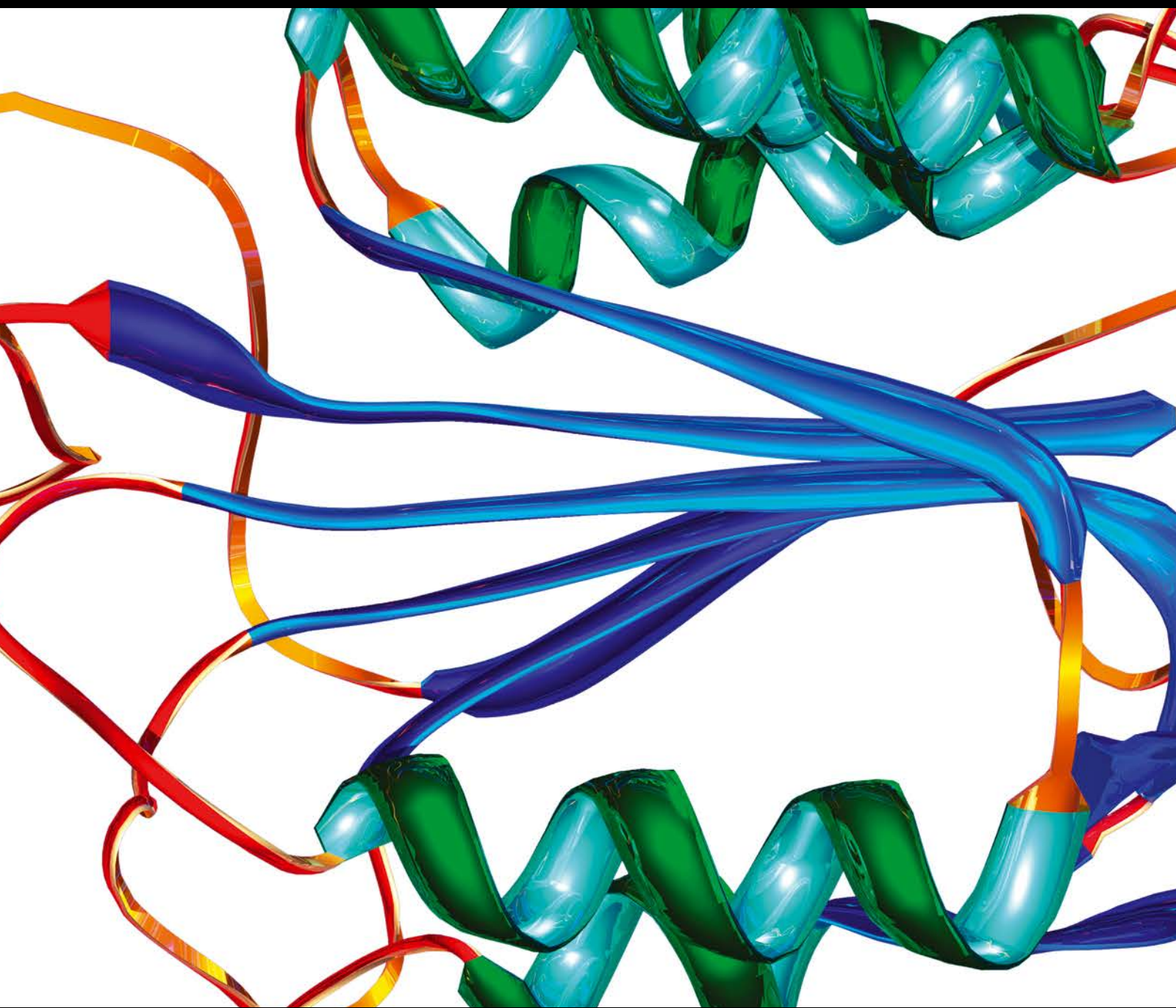
Disease Markers

Disease Biomarkers in Gastrointestinal Malignancies

Guest Editors: Omeed Moaven, Hamid Raziee, Wilbur Bowne, Mohammad Reza Abbaszadegan, and Bryan C. Fuchs



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Editorial Board

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Editorial

Disease Biomarkers in Gastrointestinal Malignancies

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Of every five newly diagnosed cancers, one is a gastrointestinal (GI) malignancy in origin. Lower GI cancers are among the top three most frequent cancers in the United States and many western countries while upper GI cancers rank as the most prevalent type in many Asian countries, especially in central and eastern Asia. GI cancers are usually diagnosed in more advanced stages and in the absence of effective early diagnostic tools and therapeutic modalities, the survival rates are generally disappointingly low.

