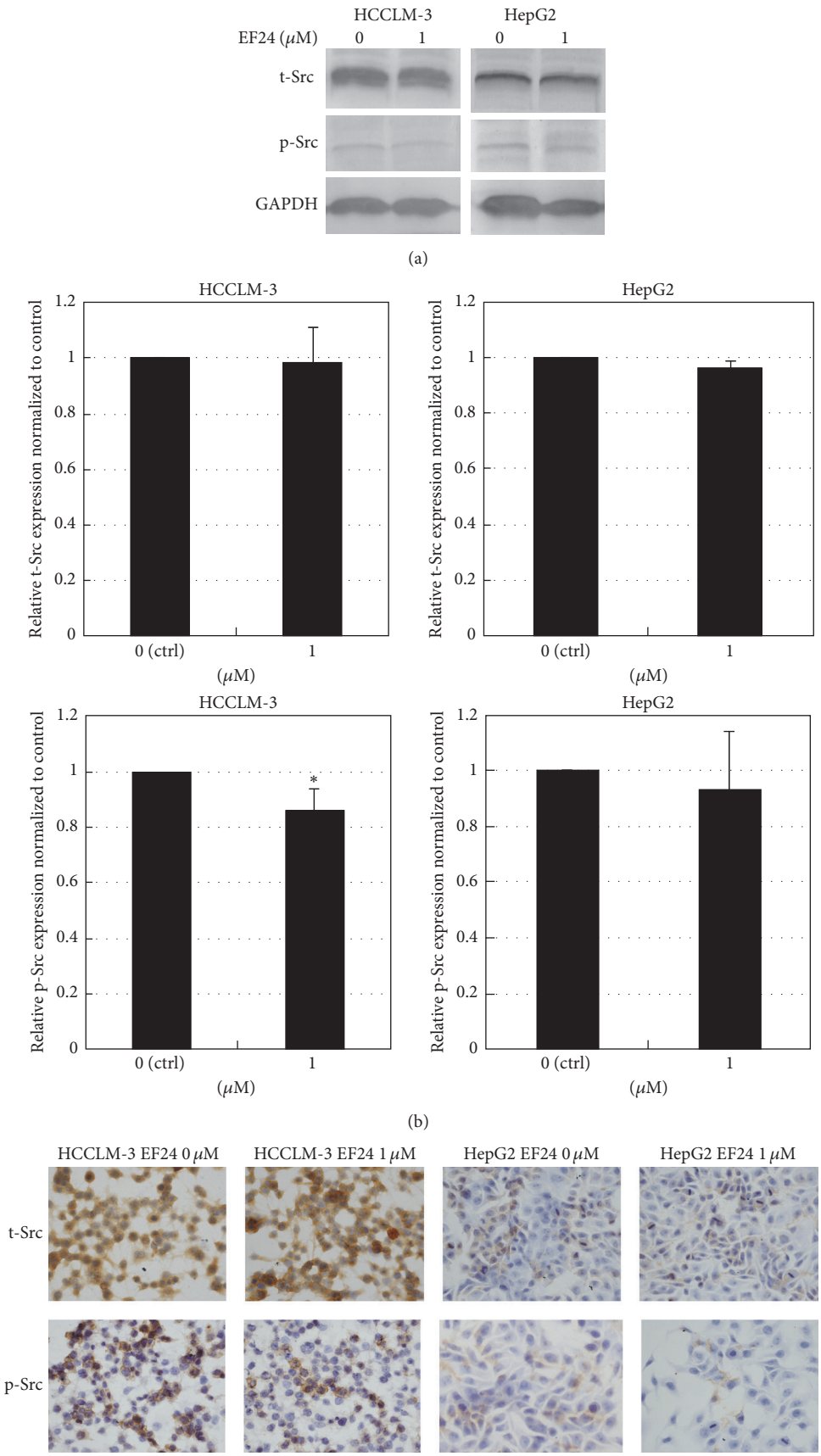

Figure 5: Continued.

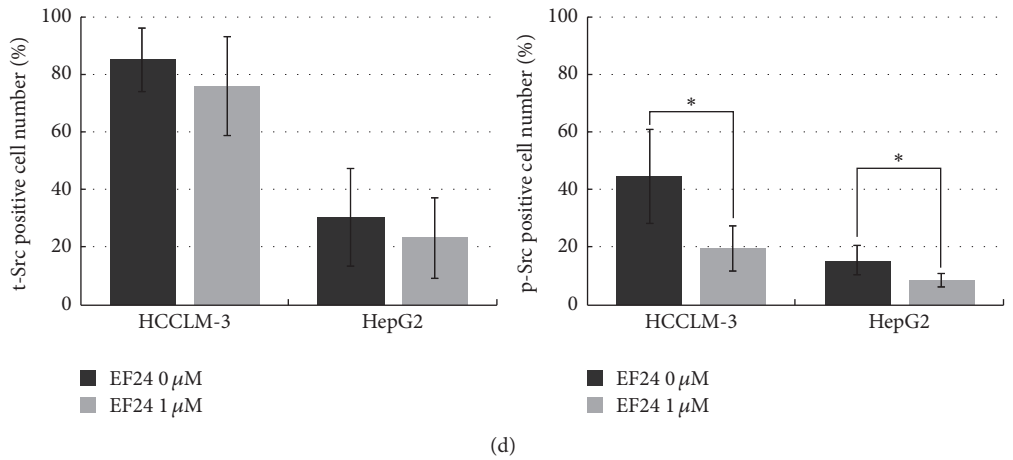

Figure 5: EF24 inhibits the phosphorylation of Src in HCC cells. (a) Detection of the expression of t-Src and p-Src in HCCLM-3 and HepG2 cells treated with 1 μM EF24 by using Western blot assay. (b) Scanning densitometric analysis of Western blot visualizing the relative levels of t-Src and p-Src in HCCLM-3 and HepG2 cells treated with 1 μM EF24. EF24 treatment reduced p-Src but not t-Src level in HCCLM-3 cells. Data were shown as mean ± SD of three independent experiments, $^*P < 0.05$. (c) Detection of the expression of t-Src and p-Src in HCCLM-3 and HepG2 cells treated with 1 μM EF24 by using immunocytochemistry staining (×400). (d) Statistical analysis of the percentage of t-Src or p-Src staining positive cells. EF24 treatment reduced p-Src but not t-Src level in both HCCLM-3 and HepG2 cells. Data were shown as mean ± SD of three independent experiments, $^*P < 0.05$.

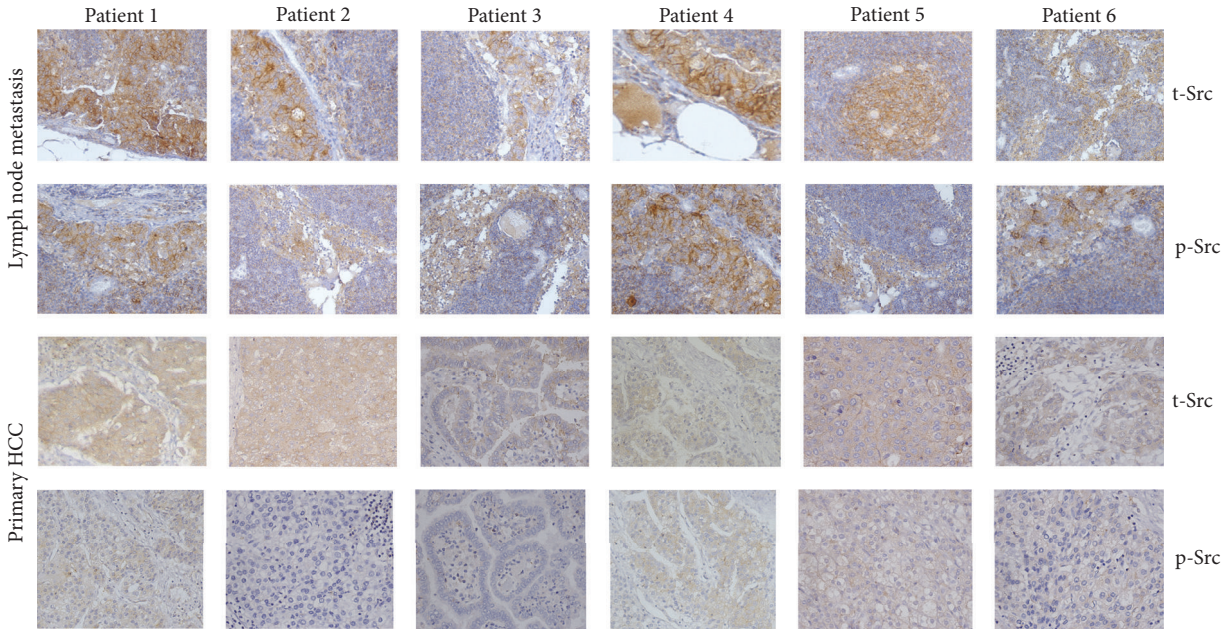

Figure 6: Representative immunohistochemistry results about Src expression in six human primary HCC patients and their paired lymph node metastasis tissues (magnification, ×200).

bioavailability [26, 27], has been shown to possess antiproliferative ability in anticancer screens [11]. However, few studies have detected the effect of EF24 on cancer metastasis. The present study demonstrates for the first time, to the best of our knowledge, that EF24 inhibits HCC cell migration and invasion.

Cellular migration is a tightly coordinated mechanism essential in physiological processes and cancer invasion and metastasis [28]. Filopodia are thin, finger-like, actin-rich membrane protrusions [29] that command the direction of the migrating cells and contribute towards cancer cell invasion [30, 31]. Notably, in this study, EF24 was found to reduce the migratory and invasion potential of HCC cells and reduce the quantity of filopodia present. This result can be supported by a previous study that demonstrated that curcumin targets breast cancer stem-like cells with microtentacles as an antimetastatic strategy [32]. In addition, a previous study observed that EF24 disrupts the microtubule cytoskeleton and inhibits hypoxia-inducible factor-1 [33]. Therefore, it is reasonable that the reduction of filopodia

may contribute to the lower migration ability under EF24 treatment in HCC cells.

Src, a nonreceptor tyrosine kinase, is a critical modulator of multiple signaling pathways mediated by integrin-extracellular matrix interactions [34]. Activated Src (p-Y416Src) initiates signaling pathways that induce cell proliferation, migration, and invasion [35]. Additionally, we previously found that high Src expression scores in HCC tissues were associated with positive lymph node metastasis status [36]. In this study, we further investigated the status of activated Src in primary HCC tissues and their paired lymph nodes with HCC metastasis. The results demonstrated that p-Y416Src is expressed significantly higher in metastatic lymph node tissue compared with that in paired primary liver HCC tissues. Curcumin was reported to regulate the expression of the Src-Akt axis via modulation of miR-203 in bladder cancer [37]. EF24 was found to inhibit migration and EMT in melanoma cells via the suppression of Src and high-mobility group AT-hook 2 [15]. Thus, in this study, we determined whether EF24 inhibits HCC metastasis by downregulating the expression or activation of Src. Then, we examined the expression and phosphorylation change of Src in HCC cells following treatment with EF24 by using Western blot and immunocytochemistry assay analysis. As expected, we demonstrated that treatment with EF24 inhibits the phosphorylation of Src instead of affecting the total level of the protein. This result is consistent with a previous study [15]. These data suggest that Src may be a potential target for HCC metastasis and that inhibition of the phosphorylation of Src may be a molecular mechanism underlying EF24 inhibiting the metastasis of HCC cells.

5. Conclusion

In summary, we demonstrated that EF24 suppresses HCC migration and invasion *in vitro*. This study provides evidence to support that EF24 may be a useful therapeutic reagent for the treatment of hepatocellular carcinoma and suggests that Src is a novel and promising therapeutic target in hepatocellular carcinoma.

Competing Interests

The authors declare no conflict of interests regarding the publication of this paper.

Authors' Contributions

Ran Zhao and Lamtin Tin equally contributed to this work.

Acknowledgments

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Review Article

Deregulated MicroRNAs in Biliary Tract Cancer: Functional Targets and Potential Biomarkers

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Biliary tract cancer (BTC) is still a fatal disease with very poor prognosis. The lack of reliable biomarkers for early diagnosis and of effective therapeutic targets is a major demanding problem in diagnosis and management of BTC. Due to the clinically silent and asymptomatic characteristics of the tumor, most patients are diagnosed at an already advanced stage allowing only for a palliative therapeutic approach. MicroRNAs are small noncoding RNAs well known to regulate various cellular functions and pathologic events including the formation and progression of cancer. Over the last years, several studies have shed light on the role of microRNAs in BTC, making them potentially attractive therapeutic targets and candidates as biomarkers. In this review, we will focus on the role of oncogenic and tumor suppressor microRNAs and their direct targets in BTC. Furthermore, we summarize and discuss data that evaluate the diagnostic power of deregulated microRNAs as possible future biomarkers for BTC.

1. Introduction to Biliary Tract Cancer and MicroRNAs

Biliary tract cancer (BTC) is a malignant disease of the biliary tract epithelia cells, the cholangiocytes. Depending on the localization of the tumor, the term BTC comprises cholangiocarcinomas (CC) of the intrahepatic (IHC) and extrahepatic (EHC) bile ducts, as well as gallbladder cancer (GBC) [1, 2]. The most common BTC is GBC, whereas, for CCs, approximately two-thirds involve the extrahepatic bile ducts [3, 4]. In general, BTC is a rare disease with 3–8 new cases per 100,000 population in the US. However, CC is the second most common hepatic malignancy after hepatocellular carcinoma and the incidence of CC has increased over the last years [5, 6]. Epidemiologic studies revealed strong regional differences in development of BTC, with the area of Khon Kaen in Thailand being the most drastic example, where CC accounts for over 85% of all cancers [3, 5]. This is due to region-specific risk factors such as consumption of undercooked fish, liver fluke infestation (*Opisthorchis viverrini*), and subsequent chronic inflammation of the liver and the bile ducts [5]. In the Western World, the major risk factors for development of BTC are primary sclerosing cholangitis (PSC) and hepatitis B and hepatitis C viruses (HBV and HCV) as well as malformations of the biliary tract. In addition, lifestyle risk factors such as alcohol consumption, smoking, obesity, or exposure to certain chemicals and toxins contribute to the development of BTC and might at least in part explain the raising incidences in Europe and the US [2, 7]. The prognosis of BTC patients is very poor: the median survival after diagnosis is 24 months, and the 5-year survival rate is only about 10% [6]. The only potentially curative therapeutic option is complete resection of the tumor. However, due to the lack of efficient follow-up therapies and high therapeutic resistance, tumor recurrence is the norm [8]. Even more problematic in management of BTC

is the long presymptomatic phase of the tumor progression which combined with the lack of potent biomarkers makes early diagnosis as the prerequisite for curative resection very difficult [9]. As a consequence, most patients are often diagnosed at already advanced stage of the disease, only allowing for palliative treatment with best supportive care. Currently, a combination of cisplatin and gemcitabine is the standard chemotherapeutic option for palliative treatment of BTC, leading to a median survival of only about one year [10, 11]. For nonresectable hilar BTC, photodynamic therapy is established as a palliative therapeutic option yielding 15-month median survival [12–14]. It is therefore evident that identification of new therapeutic options and, especially, biomarkers is of utmost importance to improve the prognosis of patients with BTC.

MicroRNAs (miRNAs) are short (20–22 nucleotides) noncoding RNAs that act as posttranscriptional regulators of gene expression via direct interaction with protein-coding mRNAs, thereby controlling several important physiological processes, such as cell proliferation, apoptosis, and cell differentiation. Biogenesis of miRNAs involves several steps. First, the miRNA gene is transcribed by RNA polymerase II, resulting in a long primary miRNA [15]. Noteworthy, miRNA genes can be transcribed as single transcriptional unit as well as polycistronically [16]. Then, the long primary miRNA transcripts are processed by the nuclear RNase Drosha resulting in shorter precursor miRNAs of a length of approximately 60 to 70 nucleotides [17]. Next, these precursors are exported from the nucleus to the cytoplasm, where they are cleaved by another RNase, called Dicer, to double-stranded miRNAs [18]. For regulation of mRNA transcripts, one strand of the mature miRNA is then incorporated in the RNA-induced silencing complex (RISC). In general, miRNAs exert their regulatory function by sequence-specific binding of their $5'$ end, called the seed region, to the $3'$ untranslated region ($3'$ UTR) of target mRNAs. Perfect match between the seed region and the $3'$ UTR leads to degradation of the mRNA, whereas imperfect match results in inhibition of translation [19]. It is speculated that more than half of the protein-coding genes fall within the regulation of miRNAs. Furthermore, one miRNA species may have up to hundreds of protein-coding mRNAs as potential targets [20]. Today, over 28,000 miRNA species are known (based on miRBase, http://www.mirbase.org/).

These facts underline the central and essential role of miRNAs in regulating the realization of genetic information. More than a decade ago, first publications described miRNAs as a RNA species that is relevant in cancer [21, 22]. Today it is clear that deregulated miRNA expression plays a major role in the development and progression of various types of cancer (for a detailed review, see [23]). MicroRNAs can act as both oncogenes and tumor suppressor genes. As oncogenes, they are overexpressed in tumors, leading to excessive degradation of mRNAs that are coding for tumor suppressor proteins. As tumor suppressive factors, miRNAs are underexpressed in tumors, resulting in insufficient degradation of their target mRNAs, which often code for oncogenes. Fulfilling both of these roles, miRNAs influence and contribute to various aspects and hallmarks of cancer such as migration and invasion, proliferation, cancer stemness, metabolism, therapeutic resistance, and angiogenesis [23–25].

2. MicroRNA and BTC

At present, a reasonable number of published studies describe such a major role of miRNA deregulation for BTC tumorigenesis. Comprehensive microarray screens revealed numerous deregulated miRNA species in BTC samples and BTC model systems [26–37]. In this review, however, we will concentrate on miRNAs that were not only shown to be deregulated in BTC tissues but also for which direct target genes were functionally identified and/or validated. An overview of the miRNAs discussed in this chapter is given in Table 1.

To generally clarify potential involvement of miRNAs in BTC, Shu et al. measured the expression of Drosha and Dicer and found significant downregulation of both enzymes in GBC tissue [38]. Since both enzymes are absolutely essential in the production of mature miRNAs, this observation (i) strongly suggests a principal role of miRNA deregulation in BTC and (ii) also might lead to the conclusion that, in general, the type of miRNA deregulation in BTC might be underexpression more often than overexpression. In fact, as shown in Table 1 and considering the currently available data, most deregulated miRNAs in BTC tissues show decreased expression when compared to their healthy counterparts. Underexpression of these miRNAs uniformly correlates directly to various unfavorable clinicopathological characteristics such as advanced clinical stage, enhanced lymph node and distant metastasis, poor differentiation of tumor cells, and poor disease-free and overall survival. Hence, these miRNAs may function as tumor suppressors and their underexpression subsequently leads to diminished negative regulatory control of transcripts that encode for oncogenes. However, miRNAs can also act as oncogenes and overexpression of these miRNA species leads to increased degradation of mRNAs that otherwise would be translated into proteins with various tumor suppressor functions. Consequently, overexpression of oncogenic miRNAs is also correlated with disadvantageous clinicopathological features. To get more insight into the functional role and direct targets of tumor suppressor and oncogenic miRNAs, several *in vitro* and *in vivo* experiments were conducted. As described in detail below, these studies validated predicted direct target genes of miRNAs by luciferase reporter assays, downstream expression analysis, and various miRNA overexpression and knockdown experiments.

2.1. Tumor-Suppressive miRNAs in BTC and Their Direct Targets. MicroRNA 34a has been described to be downregulated in BTC tissue versus nontumor tissue by two independent studies [39, 40]. Functional *in vitro* and *in vivo* studies showed that PNUTS (see Abbreviations for full gene names) is a direct target of miRNA-34a. PNUTS is a protein that regulates telomere length and its overexpression reduces telomere shortening as well as apoptotic events connected with telomere shortening [41]. Telomere shortening is a mechanism that naturally limits the number of cell divisions of healthy cells and is known to be deregulated in cancer

Table 1: MicroRNAs are deregulated in biliary tract cancer specimens.

	miRNA	Tissue	Clinicopathological characteristics associated with deregulated miRNA expression	Target	Ref.
Downregulated	26a	GBC	Advanced histologic grade	HMGA2	[62]
	34a	EHC, GBC	Poor disease-free and overall survival; increased telomere length; advanced clinical stage; lymph node metastasis	PNUTS, SMAD4	[39, 40]
	101	GBC	Enhanced tumor size; enhanced tumor invasion; higher TNM stage; poor survival		[78]
	122		Increased expression of PKM2	PKM2	[77]
	124	IHC		SMYD3	[68]
	135a-5p	GBC	Advanced histologic grade	VLDLR	[72]
	138		Increased expression of BAG-1	BAG-1	[79]
	144	CC	Increased expression of LIS1	LIS1	[66]
	145	GBC	Poor survival	MRP	[70]
	200 family	CC		SUZ12, ROCK2	[56]
	204	IHC	Lymph node metastasis	SLUG	[53]
	214		Enhanced metastatic potential	TWIST	[54]
	370	CC		WNT10B	[80]
	373	hCC	Poor cell differentiation; advanced clinical stage	MBD2	[63]
	605	IHC		PSMD10	[43]
Upregulated	20a	GBC	Local invasion; distant metastasis; poor prognosis and survival	SMAD7	[81]
	21	CC, IHC	Poor disease-free and overall survival; higher clinical stage at diagnosis; poor cell differentiation; lymph node metastasis	15-PGDH, PDCD4, TIMP3, PTPN14, PTEN	[82–85]
	26a	CC		GSK-3β	[86]
	92a			PTEN	[87]
	141	BTC	Shorter disease-free and overall survival; greater risk of angiolymphatic invasion		[88]
	155	GBC	Shorter disease-free survival; lymph node metastasis; vessel invasion		[89]
	221	EHC	Shorter disease-free survival; advanced clinical stage	PTEN	[90]

CC: cholangiocarcinoma; EHC: extrahepatic cholangiocarcinoma; GBC: gallbladder carcinoma; hCC: hilar cholangiocarcinoma; IHC: intrahepatic cholangiocarcinoma; miRNA: microRNA.

cells. Interestingly, in BTC tissues with decreased miRNA-34a expression, also increased telomere length was found [39]. The signal transduction molecule SMAD4 was identified as an additional target of miRNA-34a in BTC and was recently found to have a tumor-promoting role in hepatocellular carcinoma [40, 42]. In another study, the regulatory component of the 26S proteasome, PSMD10, was described as a target of deregulated miRNA-605 expression in BTC and, interestingly, an inverse expression pattern of miRNA-605 and PSMD10 was observed [43]. In an *in vitro* model, the authors demonstrated that overexpression of the tumor suppressor miRNA-605 resulted in suppression of BTC cell proliferation and this effect was rescued by ectopic expression of PSMD10, suggesting a direct mechanistic connection between miRNA-605 and PSMD10 as a driver of BTC cancer cell proliferation. In line with these observations, PSMD10 was shown to promote cell cycle progression and proliferation of pancreatic cancer cells [44]. Of note, enhanced PSMD10 expression was found to be directly involved in ubiquitylation and degradation of p53, a key tumor suppressor gene, which is also known to play a role in BTC [6, 45].

Detachment of cells from the primary tumor and subsequent carving through the extracellular matrix to invade the angiolymphatic system to eventually form metastasis is a hallmark of cancer in general and likewise in BTC [46–48]. The term "epithelial-mesenchymal transition" (EMT) describes a complex process that is essential for formation of secondary tumors and in which epithelial cells lose their polarity and gain mesenchymal traits including the ability to detach from the (primary) tumor [49, 50]. By controlling cell adhesion and cell-cell contact, E-Cadherin is an important epithelial and anti-EMT marker [51]. SLUG is a transcription factor that directly represses E-Cadherin, thereby activating EMT [52]. Qiu et al. demonstrated that the expression of miRNA-204 was lowered in IHC tissue and inversely correlated with the expression of SLUG. They also showed

increased incidences of lymph node metastasis in patients with diminished miRNA-204 and enhanced SLUG expression. In addition, they identified SLUG as a direct target of miRNA-204 explaining the inverse expression patterns in the tissue samples [53]. Another miRNA, whose downregulation especially in metastatic BTC specimens was demonstrated, is miRNA-214 [54]. Using an *in vitro* model of BTC, the authors demonstrated a direct inhibitory effect of miRNA-214 on cell metastasis and EMT phenotype. They show that inhibition of miRNA-214 resulted in increased expression of EMT promoter TWIST, accompanied by decrease of epithelial marker, E-Cadherin. Furthermore, they identified TWIST as a direct functional target of miRNA-214. The miRNA-200 family was also shown to participate in EMT and metastasis regulation by directly targeting E-Cadherin repressors [55]. In a miRNA microarray study, Peng et al. found members of the miRNA-200 family to be underexpressed in CC samples [56]. Furthermore, in a BTC mouse model, they showed that upregulation of miRNA-200 family members resulted in inhibition of distant metastasis, underlining the role of this miRNA family in formation of secondary tumors. Searching for possible direct targets of miRNA family, they found ROCK2 to be a target of miRNA-200b/c. ROCK2 regulates cytoskeletal signaling events and cellular motility and was already shown to promote invasion of non-small cell lung cancer cells [57]. Of note, Peng et al. noticed overexpression of ROCK2 in CC samples, which, combined with the observed underexpression of miRNA-200 family members, strengthens the functional connection between these tumor suppressor miRNAs and the prometastatic factor ROCK2 [56]. In the same study, SUZ12 was also identified as a direct target of miRNA-200 family members. SUZ12 is a core component of the Polycomb Repressive Complex 2 (PRC2), a histone methyltransferase complex that performs a specific histone methylation, thereby influencing compaction of chromatin and ultimately access to the DNA and gene transcription. Recently, we described that PRC2 plays a role in development and progression of BTC [58, 59]. PRC2 is a master epigenetic regulator, and its general overactivation as well as overexpression of its core components influences several aspects of BTC carcinogenesis [58]. Peng et al. described that silencing of SUZ12 resulted in reduced anchorage-independent growth of CC cells and that regulation of SUZ12 by miRNA-200 family members therefore is important in BTC [56].

HMGA2 is a protein that also participates in chromatin-dependent regulation of gene activity by modifying the chromatin structure [60]. Overexpression of HMGA2 can serve as a predictor of poor prognosis in IHC [61]. In a study by Zhou and coworkers, HMGA2 was identified to be a direct target of tumor suppressor miRNA-26a, a miRNA species that was found to be downregulated in GBC tissue [62]. The same study has demonstrated that HMGA2 counteracts the antitumor effects of ectopic miRNA-26a expression, which characterizes HMGA2 as a miRNA-26a-regulated oncogene in BTC.

Besides histone modification, DNA methylation is another important mechanism of epigenetic gene regulation. In this context, Chen et al. described MBD2 as a direct target of miRNA-373, a tumor miRNA species downregulated in hilar CC, which was associated with advanced clinical stage [63]. MBD2 is involved in the DNA methylation-dependent repression of gene transcription as a reader of cytosine methylation and was suggested as a marker associated with poor prognosis for patients with hepatocellular carcinoma [64, 65]. MicroRNA-144 is another miRNA species found to be downregulated in CC tissues compared to nonmalignant tissues as well as in CC cell lines versus nonmalignant cells [66]. Although no clinicopathological features that are associated with deregulated miRNA-144 expression were presented in this study, the authors clearly demonstrate an inverse expression pattern of miRNA-144 (low expression) and LIS1 (high expression) in CC patient samples. Interestingly, another study described LIS1 as a driver of cell migration and invasive potential of lung cancer cells [67]. In line with this study, Yang et al. demonstrated that LIS1 is a direct target of miRNA-144 in BTC and, furthermore, they showed that ectopic expression of miRNA-144 diminished LIS1 expression [66]. Combined with their observation that knockdown of LIS1 reduced invasion of CC cells, miRNA-144 is yet another miRNA whose downregulation in CC can result in enhanced metastatic potential. Investigating the role of HCV in development of BTC, Zeng et al. observed diminished levels of miRNA-124 in IHC patient samples [68]. Ectopic expression of miRNA-124 resulted in reduced migration and invasiveness in CC cells as well as in reduced protein levels of SMYD3 which they identified as a direct target of miRNA-124. SMYD3 is a histone methyltransferase already shown to promote invasion of cancer cells [69]. It is worth mentioning that one of the downstream targets of SMYD3 is the metalloproteinase MMP-9, an enzyme that plays a pivotal role in the process of invasion by degrading the extracellular matrix [69]. By overexpressing miRNA-124, Zeng et al. observed downregulation not only of SMYD3 itself but also of its downstream target MMP-9 in CC cells [68].

Chemoresistance is a major problem in management of BTC [2]. In a recent study on GBC by Zhan et al., a correlation between miRNA-145 expression and sensitivity towards the standard chemotherapeutic cisplatin was shown [70]. Overexpression of miRNA-145 increased efficacy of cisplatin treatment, whereas lower levels of miRNA-145 decreased sensitivity towards cisplatin treatment in an *in vitro* model of BTC. Expression analysis of miRNA-145 in GBC tissue versus corresponding noncancerous gallbladder tissue showed downregulation of miRNA-145. As a direct target of miRNA-145, the authors identified MRP1, a family member of the "ATP Binding Cassette" drug efflux pumps [70]. These proteins are often upregulated in cancer cells and contribute to multidrug resistance in various types of cancer including BTC [59]. Zhan et al. recognized high sensitivity of miRNA-145 expressing tumors to cisplatin in a BTC mouse model, potentially caused by negative transcriptional control of MRP1 expression. By correlating expression data of miRNA-145 and MRP1 versus clinicopathological features of GBC patients that received chemotherapy, they noted that low expression of miRNA-145 is indeed linked to high expression of MRP1 and poor prognosis [70]. Of note, profiling of general miRNA expression might be a predictor of therapeutic

efficiency/resistance as shown in our recent study on the mechanisms of photodynamic therapy in BTC cell lines [71]. In another study, Zhou and coworkers identified miRNA-135a-5p as an underexpressed miRNA species in GBC tissue and a negative correlation with VLDLR expression in the tested specimens [72]. VLDLR is a member of the low-density lipoprotein receptor superfamily which is involved in receptor-mediated endocytosis of specific ligands and has been reported to play a role in pathogenesis and tumor cell proliferation [73, 74]. Ectopic expression of miRNA-145 in GBC cells inhibited GBC cell growth *in vitro* and *in vivo* and this effect was VLDLR-dependent [72]. The metabolic profile of cancer cells differs significantly from their healthy counterparts. One well known phenomenon observed in the metabolism of cancer cells is that cancer cells favor aerobic glycolysis as primary ATP source instead of the far more efficient oxidative phosphorylation ("Warburg effect") [75]. PKM2 is a rate-limiting enzyme that catalyzes the last step of glycolysis in a way that supports aerobic glycolysis. Unsurprisingly, PKM2 was found to be overly expressed in numerous cancer types [76]. Lu et al. identified PKM2 as a direct target of miRNA-122 in GBC cells. They showed further that, in GBC patient samples, miRNA-122 is underexpressed, whereas PKM2 expression is enhanced [77].

Another downregulated miRNA in BTC tissue is miRNA-138 as presented by Ma and coworkers [79]. Interestingly, they found a significant inverse correlation between miRNA-138 and BAG-1 expression in GBC tissue versus adjacent nonneoplastic tissue and furthermore identified BAG-1 as a direct target of miRNA-138. BAG-1 is a known antiapoptotic protein and silencing of BAG-1 in an *in vitro* model of BTC leads to apoptotic events [79, 99]. Of note, overexpression of miRNA-138 resulted in inhibition of GBC cell growth, whereas simultaneous overexpression of BAG-1 reversed this growth inhibitory effect, thus underlining the functional connection between miRNA-138 and its direct target BAG-1 [79].

2.2. Targets of Oncogenic MicroRNAs in BTC. MicroRNA-21 is a classic oncogenic miRNA species that contributes to carcinogenesis in various tumor types [23]. Regarding BTC, several studies found an overexpression of miRNA-21 in BTC tissue and a correlation with disadvantageous clinicopathological features such as poor disease-free and overall survival, advanced clinical stage, poor cell differentiation, and lymph node metastasis [82–85]. Due to its role as an overexpressed oncogenic miRNA, targets of miRNA-21 are often tumor suppressor genes. In BTC, several direct targets of miRNA-21 have been described. PTEN is a well-known tumor suppressor gene that is often mutated in cancer and was already functionally connected to development of GBC [58, 100, 101]. Wang et al. identified PTEN as a direct target of miRNA-21 in BTC [84]. To determine the clinical significance of this result, they performed Kaplan-Meier analyses and could demonstrate that high miRNA-21 correlated with poor disease-free and overall survival, whereas high PTEN expression correlated with enhanced disease-free and overall survival. Of note, PTEN was also identified as a direct target of two other oncogenic miRNAs in BTC, namely, miRNA-221 and miRNA-92a, which belong to the 17–92 cluster [87, 90]. Another direct target of miRNA-21 is 15-PGDH, an enzyme that converts prostaglandin E2 (PGE2) to its inactive form [82, 102]. BTC often develops under inflammatory conditions [6]. PGE2 is the primary prostaglandin that is involved in inflammation in various pathogenic processes and deregulated 15-PGDH activity results in diminished PGE2 conversion and subsequently in a more inflammatory environment that facilitates carcinogenesis [102, 103]. As a consequence, 15-PGDH acts as a tumor suppressor gene as already shown for breast cancer [104]. In the study by Lu et al., they showed not only that miRNA-21 is overexpressed in CC tissue, but also that 15-PGDH is a direct target of miRNA-21 and that PGE2 drives tumorigenesis in an BTC *in vitro* model [82]. As already mentioned, degradation of extracellular matrix by metalloproteinases is a key step in development of secondary tumors. TIMP3 is an inhibitor of MMP-9, a metalloproteinase that not only was identified in BTC as a downstream target of the potentially deregulated oncogene SMYD3 [68] but also whose enhanced expression was directly correlated to poor overall survival in patients with hilar CC [105]. Interestingly, TIMP3 is another direct target of oncogenic miRNA-21, and, in addition, overexpression of miRNA-21 in CC samples is correlated with diminished levels of TIMP3 in CC specimens [83]. Two studies described PDCD4 as an additional direct miRNA-21 target in BTC [83, 85]. PDCD4 is a tumor suppressor gene that regulates various aspects of carcinogenesis in different types of cancer and whose downregulation or loss is associated with poor prognosis [106–108]. Another direct target of miRNA-21 in BTC is PTPN14 [84], which was shown to be a negative regulator of metastasis as well as of the potentially cancer-driving Hippo/YAP pathway [109, 110].

Besides miRNA-21, other oncogenic miRNA species and their direct targets were also identified in BTC. Zhang et al. described miRNA-26a as a direct regulator of GSK-3β, which itself is a negative regulator of β-Catenin signaling [86]. Accumulation of the transcription factor β-Catenin in the nucleus is a consequence of an active WNT signaling pathway [111–113], leading to activation of diverse downstream genes. GSK-3β is part of the β-Catenin destruction complex, which, in the absence of active WNT pathway signaling, marks β-Catenin for proteasome-mediated destruction [114]. In their study, Zhang and coworkers observed increased miRNA-26a expression in CC tissues and cell lines [86]. Functional cell-based experiments demonstrated direct interaction between miRNA-26a and GSK-3β; in addition, miRNA-26a-mediated reduction of GSK-3β resulted in activation of β-Catenin and expression of cancer-driving genes such as cell cycle promoters. Another upregulated oncogenic miRNA in GBC was found by Chang and coworkers [81]. In this study, elevated miRNA-20a expression in GBC tissue was correlated with local invasion and distant metastasis. A functional explanation of this observation is given by the identification of SMAD7 as a direct target of miRNA-20a as well as the inverse expression pattern of miRNA-20a and SMAD7. High expression of miRNA-20a correlated with low expression of SMAD7 and this expression pattern resulted in very poor overall survival [81]. SMAD7 was first described as an

inhibitor of TGF-β signaling but is now known as a versatile regulator of various signaling pathways. The role of SMAD7 in cancer progression is not uniform, as overexpression of SMAD7 can lead to both favorable and poor prognoses [115]. Interestingly, Huang et al. observed enhanced invasion and migration of GBC cells after SMAD7 inhibition, suggesting a more tumor suppressive role in this context, whereas, in another publication regarding BTC, SMAD7 was found to be overexpressed in CC samples and correlated with lower disease-free and overall survival [116].

Taken together, these studies show that deregulation of miRNAs plays a central role in development and progression of BTC and also translates into real clinical consequences. Overexpression or underexpression of certain miRNA species is associated with disadvantageous clinicopathological characteristics and poor disease-free and overall survival in BTC. The presented studies clearly demonstrate that individual miRNAs do act as tumor suppressors or oncogenes in BTC, depending on their actual regulatory targets. Of note, current evidence suggests that the same miRNA species can be both tumor-promoting and tumor-repressing within BTC. In the studies summarized in Table 1, miRNA-26a was found to be upregulated acting as an oncogenic miRNA, whereas it was found to be downregulated in another study, where it acted *de facto* as a tumor suppressor miRNA [62, 86]. Another example of this phenomenon is miRNA-200c, which was downregulated in CC samples in the study conducted by Peng et al. [56] but significantly upregulated in another study [117].

2.3. Regulation of miRNA Expression. Based on the presented studies, it is evident that deregulation of miRNAs influences various aspects of BTC carcinogenesis. Understanding the reasons of miRNA deregulation might therefore be of great scientific and clinical interest. As already described, miRNAs genes are transcribed by RNA polymerase II [15]. Like protein-coding genes, miRNA genes can be regulated by epigenetic events, altering the transcriptional accessibility of these genetic loci. DNA methylation at CpG islands in promoter regions is an epigenetic mechanism that leads to transcriptional repression of the respective gene. Chen et al. observed downregulation of miRNA-373 in patients with hilar cholangiocarcinoma [63]. In order to investigate the mechanism of the miRNA-373 deregulation, they analyzed the genomic surrounding of the miRNA-373 gene and identified a region at the 5′ flank that may serve as a promoter. Of note, this putative promoter harbors a potential CpG island, allowing for epigenetic regulation. Accordingly, analysis of the methylation status of this CpG island revealed high methylation rates, including homozygous methylation. Furthermore, high DNA methylation at this genomic region correlated with low miRNA-373 levels in BTC patient samples, suggesting that downregulation of miRNA-373 is a consequence of aberrant epigenetic regulation.

Another study that connects deregulated miRNA expression with DNA methylation was conducted by Zeng et al. [68]. Here, the authors investigated if the observed low expression of miRNA-124 in BTC samples is caused by enhanced DNA methylation. Inhibition of DNA methyltransferases significantly raised miRNA-124 expression. It is worth mentioning that this study was done using HCV-related IHC specimens and it is known that HCV infection is a major risk factor for development of BTC [2]. In this regard, Zeng et al. showed that HCV directly causes upregulation of DNA methyltransferases which in consequence leads to enhanced DNA methylation events and silencing of genes including miRNA-124 [68]. Further evidence of a central contribution of DNA methylation in regulation of miRNAs in BTC was presented by An et al. in a study investigating the underexpression of miRNA-370 in CC tissues [80]. Additionally, DNA demethylation in CC cells resulted in enhanced expression of miRNA-370. Looking at the imprinting status of miRNA-370, they found that the paternal allele of miRNA-370 was silenced via genomic imprinting, while the maternal allele was responsible for expression of this miRNA species. Therefore, reduction of miRNA-370, as observed in this study, has to be an implication of silencing of the maternal allele. An et al. also described overexpression of Interleukin-6 (IL-6) in their CC sample cohort, a cytokine that is well known to be a potent mitogen as well as a major proinflammatory factor in CC [118], and found that IL-6 induces DNA hypermethylation, thereby effectively suppressing the expression of miRNA-370 from the nonimprinted maternal allele [80]. This observation goes in line with another study that also described IL-6 as a promoter of DNA methylation, again leading to downregulation of the tumor suppressor miRNA-370 [119].

An interesting regulatory mechanism for miRNAs was described by Ma et al., which involves HOTAIR, a long-noncoding RNA which they found to be overly expressed in GBC patient material [120]. Silencing of HOTAIR led to an upregulation of miRNA-130a, suggesting a direct regulatory connection at the posttranscriptional level. Interestingly, this upregulation was only observed for mature miRNA-130a and not for its precursor forms. More evidence of direct regulation of miRNA-130a by HOTAIR relates to the fact that both of these RNA species were found to be present in the same RISC complex [120]. The potential oncogenic role of HOTAIR in BTC was already described for breast and colon cancer as well as for BTC [59, 121, 122].

3. MicroRNAs as Biomarkers

As mentioned in the introduction, one major problem in management of BTC is the advanced stage at time of diagnosis, excluding surgery as the only potentially curative treatment option [9]. Specific biomarkers in early stages of BTC would therefore allow rapid diagnosis and broaden the spectrum of therapeutic options available at time of diagnosis, which in turn should improve prognosis and outcome for patients with BTC. A suitable biomarker should be noninvasive, stable in fluids such as blood or bile, disease-specific, and easy to access and to measure and has to be sensitive in order to identify positive cases (high true positive rate) as well as being specific in order to distinguish positive from negative cases (low false positive rate) [123]. A common test for evaluation of a potential biomarker is the "Receiver Operating Characteristic" (ROC) analysis, in

which the true positive rate and the false positive rate of the potential biomarker are plotted. The "area under the curve" (AUC) is then used for interpretation of the diagnostic power of the tested biomarker candidate. By definition, a perfect biomarker results in an AUC of 1.0, whereas a random chance results in an AUC of 0.5 [124, 125]. The currently employed biomarkers for BTC are carcinoembryonic antigen (CEA) and CA19-9, both showing rather inferior sensitivity and specificity [126, 127]. Since the discovery that miRNAs are stable in serum and plasma and that these circulating miRNAs can serve as specific biomarkers for various types of cancer, scientists worked on the identification of miRNA expression patterns that can be used as biomarkers for BTC [128]. For BTC, bile might also be an attractive body fluid for identification of diagnostic miRNAs, as potential biomarkers may be directly secreted by malignant cholangiocytes into bile and, therefore, samples may be more meaningful and specific due to the spatial proximity of the tumor cells and this matrix [127]. Accordingly, Li et al. proved the existence of diverse miRNA species in extracellular vesicles from human bile and demonstrated high stability of miRNAs expressed in biliary extracellular vesicles [129]. In the following paragraph, we will give an overview of studies that looked for specific miRNA expression patterns in order to potentially identify biomarkers regarding BTC (see Table 2).

Circulating miRNA-106a was downregulated in serum of CC patients compared to patients with benign biliary disease (BBD) and healthy individuals, correlating with poor prognosis and lymph node metastasis [91]. Of note, a gradual decline of miRNA-106a serum levels was observed, with highest expression in healthy individuals, medium expression in BBD, and lowest expression in CC patients. The authors then evaluated the diagnostic power of circulating miRNA-106a for discrimination of CC patients versus healthy individuals and CC patients against BBD. Serum miRNA-106a was very effective in distinguishing CC from nonmalignant cases, resulting in an AUC of 0.89, and moderate in distinguishing CC from BBD with an AUC of 0.79. Compared to the standard BTC biomarker, CA19-9, the authors concluded that serum miRNA-106a shows moderate-to-superior diagnostic value [91].

As discussed above, miRNA-21 is a proven and potent oncogene in BTC carcinogenesis (see Table 1). In addition, several studies investigated the diagnostic power of this miRNA species. In plasma samples, miRNA-21 was overexpressed in BTC compared to BBD and healthy controls and, interestingly, after surgery, plasma levels of miRNA-21 significantly declined [93]. Again, an expression gradient was observable: highest expression of plasma miRNA-21 in BTC patients, medium in BBD patients, and lowest in healthy individuals. MicroRNA-21 was superior over CA19-9 in differentiating BTC patients versus healthy individuals with sensitivity of 84% (CA19-9: 36.2%). However, combination of plasma miRNA-21 and CA19-9 levels grouped as a diagnostic panel resulted in even better sensitivity (90.4%). A similar result was seen for discrimination between patients with BTC and BBD, where miRNA-21 showed approximately twice the sensitivity of CA19-9 (71.2% versus 36.1%), but, again, combination of both factors resulted in overall better sensitivity (79.8%) [93]. Further evidence that qualifies secreted miRNA-21 as a potential biomarker comes from a study conducted by Wang et al., in which they measured serum levels of miRNA-21 in patients with IHC compared to healthy individuals [84]. Serum miRNA-21 was significantly enhanced in IHC samples and, in line with the study by Kishimoto and coworkers [93], miRNA-21 markedly declined after surgery. However, this effect was only observable for potentially curative surgery and not for palliative resection [84]. Again, miRNA-21 showed robust characteristics as a biomarker for BTC: serum miRNA-21 levels discriminated IHC patients from healthy individuals with high sensitivity (87.8%) and specificity (90.5%) and an AUC of 0.908. In urine, miRNA-21 levels were increased in periductal fibrosis and CC patients compared to healthy individuals. MicroRNA-21 was able to distinguish healthy individuals from periductal fibrosis (AUC 0.735) and CC (0.820) patients [98]. Interestingly, the authors also observed enhanced levels of miRNA-192 in urine samples of *Opisthorchis viverrini*-infected patients as well as in periductal fibrosis and CC patients compared to healthy controls. The diagnostic power of miRNA-192 alone is moderate: *Opisthorchis viverrini*-infected (AUC 0.766); periductal fibrosis (0.781); CC (0.682) versus nonmalignant controls. However, combination of miRNA-21 and miRNA-192 in a biomarker panel enhanced their ability to discriminate healthy individuals from *Opisthorchis viverrini*-infected (AUC 0.812), periductal fibrosis (0.815), and CC (0.849) cases, making urine another possible noninvasive source of CC biomarkers [98]. Complementing these promising results in terms of miRNA-21 as a potential biomarker, Selaru et al. evaluated the diagnostic power of miRNA-21 expression in CC tissue and, although this study cannot contribute to the evaluation of miRNA-21 as a noninvasive diagnostic marker, the results are still valuable [83]. Selaru and coworkers saw not only significant overexpression of miRNA-21 in BTC tissue compared to nonmalignant control samples but also outstanding and nearly optimal sensitivity (95%), specificity (100%), and AUC (0.995) for miRNA-21 in distinguishing CC from normal bile duct [83].