Considering the high mortality rate, tremendous effort has been directed to address the urgent need for discovery of effective early diagnostic tools, efficient therapeutic targets, and treatment monitoring markers for GI malignancies. Biomarkers are one of these favorite tools with several potential applications in various aspects of clinical management of cancers. A plethora of biomarkers has been studied in GI cancers, of which only a handful have found their way from bench to bed. Guidelines have been published by different cancer societies and groups, such as American Society of Clinical Oncology (ASCO), European Society of Medical Oncology (ESMO), and European Group on Tumour Markers (EGTM), with recommendations regarding clinical applications of available markers for gastrointestinal tumors [1–3]. CEA, *K-RAS*, HER2, and KIT are among the biomarkers with validated clinical implications in management of colorectal cancer (CRC), gastric cancer, and gastrointestinal stromal tumors [1, 4–6]. Nonetheless, there is a growing list of emerging markers with promising clinical results that need to be validated for routine clinical applications and current data are insufficient to recommend them as part of the clinical guidelines.

The current special issue tackles this important area of cancer research. In this issue, S.-F. Chiang et al. report their investigation of bone marrow stromal antigen 2 (BST2; also known as CD317, tetherin, and HM1.24) as a plasma biomarker in 152 patients with CRC. They show that, compared to the controls, BST2 was significantly elevated in plasma samples from CRC patients. In addition, high BST2 expression in CRC tissue, as assessed by immunohistochemistry, was associated with poorer 5-year survival. BST2 has also been under investigation as a potential target for immunotherapy for over a decade [7]. In fact, a humanized monoclonal antibody targeting BST2 has been tested in Phase 1 trial of multiple myeloma (MM) but the response rate was low [8]. More recently, BST2-specific cytotoxic T lymphocytes targeting MM cells have been developed [9, 10]. Therefore, it is possible that BST2 could be a potential therapeutic target in CRC. However, given its detection in the plasma, future studies should also examine BST2 as a novel biomarker to noninvasively monitor therapeutic response.

In another study, T. Xue et al. have investigated the clinical significance of miRNA-20b as a marker in hepatocellular carcinoma (HCC) and reported its association with poor survival. They confirmed HIF-1α and VEGF as the targets of miRNA-20b *in vitro* and showed their regulation in both normal and hypoxic conditions, suggesting miRNA-20b as

an adaptation mechanism that may play a role in tumor progression. This study was performed on a small retrospective cohort and the intriguing results should be validated in future larger prospective studies. Also the functional studies need to be expanded to better understand its role in tumor progression.

Thomsen-Friedenreich (TF) antigen is one of the tumor-associated glycans (TAG), which is normally overexpressed in cancer cells, and has a role in cell adhesion to endothelium. In search of a serologic biomarker for gastric cancer, O. Kurtenkov and K. Klaamas looked into the presence and avidity of anti-TF antibodies in serum samples of cancer patients and normal controls. Drawing on their prior study showing increased sialylation of anti-TF antibodies in gastric cancer, they assessed the following: (1) serum levels of anti-TF antibodies by ELISA; (2) reactivity of anti-TF antibodies to *Sambucus nigra* agglutinin (SNA); (3) avidity of anti-TF antibodies by ELISA; and (4) avidity of SNA-reactive anti-TF antibodies in 104 patients and 49 controls. They showed, for the first time, that SNA-reactive—and therefore aberrantly sialylated—TF-specific antibodies have a significantly higher avidity in cancer patients, with a diagnostic accuracy of 73.2%, and a sensitivity of 70.3% in stage I patients. While these results provide an exciting venue of further investigation for a serum-based marker for gastric cancer, all these biomarkers need prospective evaluation and validation studies for determining the clinical impact, which is missing for many of the newly diagnosed markers. The clinical application would need stringent prospective validation of specificity, sensitivity, and cost-effectiveness.

The neutrophil-to-lymphocyte ratio (NLR) has been proposed as a potential inflammation-based prognostic indicator in various malignancies but there have been controversial reports of its prognostic values in gastric cancer. Sun et al. are here reporting the results of a meta-analysis including 19 studies with 5431 patients and concluded that pretreatment NLRs can predict the prognosis of gastric cancer. The clinical significance of these findings still needs to be validated in a larger independent study.

In an effort to highlight the implications of HER2, a marker which is now accepted as part of practice guidelines in advanced gastric cancer, A. Ieni et al. have reviewed the HER2 status in various stages of gastric tumorigenesis and their clinical significance, suggesting a potential role in early steps of gastric carcinogenesis and offering potential clinical implications in both early and advanced gastric adenocarcinoma.

Further on serum-based markers H. Kishikawa et al. review the current evidence about the use of "ABC method," a combination of anti-*Helicobacter pylori* antibody and serum pepsinogen (PG), for gastric cancer screening. In this method, based on *H. pylori* (HP) titre and PG, subjects are subdivided into 4 groups (A, HP−/PG−; B, HP+/PG−; C, HP+/PG+; D, HP−, PG+), with recommendation for endoscopy surveillance in B, C, and D groups every 3, 2, and 1 year, respectively. After discussing the available evidence, the authors conclude that gastric cancer risk is not the same in each of the above categories and recommend that HP antibody titre measurement should be done to categorize patients into low-negative, high-negative, low-positive, and high-positive groups. They further recommend endoscopic surveillance in high-negative antibody titres in group A every 3 years, high-positive titres in group B every 2 years, and low-positive titres in group C every year.

Recommending a tumor marker as part of a practice guideline requires a multistep complex process that starts with discovery and introduction of the biomarker in preclinical phase followed by a rigorous analytical validation that comprises assay development, strong methodology, and robust statistical and bioinformatics tools. The ultimate path toward FDA or other regulatory approval is an unequivocal clinical validation with independent prospective studies. This process can take two to three decades and there are many examples of overoptimistic interpretation of the promising early results [11–13], which eventually failed to succeed achieving FDA clearance due to lack of accuracy or robustness in at least one of the above-mentioned steps. While we all review, observe, and contribute to the expanding body of literature of the emerging tumor markers, learning the lessons from the stories of failures and successes will create a pragmatic and realistic path toward the ultimate goal of recognizing a tumor marker as an effective tool with a significant clinical outcome.

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Review Article

Can the Neutrophil to Lymphocyte Ratio Be Used to Determine Gastric Cancer Treatment Outcomes? A Systematic Review and Meta-Analysis

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The prognostic role of neutrophil to lymphocyte ratio (NLR) in gastric cancer remains controversial. We aimed to quantify the prognostic role of peripheral blood NLR in gastric cancer. A literature search was conducted in PubMed, EMBASE, and Cochrane databases. The results for overall survival (OS) and progression-free survival (PFS)/disease-free survival (DFS) are expressed as hazard ratios (HRs) with 95% confidence intervals (CIs). 19 studies with 5431 patients were eligible for final analysis. Elevated NLRs were associated with a significantly poor outcome for OS (HR = 1.98; 95% CI: 1.75–2.24, $p < 0.001$) and PFS (HR = 1.58; 95% CI: 1.32–1.88, $p < 0.001$) compared with patients who had normal NLRs. The NLR was higher for patients with late-stage compared with early-stage gastric cancer (OR = 2.76; 95% CI: 1.36–5.61, $p = 0.005$). NLR lost its predictive role for patients with stage IV gastric cancer who received palliative surgery (HR = 1.73; 95% CI: 0.85–3.54, $p = 0.13$). Our results also indicated that prognoses might be influenced by the NLR cutoff values. In conclusion, elevated pretreatment NLRs are associated with poor outcome for patients with gastric cancer. The ability to use the NLR to evaluate the status of patients may be used in the future for personalized cancer care.

1. Introduction

Gastric cancer is the second most common cause of cancer mortality worldwide, in part because most patients are diagnosed with advanced, inoperable disease [1]. Early detection, surgical resection, and adjuvant therapy have improved the survival of patients with early-stage gastric cancer. Even for patients with advanced gastric cancer who receive potentially curative resections, the 5-year survival remains at still 30–50% [2]. In addition, many patients experience side effects from surgery and adjuvant therapy [3, 4]. Treatment strategies are determined by TNM staging system. However, many patients of the same TNM stage have different prognoses [5]. It is important to identify factors that predict the treatment response and survival of gastric cancer patients.

Recently, an increasing number of studies have focused on tumor microenvironment, which is associated with the systemic inflammatory response and may play an important role in cancer tumorigenesis and progression [6, 7]. Many markers of systemic inflammation response to tumors have been investigated as prognostic and predictive biomarkers, such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) [8, 9]. The neutrophil-to-lymphocyte ratio (NLR) is a potential inflammation-based prognostic indicator for several types of cancer, such as renal cell carcinoma [10], hepatocellular carcinoma [11], and colorectal carcinoma [12]. Some studies have indicated that elevation in the NLR for patients with gastric cancer may predict worse prognosis [13]. However, other studies [14] have shown no such association. The association between the NLR and clinicopathological characteristics and prognosis function of patients with gastric cancer remains unclear.

In this study, we conducted a systematic review and meta-analysis to quantify the prognostic role of the peripheral

blood NLR in gastric cancer. We also aimed to determine the correlation between the NLR and clinicopathological factors for patients with gastric cancer.

2. Materials and Methods

2.1. Systematic Search Strategy. This study was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines [15]. A sensitive search strategy was developed for all English-language literature published before November 2014 using PubMed, EMBASE, and the Cochrane Database of Systematic Reviews. The search strategy included the keywords "neutrophils", "lymphocytes", "neutrophil-to-lymphocyte ratio", "NLR", and "stomach neoplasms". Review articles and bibliographies of other relevant articles were individually examined to identify additional studies.

2.2. Inclusion and Exclusion Criteria. All of the studies included were comparative studies of patients with gastric cancer who had a high or low peripheral blood NLR. Treatments included curative surgery, palliative resection, or palliative chemotherapy. The hazard ratio (HR) and 95% confidence intervals (CIs) or survival curves for overall survival (OS), progression-free survival (PFS), or disease-free survival (DFS) were required. Articles lacking full text and data that could not be acquired from the authors were excluded. When multiple studies were reported by the same team from the same institute and were performed at the same time, only the latest article or the one with the largest data set was included.