Besides miRNA-21, serum miRNA-26a was also suggested to provide accuracy as a diagnostic tool for BTC [94]. Levels of miRNA-26a were increased in serum of CC patients compared to healthy control subjects and correlated with short progression-free and overall survival. Importantly, serum miRNA-26a was able to differentiate between patients with CC and healthy individuals with sensitivity and specificity values above 80% and an AUC of 0.899, thereby being a superior biomarker compared to CA19-9, which only displayed an AUC of 0.723. Of note, serum levels of miRNA-26a declined significantly in patients who underwent potential curative surgery [94]. In a large screen for differentially expressed miRNAs in serum of patients with BTC or PSC versus healthy individuals, Bernuzzi et al. identified miRNA-200c as being deregulated in PSC with an AUC value of 0.74 for distinguishing PSC from healthy control [95]. For BTC, they found serum levels of miR-483-5p and miR-194 to be enhanced compared to both PSC and controls (with control samples displaying the lowest expression levels). ROC curve

Table 2: MicroRNAs as potential biomarkers for BTC.

	miRNA (source)	Groups	Clinicopathological characteristics associated with deregulated miRNA expression	AUC	Ref.
Downregulated	106a (serum)	CC versus control	Lymph node metastasis; poor prognosis	0.89	[91]
	106a (serum)	CC versus BBD		0.79	[91]
	1537 (bile)	PSC versus PSC/CC		0.78	[92]
	412 (bile)			0.81	[92]
	640* (bile)			0.81	[92]
	3189 (bile)			0.80	[92]
Upregulated	21 (plasma)	BTC versus control	Decline of miRNA-21 plasma levels after surgery	0.93	[93]
	21 (plasma)	BTC versus BBD		0.83	[93]
	150 (plasma)	IHC versus control		0.791; 0.920 (+CA19-9)	[26]
	21 (tissue)	CC versus control		0.995	[83]
	21 (tissue)	IHC versus control	Decline of miRNA-21 serum levels after potentially curative surgery	0.908	[84]
	26a (serum)		Decline of miRNA-26a serum levels after potentially curative surgery; shorter progression-free and overall survival	0.899	[94]
	483-5p (serum)	CC versus control		0.77; 0.81 (+miRNA 194)	[95]
	194 (serum)			0.74; 0.81 (+miRNA 483-5p)	[95]
	192 (serum)		Lymph node metastasis; shorter survival	0.803	[96]
	200c (serum)	PSC versus control		0.74	[95]
	1281 (serum)	PSC versus CC		0.83	[92]
	126 (serum)			0.87	[92]
	26a (serum)			0.78	[92]
	30b (serum)			0.78	[92]
	122 (serum)			0.65	[92]
	9 (bile)	BTC versus BBD		0.975	[97]
	145* (bile)			0.975	[97]
	944 (bile)			0.765	[97]
	21 (urine)	CC versus control		0.820; 0.849 (+miRNA 192)	[98]
	192 (urine)			0.682; 0.849 (+miRNA 192)	[98]

AUC: area under curve; BBD: benign biliary diseases; BTC: biliary tract cancer; CC: cholangiocarcinoma; IHC: intrahepatic cholangiocarcinoma; miRNA: microRNA; PSC: primary sclerosing cholangitis; PSC/CC: cholangiocarcinoma complicating primary sclerosing cholangitis.

analysis for both of these miRNA species resulted in AUC of 0.77 for miRNA-483-5p and AUC of 0.74 for miRNA-194. However, combination of these two miRNAs significantly increased the AUC value to 0.81 [95]. MicroRNA-192, which was already found to be upregulated in urine samples of CC patients as mentioned above [98], is another circulating miRNA of potential diagnostic value as published by Silakit and others [96]. This miRNA species was found to be upregulated in CC serum samples versus serum of healthy control subjects and associated with disadvantageous clinicopathological characteristics. In distinguishing CC patients from control individuals, miRNA-192 achieved sensitivity of 74%, specificity of 72%, and an AUC of 0.803 [96]. A similar AUC (0.791) was calculated for circulating miRNA-150 in plasma to differentiate between IHC patients and healthy controls and, compared to the calculated AUC of CA19-9 (0.747), the potential of plasma miRNA-150 was superior [26]. Again, a combination of plasma miRNA-150 and CA19-9 was significantly more powerful as a diagnostic tool for BTC than either factor alone, resulting in an AUC of 0.92 [26]. Concerning bile as a potential source of BTC biomarkers, Shigehara and coworkers confirmed presence and stability of endogenous miRNAs in bile, making bile a potentially attractive source of biomarkers for BTC, and therefore compared in a comprehensive microarray study the expression levels of miRNAs in bile of patients with BTC with those of patients suffering from BBD [97]. They found numerous deregulated miRNAs, including three that were markedly upregulated in BTC versus BBD: miRNA-9, miRNA-145*, and miRNA-944. For miRNA-9 and miRNA-145*, the outstanding AUC value of 0.975 suggests high potential as specific markers to discriminate BTC from BBD. The prognostic power of miRNA-944 was lower, albeit still resulting in an AUC value of 0.765 [97]. In another study using bile as source of putative diagnostic relevant miRNA species, Voigtländer et al. performed a large screen in bile and serum of patients with PSC and CC and observed different miRNA profiles between both diseases [92]. In serum, they found five upregulated miRNAs in PSC versus CC which showed good-to-moderate diagnostic power in distinguishing PSC from CC: miRNA-1281, miRNA-126, miRNA-26a, miRNA-30b, and miRNA-122. In bile, potential biomarker miRNAs that were able to distinguish the two diseases were downregulated in PSC versus CC: miRNA-1537, -412, -640, and -3189. Of note, AUC values of each of those four candidates were relatively equal, around 0.80 [92].

4. Discussion and Outlook

MicroRNAs are of great scientific and clinical interest, as it becomes more and more clear that this noncoding RNA species is a major factor in cancer diseases [23]. Their sheer number combined with their ability to potentially target multiple protein-coding transcripts gives an idea of the overall regulatory power of miRNAs [20]. For BTC, not only might miRNAs constitute new therapeutic targets themselves but also their function helps shed more light on the cellular and pathologic processes contributing to BTC carcinogenesis. As summarized in this review and in Figure 1(a), identification of direct targets of deregulated miRNAs in BTC can provide valuable knowledge about functional associations and new starting points for therapeutic strategies (Table 1). As one intensively investigated example, miRNA-21 was found to be frequently deregulated in BTC samples with poor clinicopathological features (Table 1). By directly promoting tumor growth and aggressiveness via direct transcriptional suppression of known tumor suppressor genes, miRNA-21 acts as a potent oncogenic miRNA.

Diagnosis of BTC at early stages is one important factor to improve prognosis. However, up to now, available biomarkers are not sensitive and specific enough [127]. MicroRNAs have been shown to circulate in a stable form in serum and plasma [128] and, in addition, are also detectable in bile fluid [129]. Therefore, several studies have already investigated expression patterns of miRNAs in plasma, serum, urine, and bile of BTC patients compared to healthy individuals. They found that circulating miRNAs obtained from body fluids have the potency to be sensitive and specific noninvasive biomarkers for BTC for diagnosing the tumor as well as for discriminating BTC from BBD (Table 2). Of note, the calculated diagnostic power of certain miRNAs exceeds the diagnostic power of the standard BTC marker, CA19-9, as illustrated in Figure 1(b). However, following the results of the presented studies, it may be useful to not just concentrate on one biomarker but rather combine a certain number of candidate biomarkers in a group to achieve maximum sensitivity, specificity, and diagnostic accuracy [26, 93, 95]. Again, for the mentioned miRNA-21, its aberrant expression in plasma, serum, urine, and tissue has superior diagnostic power in differentiating BTC from BBD and healthy controls, thus qualifying this miRNA as a potential biomarker (Table 2). In this regard, it will be interesting to determine the expression levels of miRNA-21 in bile of BTC patients and healthy patients to estimate a potential biomarker function also in this body fluid. Further, more detailed investigation of the role of miRNA-21 in early events of BTC development and carcinogenesis will be helpful to evaluate miRNA-21 as a biomarker for BTC.

Abbreviations

15-PGDH: Hydroxyprostaglandin Dehydrogenase 15-(NAD)
3′ UTR: 3′ untranslated region
AUC: Area under the curve
BAG-1: BCL2-associated athanogene 1
BBD: Benign biliary disease
BTC: Biliary tract cancer
CC: Cholangiocarcinoma
EHC: Extrahepatic cholangiocarcinoma
EMT: Epithelial-mesenchymal transition
GBC: Gallbladder cancer
GSK-3β: Glycogen synthase kinase 3 beta
HBV: Hepatitis B virus
hCC: hilar cholangiocarcinoma
HCV: Hepatitis C virus
HMGA2: High-mobility group AT-hook 2
IHC: Intrahepatic cholangiocarcinoma

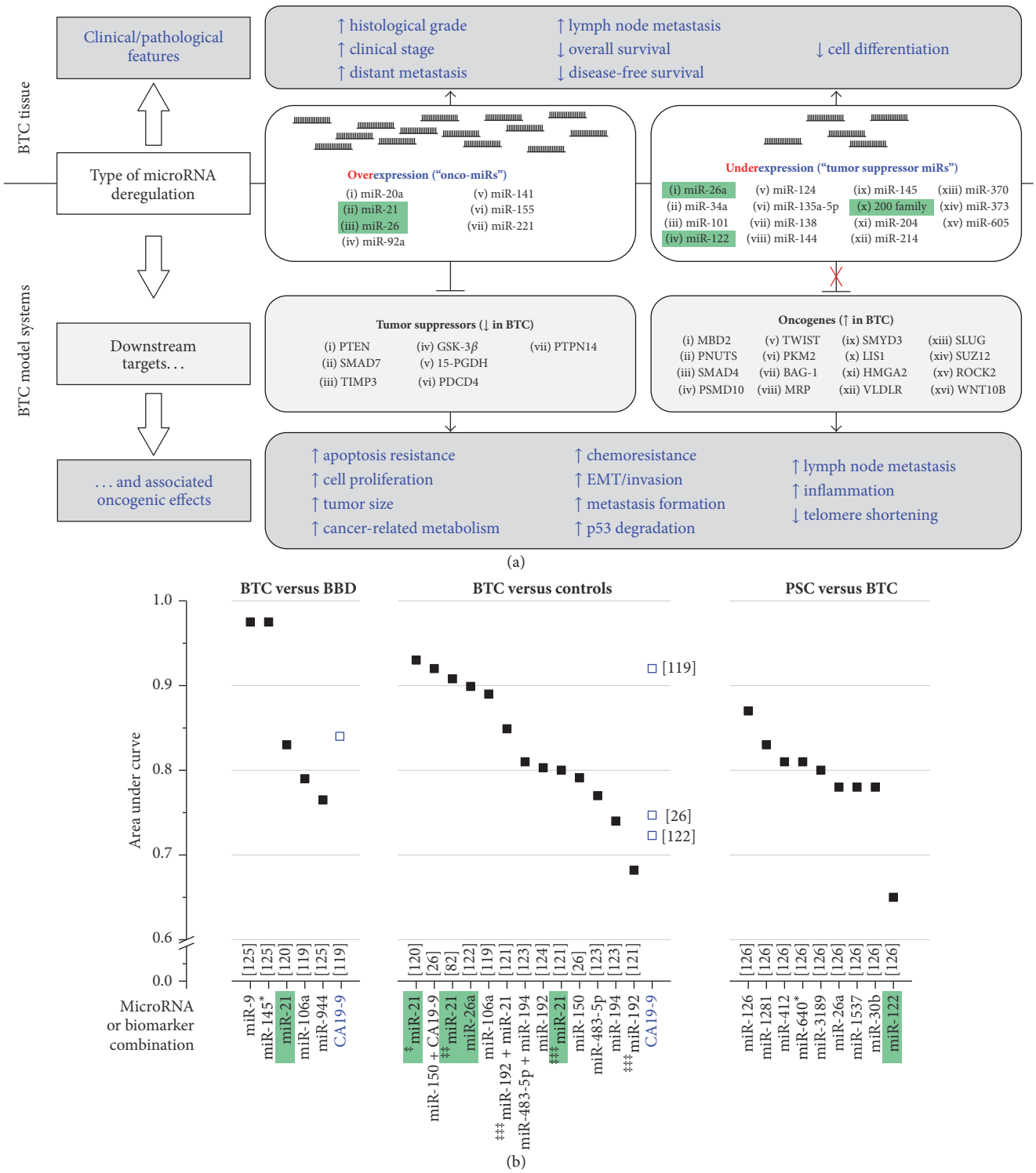

FIGURE 1: Role of deregulated miRs in BTC and their potential use as biomarkers. (a) Deregulation of miR expression in BTC tissue versus healthy controls results in unfavourable clinicopathological characteristics as well as poor outcome (upper part). Validated direct targets of deregulated miRs in BTC model systems include known tumor suppressors and oncogenes. Overexpression of oncogenic miRs results in aberrant downregulation of target tumor suppressors, whereas underexpression of tumor suppressor miRs results in insufficient negative transcriptional control of oncogenes (marked as red "X"), leading to their upregulation. Both of these events eventually cause diverse oncogenic effects (lower part). Figure based on Table 1. (b) Summary of distinguishing power of individual miRs regarding their use as potential biomarkers. The area under curve values from individual studies (reference numbers in square brackets; for details, see Table 2) indicate the quality of the respective miR as a biomarker (between 0.5 and max. 1.0), compared to CA19-9 as a conventional marker. Green boxes indicate miRs for which deregulation in BTC tissue as well as the use as a biomarker for BTC has been investigated. ‡: from plasma; ‡‡: from serum; ‡‡‡: from urine. For full gene names, see Abbreviations. BBD: benign biliary disease; BTC: biliary tract cancer; EMT: epithelial-mesenchymal transition; miR: microRNA; PSC: primary sclerosing cholangitis.

LIS1: Platelet activating factor acetylhydrolase 1b regulatory subunit 1
MBD2: Methyl-CpG binding domain protein 2
miRNA: MicroRNA
MMP-9: Matrix metallopeptidase 9
MRP1: ATP Binding Cassette subfamily C member 1
PDCD4: Programmed cell death 4
PGE2: Prostaglandin E2
PKM2: Pyruvate kinase, muscle
PNUTS: Protein phosphatase 1 regulatory subunit 10
PRC: Polycomb Repressive Complex
PSC: Primary sclerosing cholangitis
PSMD10: Proteasome 26S subunit, non-ATPase 10
PTEN: Phosphatase and tensin homolog
PTPN14: Protein tyrosine phosphatase, nonreceptor type 14
RISC: RNA-induced silencing complex
ROC: Receiver Operating Characteristic
ROCK2: Rho associated coiled-coil containing protein kinase 2
SLUG: Snail family zinc finger 2
SMAD4: SMAD family member 4
SMAD7: SMAD family member 7
SMYD3: SET and MYND domain containing 3
SUZ12: SUZ12 polycomb Repressive Complex 2 subunit
TGF-β: Transforming growth factor-beta
TIMP3: TIMP metallopeptidase inhibitor 3
TWIST: Twist family BHLH transcription factor 1
VLDLR: Very low-density lipoprotein receptor
WNT10B: Wnt family member 10B
YAP: Yes-associated protein 1.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Expression and Clinical Significance of the Novel Long Noncoding RNA ZNF674-AS1 in Human Hepatocellular Carcinoma

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Long noncoding RNAs (lncRNAs) play crucial roles in cancer occurrence and progression. However, the relationship between the expression levels of lncRNAs and the hepatocellular carcinoma (HCC) process is unclear. The goal of this study was to determine the expression level of ZNF674-AS1, a newly found lncRNA, in HCC and its clinical association. The expression of ZNF674-AS1 in 137 pairs of tumorous and adjacent normal tissues from patients with HCC was detected by quantitative real-time reverse transcription polymerase chain reaction. Additionally, the potential associations between its level in HCC tissue and clinicopathological features were analyzed. The expression of ZNF674-AS1 in the HCC cell lines HepG2, HCCLM3, SK-Hep1, HuH7, Hep3B, and MHCC97H was significantly downregulated compared with that in the normal liver cell line QSG-7701. The expression of ZNF674-AS1 was downregulated in 72% (99/137) of HCC tissues compared with that in paired adjacent normal tissues ($p < 0.01$). The results showed that the ZNF674-AS1 expression level was significantly correlated with metastasis ($p = 0.041$), clinical stage ($p = 0.039$), and histopathologic grading ($p = 0.045$). In addition, the Kaplan–Meier survival curves revealed that low ZNF674-AS1 expression was associated with poor prognosis in patients with HCC. Our data suggest that ZNF674-AS1 may play some role during cancer occurrence and progression and may be a new biomarker for HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is a leading cause of cancer death in many Asian and African countries [1]. HCC causes approximately 662,000 deaths each year worldwide and about half of them occur in China [2]. The major causes of HCC are viral infections and alcohol and tobacco use.

Intensive investigations over the last few decades have focused on the role of protein-coding genes in the pathogenesis of HCC, and efforts have been made to identify appropriate prognostic markers for HCC [3–6], including primary tumor size, elevated alpha-fetoprotein levels, and gene expression markers in the primary tumor. However, these methods have not proven to be adequate in predicting the prognosis of all patients with HCC. In addition, recent studies have indicated that several long noncoding RNAs (lncRNAs) are dysregulated in HCC, and their aberrant expression levels are associated with tumorigenesis, metastasis, prognosis, or diagnosis [7–12].

lncRNAs are non-protein-coding transcripts longer than 200 nucleotides that lack an open reading frame of significant length [13–17]. As a new type of regulatory RNA

molecule, lncRNA has a diverse subcellular location and plays important roles in many aspects of cell activity. Further, lncRNAs regulate gene expression at the epigenetic, transcriptional, posttranscriptional, and translational levels during cancer development [13, 18]. ZNF674 antisense RNA 1 (ZNF674-AS1, NR_015378) is an lncRNA that was first identified from the lncRNA expression profile of HCC identified by microarray analysis [10]. However, its association with HCC is unclear. The goal of the present study was to determine the expression level of ZNF674-AS1 in HCC and then to evaluate the relationship between its expression levels and clinical pathological characteristics of patients with HCC.

2. Materials and Methods

2.1. Patient Data and Tissue Samples. One hundred and thirty-seven fresh HCC tissue samples and matched normal adjacent tissue samples from 2010 to 2014 were selected from patients who underwent resection of primary HCC at our cancer center in the Department of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital, College of Medicine, Zhejiang University, China. Tumor tissues and paired adjacent nontumorous tissues 5 cm from the edge of the tumor were obtained during surgery. None of the patients received preoperative therapy. The resected tumor and paired nontumor tissue specimens were immediately frozen in liquid nitrogen and kept at −80°C until analysis [12]. The diagnosis of each specimen was confirmed histopathologically. All of the clinical data were collected by physicians, and the researchers were blinded to the clinical data. The study was approved by the Human Research Ethics Committee of The First Affiliated Hospital, College of Medicine, Zhejiang University. Written informed consent was obtained from all of the subjects.

2.2. Total RNA Preparation and Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Detection. Total RNA was isolated using TRIzol® Reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol, and cDNA was synthesized (Bio-Rad, Hercules, CA, USA). Real-time PCR was performed according to the manufacturer's instructions.

The expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ZNF674-AS1 were evaluated using real-time qRT-PCR. The primers were as follows: ZNF674-AS1 forward (5′-CAAAGCCTGTGGCCGATGTG-3′) and reverse (5′-ATGGTCACACATTCCTTCTCCC-3′) and GAPDH forward (5′-AGAAGGCTGGGGCTCATTTG-3′) and reverse (5′-AGGGGCCATCCACAGTCTTC-3′). The cDNAs were amplified using an Applied Biosystems 7500-fast PCR machine; the reaction was performed according to the PCR kit instructions. The cycling conditions were as follows: denaturation at 94°C for 5 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 20 s. All of the experiments were conducted three times, and the average was determined. The $2^{-\Delta\Delta Ct}$ formula was used to calculate differential gene expression [19].

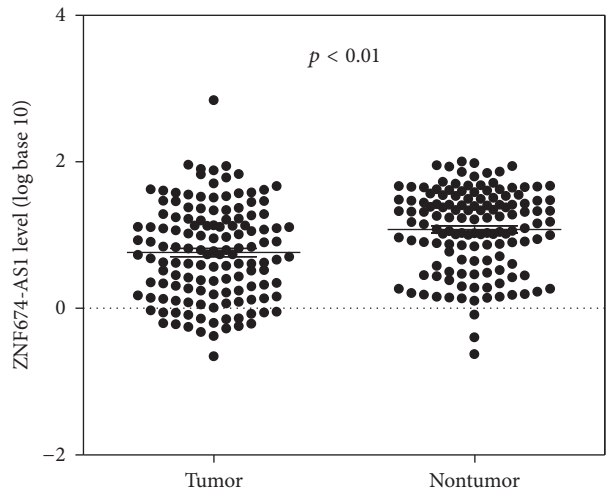

Figure 1: In all 137 tissue pairs, the downregulation of ZNF674-AS1 expression was significant in tumors compared to adjacent noncancerous tissues.

2.3. Cell Lines and Cell Culture. Six HCC cell lines (HepG2, HCCLM3, SK-Hep1, HuH7, Hep3B, and MHCC97H) and one normal liver cell line (QSG-7701), all of which are maintained at our institution, were used in this study. All of the cell lines were maintained in a humidified atmosphere containing 5% CO_2 at 37°C and were passaged using standard cell culture techniques [19].

2.4. Statistical Analysis. The relationship between ZNF674-AS1 expression and clinicopathological variables was assessed using a χ^2 test. All of the statistical analyses were performed using SPSS 19.0 for Windows (SPSS, Chicago, IL, USA) and GraphPad Prism® 5.0 (GraphPad Software, La Jolla, CA, USA). A one-way analysis of variance (ANOVA) and Student's *t*-tests were used as appropriate. Overall survival curves were plotted according to the Kaplan–Meier method. A p value less than 0.05 was deemed to indicate statistical significance.

3. Results

3.1. ZNF674-AS1 Was Downregulated in HCC Cell Lines and Tissues. Using qRT-PCR, we detected the expression levels of ZNF674-AS1 in HCC cell lines and tissues. We found that the expression level of ZNF674-AS1 in cancer tissues from patients with HCC was significantly lower than those in matched normal tissues ($p < 0.01$; Figure 1). Furthermore, the expression of ZNF674-AS1 was decreased in 72% (99/137) of HCC tissues compared with that in matched normal tissues (Figure 2). The expression of ZNF674-AS1 in five HCC cell lines (HCCLM3, SK-Hep1, HuH7, Hep3B, and MHCC97H) was significantly downregulated compared with that in the normal liver cell line QSG-7701 (Figure 3).

3.2. Relationship between the ZNF674-AS1 Levels in Cancer Tissues and Clinicopathological Factors in Patients with HCC. Next, we explored whether the ZNF674-AS1 expression levels were associated with the clinicopathological factors of HCC.

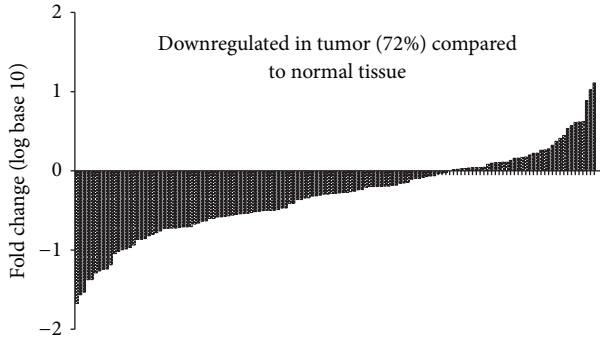

Figure 2: The ZNF674-AS1 expression levels in hepatocellular carcinoma (HCC) tissues was reduced (72%).

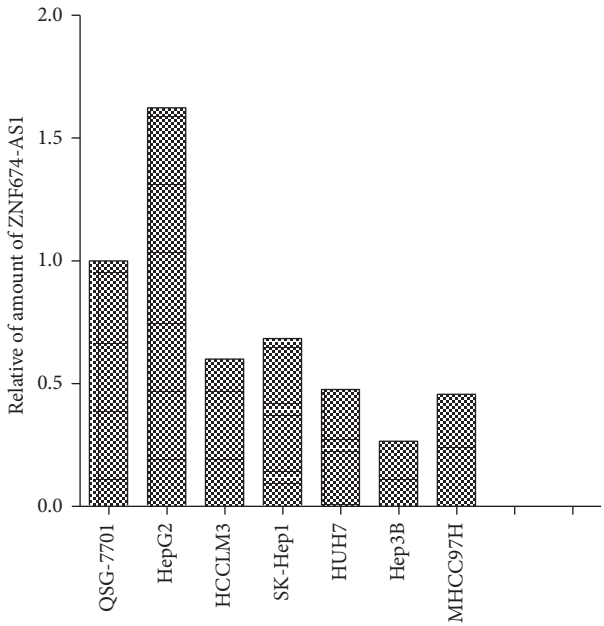

Figure 3: The ZNF674-AS1 expression level in HCC cell lines and a normal liver cell line QSG-7701.

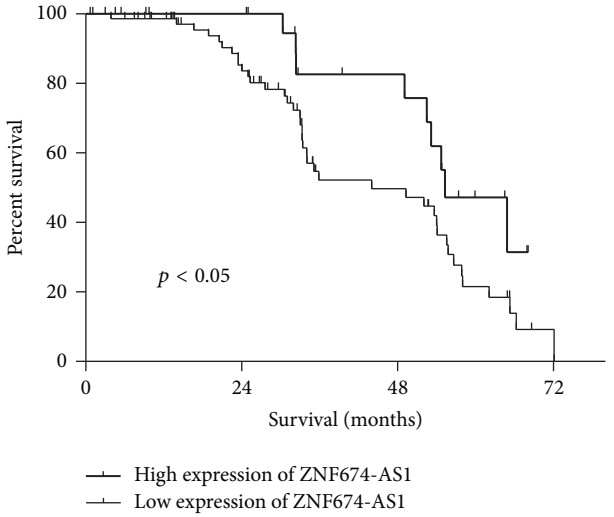

Figure 4: Kaplan–Meier curves estimating the 5-year recurrence-free survival rates according to the expression of the ZNF674-AS1 in patients with HCC who underwent hepatic resection.

As shown in Table 1, the ZNF674-AS1 levels were associated with clinical stage ($p = 0.039$), histopathologic grade ($p = 0.045$), and cancer distal metastasis ($p = 0.041$). However, there was no significant correlation between ZNF674-AS1 expression and other clinicopathological features, such as age, gender, tumor diameter, hepatitis B, and liver cirrhosis. Finally, the assessment of overall survival in HCC patients revealed that a lower expression of ZNF674-AS1 was correlated with the adverse survival of patients with HCC (Figure 4).

4. Discussion

With the advances in high-resolution microarray and massively parallel sequencing technology, it has been well accepted that at least 90% of the human genome is actively transcribed into ncRNAs, while less than 2% of the genome sequences encode proteins [13, 15, 20]. Recent studies have highlighted that lncRNAs, larger than 200 nucleotides, are a new class of noncoding RNAs that might play critical roles in HCC progression [9, 12, 21].

ZNF674 antisense RNA 1 (ZNF674-AS1, NR_015378), located at Xp11.23, is a novel lncRNA that was identified from a lncRNA microarray analysis [10]. Zhu et al. demonstrated for the first time that ZNF674-AS1 was upregulated in 19 pairs of HCC samples compared with adjacent tumor samples [10]. However, in our study, we found that ZNF674-AS1 levels in 137 cancer tissues from patients with HCC were significantly lower than those in corresponding normal tissues. Further, the expression of ZNF674-AS1 in five HCC cell lines (HCCLM3, SK-Hep1, HuH7, Hep3B, and MHCC97H) was also significantly downregulated compared with the normal liver cell line QSG-7701. The differences between our study and Zhu et al.'s may be due to the number of HCC samples; 19 pairs of HCC samples are not sufficient to determine the expression level of ZNF674-AS1 in HCC.

To investigate the clinical value of ZNF674-AS1 in HCC diagnosis and prognosis, we investigated the correlation between the expression of ZNF674-AS1 and clinicopathological features of HCC. The statistical analysis indicated that low ZNF674-AS1 expression was significantly associated with clinical stage, histopathologic grading, and cancer distal metastasis. The Kaplan–Meier analysis showed that patients with low levels of ZNF674-AS1 expression tended to live a shorter life than those with high levels.

The present study has some limitations. Although ZNF674-AS1 may be a tumor suppressor gene, we have neither verified its role in vivo and in vitro nor found the underlying mechanism in the progression of HCC.

5. Conclusion

These results suggest that the aberrant expression of ZNF674-AS1 might be involved in the biological characteristics of

Table 1: Clinicopathological correlation of ZNF674-AS1 expression in human hepatocellular carcinoma (HCC). AFP: Alpha Fetal Protein; PVTT: Portal Vein Tumor Thrombus; $^{*}p < 0.05$.

Parameters	Group	Total	ZNF674-AS1 expression		p value
			Low	High	
Gender	Male	118	85	33	0.88
	Female	19	14	5	
Age	<60	88	64	24	0.87
	≥60	49	35	14	
Hepatitis B	Absent	40	31	9	0.61
	Present	97	68	29	
Liver cirrhosis	Absent	40	31	9	0.38
	Present	97	68	29	
AFP	Negative	51	36	15	0.74
	Positive	86	63	23	
Tumor size	≤3	27	20	7	0.82
	>3	110	79	31	
Tumor number	Single	105	79	26	0.16
	Multiple	32	20	12	
PVTT	Absent	94	65	29	0.23
	Present	43	34	9	
Metastasis	Absent	121	84	37	0.04*
	Present	16	15	1	
Clinical stage	I-II	102	69	33	0.039*
	III-IV	35	30	5	
Histopathologic grading	Poorly	102	79	23	0.045*
	Well + moderately	35	20	15	

HCC and might be a novel biomarker for predicting the free survival of HCC.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Lufei Zhang and Tianyu He contributed equally to this work.

Acknowledgments

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Review Article

Prognostic Role of the Circulating Tumor Cells Detected by Cytological Methods in Gastric Cancer: A Meta-Analysis

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Objective. We performed a meta-analysis of available studies to assess the prognostic value of circulating tumor cells detected by cytological methods for patients with gastric cancer. *Methods*. Two authors systematically searched the studies independently with key words in PubMed, MEDLINE, EMBASE, Science Citation Index Expanded, and Cochrane Library (from inception to April 2016). The estimated hazard ratio, risk ratio, odds ratio, and their 95% confidence intervals were set as effect measures. All analyses were performed by STATA 12.0. *Results*. Sixteen studies were included in this meta-analysis. CTCs-high status was significantly associated with poor overall survival (HR = 2.23, 95% CI: 1.86–2.66) and progression-free survival (HR = 2.02, 95% CI: 1.36–2.99). CTCs-high status was also associated with depth of infiltration (OR = 2.07, 95% CI: 1.16–3.70), regional lymph nodes metastasis (OR = 1.85, 95% CI: 1.26–2.71), and distant metastasis (OR = 2.83, 95% CI: 1.77–4.52). For unresectable gastric cancer patients, CTCs-high status was significantly associated with poor overall survival, progression-free survival, and disease control rate before and during chemotherapy group. *Conclusions*. Our meta-analysis has evidenced the significant prognostic value of CTCs detected for both PFS and OS in gastric cancer patients. For patients treated with chemotherapy alone, we proved that CTCs detected by cytological method showed a significant prognostic value and poor response to chemotherapy.

1. Introduction

Gastric cancer is the fifth most common malignant neoplasm and the third leading cause of death from cancer [1]. Most patients relapse after a prior curative surgical approach [2]. So far, pathological stage, histological type, lymphatic vessels, and vascular infiltration were widely used as prognostic factors of patients with gastric cancer. But all of them had limitations, and new and better predictors of survival of patients with gastric cancer were needed. Since the circulating tumor cells (CTCs) were discovered in peripheral blood of the patient with cancer in 1896, CTCs have been used in many aspects of cancer management, such as monitoring tumor recurrence and treatment efficacy, determining drug-selection strategies, and predicting the survival of cancer patients [3]. Recently, meta-analyses of CTCs' prognostic value have been confirmed in patients with lung cancer [4], breast cancer [5], and colorectal cancer [6].

Due to the low concentration in peripheral blood and the limited technology on CTCs detection, general inspection methods find it difficult to detect the rare cells and there is no widely accepted method in detecting the CTCs in gastric cancer. Currently, the major techniques used to identify CTCs can be divided into two aspects, the cytological methods (such as CellSearch, immunocytochemistry, flow cytometry, and immune-magnetic and fluorescence-activated cell sorter) and the molecular methods (mainly the PCR) [3]. Although meta-analyses have shown that the presence of CTCs in peripheral blood of patients with gastric cancer was associated with poor prognosis and clinical characteristics [7–9], most studies involved in these meta-analyses used the molecular methods and the prognostic value of CTCs detected by cytological methods remains controversial. The pooled HR on OS from two meta-analyses showed different results for CTCs positive patients when detected by cytological methods (HR = 2.00, 95% CI: 0.1.28–3.13 [7]; HR = 1.67,

95% CI: 0.57–4.92 [9], resp.) and the number of the involved studies was very little (two and three, resp.). So there were limitations in them. Besides, new studies using the cytological methods have been reported recently. Therefore, it is necessary to carry out a new meta-analysis on the prognostic role of CTCs in patients with gastric cancer.

With the controversies existing in the prognostic role of CTCs for gastric cancer, here, we conducted the meta-analysis of published literature on this topic to summarize the evidence of the clinical and prognostic role of CTCs detected by cytological methods in gastric cancer patients.

2. Methods

2.1. Search Strategy. Two authors systematically searched the studies independently with key words "gastric cancer", "circulating tumor cells", "prognosis", and "peripheral blood" in PubMed, MEDLINE, EMBASE, Science Citation Index Expanded, and Cochrane Library (from inception to April 2016). An additional search through Google Scholar and the clinical trial registration website was conducted to obtain information identifying other potentially relevant publications. Discrepancies were resolved by the third author. In order to ensure the integrity of the retrieval, we also conducted a manual search.

2.2. Inclusion and Exclusion Criteria. The inclusion criteria were as follows: (1) studies using any kind of cytological methods to evaluate the association between the circulating tumor cells and overall survival (OS), progression-free survival (PFS), or clinic-pathological characteristics of gastric cancer; (2) sufficient data to calculate a hazard ratio (HR), risk ratio (RR), or odds ratio (OR) and 95% confidence interval (95% CI) being available; (3) at least 20 patients being involved in the studies; (4) samples being collected from the peripheral blood.

The exclusion criteria were as follows: (1) samples coming from lymph nodes, the peritoneal cavity, or bone marrow; (2) studies based on overlapping patients; (3) meta-analysis, review, single test, case report, conference reports and experiments, reporting the expert experience; (4) outcome being unclear or the apparent paradox existence; (5) unattainability of enough data after contacting the original author or magazine.

2.3. Data Extraction. Data retrieved from the studies included the first author's name, year of publication, number of patients, detection method, CTCs-high number, country (or area) of patients, sampling times (before the initiation of surgery and chemotherapy ["baseline"] or after the initiation of chemotherapy ["during chemotherapy"]), population of the patients (resectable or unresectable) and prognostic value (OS and PFS), disease control rate (DCR) to chemotherapy, tumor clinic-pathological characteristics, and hazard ratio (HR). For studies with multiple arms (i.e., resectable and unresectable groups), each of the subgroups was considered an independent data set. For studies with multiple time points (i.e., baseline and during chemotherapy), we used data from "baseline" samples prior to the data from "during chemotherapy" samples because those data were usually dependent. If the HR and its 95% CI were not reported directly in the original study, these values were calculated from available reported data using software designed by Tierney et al. [26]. All data was extracted independently by two investigators. The discrepancy between the reviewers was finally achieved through consultation. We used the Newcastle-Ottawa scale (NOS) [27] to assess the quality of cohort studies which was recommended by the Cochrane Library for observational studies, where a score of 5–9 means high quality and a score of 1–4 means low quality. This article follows the QUORUM and the Cochrane Collaboration guidelines (http://www.cochrane.de) for reporting meta-analysis (PRISMA statement) [28].

2.4. Statistical Analysis. All analyses of the data in our meta-analysis were performed using the STATA 12.0 package (StataCorp, College Station, TX, USA). The estimated HR was used to evaluate the prognostic effect (PFS and OS) as demonstrated by Parmar et al. [29] and HR > 1 reflected more deaths or progression in the CTCs-high arm. Besides, the estimated odds ratio (OR) was used to summarize the association between CTCs detection and gastric tumor clinic-pathological characteristics, and the estimated risk ratio (RR) was used to evaluate the efficacy of chemotherapy (DCR). All statistical values (pooled HR, RR, and OR) were combined with a 95% CI and the P value threshold was set at 0.05. Heterogeneity was assessed by I^2 inconsistency test and χ^2 based Cochran's Q statistic test [30] in which $I^2 > 50\%$ or $P < 0.1$ indicated significant heterogeneity. When $I^2 < 50\%$ and $P > 0.1$, the fixed effect model was used, or the random effects model was used conversely [31]. Publication bias was detected by Begg's test and Egger's test [32]. $P < 0.05$ was considered of significant publication bias. Furthermore, subgroup analyses were made according to the difference of the data retrieved such as country, methodology, population of the patients, CTC-high number, and quality of the studies. Subgroup analyses were performed only when there were two or more studies included in the subgroups. And in order to explore the potential sources of heterogeneity, we also did univariate metaregression analyses (random effects) on the same factors.

3. Result

3.1. Baseline Study Characteristics. According to the mentioned retrieval method, 581 potentially relevant studies were assessed. Detailed steps of the search were shown (Figure 1). After the selection procedure, 16 cohort studies with a total of 1110 gastric cancer patients were included [10–25]. The basic characteristics and the quality assessment of these studies were shown in Table 1. These studies were from seven countries (China, Japan, Korea, Poland, USA, UK, and Netherlands) and were published between 2007 and 2016. Four of the retrieved studies only provided the association between the CTCs and clinic-pathological characteristics.

12 studies mentioned the prognostic significance of the CTCs; and seven of the studies used the CellSearch method, eight provided the prognostic information of the unresectable gastric cancer patients treated with chemotherapy alone, and

Table 1: Baseline characteristics and quality assessment by the Newcastle-Ottawa scale of eligible studies.

Study	Number	Methodology	CTC-high number	Country	Population	Time points	End point	Stars
Li et al. 2016 [10]	136	CellSearch	≥3	China	UR	Baseline	OS/PFS	7
						During chemotherapy	OS/PFS	
Meulendijks et al. 2016 [11]	24	FACS-ICC	≥2	Netherlands	UR	Baseline	OS/PFS	5
Lee et al. 2015 [12]	100	CellSearch	≥5	Korea	UR	Baseline	OS/PFS	7
Okabe et al. 2015 [13]	136	CellSearch	≥1	Japan	R + UR	Baseline	OS/PFS	6
Xia et al. 2015 [14]	36	Flow cytometry	≥1	China	R	Baseline	OS	4
Kubisch et al. 2015 [15]	62	Immune-magnetic	≥1	USA	UR	Baseline	OS/PFS	7
						During chemotherapy	OS/PFS	7
Sclafani et al. 2014 [16]	22	CellSearch	≥2	UK	UR	Baseline	OS/PFS	4
Uenosono et al. 2013 [17]	251	CellSearch	≥1	Japan	UR	Baseline	OS	6
					R	Baseline	OS/RFS	
Ito et al. 2012 [18]	65	ICC	≥5	Japan	R	Baseline	OS	5
Matsusaka et al. 2010 [19]	52	CellSearch	≥4	Japan	UR	Baseline	OS/PFS	6
						During chemotherapy	OS/PFS	
Hiraiwa et al. 2008 [20]	27	CellSearch	≥2	Japan	UR	Baseline	OS	4
Pituch-Noworolska et al. 2007 [21]	57	FACS-ICC	≥1	Poland	R	Baseline	OS	7
Kolostova et al. 2015 [22]	22	MetaCell ICC	≥1	Poland	R + UR	Baseline	NR	3
Li et al. 2015 [23]	44	FACS	≥1	China	R	Baseline	NR	4
Li et al. 2014 [24]	45	FACS	≥1	China	R + UR	Baseline	NR	4
Yuan et al. 2015 [25]	31	FACS	≥1	China	R + UR	Baseline	NR	4

FACS: fluorescence-activated cell sorter; ICC: Immunocytochemistry; UR: unresectable; R: resectable; NR: unreported; Stars: 0–4 means low quality; 5–9 means high quality.

one had two independent data sets with multiple arms. Besides, three of the studies had two data sets with multiple time points. As to the quality assessment shown in Table 1, three of the 12 studies were of low quality and the other 9 studies were of high quality.

3.1.1. The Prognostic Effect (OS and PFS) of CTCs Detection. All 12 studies were available for the overall survival, and seven studies were available for the progression-free survival. There was no significant heterogeneity between these studies when pooling the HR on OS (I^2 = 28.6%, P = 0.157) and a fixed model was used; the pooled HR for OS was 2.23 (95% CI: 1.86–2.66) (Figure 2(a)). However, the heterogeneity for PFS (I^2 = 59.3%, P = 0.022) was significant, the random effects model was used, and the pooled HR was 2.02 (95% CI: 1.36–2.99) (Figure 2(b)). The pooled results showed that CTCs-high status detected by cytological methods was a significant prognostic factor for gastric cancer patients, and there were more deaths or progression in the CTCs-high arm than in the CTCs-low arm.

Furthermore, we stratified the included studies based on variables (such as country, population, methodology, CTCs-high number, and quality); the results were shown in Table 2. The results showed a significant prognostic effect for OS and PFS and demonstrated a higher risk of deaths or progression in the CTCs-high arm than in the CTCs-low arm for all subgroups. For PFS, the heterogeneity dropped to insignificant level when studies were stratified by methodology (I^2 = 39.9%, P = 0.155; and I^2 = 0.0%, P = 0.667, resp.). For OS, heterogeneity was eliminated in subgroups by exclusion of studies coming from non-East Asia countries, resectable patients, or non-CellSearch methods.

3.1.2. OS, PFS, and DCR with CTCs Detection in Unresectable Patients. Eight of the involved studies were designed for patients with unresectable or recurrent gastric cancer patients. As shown in Figure 3, eight data sets from baseline (before chemotherapy) samples of these studies were available for OS; the pooled analysis showed a prognostic effect of CTCs-high status (HR = 2.16, 95% CI: 1.72–2.71), with no significant heterogeneity between the studies (I^2 = 0.0%, P = 0.690) (Figure 3(a)). Six data sets with an significant heterogeneity (I^2 = 66.1%, P = 0.011) were available for PFS. The pooled HR for PFS was 2.03 (95% CI: 1.26–3.26) (Figure 3(c)). For the disease control rate (DCR), 4 studies were available. The pooled RR was 0.71 (95% CI: 0.61–0.82) With an significant heterogeneity (I^2 = 88.9%, P < 0.001) (Figure 3(e)). These results showed a poor prognosis and response

Table 2: Results of subgroup analyses on PFS and OS.

Variables	OS				PFS			
	HR [95% CI]	n	I^2 (%)	P^d	HR [95% CI]	n	I^2 (%)	P^d
Country								
East Asia	2.30 [1.89–2.80]	9	0.0	0.557	1.74 [1.21–2.50]	4	44.4	0.145
Non-East Asia	1.89 [1.23–2.90]	4	67.8	0.025	2.72 [1.04–7.13]	3	68.5	0.042
Population								
Resectable	2.38 [1.40–4.06]	5	66.3	0.018	—	1	—	—
Unresectable	2.16 [1.72–2.71]	8	0.0	0.690	2.03 [1.26–3.26]	6	66.1	0.011
Methodology								
CellSearch	2.17 [1.78–2.65]	8	0.0	0.870	1.69 [1.31–2.20]	5	39.9	0.155
Non-CellSearch	2.86 [1.39–5.90]	5	70.0	0.010	4.43 [2.39–8.23]	2	0.0	0.667
CTC-high $n \geq 3$								
Yes	2.03 [1.49–2.77]	4	11.0	0.338	1.65 [1.02–2.68]	3	60.8	0.078
No	2.33 [1.87–2.90]	9	38.1	0.114	2.52 [1.32–4.79]	4	58.6	0.064
Quality								
High	2.15 [1.79–2.60]	10	38.2	0.103	2.17 [1.45–3.27]	6	60.9	0.026
Low	3.03 [1.71–5.37]	3	0.0	0.603	—	1	—	—
Overall	2.23 [1.86–2.66]	13	28.6	0.157	2.02 [1.36–2.99]	7	59.3	0.022

The superscript "d" refers to heterogeneity.

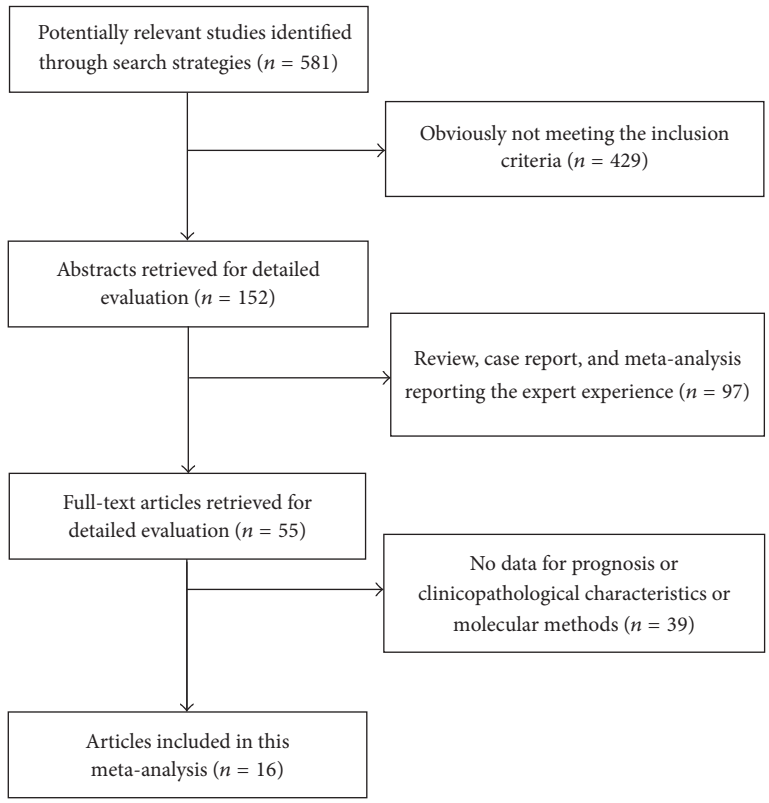
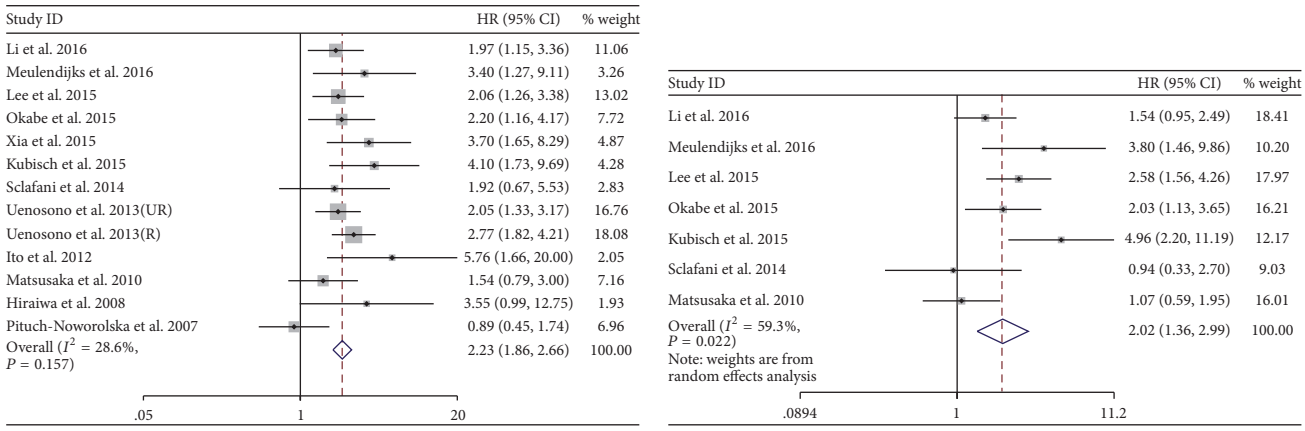


Figure 1: Selection of the included studies.

to chemotherapy in the unresectable gastric cancer patients with CTCs-high status detected at baseline.

Besides, three studies also reported the prognostic value and the DCR for the CTCs-high status detected during chemotherapy. We pooled these data separately, and the results were shown in Figure 3. A poor prognosis and response to chemotherapy were found in CTCs-high status arm (OS: HR = 4.33, 95% CI [2.77–6.76]; PFS: HR = 4.94, 95% CI [1.83–13.28]; DCR: RR = 0.62, 95% CI [0.49–0.77]) (Figures 3(b), 3(d), and 3(f)).