2.3. Data Extraction and Quality Assessment. Data collection and analyses were performed by two researchers using predefined tables, which included author, publication time, sample size, age, treatment, follow-up, tumor differentiation, TNM stage, tumor size, and cutoff value used to define the elevated NLR, OS, PFS, and DFS. If a study did not provide a HR for the OS, PFS, or DFS, we used Engauge Digitizer version 4.1 to distinguish survival curves and calculate HRs and 95% CIs. The first reviewer (Jingxu Sun) extracted data and another reviewer (Xiaowan Chen) checked the data with any disagreements resolved by discussion and consensus.

Two reviewers (Jingxu Sun and Xiaowan Chen) performed quality assessment of the observational studies using the Newcastle-Ottawa scale [16]. Articles with NOS scores ≥6 were considered to be of high quality because standard validated criteria for important end points have not been established. The mean value for all included articles was 6.1 and the details are shown in Table 2.

2.4. Statistical Analysis. Meta-analysis was performed with Review Manage version 5.2 (Cochrane Collaboration, Copenhagen, Denmark) and Stata version 12.0 (Stata, College Station, TX, USA), and Microsoft Excel 2010 (Microsoft, Santa Rosa, CA, USA) was used for statistical analysis. If there was any disagreement, discussion among the authors was required. The HRs and 95% CIs for available data were calculated to identify potential associations with the OS, PFS, or DFS in two groups, using the method reported by Tierney et al. [17]. The odds ratios (ORs) and 95% CIs were calculated as effective values of the results of the analysis between NLR and clinicopathological characteristics. Statistical heterogeneity among studies was quantified using the χ^2 and I^2 statistic. The I^2 statistic was derived from the Q statistic ($[Q - \mathrm{df}/Q] \times 100$), and it provides a measure of the proportion of the overall variation attributable to heterogeneity among studies. If the heterogeneity test was statistically significant, then the random effects model was used. The source of heterogeneity was investigated by meta-regression and subgroup analysis. The p value threshold for statistical significance was set at 0.05 for effect sizes. Publication bias was analyzed by Begg's test and Egger's bias indicator test, and the results were then expressed in a funnel plot.

3. Results

3.1. Studies Included and Methodological Quality. The initial search strategy identified 82 articles, including 26 that were further evaluated after initial review of the titles and abstracts. After further consideration of the remaining articles, 19 studies [13, 14, 18–34] involving 5431 patients were included in our meta-analysis. All of the included articles were observational cohort studies and all of the NLRs were tested before treatment. A flowchart of the search strategy is shown in Figure 1. The study characteristics are summarized in Table 1. Six were studies were from Japan, six were from Korea, three were from China, two were from Italy, one was from Turkey, and one was from Egypt. Ten of these articles had <200 patients and another nine had >200 patients. All of the included articles provided the TNM stage of patients, and four only studied patients in stage IV. The NOS score was summarized in Table 2.

3.2. OS and NLR for Patients with Gastric Cancer. Survival was significantly longer for patients with a low NLR than those with a high NLR with a pooled HR of 1.98 (95% CI: 1.75–2.24, $p < 0.001$; Figure 2) and the heterogeneity was significant ($p = 0.003$, $I^2 = 53\%$).

We performed meta-regression and subgroup analysis to explore heterogeneity by country, year of publication, sample size, cut-off value for NLR, and whether patients underwent surgery. Almost all of the subgroup analyses had no influence on the heterogeneity of the pooled analysis with the exception of the subgroup distinguished by sample size (Table 3). Meta-regression also demonstrated that sample size may explain the source of heterogeneity ($p = 0.021$).

3.3. PFS, DFS, and NLR for Patients with Gastric Cancer. There were four studies [20, 22, 25, 29] that reported a correlation between the PFS and NLR, and three studies [21, 28, 30] provided data regarding DFS and NLR. The pooled results show that patients with an elevated NLR have shorter PFS and DFS after treatment compared with patients with a normal NLR (HR = 1.58; 95% CI: 1.32–1.88, $p < 0.001$; Figure 3). There was no evidence of statistical heterogeneity ($p = 0.78$, $I^2 = 0\%$). For PFS, the pooled HR was 1.61 (95% CI: 1.31–1.97, $p < 0.001$) with no significant heterogeneity. For