(a) OS (b) PFS

Figure 2: Hazard ratio (HR) for overall survival (OS) and progression-free survival (PFS) of the included studies.

3.1.3. Correlation between Detection of CTCs and Clinic-Pathological Characteristics. We extracted clinic-pathological characteristics from the included studies. The potential correlation between detection of CTCs and clinical variables was investigated and showed in Figure 4, when the clinical variables were mentioned at least in 5 studies. The pooled odds ratio demonstrated that the incidence of CTCs was significantly different between the T3/T4 and T1/T2 groups (OR = 2.07, 95% CI: 1.16–3.70, $n = 5$) (Figure 4(c)), region lymph node metastasis positive and negative groups (OR = 1.85, 95% CI: 1.26–2.71, $n = 10$) (Figure 4(d)), or distant metastasis positive and negative groups (OR = 2.83, 95% CI: 1.77–4.52, $n = 10$) (Figure 4(e)). However, the pooled OR showed no significant difference between female and male, III/IV and I/II, or peritoneum metastasis positive and negative groups (Figures 4(a), 4(b), and 4(f)).

3.1.4. Evaluation of Heterogeneity and Publication Bias. To explore the potential sources of heterogeneity, we conducted a meta-regression that considered the covariates of country, population, methodology, CTCs-high number, and quality for data from baseline samples. The results were shown in Table 3. In a univariate analysis, methodology showed a borderline explanatory variable that influenced the heterogeneity of estimated HR for PFS (coefficient = 0.980, standard error = 0.387, and $P = 0.053$), and it explained 73.92% proportion of between-study variance. However, other covariates were not significantly correlated with the heterogeneity across studies on PFS. For OS, none of these covariates was significantly correlated with the heterogeneity across studies on OS; this was in accordance with the little heterogeneity ($I^2 = 28.6\%$, $P = 0.157$) and may indicate the consistency between the involved studies.

For the data from during chemotherapy samples, we performed sensitivity analyses to explore the potential sources of heterogeneity and test whether the results were stable. And the results were showed in Table 4. Sensitivity analyses indicated that the study by Matsusaka et al. [19] was the main origin of the heterogeneity for PFS. After the exclusion of the study, the heterogeneity for PFS was removed. This may be due to the limited CTC-high patients of the study by Matsusaka et al. (only nine patients). And while we deleted any one of the studies from the overall pooled analysis each time, the pooled HR for OS and PFS still remained significant. This indicated that the pooled results were stable.

Publication bias was detected by Begg's test and Egger's test. $P < 0.05$ confirmed the existence of publication bias. No publication bias was shown in OS (Begg's $P = 0.300$, Egger's $P = 0.311$) and PFS (Begg's $P > 0.999$, Egger's $P = 0.672$).

4. Discussion

Gastric cancer is a very common disease with high rates of prevalence and mortality in the world [1]. Although great progress has been made in the treatment of gastric cancer, the five-year survival rate was still below 30% [2]. Recently, CTCs have been shown to have an important role in tumor metastasis, and their significant prognostic value has also been demonstrated in several cancers [4–6]. In this meta-analysis, we provided strong evidence that CTCs detected by cytological methods in peripheral blood were significantly associated with poor PFS and OS of gastric cancer patients, irrespective of the geographical, population, and detection methods and CTCs-high number differences.

The result of our meta-analysis solved the controversies from two independent meta-analyses [7, 9] and demonstrated the prognostic role of CTCs detected by cytological methods in gastric cancer. Cytological methods may avoid false positive results from nonneoplastic and contaminated samples which was frequent in molecular methods, and they were able to count the number of CTCs and recognize viable and functional CTCs [33], so they may provide us with a more accurate result by using the cytological methods. Besides, to our knowledge, this was the first meta-analysis that assessed the prognostic and predictive value of CTCs in unresectable gastric cancer patients treated with chemotherapy alone.

According to the results in our meta-analysis, CTCs detected by cytological method have shown an significant

Table 3: Results of metaregression on OS and PFS.

Variables	OS				PFS			
	Coef.	Std. err.	*P*	Adj *R*-squared	Coef.	Std. err.	*P*	Adj *R*-squared
Country	−0.1778	0.2920	0.555	−9.31%	0.4922	0.4600	0.333	18.62%
Score	0.3353	0.3580	0.369	14.16%	−0.8383	0.7327	0.304	2.50%
Methodology	0.1810	0.2714	0.519	−79.51%	0.9800	0.3875	0.053	73.92%
Population	0.0445	0.2507	0.862	−70.30%	0.0001	0.6618	>0.999	−47.68%
CTC-high *n*	0.1265	0.2551	0.630	−47.27%	0.4266	0.4353	0.372	1.48%

Adj *R*-squared: proportion of between-study variance explained; *n*: number; Coef.: coefficient; Std. err.: standard error.

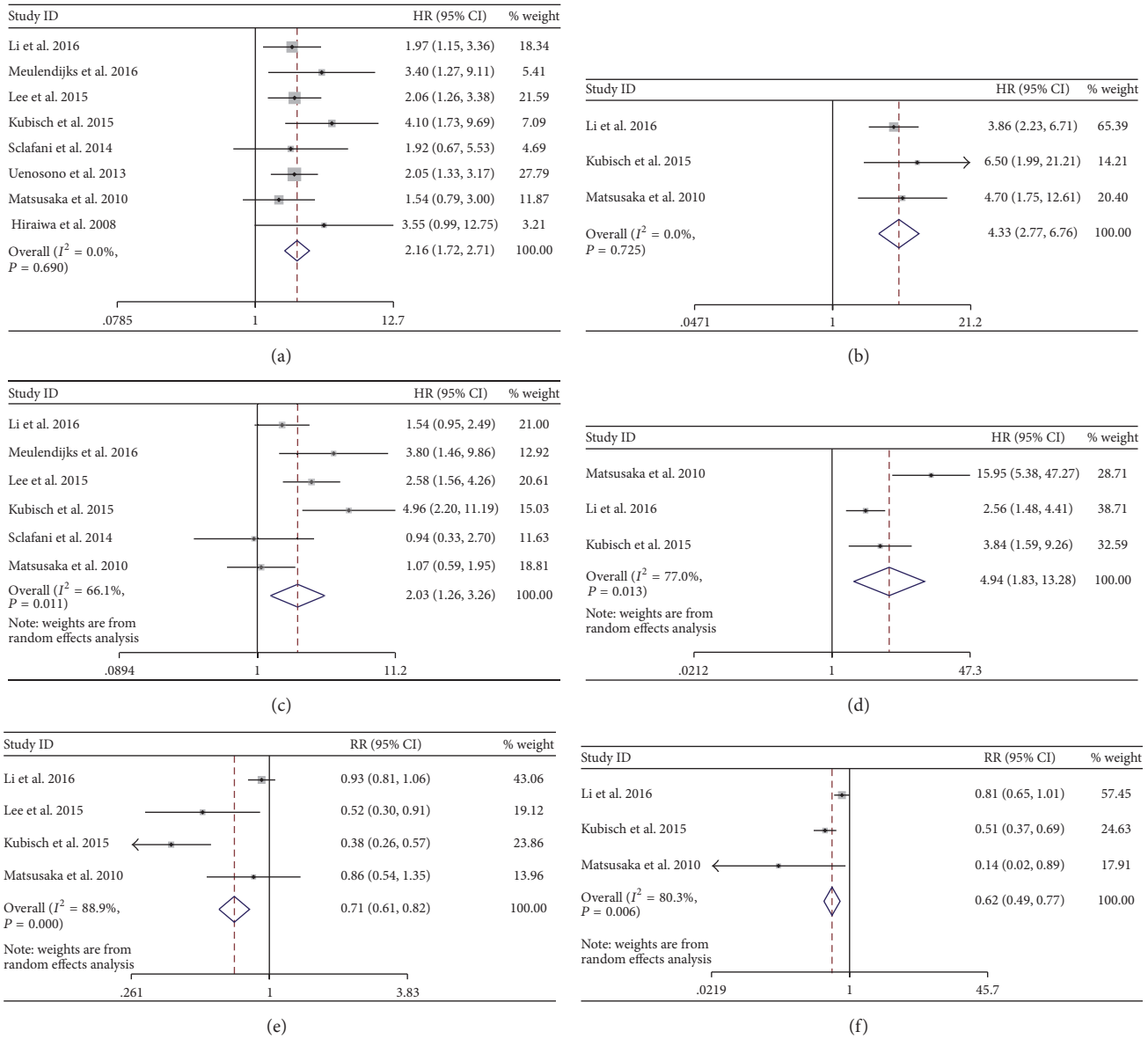


Figure 3: Hazard ratio (HR) for OS at baseline (a), OS in during chemotherapy (b), PFS at baseline (c), PFS in during chemotherapy (d), risk ratio (RR) for DCR at baseline (e), and DCR in during chemotherapy (f). OS: overall survival; PFS: progression-free survival; DCR: disease control rate.

Table 4: Sensitivity analyses for data during chemotherapy samples.

Study omitted	OS				PS			
	HR	95% CI	I^2 (%)	P^d	HR	95% CI	I^2 (%)	P^d
Li et al. 2016 [10]	5.37	2.52–11.45	0.0	0.680	7.54	1.87–30.38	74.9	0.046
Kubisch et al. 2015 [15]	4.05	2.50–6.55	0.0	0.734	6.00	1.00–35.92	88.5	0.003
Matsusaka et al. 2010 [19]	4.24	2.57–6.99	0.0	0.435	2.86	1.80–4.55	0.0	0.441
Combined	4.33	2.77–6.76	0.0	0.725	4.94	1.83–13.28	77.0	0.013

The superscript "d" refers to heterogeneity.

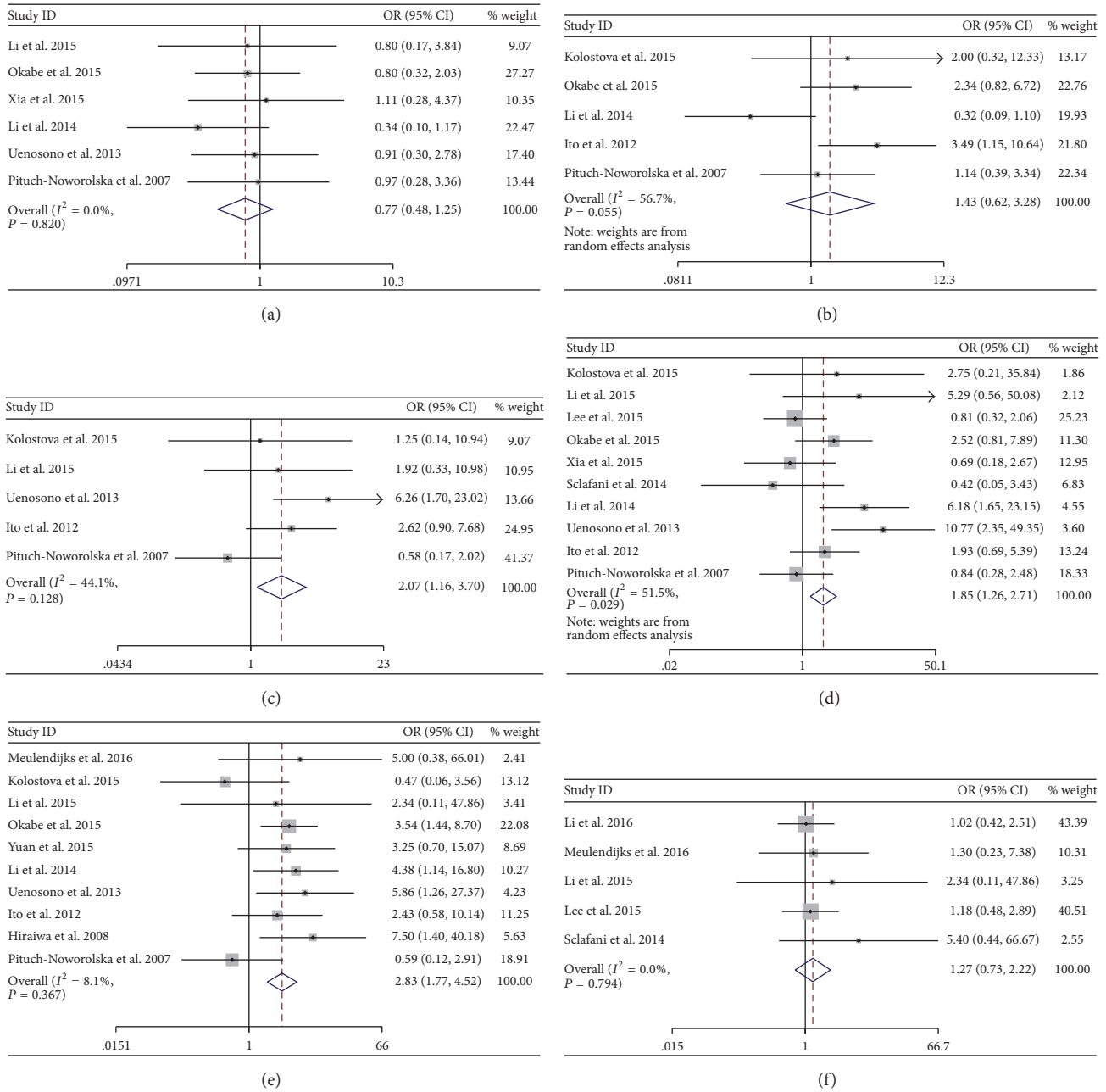


Figure 4: Odds ratio (OR) for sexuality (a), TNM stage (b) (III/IV versus I/II), depth of infiltration (c) (T3/4 versus T1/2), RLNs metastasis (d), distant metastasis (e), and peritoneum metastasis (f) associated with CTCs-high status. RLNs: regional lymph nodes.

prognostic value and association with some of the clinic-pathological characteristics in gastric cancer patients. The pooled results showed more deaths or progression in the CTCs-high arm than in the CTCs-low arm, and this result was also found in all subgroups when we stratified the included studies based on variables (Table 2). These results demonstrated that CTCs-high status detected at baseline indicated poor prognosis in gastric cancer patients and these patients may need more aggressive treatment and frequently efficacy assessments.

Furthermore, a significant heterogeneity was found in PFS ($I^2 = 59.3\%$, $P = 0.022$). To explore the potential sources of heterogeneity, we made subgroup analyses and found that the heterogeneity dropped to insignificant level when studies were stratified by methodology ($I^2 = 39.9\%$, $P = 0.155$; and $I^2 = 0.0\%$, $P = 0.667$, resp.). Then, in meta-regression, methodology also showed a borderline explanatory variable for the heterogeneity on PFS (coefficient = 0.980) and explained 73.92% proportion of between-study variance. So we finally confirmed methodology had positively contributed to heterogeneity on PFS. This may be explained by the multiple cytological methods. The approaches for CTC isolation/enrichment and techniques for CTC detection/identification used in those cytological methods were different; then, the specificity and reliability of its detection were also different. And as CTCs are generally thought to be quite heterogeneous in both phenotype and genotype, some specific CTCs may be ignored in some methods; for example, the CTCs that had undergone the epithelial-to-mesenchymal transition could hardly be detected by using CellSearch method and may be detected by using other methods. Besides, although heterogeneity was eliminated in subgroups by exclusion of studies coming from non-East Asia countries, resectable patients, or non-CellSearch methods, the meta-regression on OS showed none of the covariates was significantly correlated with the heterogeneity. Taking into account the little heterogeneity on OS ($I^2 = 28.6\%$, $P = 0.157$), we confirmed the consistency on OS between the involved studies.

Moreover, we assessed correlation between detection of CTCs and clinic-pathological characteristics, and we found that CTCs were more frequent in T3/T4, lymph node metastasis positive, and distant metastasis positive gastric cancer patients. But no significant difference was found between female and male, III/IV and I/II, or peritoneum metastasis positive and negative groups. The negative results for peritoneum metastasis and TNM staging were mainly caused by the limited studies (only five studies involved). So more studies assessing correlation between CTCs and clinic-pathological characteristics were needed.

In this meta-analysis, we proved that CTCs-high status showed a significant prognostic value and poor response to chemotherapy in gastric cancer patients treated with chemotherapy alone. As the data used in the initial analysis was only from prechemotherapy samples, we also made an independent analysis for the data from during chemotherapy samples. And we found coincident result of poor prognosis and response to chemotherapy for CTCs-high status patients in prechemotherapy and during chemotherapy group. The CTCs-high status before/during chemotherapy can be used as a prediction marker for the prognosis and response to chemotherapy.

At the same time, we found a more conspicuous result for both prognostic effect and the response to chemotherapy (DCR) in during chemotherapy group than prechemotherapy group. The same result for prognostic effect was also observed in other studies [11, 15, 19]. As for the conspicuous result in during chemotherapy group, we thought it may be because CTCs can be eliminated by chemotherapeutic drugs through direct and indirect mechanisms, such as cytotoxic and antimetabolic effects. And the remaining CTCs after chemotherapy may be more aggressive than before, and it may be easy to form metastases or cause recurrence. According to the results in our meta-analysis, we thought that CTCs-high status exhibited during chemotherapy may indicate more resistance to the chemotherapy and be useful for monitoring therapeutic effect. Furthermore, for data from during chemotherapy, we confirmed that the pooled results were stable and the heterogeneity was caused by the study of Matsusaka et al., and this may be explained by the limited CTC-high patients number in Matsusaka et al. [19]. Generally, CTCs-high during chemotherapy could provide earlier opportunities for early intervention or for the adjustment of chemotherapy by changing the chemotherapeutic regimen, intensity, and/or period.

Besides, as shown in another meta-analysis for colorectal cancer [34] and one study [10] involved in our meta-analysis, fluctuations in CTC levels before and during chemotherapy were closely associated with the tumor response to chemotherapy and prognosis, and the decreases and increases of CTC number in posttherapy were associated with superior and inferior survival, respectively. But for the lack of the related data, we failed to analyze the fluctuations of CTCs. So, for the predicted role of the changes in CTCs, more high quality related articles were needed.

There are some limitations in our meta-analysis. Firstly, the meta-analysis used the pooled data which was extracted from heterogeneous studies, not original data from the individual patients. The total number of patients from the involved studies was relatively small. Large prospective studies for gastric cancer were absent in this meta-analysis. Secondly, multiple methods for CTCs detection were used in our meta-analysis, and the standard for CTCs-high status in our retrieved studies was also different. These may contribute to the heterogeneity and limit its uses. Besides, approaches based on cytological method have biologic specificity and can quantify the number of CTCs, but the efficiency and sensitivity for the detection of CTCs are relatively low compared with the molecular methods. At last, little studies were designed for the predicted role of the fluctuations in CTC, so this meta-analysis did not carry out the relative conclusion.

5. Conclusion

In conclusion, our meta-analysis has evidenced the significant prognostic value of CTCs detected for both PFS and OS in gastric cancer patients, and the detection of CTCs was associated with some clinic-pathological characteristics. For

the patients treated with chemotherapy alone, we proved that CTCs detected by cytological method showed a significant prognostic value and poor response to chemotherapy. But, large prospective studies are needed to validate the prognostic values of the changes in CTC. Meanwhile, more high quality randomized controlled trials are needed to provide more information. And the same standardized detection platforms and number of the favorable CTCs are expected to normalize and reduce the inconsistencies across studies.

Abbreviations

CTCs: Circulating tumor cells
PFS: Progression-free survival
OS: Overall survival
DCR: Disease control rate
HR: Hazard ratio
RR: Risk ratio
OR: Odds ratio
95% CI: 95% confidence intervals.

Disclosure

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper. All authors have read and approved the final manuscript.

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Review Article

Predictors and Modulators of Synthetic Lethality: An Update on PARP Inhibitors and Personalized Medicine

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Poly(ADP-ribose) polymerase (PARP) inhibitors have proven to be successful agents in inducing synthetic lethality in several malignancies. Several PARP inhibitors have reached clinical trial testing for treatment in different cancers, and, recently, Olaparib (AZD2281) has gained both United States Food and Drug Administration (USFDA) and the European Commission (EC) approval for use in *BRCA*-mutated advanced ovarian cancer treatment. The need to identify biomarkers, their interactions in DNA damage repair pathways, and their potential utility in identifying patients who are candidates for PARP inhibitor treatment is well recognized. In this review, we detail many of the biomarkers that have been investigated for their ability to predict both PARP inhibitor sensitivity and resistance in preclinical studies as well as the results of several clinical trials that have tested the safety and efficacy of different PARP inhibitor agents in *BRCA* and non-*BRCA*-mutated cancers.

1. Introduction

DNA damage can be acquired through endogenous and exogenous sources that, if left unrepaired, can contribute to genomic instability and oncogenesis. Indeed, defects in the DDR signaling pathway are often found in various human cancers [1–3]. The concept of "synthetic lethality" between two genes becomes relevant when a mutation to either separately is still compatible with viability, but mutations to both genes lead to death [4]. If an oncogenetic gene mutation is viewed as the first "hit," targeting a partner gene or gene product should theoretically induce synthetic lethality in neoplastic cells. This therapy would also have minimal side effects on healthy cells with normal gene function [5]. A relevant example of synthetic lethality quickly moving to clinical application is the use of poly(ADP-ribose) polymerase (PARP) inhibitors for the treatment of *BRCA*-associated cancers. *BRCA1* and *BRCA2* are tumor suppressor genes encoding proteins that play important roles for DNA double-stranded break (DSB) detection for the homologous recombination (HR) repair pathway [6, 7]. Deficiencies in *BRCA1/2* function are associated with compromised HR repair, genomic instability, and oncogenesis [8–10]. PARP is a nuclear protein in the base excision repair (BER) pathway that recruits BER machinery to DNA single-stranded break (SSB) sites [5]. Inhibition of PARP would cause a collapse in the BER pathway and result in the accumulation of SSBs that break down to DSBs upon undergoing DNA replication [11–13]. In healthy cells, PARP inhibition would be of no large consequence because of effective DSB repair. However, in the context of *BRCA*-mutated cancers with compromised HR repair, breakdown of the BER pathway brought on by PARP inhibition would kill tumor cells from the buildup of DSBs [13–15]. The efforts to take advantage of synthetic lethality with PARP inhibitors have led to drug development for the treatment of patients with germline mutations in *BRCA1/2*. Olaparib is a PARP1/2 inhibitor that has gained approval by both the FDA and EC for use in patients with *BRCA*-mutated advanced, recurrent, platinum-sensitive serous ovarian cancer [16]. Meanwhile, Veliparib, Niraparib,

Table 1: PARP inhibitors currently undergoing development.

PARP inhibitors	Tumor types	Most advanced developmental stage in progress	Key clinical trials	References
Olaparib	FDA approved for ovarian cancer Being tested for breast, prostate, and pancreatic cancers	FDA approved	Phase II trial found that Olaparib monotherapy in patients with BRCA1/2 mutated ovarian cancer following ≥3 chemotherapy treatments resulted in 31% response rate Phase II trial found that Olaparib maintenance therapy in patients with platinum- (Pt-) sensitive recurrent serous ovarian cancer with mutated BRCA1/2 resulted in median progression free survival of 6.9 months longer than those receiving placebo	[150, 151]
Talazoparib	Being tested for ovarian, breast, and various advanced/metastatic solid cancers (primary peritoneal carcinoma, fallopian tube carcinoma, etc.)	Phase III	Phase II trial for Talazoparib monotherapy in patients with deleterious BRCA1/2 mutated ovarian cancer who had prior PARP inhibitor treatment currently recruiting Phase III trial for Talazoparib monotherapy in patients with BRCA1/2 mutated, advanced, or metastatic breast cancer currently recruiting	[152, 153]
Veliparib	Being tested for breast, pancreatic, non-small-cell lung cancers, lymphoma, and multiple myeloma, mostly in combination with chemotherapy	Phase III	Phase I/II trial of Veliparib and Topotecan for relapsed ovarian cancer of negative or unknown BRCA status completed Phase II trial of Veliparib alone or with Gemcitabine and Cisplatin in patients with locally advanced or metastatic pancreatic cancer currently recruiting Phase I/II trial for Veliparib, Bendamustine HCl, and Rituximab in patients with relapsed lymphoma and multiple myeloma completed	[154–156]
Rucaparib	Being tested for ovarian and pancreatic cancers	Phase III	Phase III trial for Rucaparib maintenance therapy in patients with Pt-sensitive recurrent ovarian cancer, fallopian tube, or primary peritoneal cancers currently recruiting Phase II trial for Rucaparib monotherapy in patients with BRCA1/2 mutated, locally advanced, or metastatic pancreatic cancer currently ongoing	[157, 158]
Niraparib	Being tested for ovarian and breast cancers and Ewing sarcoma	Phase III	Phase III trial for Niraparib monotherapy in patients with HER2 negative, BRCA1/2 mutated breast cancer currently recruiting Phase II trial for Niraparib monotherapy in patients with ovarian cancer following ≥3 chemotherapy treatments currently recruiting	[159–161]

Rucaparib, CEP9722, and BMN673 are all undergoing clinical trials to oversee their potential for treating common *BRCA*-associated cancers (Table 1) [17–21]. Several other PARP inhibitors that are mentioned in this review are being used in *in vitro* studies but have not yet been tested clinically.

In spite of the push to develop PARP inhibitors, opportunities for their optimal application remain largely unclarified. While defects in HR pathways signify opportunities for synthetic lethality, there is a push to look beyond *BRCA* mutational status to assess HR dysfunction, especially since only 15% of ovarian epithelial cancers are deficient in HR due to mutations of *BRCA1/2* [22, 23] and only 5–10% of breast and ovarian cancers are associated with *BRCA* germline mutation [24]. Meanwhile, it is increasingly apparent that HR

defects are not always predicted by germline *BRCA* status. For example, several phase II clinical trials that stratified patients according to *BRCA1/2* germline mutational status showed less than 50% objective response rate (ORR) to Olaparib compared to control [25, 26]. It appears that a significant subset of sporadic cancers with "BRCAness," a *BRCA*-like phenotype resulting from HR deficiencies, are also hypersensitive to PARP inhibitors. For example, Gelmon et al. showed that a significant fraction of ovarian and breast cancer patients with an intact *BRCA* gene responded to PARP inhibitors [27]. However, this expanded arsenal for PARP inhibitor therapy will remain untapped unless effective strategies are in place for patient stratification. Given the fact that BRCAness is a prerequisite for hypersensitivity to PARP inhibitors, the optimization of synthetic lethality relies on having biomarkers to predict BRCAness.

In this review, we detail select predictive and modulatory biomarkers for PARP inhibitors of clinical-translational significance that will help reap the benefits of personalized cancer therapy.

2. Biomarkers in the HR Pathway

2.1. Partner and Localizer of BRCA2 (PALB2). PALB2 is a tumor suppressor [28] and binding partner of BRCA2 that facilitates the nuclear localization and HR capabilities of BRCA2 [28]. During HR, PALB2 association with RAD51 and DNA stimulates strand invasion [28]. Mutations in *PALB2* have been demonstrated in 1.1% of patients with familial breast cancer [29] and the c.1592delT frameshift mutation has been linked to a 6-fold increase in likelihood of developing breast cancer [30]. *PALB2* mutations were also identified in 0.6% of patients with familial pancreatic cancer [31]. *PALB2*-deficient lymphoblasts EUFA1341 cells displayed increased cytotoxicity in response to Olaparib compared to their controls [28]. Due to the fact that PALB2 helps to regulate BRCA2/RAD51-mediated HR and has demonstrated its ability to induce synthetic lethality in the presence of PARP inhibition, PALB2 deficiency in tumors is an interesting prospect for future clinical trials regarding PARP inhibitor sensitivity.

2.2. Fanconi Anemia (FA) Complementation Group (FANC). FANC members include FANCD1 (BRCA2), FANCD2, FNAC31, and FANCN and play a major role in HR [32]. These proteins are related by their common association in a nuclear complex. After DNA damage, activation of the FA repair pathway involves the colocalization of FANCD2 with BRCA1 [33] in a manner dependent on monoubiquitination [34]. Thus, the functional biomarker of the FA pathway activation is nuclear FA protein/BRCA foci formation. The impairment of nuclear FA protein/BRCA foci formation after DNA damage is a powerful method for assessing functionality of the FA repair pathway [32, 35] and an important biomarker for HR defects. Powerful metrics are available to detect FANCD2/BRCA1 foci formation, such as the FA triple-staining immunofluorescence based method (FATSI), which identified a subset of non-small-cell lung cancer (NSCLC) tumors that were deficient in FANCD2/BRCA1 foci and thus were repair deficient [32]. Subsequently, these NSCLC cells were hypersensitive to Veliparib, BMN673, and ABT263 [32]. It was also shown that HeLa cervical cancer cells with defective FANCD2, FANCA, or FANCC exhibited cellular hypersensitivity to KU0058948 [4]. Thus, deficient FANCD2 manifested by absent foci formation after DNA damage may be a valuable biomarker to predict PARP inhibitor sensitivity.

2.3. Rad51. Rad51 is crucial for repair of DSBs via the HR pathway. RAD51 nucleates on single-stranded DNA molecules (ssDNA), which initiates the search for its homologous sequence and strand invasion [32, 36]. RAD51 also interacts with PALB2, BRCA1, and BRCA2 during HR [37, 38]. Formation of RAD51 nuclear foci in response to DNA damage is a functional biomarker for intact HR [39, 40] and lack of foci predicts deficient HR and breast cancer sensitivity to chemotherapy [35, 41]. Graeser et al. showed that sporadic breast cancers with lower Rad51 scores (Rad51 foci formation following anthracycline-based chemotherapy) showed decreased HR and increased sensitivity to anthracycline-based chemotherapy [42]. Furthermore, Rad51 paralog C deficiency caused Olaparib sensitivity in a gastric cancer xenograft model [43]. Increased sensitivity to KU0058948 was also observed in HeLa cells with deficient Rad51 and Rad54 [4]. Mukhopadhyay et al. demonstrated that 93% of ovarian cancer cells that showed no increase in Rad51 foci upon exposure to Rucaparib, and thus had deficient HR, subsequently showed cytotoxicity. Conversely, ovarian cancer cells that showed increased Rad51 foci, and thus had adequate HR, did not demonstrate cytotoxicity [44]. These results show that lack of Rad51 foci in response to DNA damage is a predictor of defective HR and thus can predict sensitivity to PARP inhibition.

3. Biomarkers in the DDR Pathways

3.1. Ataxia Telangiectasia Mutated (ATM). ATM is autophosphorylated on Ser1981 in response to DNA DSBs and phosphorylates several proteins within the nucleus of mitotic cells, including BRCA1, p53, CHK2, RAD17, and RAD9, resulting in DSB repair and arrest of the cell cycle [45]. McCabe et al. showed that HeLa cells treated with an ATM kinase inhibitor or siRNA targeting ATM were hypersensitive to KU0058948 [4]. Furthermore, Williamson et al. showed that Granta519 and UPN2 mantle cell lymphoma cells with low ATM expression levels were hypersensitive to Olaparib compared to their controls [46]. Interestingly, *ATM* deficiency predicted PARP1 inhibitor sensitivity in p53-null gastric cancer cells, and it was speculated that combined inhibition of ATM and PARP1 is a potential therapy for *p53*-disrupted gastric cancer [47].

3.2. Serine-Threonine Protein Phosphatase (PP2A). PP2A is a phosphatase in the Ser/Thr protein family with 4 regulatory subunits, PPP2R2A, PPP2R2D, PPP2R5A, and PPP2R3C. It is vital to DSB repair and activation of cell cycle checkpoints due to DNA damage [48] but has also been shown to negatively regulate ATM, CHK1/2, and other proteins necessary for DSB

repair [48]. This may be explained by the fact that different PP2A complexes have different functions at different points of the repair process [48]. PPP2R2A dephosphorylates ATM at S367, S1893, and S1981, which mediates its retention at sites of DSBs and facilitates HR [48]. Kalev et al. showed that 40% of NSCLCs exhibited decreased PPP2R2A levels and consequently had increased phosphorylation of ATM at S1981, decreased retention at sites of DSBs, and decreased HR [48]. Also, HeLa cells treated with shRNAs specific for PPP2R2A and lung carcinoma cell lines with intrinsically decreased levels of PPP2R2A showed increased sensitivity to Veliparib in comparison to their respective controls [48]. These facts demonstrate the importance of PPP2R2A in maintaining ATM function integrity and the potential usage of decreased PPP2R2A expression as a predictor of PARP inhibitor sensitivity.

3.3. Mre11. Mre11 is part of the Mre11-Rad50-Nbs1 (MRN) complex, which contributes to DSB sensing and scaffolding of HR effector proteins at DSB sites [49]. Deficiency in *MRE11* is commonly found in endometrial cancer, and Koppensteiner et al. found that these *MRE11*-deficient endometrial cancers are hypersensitive to BMN673 [50]. Loss of Mre11 in head and neck cancer cells confers hypersensitivity to GPI15427 both *in vitro* and *in vivo* using a mouse xenograft [51]. Furthermore, Cal51 breast cancer cells [50] and various acute myeloid leukemia (AML) cell lines [52] with deficient Mre11 showed hypersensitivity to KU58948 and BMN673, respectively.

3.4. Tumor Protein p53 (TP53). TP53 is a tumor suppressor in the DDR pathway that causes transient cell cycle arrest, senescence, and apoptosis in response to DNA damage [53]. Almost all *BRCA1*-mutated breast cancers have a deleterious *TP53* mutation, resulting from genomic instability-mediated complex and truncating mutations [54]. This suggests that *TP53* deficiency may represent a biomarker for BRCAness and hypersensitivity to PARP inhibitors [54, 55]. Furthermore, over 90% of basal-like breast cancers (triple-negative, high-grade breast carcinomas) have a deleterious *TP53* mutation and exhibit a molecular phenotype reminiscent of *BRCA1*-deficient breast cancer [54]. A recent study showed that depletion of TP53 in various breast cancer cell lines displayed hypersensitivity to the PARP inhibitor IQD in comparison to their respective controls [56].

3.5. γH2AX. γH2AX is a variant of the H2A histone family that is phosphorylated on Ser139 by ATM and ATM-Rad3-related (ATR) in the PI3K pathway of DNA repair and functions to recruit other DNA repair proteins in response to DNA damage [57, 58]. Importantly, γH2AX foci form in response to DSBs [57], and the presence of foci can be utilized as a biomarker to measure DNA damage induced by PARP inhibition [59]. *BRCA1*-mutated acute myeloid leukemia cells that were exposed to Olaparib subsequently formed γH2AX foci, suggesting that γH2AX foci formation may be a useful biomarker for successful PARP inhibition [59]. Furthermore, there have been two completed phase I trials for Veliparib in which investigators found that γH2AX was a reliable biomarker to measure sensitivity to PARP inhibition of circulating tumor cells of metastatic solid tumors or lymphomas [60, 61].

4. Biomarkers in the BER Pathway

4.1. Poly(ADP-Ribose) (PAR). PAR chains are linear and branched chains of up to 200 ADP-ribose units whose formation is catalyzed by PARP1/2 [62, 63]. PARPs play a significant role in BER, and PARylation acts as a specific indicator of PARP activity in DNA repair. PARylation also plays a role in chromatin modification, transcription, telomere cohesion, cell death, insulator function, mitotic apparatus function, and energy metabolism [62, 64], which affect genome stability, inflammation, neuronal function, aging, and carcinogenesis [64]. A subset of head and neck cancers has an elevation in basal PARylation [65]. Interestingly, head and neck cancer cells with elevated PAR are hypersensitive to Veliparib [65], suggesting that high PAR levels predict sensitivity to PARP inhibition. Further studies should be done to determine the significance of elevated PAR in different tumors.

4.2. PARP1-Binding Protein (PARP-BP). PARP1-binding protein (PARP-BP) is encoded by the gene C12orf48 and directly interacts with PARP1 to enhance its activity and the repair of DNA breaks [66]. Its expression is upregulated in pancreatic ductal adenocarcinomas (PDACs) and a number of other malignancies, which indicates increased PARP activity. Knockdown of C12orf48 in PDACs decreased PARP-BP expression, which subsequently caused decreased PARP1 activity and cell viability, while increasing sensitivity to Adriamycin, UV irradiation, and hydrogen peroxide [66]. This highlights the importance of PARP1 activity in the viability of PDACs with upregulated PARP-BP and should be further explored to determine if these tumors may be hypersensitive to PARP inhibitors.

4.3. X-Ray Repair Cross-Complementing 1 (XRCC1). XRCC1 is a key player in the DNA BER pathway, which is recruited in response to PAR chain formation at SSB sites by PARP1 [67]. *XRCC1* is deficient in 16% of breast cancers and is associated with high grade, triple negativity, loss of hormone receptors, and basal-like breast cancers [68]. *XRCC1*-deficient Chinese hamster ovary (CHO) EM9 cells showed accumulation of SSBs [67] and were hypersensitive to PARP inhibitors due to the supplementary effect of PARP inhibitors in preventing DNA ligation [67]. Mouse fibroblasts deficient in *XRCC1* were hypersensitive to the PARP inhibitor 4-amino-1,8-naphthalimide (4-AN) [69], and XRCC1 knockdown breast cancer cells were hypersensitive to KU0058948 [70].

5. "Other" Biomarkers

Lastly, there are other proteins and abnormalities in DNA expression that do not play a direct role in HR or DDR but can indirectly affect the process. These "other biomarkers" indirectly affect DNA repair through regulation of BRCA1/2, ATM, or other proteins responsible for its execution. Thus,

their abnormal expression may be predictive biomarker for PARP inhibitor sensitivity.

5.1. E26 Transformation Specific or E-Twenty-Six (ETS). *ETS* genes belong to a large family of transcription factors that regulate cell differentiation, proliferation, migration, cell cycle control, apoptosis, invasion, and angiogenesis [71, 72]. *ETS* gene fusions occur widely in many cancers including Ewing's sarcoma, acute myeloid leukemia (AML), and prostate cancer [73]. Baker et al. found that ETS-2 complexes with components of SWI/SNF repress *BRCA1* in MCF7 cells [74]. ETS-1 expression is a poor prognostic marker for breast, lung, colorectal, and ovarian cancer [71, 75, 76]. Interestingly, Legrand et al. revealed that ETS-1 activates the catalytic activity of PARP1, which then PARylates ETS-1 [76], revealing a novel link between ETS-1 and DDR pathways. They also found that PARP inhibition upregulates *ETS-1* transcriptional activity and led to its nuclear accumulation and selective cytotoxicity in ETS-1 expressing HeLa cells [76]. This suggests that nuclear ETS-1 expression may be a predictive biomarker for PARP inhibitor sensitivity. However, in a phase 1 dose-escalation study, no correlation was found between *ETS* gene rearrangement and sensitivity to Niraparib in prostate cancer [20]. Further studies should be performed to determine the significance of nuclear ETS-1 expression in PARP inhibitor sensitivity.

5.2. Transforming Growth Factor β (TGFβ). TGFβ is a cytokine whose presence at tumor sites has classically been associated with poor prognosis [77]. TGFβ has been shown to inhibit the expression of ATM, mutS homolog 2 (MSH2), and BRCA1 in BT474 breast cancer cells through microRNA, specifically the miR-181 family [77], inducing a BRCAness phenotype. Similarly, treatment of MDA-MB-231, MDA-MB-468, and BT474 breast cancer cells with TGFβ caused increased sensitivity to Veliparib [77]. Thus, the presence of increased TGFβ signaling may be an indicator of BRCAness and subsequent hypersensitivity to PARP inhibition.

5.3. MicroRNAs (miRNAs). miRNAs are small noncoding RNAs that mediate posttranscriptional repression and degradation of mRNA transcripts [77, 78]. Usage of miRNA is an effective and clinically tolerable method for inducing BRCAness and hypersensitivity to PARP inhibitors. The miR-181 family is induced by TGFβ to suppress ATM, MSH2, and BRCA, promoting BRCAness, as previously described [77]. Furthermore, more aggressive breast cancers exhibited increased expression of miR-181 [79]. Similarly, miR-182 downregulates *BRCA1* expression in various breast cancer cell lines *in vitro* and *in vivo*, resulting in defective HR-mediated repair and increased sensitivity to irradiation and Olaparib [80]. Mouse xenograft of MDA-MB-231 cells stably expressing miR-182 showed increased PARP inhibitor sensitivity to 4-amino-1,8-naphalamide (ANI) and Veliparib compared to their controls [80]. miR-103 and miR-107 target Rad51 and inhibit formation of Rad51 foci in response to DNA damage in osteosarcoma cells [81, 82] and subsequently increase sensitivity to Olaparib [81]. Furthermore, it was demonstrated that ovarian cancer cells with high levels of hsa-miR-107 were sensitive to Olaparib, and inhibition of hsa-miR-107 eliminated this sensitivity. Similarly, overexpression of miR-96 in osteosarcoma U2OS cells reduced the levels of Rad51 by directly targeting its coding region [83], decreasing the efficiency of HR and enhancing sensitivity to Olaparib.

5.4. Lysine-Specific Demethylase 1 (LSD1). LSD1 is an epigenetic regulator of gene expression that demethylates histones H3K4 and H3K9 [84]. LSD1 regulates genes associated with proliferation including those for p21, ErbB2, and Cyclin A2 [84]. LSD1 is upregulated in many cancers and is a predictive biomarker for aggressive biology in breast cancer and prostate cancer [84]. Increased LSD1 levels show a positive correlation with progression, proliferation, and invasion of breast cancer cells [85], and pharmacological inhibition of LSD1 results in growth inhibition [84]. One study found that LSD1 is recruited to sites of DNA damage in a manner dependent on Ring Finger Protein 168 (RNF168), suggesting its potential role in DDR downstream of RNF168 [86]. Interestingly, ectopic expression of LSD1 in basal-like breast cancer cells promoted downregulation of BRCA1 and hypersensitivity to Olaparib [87]. Thus, because LSD1 is upregulated in various cancers, including breast and prostate, and there is preliminary evidence of LSD1-dependent BRCA1 suppression and PARP inhibitor sensitivity, further clinical validation is required to explore LSD1 as an official biomarker for responsiveness to PARP inhibitor.

5.5. Cyclin-Dependent Kinase 12 (CDK12). CDK12 is a kinase that regulates cell cycle checkpoints and positively regulates BRCA1 [88]. It is mutated in nearly 3% of high-grade serous ovarian cancers, resulting in reduced BRCA1 levels and compromised HR repair. CDK12 is also a key regulator in the transcription of several other genes involved in DNA repair including ATM/ATR, FANCL, and BRCA2 [89]. As such, tumors with mutated *CDK12* should show "BRCAness" and are candidates for PARP inhibitor therapy [90]. A study has shown that *CDK12*-deficient ovarian cancer cells are more sensitive to Veliparib compared to their controls [90]. Furthermore, serous ovarian carcinoma cells with mutated *CDK12* exhibit hypersensitivity to Olaparib [91], platinum derivatives, and alkylating agents [89]. This provides evidence that *CDK12*-deficient ovarian cancers could be targets for PARP inhibitor therapy, and further work should be done to evaluate PARP inhibitor efficacy in other CDK12-deficient tumors.

5.6. Transforming Acidic Coiled-Coil Containing Protein 3 (TACC3). TACC3 is a member of the TACC family, which consists of proteins that localize at centrosomes to facilitate microtubule assembly and stabilization, enabling chromosomal integrity during mitosis [92]. TACC3 has been shown to regulate microtubule nucleation by interacting with γ-tubulin ring complex proteins [93] and promoting plus-end microtubule growth [94]. Both upregulation and downregulation of TACC3 are found in human solid tumors [95, 96]. Silencing of TACC3 results in microtubule destabilization and chromosome misalignment [92]. Overexpression of TACC3 has

been shown to lead to accumulation of DSBs and negative regulation of *ATM* and subsequent DDR signaling in U2OS cells [97]. Overexpression of TACC3 also impairs HR, NHEJ, and normal cell cycle checkpoint function in U2OS cells [97]. Moreover, it was found that nontumorigenic human mammary epithelial MCF10A cells with elevated levels of TACC3 showed hypersensitivity to Olaparib and NU1025 [97], suggesting its potential role in conferring synthetic lethality. Further studies with different cell lines should be employed to determine the role of TACC3 overexpression in conferring hypersensitivity to PARP inhibition.

5.7. Aurora Kinase A (Aur A). Aur A is a protein kinase necessary for construction of the mitotic spindle [98] and phosphorylates cell division cycle 25 homolog B (CDC25B) at the G2/M checkpoint, causing CDK1 activation and mitotic entry [99]. Cazales et al. showed that, during DNA damage-induced activation of the G2/M checkpoint, Aur A was not activated, and the cell cycle did not progress in U2OS cells [99]. However, ectopic expression of activated Aur A resulted in a bypass of this checkpoint [99]. Aur A is overexpressed in various solid tumors, including ovarian cancer [100], cervical cancer [101], and colon cancer [102]. Sourisseau et al. demonstrated that overexpression of Aur A impairs formation of Rad51 foci in MCF10A cells and HR in human embryonic kidney 293 (HEK293) cells. Overexpression of Aur A in PIR12 (Capan 1-derived PARP inhibitor resistant cell line) also induced sensitivity to KU0058948 [98]. Taken together, these data suggest that Aur A overexpression may predict sensitivity to PARP inhibition.

5.8. Phosphatase and Tensin Homolog (PTEN). PTEN is a tumor suppressor that inactivates the P13K/AKT pathway whose signaling is important for propagation of the cell cycle [103]. *PTEN* deficiency is associated with many malignancies, including breast [104] and prostate cancers [105], and disrupts chromosomal integrity by causing centromere breakage and translocations [106]. Mendes-Pereira et al. showed that *PTEN* deficiency leads to impairment of HR, which subsequently leads to increased sensitivity to KU0058948 in HCT116 colorectal carcinoma and HEC1A endometrial adenocarcinoma cells [107]. There are numerous reports of *PTEN*-deficient cancer cell lines that show decreased Rad51 levels [106, 107] and increased nuclear H2AX foci [106, 108, 109], suggesting deficient HR that leads to accumulation of foci. Furthermore, Shen et al. showed that PTEN potentiates activation of the *Rad51* promoter by E2F-1 in PC3 prostate cancer cells [106]. However, Fraser et al. showed that *PTEN*-deficient prostate cancer cells do not have decreased Rad51, have sufficient HR, and are insensitive to PARP inhibitors [108, 110]. This discrepancy may be explained by the fact that prostate cancer cells with an intrinsically null *PTEN* genotype have other genomic aberrations that are not present in prostate cancer cells with experimentally silenced PTEN, which can cause different behaviors [111]. Regardless, further studies should be pursued to examine the role of PTEN in HR in prostate cancer to determine its utility as a biomarker for predicting PARP inhibitor sensitivity.

5.9. Mitochondrial DNA (mtDNA). There is emerging evidence linking mtDNA depletion with BRCA2 depletion. mtDNA is depleted in breast, prostate, and thyroid transformed cells [112], which promotes activation of calcineurin/PI3Kinase/AKT signaling that causes upregulation of miR-1245 and ubiquitin ligase Skp2, negative regulators of BRCA2 [112]. This promoted HR deficiency and increased sensitivity to Rucaparib [112]. Further studies with a larger sample size are needed to further validate this promising correlation and to determine whether mtDNA depletion can be used as a biomarker for PARP inhibitor sensitivity.

5.10. Genomic Scar. A genomic scar is defined as a genomic abnormality [113] that is present in a wide variety of cancers, including breast, ovarian, pancreatic, esophagus, lung, and prostate cancers [113]. Several known genomic scars have been associated with BRCA1/2 dysfunction and homologous repair dysfunction [113]. Telomeric allele imbalance (N_{tAi}), loss of heterozygosity (LOH) clustering, mutational signature 3 (mutational signature D), and total number of somatic, synonymous, and nonsynonymous coding mutations (N_{mut}) are genomic scars that are predictive of BRCA1/2 dysfunction [113] and thus may predict responsiveness to PARP inhibitors. Homologous recombination defects (HRD) and large-scale transitions are genomic scars predictive of general HR dysfunction and may also indicate responsiveness to PARP inhibitors [113–116]. Further studies to evaluate the direct relationship between these genomic scars and sensitivity to PARP inhibitors should be performed.