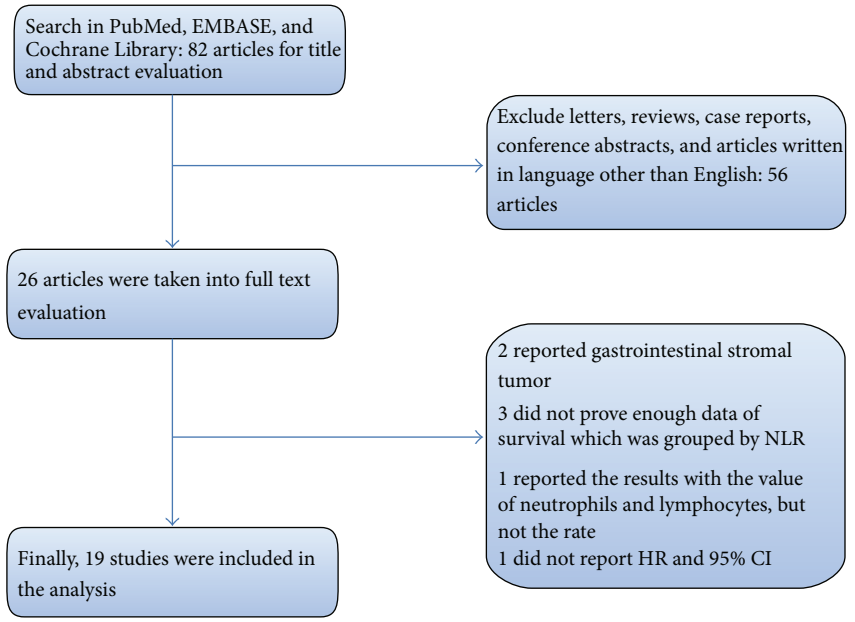


Figure 1: PRISMA flow diagram for the meta-analysis.

Study or subgroup	log[hazard ratio]	SE	Weight	Hazard ratio IV, random, 95% CI
Aurello et al. 2014	0.41	0.4	2.2%	1.51 [0.69, 3.30]
Cho et al. 2014	0.45	0.13	9.2%	1.57 [1.22, 2.02]
Dirican et al. 2013	1.01	0.17	7.2%	2.75 [1.97, 3.83]
El Aziz 2014	1.18	0.53	1.3%	3.25 [1.15, 9.20]
Graziosi et al .2015	0.53	0.26	4.3%	1.70 [1.02, 2.83]
Jeong et al. 2012	0.76	0.04	14.2%	2.14 [1.98, 2.31]
Jiang et al. 2014	0.47	0.22	5.4%	1.60 [1.04, 2.46]
Jin et al. 2013	0.85	0.37	2.5%	2.34 [1.13, 4.83]
Jung et al. 2011	0.48	0.17	7.2%	1.62 [1.16, 2.26]
Kim and Choi 2012	1.32	0.76	0.7%	3.74 [0.84, 16.60]
Kunisaki et al. 2012	0.53	0.31	3.3%	1.70 [0.93, 3.12]
Lee, D et al. 2013	1.01	0.38	2.4%	2.75 [1.30, 5.78]
Lee, S et al. 2013	0.81	0.14	8.7%	2.25 [1.71, 2.96]
Mohri et al. 2010	0.76	0.3	3.5%	2.14 [1.19, 3.85]
Mohri et al. 2014	0.83	0.24	4.8%	2.29 [1.43, 3.67]
Shimada et al. 2010	0.61	0.2	6.0%	1.84 [1.24, 2.72]
Ubukata et al. 2010	1.73	0.43	1.9%	5.64 [2.43, 13.10]
Wang et al. 2012	0.91	0.36	2.6%	2.48 [1.23, 5.03]
Yamanaka et al. 2007	0.42	0.07	12.7%	1.52 [1.33, 1.75]
Total (95% CI)			**100.0%**	**1.98 [1.75, 2.24]**

Heterogeneity: $\tau^2 = 0.03$; $\chi^2 = 38.39$, df = 18 ($p = 0.003$); $I^2 = 53\%$
Test for overall effect: $Z = 10.76$ ($p < 0.00001$)

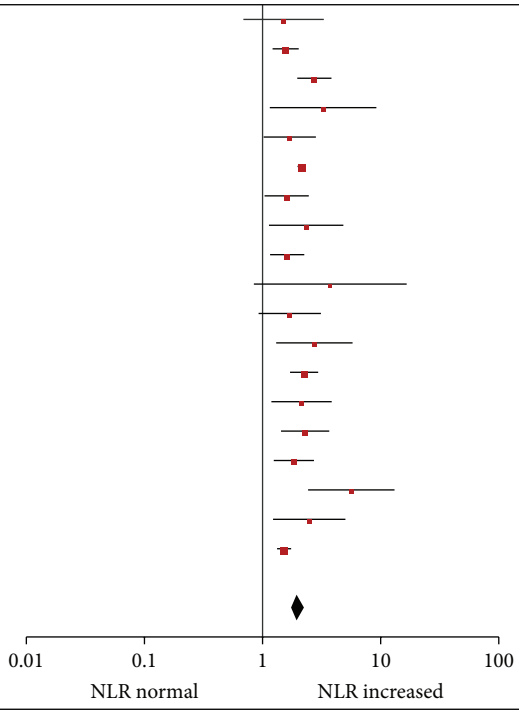


Figure 2: Hazard ratiofor overall survival.