6. Discussion

It is accepted that PARP inhibition mediates synthetic lethality in tumors with inherited BRCA deficiencies [117] and that BRCA1 hypermethylation can predict sensitivity to PARP inhibition [118–121]. However, it is increasingly clear that deficient *BRCA1/2* germline status is not enough to predict PARP inhibitor sensitivity [48, 122–124]. This growing sense is supported by clinical trials, which have shown that not all *BRCA1* mutation carriers are responsive to PARP inhibition [26, 125]. Altogether, these results further underline the need for nuanced biomarkers predictive of PARP inhibitor hypersensitivity. This entails a paradigm shift away from reliance on single predictive biomarkers for PARP inhibitor hypersensitivity (such as deficient *BRCA1* gene status) and towards the idea of predictive algorithms and biomarker codes that characterize various manifestations of "BRCAness" [126].

Additionally, we need to understand the roles of PARP1/2 outside of BER that contribute to the "off-target" effects of PARP inhibition, which induce cytotoxicity through mechanisms separate from dysfunctional HR repair, and can modulate or amplify the net synthetic lethal effect of PARP inhibitors. Even though synthetic lethality is mediated by PARP inhibition, not all benefits of PARP inhibitors are mediated through defects in HR repair. For example, while the canonical role of PARP is through the DNA damage repair pathway, PARP also plays a role in various networks including tumor-promoting inflammation, cell cycle

checkpoint regulation, senescence, angiogenesis, epithelial-mesenchymal transition (EMT), PARylation and remodeling of chromatin during transcription, and programmed cell death and metastasis [14, 127–130]. These off-target mechanisms may modulate the tumor microenvironment through a scenario known as "contextual" synthetic lethality that further sensitizes tumor cells to PARP inhibition [131]. This concept is exemplified in the emerging link between PARP inhibition and suppression of angiogenesis [130, 132–134]. Interestingly, hypoxic conditions deregulate DNA repair pathways and promote genomic instability through deregulation of BRCA1/2 [135–137]. Meanwhile, it is also postulated that PARP inhibitors inhibit BER to cause transient stalling of replication forks that degenerate into DSBs [138, 139].

It is also important to consider the way PARP inhibition relates to nononcogenic addiction, which is defined as the hyperreliance on secondary regulatory pathways in response to stressful conditions brought on by oncogene activation and tumor progression [140, 141]. Interestingly, nononcogenic addiction may represent underlying HR defects that can be targeted through synthetic lethality. For example, elevated PAR levels indicate PARP hyperactivity in response to underlying HR defects [65, 142, 143]. Thus, high PAR levels may indicate compensatory dependence on the BER pathway and therefore hypersensitivity to PARP inhibition [144]. As such, awareness of nononcogenic addiction events may enable us to distinguish between biomarkers of primary HR deficiencies versus biomarkers of secondary compensatory events both of which may predict hypersensitivity to PARP inhibition.

While the contextual parameters of PARP inhibition may be leveraged to our advantage, this may be a double-edged sword due to the addition of a new layer of complexity to the development of predictive biomarkers. For example, increased TGFβ signaling caused hypersensitivity to PARP inhibition in BT474 but not in MCF7 cells [145]. Meanwhile, knockdown of *ATM* by siRNA significantly increased sphere-forming efficiency (SFE) in BT474 and MDA361 but not in MCF7 cells. While these variations showcase the cell type-dependent outcomes of ATM regulation by TGFβ [146], they also highlight the possibility that contextual variables may inflate the perceived efficacy of PARP inhibition. Awareness of such possibilities helps guard against falsely attributing net cytotoxic effects to a single biomarker, since the observed efficacy of PARP inhibition may really be the sum effect of multiple mechanisms, not necessarily all due to synthetic lethality due to HR defects.

It is crucial to proceed with caution when identifying candidate predictive biomarkers. One example is the upregulation of EMSY, a putative oncogene that transcriptionally silences exon 3 of *BRCA2* that links the BRCA2 pathway to sporadic breast and ovarian cancer [147]. It was suggested that because EMSY amplification could mimic a *BRCA2* mutated state [148], it could account for BRCAness in sporadic breast and ovarian cancers with intact *BRCA2* [122] and possibly predict hypersensitivity to PARP inhibitors. However, it was recently shown that cells with an amplified EMSY had the same RAD51 foci formation efficacy, as well as sensitivity to PARP inhibitors, as cells without EMSY amplification [149]. Taken together, these results underscore the importance of triangulating BRCAness through a variety of biomarkers in order to detect opportunities for synergism, avoid conflation of various cytotoxic mechanisms, and customize treatment.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Stephen Murata and Catherine Zhang contributed equally to the paper.

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Research Article

Circular RNA-ITCH Suppresses Lung Cancer Proliferation via Inhibiting the Wnt/β-Catenin Pathway

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As a special form of noncoding RNAs, circular RNAs (circRNAs) played important roles in regulating cancer progression mainly by functioning as miRNA sponge. While the function of circular RNA-ITCH (*cir-ITCH*) in lung cancer is still less reported, in this study, we firstly detected the expression of *cir-ITCH* in tumor tissues and paired adjacent noncancer tissues of 78 patients with lung cancer using a TaqMan-based quantitative real-time PCR (qRT-PCR). The results showed that the expression of *cir-ITCH* was significantly decreased in lung cancer tissues. In cellular studies, *cir-ITCH* was also enhanced in different lung cancer cell lines, A549 and NIC-H460. Ectopic expression of *cir-ITCH* markedly elevated its parental cancer-suppressive gene, ITCH, expression and inhibited proliferation of lung cancer cells. Molecular analysis further revealed that *cir-ITCH* acted as sponge of oncogenic miR-7 and miR-214 to enhance ITCH expression and thus suppressed the activation of Wnt/β-catenin signaling. Altogether, our results suggested that *cir-ITCH* may play an inhibitory role in lung cancer progression by enhancing its parental gene, ITCH, expression.

1. Introduction

Lung cancer is the most common incident cancer and the leading cause of cancer-related death in China [1]. Although continuous efforts have been devoted to improving the therapeutic response and treatments for stage I lung cancer have demonstrated survival benefits [2, 3], the overall five-year survival rate of advanced lung cancer is still less than 15% [4–6]. Therefore, the development of finding novel therapeutic targets is of particular importance for the treatments of lung cancers, and a further understanding of the molecular mechanisms underlying lung cancer is essential to achieve this goal.

Circular RNAs (circRNAs) represent a large class of endogenous RNAs with covalently closed continuous loop [7]. For decades, circRNAs were mostly misinterpreted as splicing errors that result from splicing artefacts or gene rearrangements [8]. But recently (from 2012/2013), circRNAs were rediscovered from RNA sequencing (RNA-seq) data and shown to be ubiquitous in mammalian cells and more abundant (certain circRNAs are up to 200 times) than their linear counterparts [9, 10]. Tissue, as well as development-specific expression of circRNAs, further indicates that they originate from nonrandom back-splice events [7, 11]. With regard to their function, several studies reported that circRNAs mainly serve as miRNA sponges to regulate gene expression [7, 12]. For at least one specific circRNA, ciRS-7, which harbors more than 70 conventional miR-7 binding sites, impairs the regulatory effect of miR-7 *in vivo* [12]. miRNAs regulate a variety of essential biological functions such as cellular differentiation, apoptosis, and proliferation and thus play critical role in cancer progression [13]. Based on these clues, circRNAs were found to be closely related to development of different cancers, including esophageal squamous cell carcinoma, colorectal cancer, gastric cancer, and ovarian cancer [14–17]. Specifically, Hsa_circ_002059 expression levels are significantly correlated with distal metastasis and TNM stage of gastric cancer and thus may be a potential novel and stable biomarker for the clinical diagnosis of gastric cancer [17].

Aberrant activation of the Wnt/β-catenin pathway plays a critical role in tumor initiation, progression, and metastasis of lung cancer [18–20]. The E3 ubiquitin (Ub) protein ligase (ITCH) inhibits Wnt/β-catenin signaling in cancers mainly by promoting the ubiquitination and degradation of phosphorylated disheveled 2 (Dvl2) [21]. Circular RNA-ITCH

Table 1: Baseline demographic and clinical characteristics of study populations.

Characteristics	Population		cir-ITCH relative expression[#]	p value
	N	(%)		
Age (years)				
≤40	10	12.82	0.0607 ± 0.0169	0.0076*
40–60	31	39.74	0.0466 ± 0.0307	
≥60	37	47.44	0.0357 ± 0.0155	
Sex				
Male	45	57.7	0.0432 ± 0.0257	0.9704
Female	33	42.3	0.0434 ± 0.0225	
Family history				
Yes	23	29.49	0.0441 ± 0.0290	0.8437
No	55	70.51	0.0429 ± 0.0221	
Smoking				
Never	53	67.95	0.0487 ± 0.0283	0.1713
Ever	25	32.05	0.0407 ± 0.0230	
Drinking				
Never	33	42.3	0.0478 ± 0.2352	0.1568
Ever	45	57.7	0.0399 ± 0.0244	
Tumor type				
Adenocarcinoma	29	37.18	0.0443 ± 0.0267	0.9183
Large cell carcinoma	25	32.05	0.0436 ± 0.0250	
Squamous cell carcinoma	24	30.77	0.0416 ± 0.0218	
Stage				
I	18	23.08	0.0605 ± 0.0273	0.0011*
II	21	26.92	0.0444 ± 0.0260	
III	22	28.21	0.0375 ± 0.0182	
IV	17	21.79	0.0309 ± 0.0142	

* $p < 0.05$ means statistically significant difference existed within subgroups.
[#] Relative expression value was normalized to GAPDH expression level.

(*cir-ITCH*) shared some miRNAs binding sites with the 3′-untranslated region (UTR) of ITCH, including those for miR-7, miR-17, miR-214, miR-128, and miR-216b [7, 12, 15]. As sponge of oncogenic miR-7, miR-17, and miR-214, *cir-ITCH* increases the level of ITCH and thus indirectly inhibits the activation of Wnt/β-catenin pathway; these effects finally result in the suppression of esophageal squamous cell carcinoma [16] and colorectal cancer [15]. However, there are no reported studies on the functional roles of *cir-ITCH* in lung cancer.

As two oncogenic miRNAs, miR-7 and miR-214 are overexpressed in lung cancer cells, enhance radiotherapy response, and promote the progression of lung cancer [22, 23]. Thus, in this study, we hypothesized that *cir-ITCH* might compete with ITCH to bind to miR-7 and miR-214 and may be involved in lung cancer development. To address this hypothesis, we detected the expression of *cir-ITCH* in primary tumor tissues and different lung cancer cell lines. Then, the functional relevance of *cir-ITCH* with lung cancer was further examined by biochemical assays.

2. Materials and Methods

2.1. Participants and Tissue Samples. The study was approved by the Ethical Review Board for Research in Tongji Hospital, affiliated to Tongji Medical College of Huazhong University of Science and Technology. 78 lung cancer biopsy specimens and paired adjacent normal tissues were obtained from Department of Pathology of Tongji Hospital. Tissues were acquired and immediately stored at liquid nitrogen until use. There were no limitations on the age, sex, histology, or stage of lung cancer. The patients' characteristics were summarized in Table 1.

2.2. Cell Culture. Human lung cancer cell lines A549 and NCI-H460 were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, Shanghai Institute of Cell Biology. Cells were cultured in DMEM medium (Gibco, CA, USA) and supplemented with 10% fetal bovine serum (Gibco), 2 μM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

2.3. Circular RNA Plasmid Construction. Human *cir-ITCH* cDNA was synthesized by GeneWiz (Suzhou, China) and cloned into pcDNA3.1 (Invitrogen, CA, USA) as previously described [15, 16]. Recombinant plasmid pcDNA3.1-*cir-ITCH* was verified by direct sequencing.

Table 2: The sequences of primers used in this study.

Gene	Forward (5′-3′)	Reverse (5′-3′)	Probe
cir-ITCH	GCAGAGGCCAACACTGGAA	TCCTTGAAGCTGACTACGCTGAG	CCGTCCGGAACTATGAACAACAATGGCA
GAPDH	CCATGACCCCTTCATTGACC	TTGATTTTGGAGGGATCTCG	CTGAGAACGGGAAGCTTGTC
Linear ITCH	TAGACCAGAACCTCTACCTCCTG	TTAAACTGCTGCATTGCTCCTTG	
Circular ITCH	ACAGAGACAACCGAGAAACAGTG	GCCTTGATACTTGTTACCGTCGA	
c-Myc	TTCGGGTAGTGGAAAACCAG	CAGCAGCTCGAATTTCTTCC	
cyclinD1	GAGGAGCAGCTCGCCAA	CTGTCAAGGTCCGGCCAGCG	
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	

2.4. RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction. Total RNA was isolated from cells and tissues using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was reversely transcribed into cDNA using First Strand cDNA Synthesis Kit (Toyobo). The relative gene expression of *cir-ITCH* was quantified using a real-time RT-PCR with the TaqMan probe. GAPDH was used as an internal control [24], and all reactions were performed in triplicate. The primers used for polymerase chain reaction (PCR) amplification are listed in Table 2.

2.5. RNase R Digestion. The RNase R digestion reaction was performed following previously published procedures. The digestion and precipitation reactions were repeated twice with a ratio of 3 U of enzyme/1 mg of RNA [25].

2.6. Transient Transfections and Luciferase Assays. A549 and NCI-H460 cells were seeded in 24-well plates (1×10^5 cells per well) and cultured to about 70% confluence before transfection. Then, cells were transfected with 800 ng of the reporter plasmids described above using Lipofectamine 2000 (Invitrogen). Cells were cotransfected with the miRNAs according to the manufacturer's instructions [26]. Each group included 6 replicates, and triplicate independent experiments were performed. 24 h after transfection, the cells were collected using 100 μL passive buffer, and Renilla luciferase activity was detected using the Dual-Luciferase Reporter Assay System (Promega); the results were normalized against the activity of the Renilla luciferase gene [16].

2.7. Actinomycin D Assay. A549 and NCI-H460 cells were seeded at 5×10^4 cells per well in 24-well plate overnight and then transiently transfected with 1 or 40 pmol of miRNA mimics (Ambion) using Lipofectamine 2000 with or without 40 pmol of miRNA inhibitor as indicated. 24 h later, cells were then exposed to 2 mg/L actinomycin D (Sigma) for 1, 2, and 3 h. The cells were harvested and the stability of the *cir-ITCH* mRNA was analyzed using quantitative reverse transcription PCR (qRT-PCR).

2.8. Western Blotting. Protein was isolated from cell lysis using Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Rockville, MD, USA). Equivalent amount of protein was loaded on 10% SDS-PAGE gel (Invitrogen) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The PVDF membranes were blocked with 5% nonfat milk for 1 hour at 37°C. Membranes were incubated overnight at 4°C with anti-Wnt3a antibody (1 : 1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-β-catenin antibody (1 : 1000 dilution, Santa Cruz Biotechnology), or β-actin (1 : 5000 dilution, Abcam, Cambridge, MA, USA) and then incubated with secondary HRP-goat anti-rabbit/mouse antibodies (1 : 10000 dilution, Santa Cruz Biotechnology). Signals were detected using ECL detection reagent (Millipore) following the manufacturer's instructions.

2.9. Cell Viability Assay. Cell viability assay was carried out with a Cell Counting Kit-8 (Beyotime, Shanghai, China) according to the manufacturer's instructions [27]. 1×10^4 cells in 100 μL of A549 and NCI-H460 cells were seeded into 96-well plates (BD Biosciences), respectively. 24 h after transfection, cells were incubated for another 1, 2, and 3 days. The numbers of cells per well were detected by the absorbance (450 nm) of reduced WST-8 at the indicated time points. The absorbance (450 nm) was measured by using SpectraMax® i3x microplate reader (Molecular Devices, Sunnyvale, CA, USA). There were 6 replicates for each group, and the experiments were repeated at least 3 times.

2.10. Statistical Analysis. All data are presented as mean ± SD and analyzed by using the GraphPad Prism version 5.00 software (GraphPad Software, CA, USA). Spearman correlation test was used to assess the association between *cir-ITCH* expression and the mRNA expression of ITCH in lung cancer tissues. Comparison between two groups for statistical significance was performed with two-tailed Student's t-test. For more groups, one-way ANOVA followed by Newman-Keuls post hoc test was used. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of the Circular RNA. We designed two sets of primers for ITCH detection: a divergent set that was expected to amplify only the circular form and an opposite-directed set to amplify the linear forms. cDNA and genomic DNA were used as templates. The circular form, namely, *cir-ITCH*, was amplified by using the divergent primers on cDNA (Figure 1(a), upper panel). And as expected, there was no

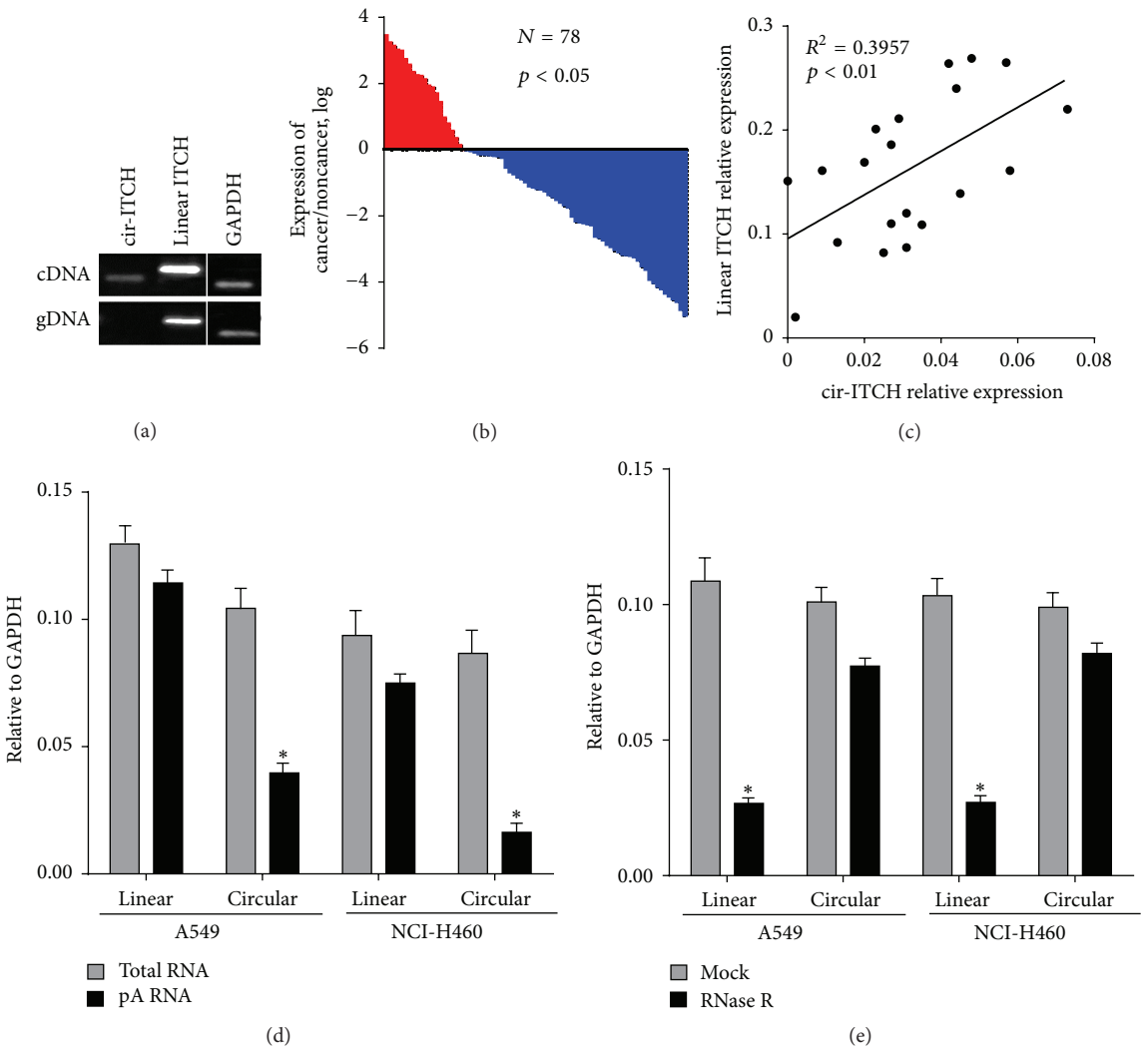

FIGURE 1: The expression level of *cir-ITCH* is closely related to lung cancer. (a) *cir-ITCH* was amplified by RT-PCR with divergent primers in cDNA but was not amplified in genomic DNA (gDNA). GAPDH, linear control. (b) qRT-PCR based on TaqMan probe was used to analyze the expression level of *cir-ITCH* in lung cancer tissues and paired noncancerous tissues. GAPDH was used as endogenous control. (c) The linear correlations between the *cir-ITCH* expression levels and linear ITCH were tested by Spearman analysis. The relative expression value was normalized by GAPDH expression level. (d) Random primers and oligo dT primers were used, respectively, in the reverse transcription experiments. The predicted circular RNA is absent in poly(A)-enriched samples. (e) The predicted circular RNA is resistant to RNase R treatment. Data are presented as mean ± SEM from three independent experiments. $^{*}p < 0.05$.

amplification when performing RT-PCR with the divergent primers on genomic DNA (Figure 1(a), lower panel). Linear-ITCH was amplificated from both of cDNA and genomic DNA templates (Figure 1(a)). GAPDH was used as a linear control (Figure 1(a)). Thus, we confirmed that *cir-ITCH* is specifically amplified with divergent primers on cDNA.

3.2. cir-ITCH Is Overexpressed and Positively Correlated with ITCH Expression in Lung Cancer Tissues. Next, cDNA of cancer tissues and paired noncancerous tissues of 78 lung cancer patients was extracted, and then the expression level of *cir-ITCH* was evaluated with the divergent primer set. *cir-ITCH* was expressed at a lower level in approximately 73.08% of the lung cancer tissues compared to that of the paired noncancerous samples (Figure 1(b)). Simultaneously, we further evaluated the association between *cir-ITCH* expression in lung cancer tissues and clinical characteristics of lung cancer patients. As shown in Table 1, *cir-ITCH* expression in lung cancer tissues was significantly associated with age ($p = 0.0076$); however, it is not correlated with other clinical characteristics including sex ($p = 0.9704$), family history ($p = 0.8437$), smoking ($p = 0.1713$), drinking ($p = 0.1568$), tumor type ($p = 0.9183$), and TNM stage ($p = 0.2531$) in lung cancer patients. To study the correlation between *cir-ITCH* and ITCH in lung cancer, we assessed the expression of *cir-ITCH* in randomly selected 20 pairs of tissue samples from the 78 patients. The results showed that patients with higher *cir-ITCH* expression levels in lung cancer tissues had a

Table 3: The sequence of the predicted miRNA binding sites on the 3′-UTR region of ITCH and *cir-ITCH*.

MicroRNA	miRNA binding sites 3′-UTR	miRNA binding sites in *cir-ITCH*
miRNA-7	GUGGCCACAUGUAUAUAGUCUUCCC	UGAGGUAGUAGGUUGUAUAGUU
miRNA-214	UGUAUAUGUCUUCCCUGCUGU	ACAGCAGGCACAGACAGGCAGU

substantial upregulation of linear ITCH ($R^2 = 0.33$, $p < 0.01$; Figure 1(c)).

3.3. Characterization of cir-ITCH in Lung Cancer Cells. To further study the role of *cir-ITCH* in lung cancer progression, we constructed a recombinant vector to express *cir-ITCH* in lung cancer cell lines according to the previous studies [15, 16]. Then, the constructed plasmid was transiently transfected into A549 and NCI-H460 cells. Next, random primers and oligo (dT) primers were used to reverse total RNA and mRNA into cDNA, respectively. In contrast to the linear products, we thought that circular products amplified with the divergent primer set would be depleted in the poly(A)-enriched RNA [28]. In our results, the expression of linear ITCH (normalized to GAPDH) showed no difference between total RNA and poly(A)-enriched RNA in both A549 cells and NCI-H460 cells (Figure 1(d)), while the expression of *cir-ITCH* was significantly decreased in poly(A)-enriched RNA compared with total RNA in these two lung cancer cell lines (Figure 1(d)).

Circular RNAs are resistant toward exonucleases for the reason of lacking free ends [29, 30]. To further confirm the circular characteristics of *cir-ITCH* in A549 cells and NCI-H460 cells, the enzyme RNase R, a highly processive 3′ to 5′ exoribonuclease that does not react on circular RNAs, was used to digest total RNA [31, 32], and then we performed RT-PCR to evaluate linear ITCH and *cir-ITCH* expression. As expected, in contrast to the linear ITCH, the predicted *cir-ITCH* was resistant to the RNase R treatment (Figure 1(e)).

3.4. Interaction between cir-ITCH and miRNA. miR-7 and miR-214 can bind to the 3′-untranslated region (UTR) of ITCH and *cir-ITCH* [16], and binding sites of these two miRNAs were presented in Table 3. The stability of *cir-ITCH* was firstly investigated with the presence of miRNA mimic or inhibitor via actinomycin D assay. A549 and NCI-H460 cells were cotransfected with the *cir-ITCH* plasmid and miRNA mimic or inhibitor, respectively. And, then, cells were treated with actinomycin D, a transcription inhibitor. Total RNA was extracted at indicated time points and the relative expression of *cir-ITCH* was evaluated. There was almost no change in *cir-ITCH* levels in both A549 cells and NCI-H460 cells with miRNA mimic or inhibitor treatment (Figures 2(a) and 2(d)), while the *cir-ITCH* levels in cells transfected with empty vector remained only 20–30% (Figures 2(b) and 2(e)), which were significantly lower than that in cells transfected with the *cir-ITCH* plasmid ($p < 0.01$). These results suggest that miR-7 and miR-214 can degrade *cir-ITCH* in lung cancer cells.

Next, the ITCH binding sequences of miR-7 and miR-214 were inserted into psiCHECK-2 vector, respectively. The constructed luciferase reporter of miR-7 or miR-214 and *cir-ITCH* plasmid were transiently cotransfected into lung cancer cells, and the luciferase activity was subsequently detected. In both A549 cells (Figure 3(a)) and NCI-H460 cells (Figure 3(b)) transfected with empty vector (control of *cir-ITCH* plasmid), luciferase activity was significantly decreased in a concentration-dependent manner with the presence of miR-7/miR-214 mimic. However, there were no significant differences in luciferase activity of miR-7/miR-214 mimic in cells with *cir-ITCH* hyperexpression (Figures 3(a) and 3(b)). Thus, *cir-ITCH* can act as sponge of ITCH to interact with miR-7 and miR-214 in lung cancer cells.

3.5. cir-ITCH Inhibits the Activation of Wnt/β-Catenin Signaling Pathway. ITCH protein promotes the degradation of phosphorylated Dvl2, which is an important regulator for Wnt/β-catenin signaling activation [21]. *cir-ITCH*, acting as a sponge of oncogenic miRNAs, can competitively inhibit these miRNAs' bind to ITCH and thus indirectly suppresses the activation of Wnt/β-catenin signaling in esophageal squamous cell carcinoma [16]. To further confirm whether *cir-ITCH* regulates the Wnt/β-catenin signaling pathway in lung cancer cells, we used a β-catenin/T-cell factor- (TCF-) responsive luciferase reporter assay [33]. As shown in Figure 4(a), overexpression of *cir-ITCH* significantly suppressed relative TCF transcriptional activity in both A549 cells and NCI-H460 cells, which suggests that *cir-ITCH* inhibits β-catenin expression. The expression level of β-catenin in lung cancer cells with *cir-ITCH* hyperexpression was further confirmed by western blotting analysis (Figure 4(b)), and it was discovered that there was an obvious decrease in β-catenin levels, while no change in Wnt3a expression was shown. Oncogene c-Myc and cell cycle regulator cyclinD1 are two important downstream targets of β-catenin [33, 34]; then, we investigated the effect of *cir-ITCH* on the mRNA expression of these two proteins. In lung cancer cells transfected with *cir-ITCH*, mRNA expression of c-Myc and cyclinD1 was significantly suppressed compared to empty vector control (Figure 4(c)).

3.6. cir-ITCH Inhibits Cellular Proliferation of Lung Cancer. *cir-ITCH* inhibits cell proliferation in both esophageal squamous cell carcinoma and colorectal cancer [15, 16]. To further confirm the role of *cir-ITCH* in lung cancer cell proliferation, lung cancer cells were transfected with *cir-ITCH* with or without miR-7 and miR-214. We noticed that miR-7 and miR-214 significantly promoted cell proliferation of A549 cells and NCI-H460 cells compared to simple empty vector transfection, while cell proliferation was dramatically decreased when *cir-ITCH* was overexpressed with the presence of miR-7 and miR-214 treatment compared to the controls. In lung cancer cells transfected with *cir-ITCH* with or without miR-7 and miR-214, treatment showed no significant difference (Figures 4(d) and 4(e)).

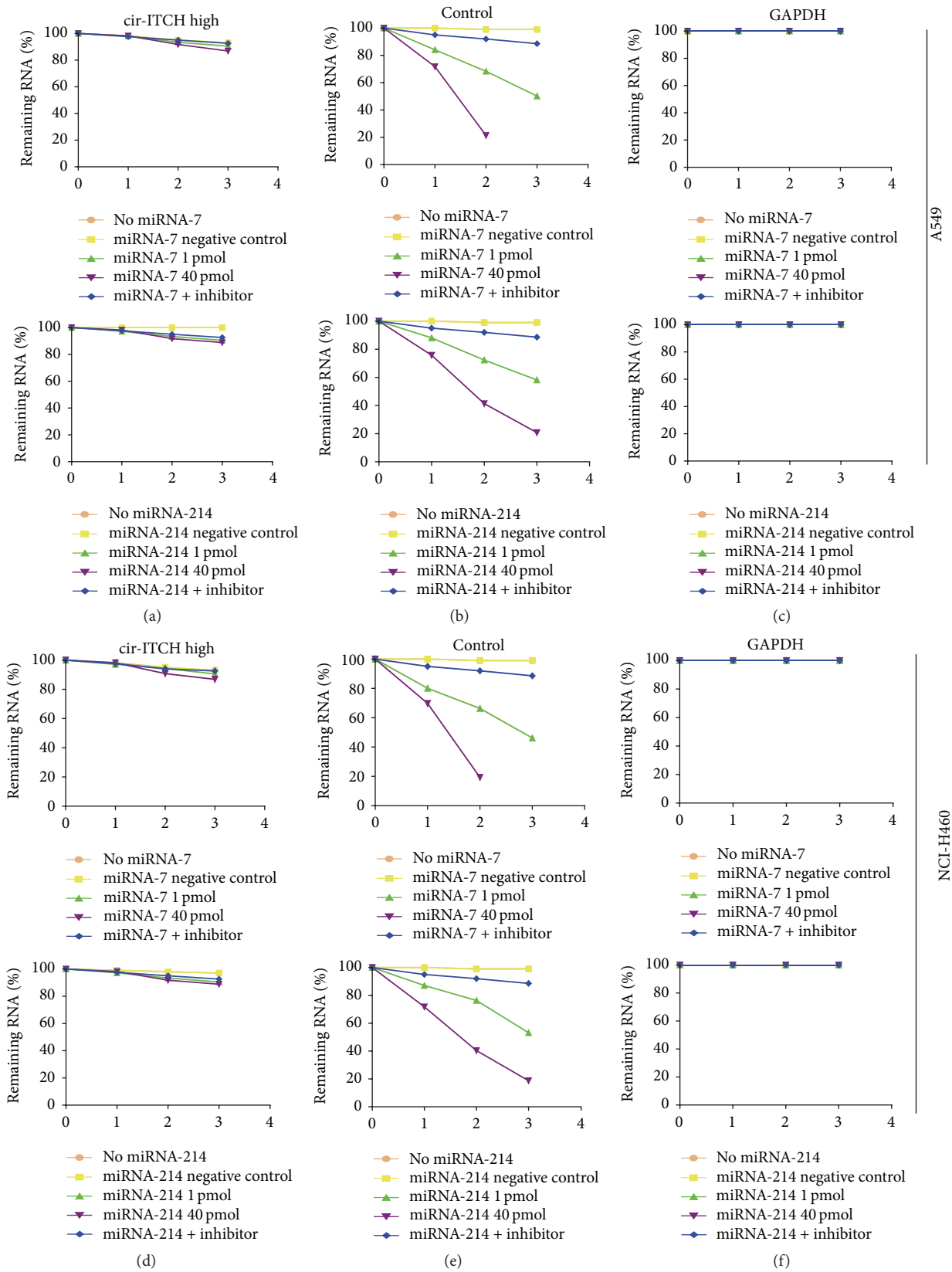

Figure 2: *cir-ITCH* inhibits the expression of miR-7 and miR-214. A549 cells were, respectively, transfected with *cir-ITCH* (a) and empty control vector (b) and simultaneously treated with miR-7 (upper) and miR-214 (lower) for 24 h. Cells were then further exposed to actinomycin D for 1, 2, and 3 h. The stability of *cir-ITCH* mRNA was analyzed by qRT-PCR relative to 0 h after actinomycin D treatment. (c) GAPDH as endogenous control. ((d)–(f)) Similar to A549 cells, the stability of *cir-ITCH* mRNA in NCI-H460 cells was evaluated by qRT-PCR. Data are presented as mean ± SEM, normalized to GAPDH.

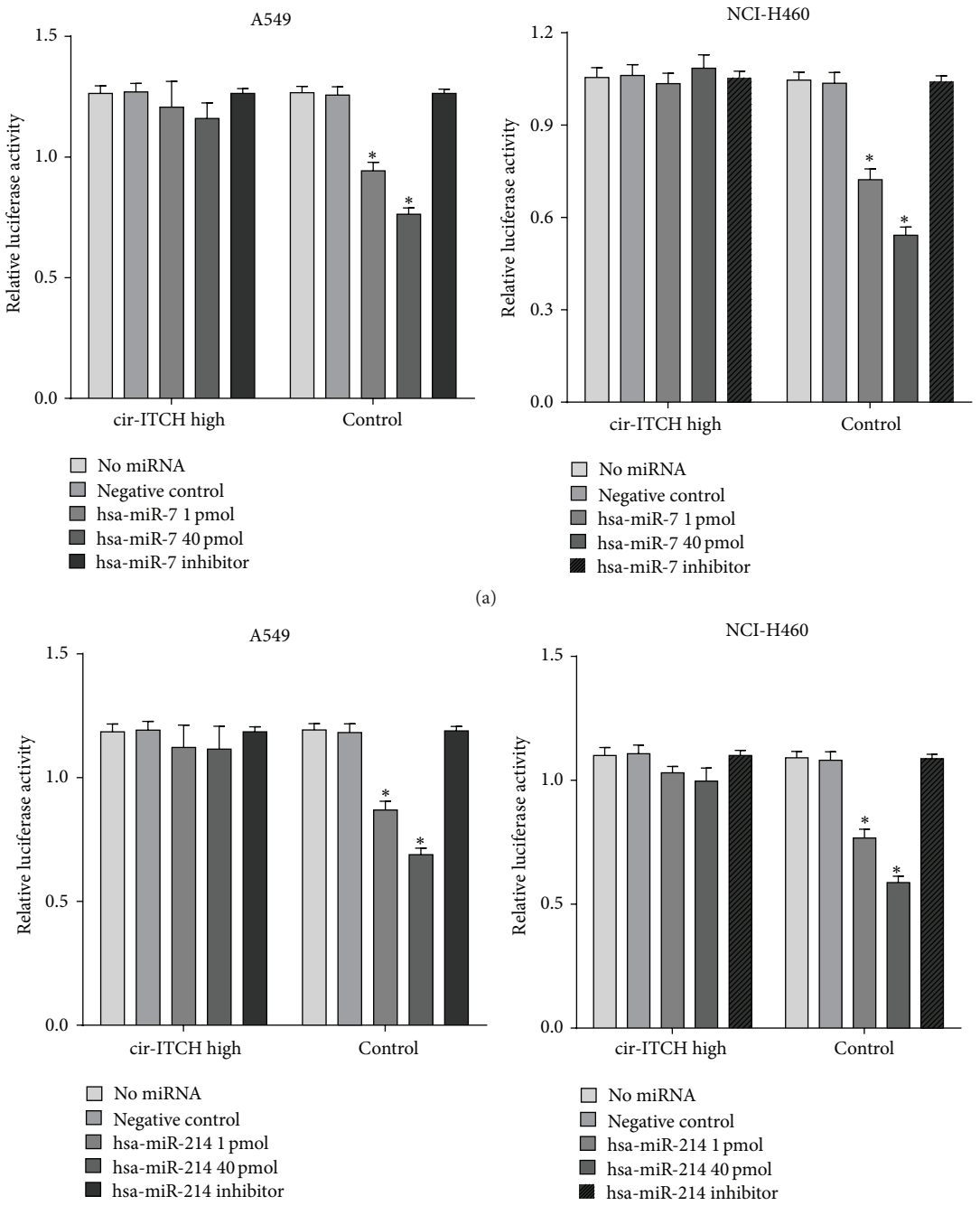

Figure 3: *cir-ITCH* acts as microRNA sponges of miR-7 and miR-214. (a) A549 (left) and NCI-H460 (right) cells were cotransfected with psiCHECK-2-ITCH constructs and *cir-ITCH* or empty control vector, respectively. Cells were further simultaneously treated with miR-7 mimic or inhibitor for 24 h. Then, relative luciferase activity of psiCHECK-2-ITCH constructs was evaluated. (b) Similar to miR-7, relative luciferase activity of the psiCHECK-2-ITCH constructs in A549 and NCI-H460 cells with the presence of miR-214 was evaluated. Data are presented as mean ± SEM from six replicates for each group. $^{*}p < 0.05$.

4. Discussion

Recently, many studies have confirmed the widespread and abundant presence of circular RNA in eukaryotic cells [35–38]. Specifically, *cir-ITCH* has been reported to inhibit the progression of esophageal squamous cell carcinoma and colorectal cancer mainly by regulating the Wnt/β-catenin pathway [15, 16]. However, the function of *cir-ITCH* in lung cancer is still unclear. In this study, we compared the expression level of *cir-ITCH* in lung cancer issues by using a TaqMan-based RT-PCR and found that *cir-ITCH* was dramatically decreased in lung cancer tissues, indicating *cir-ITCH* may play a role in regulating lung cancer progression.

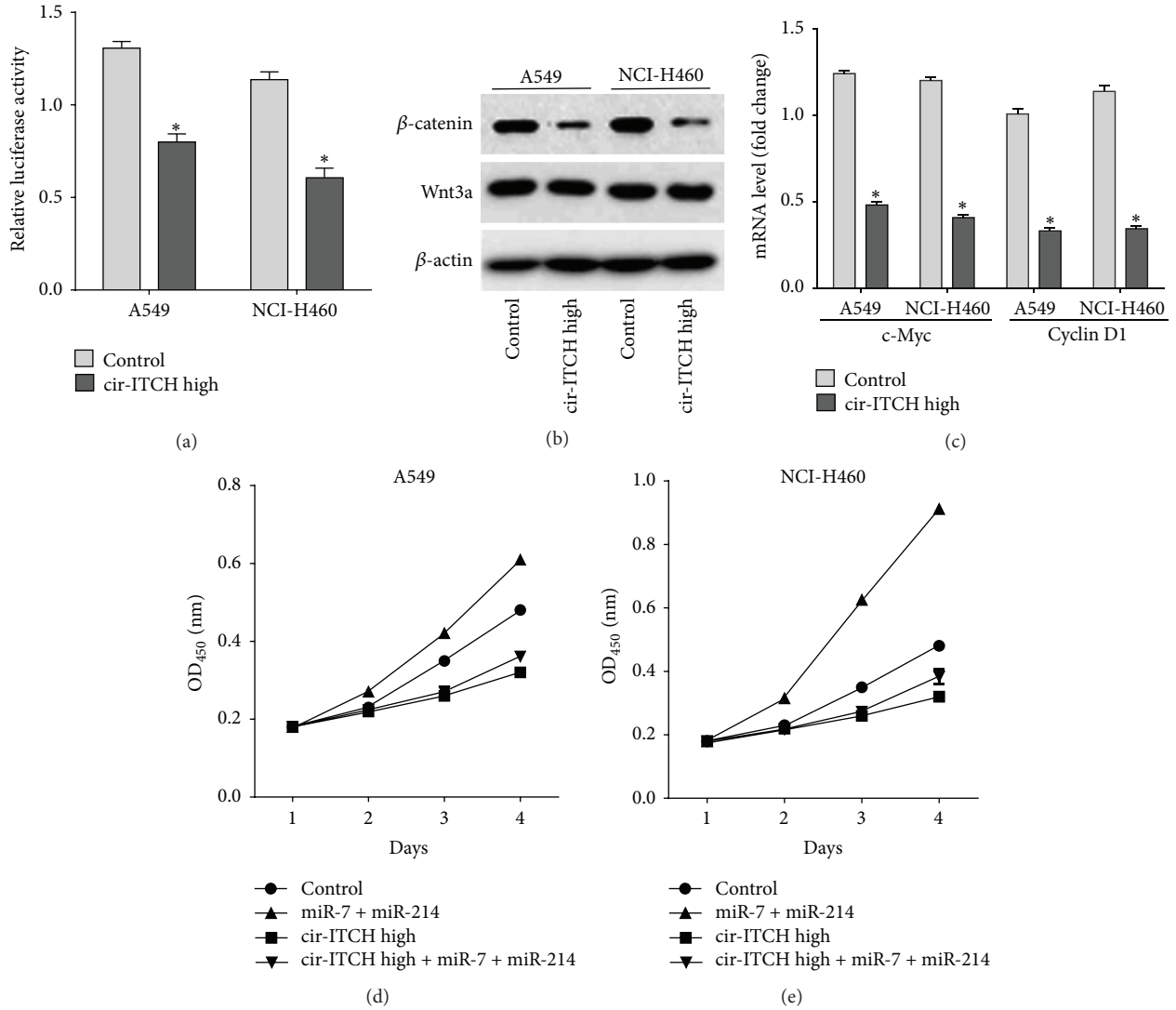

FIGURE 4: *cir-ITCH* can regulate the Wnt/β-catenin signaling pathway and cellular proliferation of lung cancer cells. (a) A549 and NCI-H460 cells were transfected with *cir-ITCH* or empty control vector; then, a β-catenin/T-cell factor- (TCF-) responsive luciferase reporter assay was performed. The luciferase activity was normalized to the Renilla luciferase activity. (b) The protein levels of Wnt3a and β-catenin were assessed in A549 cells and NCI-H460 cells by western blotting. β-actin was used as endogenous control. (c) The mRNA level of c-Myc and cyclinD1 in A549 cells and NCI-H460 cells was detected by qRT-PCR after being transfected with *cir-ITCH* or empty control vector. (d) A549 and (e) NCI-H460 cells were seeded in 96-well plates after being transfected with *cir-ITCH* and empty control vector, and cell proliferation was detected daily for 3 days by using the CCK-8 assay. Data are presented as mean ± SEM from six replicates for each group. $^{*}p < 0.05$.

cir-ITCH, located on chromosome 20q11.22, is aligned in a sense orientation to the known protein-coding gene, ITCH, a member of the E3 ubiquitin ligases [16]. Correlation analysis showed that *cir-ITCH* expression in lung cancer tissues was not correlated with clinicopathological characteristics except age. This is likely to be because the incidence of lung cancer increases with age, particularly after the age of 60 [39], and we still need to confirm this result in a larger sample population. As parental gene of *cir-ITCH*, ITCH also decreased in lung cancer patients and positively correlated with *cir-ITCH*. Hence, we speculated that ITCH may play a tumor suppressive role in lung cancer, and there is a possible connection between ITCH and *cir-ITCH*. ITCH involved in cancer progression mainly depends on its ability to regulate protein stability [40–42] and the target proteins including p63 [43], p73 [44], Notch1 [45], Dvl2 [21], RASSF5 [42], and LATS1 [41]. These proteins usually associated with tumor formation and chemosensitivity serve as either tumor suppressor or enhancer; thus, the role of ITCH in tumor progression is complicated.

Concerning the function of *cir-ITCH*, it is speculated that *cir-ITCH* serves as epigenetic miRNA sponges to competitively block the bind between miRNA and ITCH. Previous research has shown that miR-216b, miR-17, miR-214, miR-7, miR-20a, and miR-128 could bind to the 3′-UTR of ITCH and cir-ITCH [15, 16]. In our study, we found that miR-7, miR-214, and miR-128 (data not shown) decreased ITCH expression by binding to its 3′-UTR in lung cancer cell lines,

and *cir-ITCH* acts as a sponge for miR-7 and miR-214, except miR-128. These results were not fully consistent with study in cell lines of esophageal squamous cell carcinoma [16], in which *cir-ITCH* acts as a sponge for five miRNAs: miR-216b, miR-17, miR-214, miR-7, and miR-128. The reason may be owing to the difference of cancer origin, as the expression and function of circRNAs occupy tissue- and development-specific properties [11]. *cir-ITCH* also did not act as a sponge for miR-214 but for miR-20a in colorectal cancer [15]. As two oncogenic miRNAs, miR-7 and miR214 are involved in the progression of many cancers including lung cancer [22, 23, 46–49]. In lung cancer cells, we found that miR-7 and miR214 promoted cell proliferation; this activity was totally abrogated with ectopic *cir-ITCH* hyperexpression. Thus, *cir-ITCH* is involved in lung cancer progression by interacting miRNAs. However, with regard to other tumor-related activities, like migration, invasion, and colony formation, the antitumor effects of *cir-ITCH* in lung cancer still need to be further investigated.

ITCH is crucial in the control of proteasome degradation of Dvl2, which inhibits Wnt/β-catenin signaling [21, 50]. Deregulated Wnt/β-catenin signaling with cancers has been well documented in tumor initiation, progression, and metastasis, including lung cancer [18–20, 51, 52]. Blocking β-catenin signaling for cancer treatment has thus generated significant interests [53]. The beneficial effect of nonsteroidal anti-inflammatory drugs (NSAIDs) in cancer prevention and therapy has been attributed partially to the perturbation of β-catenin signaling [54]. In our study, *cir-ITCH* inhibits Wnt/β-catenin signaling in lung cancer cells with the evidence that hyperexpression of *cir-ITCH* significantly suppressed relative TCF transcriptional activity in β-catenin/TCF-responsive luciferase reporter assay. This result was further confirmed by western blotting analysis.

At last, we examined the impacts of *cir-ITCH* on two important downstream targets of Wnt/β-catenin pathway, c-Myc and cyclinD1, which are continually overexpressed in many cancers and have crucial roles in regulating cell growth, apoptosis, and differentiation [55]. Hyperexpression of *cir-ITCH* significantly suppressed the mRNA expression of c-Myc and cyclinD1 in lung cancer cells. Combined with previous studies [15, 16], we are able to conclude that *cir-ITCH* has an antitumor role in lung cancer by controlling miRNA activity, which increases the concentration of ITCH and results in suppression of the canonical Wnt/β-catenin pathway.

In conclusion, our study demonstrates that the *cir-ITCH* acts as a sponge for miR-7 and miR-214, promotes the expression of their target gene ITCH, and thus regulates lung cancer cell proliferation by indirectly inhibiting the activation of Wnt/β-catenin pathway. Further characterization of the function of circular RNAs in cancer progression will have great implication for the development of new RNA-based cancer diagnosis and therapy.

Competing Interests

The authors declare no conflict of interests.

Acknowledgments

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