DFS, the pooled HR was 1.48 (95% CI: 1.05–2.09, $p < 0.001$) with no significant heterogeneity.

3.4. TNM Stage and NLR of Patients with Gastric Cancer. Four studies [13, 19, 24, 31] reported data on the TNM stage and NLR for patients with gastric cancer. We classified TMN stage I/II in one group and stage III/IV to another group to evaluate the role of NLR. The pooled OR produced by a random-effect model was 2.76 (95% CI: 1.36–5.61, $p = 0.005$), and the significant heterogeneity was observed ($p = 0.002$, $I^2 = 80\%$; Table 3). Patients with higher NLR tended to have advanced gastric cancer.

Table 1: Characteristics of included studies.

Name	Year	Country	Patients (female/male)	Age (range)	Treatment	Follow-up (month)	TMN (I/II/III/IV)	Tumor size[a]	CEA[b]	Tumor differentiation (well/poor)	Cutoff value[c]	Number of elevated NLR
Mohri et al. [18]	2014	Japan	123 (38/85)	Median: 66 (18–94)	Resection + chemotherapy	9.3	0/0/0/123	NA	NA	45/78	3.1	118
Jiang et al. [13]	2014	China	377 (124/253)	Median: 64 (25–80)	Resection + chemotherapy	42	37/99/241/0	140/237 (5 cm)	NA	97/280	1.44	309
Cho et al. [20]	2014	Korea	268 (93/175)	Mean: 55.4	Chemotherapy	11.3	0/0/0/268	NA	NA	95/173	3	138
Graziosi et al. [19]	2015	Italy	156 (92/64)	Median: 74 (39–91)	Resection + chemotherapy	23	42/29/62/23	NA	NA	NA	2.3	80
Aurello et al. [21]	2014	Italy	102 (40/62)	Median: 69	Resection	96	34/15/35/18	NA	NA	NA	5	28
El Aziz [22]	2014	Egypt	70 (23/47)	Median: 53 (30–70)	Resection	NA	0/0/49/21	NA	NA	NA	3	40
Lee et al. [23]	2013	Korea	174 (60/114)	Median: 55 (22–74)	Resection + Chemotherapy	14.9	7/22/41/101	NA	58/118	NA	3	62
Lee et al. [24]	2013	Korea	220 (71/149)	Mean: 57 (23–89)	Resection	NA	120/35/62/3	59/161	22/195	NA	2.15	56
Jin et al. [25]	2013	China	46 (10/36)	Median: 60 (37–77)	Resection + chemotherapy	NA	0/0/40/6	NA	NA	15/31	2.5	20
Dirican et al. [26]	2013	Turkey	236 (74/162)	Median: 58 (30–86)	Resection + chemotherapy	NA	6/20/105/105	NA	NA	NA	3.8	89
Wang et al. [14]	2012	China	324 (99/225)	NA	Resection + chemotherapy	39.9	0/0/324/0	158/168 (5 cm)	NA	NA	5	11
Kunisaki et al. [27]	2012	Japan	83 (26/57)	Mean: 67.7 (37–91)	Resection + chemotherapy	14.5	0/0/22/61	10/73 (5 cm)	NA	35/48	5	18
Kim and Choi [28]	2012	Korea	93 (36/57)	NA	Resection + chemotherapy	NA	44/16/33/0	60/33 (5 cm)	NA	44/49	1.8	36
Jeong et al. [29]	2012	Korea	104 (35/69)	Median: 52.5 (28–82)	Chemotherapy	11.9	0/0/0/104	NA	NA	27/75	3	55
Jung et al. [30]	2011	Korea	293 (100/193)	Median: 63 (21–96)	Resection + chemotherapy	27.2	0/0/143/150	NA	NA	73/220	2	155
Ubukata et al. [31]	2010	Japan	157 (51/106)	Mean: 65.27 (29–84)	Resection	NA	45/30/39/43	42/115	NA	58/99	5	70
Shimada et al. [32]	2010	Japan	1028 (319/709)	Median: 65 (26–89)	Resection	23	584/132/153/159	NA	NA	521/507	4	127
Mohri et al. [33]	2010	Japan	357 (112/245)	Median: 63.4 (32–87)	Resection	68	232/57/68/0	NA	NA	198/159	2.2	130
Yamanaka et al. [34]	2007	Japan	1220 (351/869)	NA	Chemotherapy	15.6	0/0/0/1220	NA	NA	NA	2.5	644

[a]Tumor size ⩾ cutoff value/tumor size < cutoff value; [b]CEA ⩾ cutoff value/CEA < cutoff value; [c]the cutoff value of NLR; NLR: neutrophil to lymphocyte ratio; NA: not applicable; TNM: tumor node metastasis stage; CEA: carcinoembryonic antigen.

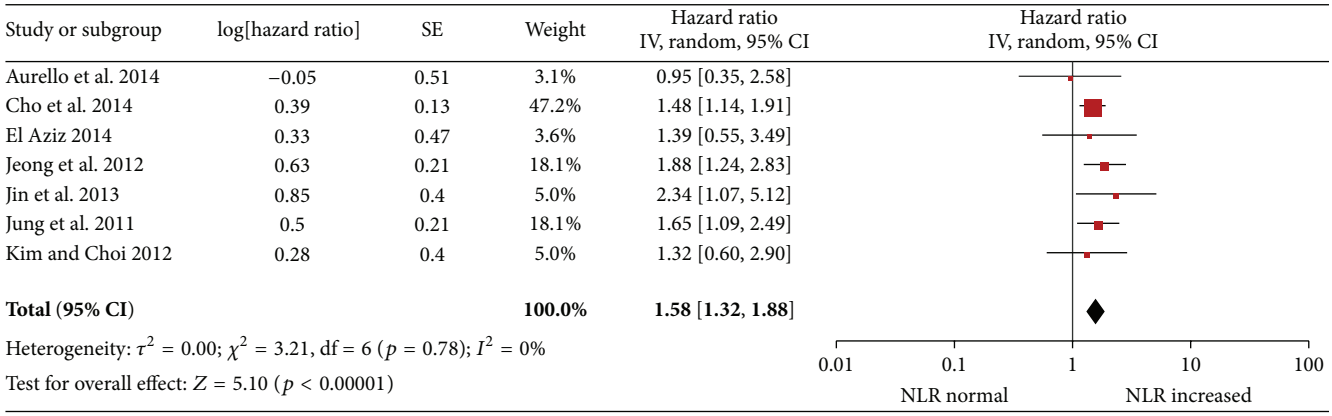


Figure 3: Hazard ratio for disease-free survival.

Table 2: Quality assessment of included studies based on the Newcastle-Ottawa scales.

Name	A	B	C	D	E	F	G	H	Scroe
Mohri et al. [18]	*	*	*	*	*	*		*	7
Jiang et al. [13]	*	*	*	*	*	*	*	*	8
Cho et al. [20]	*	*	*	*	*	*	*		7
Graziosi et al. [19]	*	*	*	*		*	*	*	7
Aurello et al. [21]	*		*	*		*	*		5
El Aziz [22]	*	*	*	*		*	*		6
Lee et al. [23]			*	*		*	*		4
Lee et al. [24]			*	*		*			3
Jin et al. [25]	*	*	*	*		*	*		6
Dirican et al. [26]	*	*	*	*		*	*		6
Wang et al. [14]	*	*	*	*	*	*	*	*	8
Kunisaki et al. [27]	*	*	*	*		*	*		6
Kim and Choi [28]			*	*	*	*	*		5
Jeong et al. [29]	*	*	*	*	*	*	*	*	8
Jung et al. [30]	*	*	*	*		*	*		6
Ubukata et al. [31]	*	*	*	*	*	*	*		7
Shimada et al. [32]	*	*	*	*	*	*	*		7
Mohri et al. [33]	*	*	*	*		*	*		6
Yamanaka et al. [34]			*	*		*	*	*	5

A: representativeness of the exposed cohort; B: selection of the nonexposed cohort; C: ascertainment of exposure; D: demonstration that outcome of interest was not present at start of study; E: comparability of cohorts on the basis of the design or analysis; F: assessment of outcome; G: follow-up long enough for outcomes to occur; H: adequacy of follow-up of cohorts.

3.5. NLR for Patients with Stage III and IV Gastric Cancer. Five studies [13, 14, 26, 30, 32] reported the NLR and OS of patients with stage III gastric cancer. Elevated NLR was associated with worse outcome (HR = 2.17; 95% CI: 1.67–2.83, $p < 0.001$), and there was no significant heterogeneity ($p = 0.29$, $I^2 = 20\%$).

Seven studies [18, 20, 26, 29, 30, 32, 34] reported NLR for patients with stage IV gastric cancer. Two of these studies [30, 32] provided data about patients who received palliative gastrectomy with or without metastasis resection. Three studies [20, 30, 34] reported patients with stage IV gastric cancer who underwent palliative treatment. For patients with stage IV gastric cancer, high NLRs were associated with poor prognosis (HR = 1.81; 95% CI: 1.50–2.18, $p < 0.001$). We performed subgroup analysis to determine whether the NLR could be a marker for different treatments such as resection or palliative chemotherapy. Patients who underwent resection had a HR of 1.73 (95% CI: 0.85–3.54, $p = 0.13$), and patients who received palliative chemotherapy had a HR of 1.83 (95% CI: 1.49–2.24, $p < 0.001$). All of the above results are shown in Table 3.

3.6. Tumor Differentiation and the NLR of Patients with Gastric Cancer. Three studies [13, 20, 30] reported the level of tumor differentiation and the NLR in gastric cancer. The combined OR was 1.05 (95% CI: 0.77–1.43, $p = 0.75$; Table 3) with no heterogeneity ($p = 0.38$, $I^2 = 0\%$), and the pooled results indicated that there was no correlation between tumor differentiation and NLR for patients with gastric cancer.

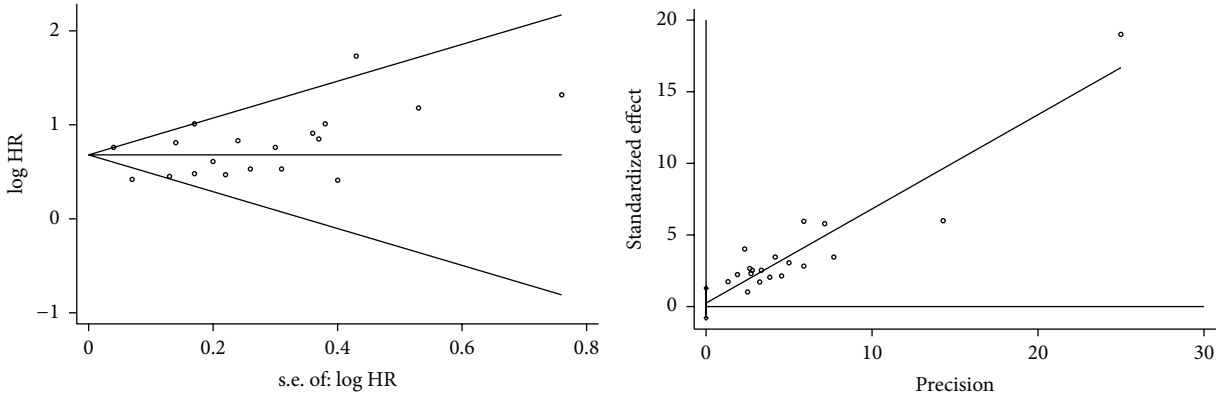
3.7. Carcinoembryonic Antigen (CEA) and NLR for Patients with Gastric Cancer. Two studies [23, 24] have presented data on the CEA level and NLR for patients with gastric cancer. There was no significant correlation between CEA and NLR for gastric cancer patients, with an OR of 1.43 (95% CI: 0.64–3.21, $p = 0.37$; Table 3).

3.8. Cutoff Value for the NLR for Patients with Gastric Cancer. All of the studies reported cutoff values for the NLR. We collected all the cutoff values for the NLR and divided the studies into four groups based on the quartiles of their cutoff values. The three quartiles were as follows: 2.20, 3.00, and 4.00. The HR in Subgroup 1 (cutoff value of NLR < 2.20) was 1.80 (95% CI: 1.43–2.26, $p < 0.001$), 1.88 in Subgroup 2 (2.20 ⩽ cutoff value of NLR < 3.00; 95% CI: 1.56–2.26, $p < 0.001$), 2.31 in Subgroup 3 (3.00 ⩽ cutoff value of NLR < 4.00; 95% CI: 1.81–2.94, $p < 0.001$), and 2.36 in Subgroup 4 (cutoff value of NLR ⩾ 4.00; 95% CI: 1.38–4.03, $p < 0.001$; Table 3).

3.9. Publication Bias. Publication bias was demonstrated using Begg's funnel plot and Egger's test. Begg's funnel plot

Table 3: Summary of the meta analysis results.

Analysis	N	References	Fixed-effect model		Random-effect model		Heterogeneity		Meta regression
			HR (95% CI)	p	HR (95% CI)	p	I^2	p	p
Subgroup analysis for OS									
Subgroup: treatments									
Surgery	12	[13, 14, 18, 19, 21, 22, 24, 25, 28, 30–32]	2.01 (1.71–2.37)	<0.001	2.11 (1.72–2.57)	<0.001	26%	0.19	
Chemotherapy	4	[20, 23, 29, 34]	1.95 (1.83–2.08)	<0.001	1.84 (1.48–2.28)	<0.001	86%	<0.001	0.207
Mutlitherapy	3	[26, 27, 33]	2.39 (1.84–3.11)	<0.001	2.39 (1.84–3.11)	<0.001	1%	0.37	
Subgroup: region									
Western	3	[19, 21, 26]	2.26 (1.74–2.94)	<0.001	2.10 (1.42–3.10)	<0.001	44%	0.17	0.543
Eastern	16	[13, 14, 18, 20, 22–25, 27–34]	1.96 (1.85–2.08)	<0.001	1.96 (1.71–2.24)	<0.001	56%	0.004	
Subgroup: sample size									
Sample size ≥ 200	9	[13, 14, 20, 24, 26, 30, 32–34]	1.69 (1.53–1.86)	<0.001	1.82 (1.55–2.13)	<0.001	45%	0.07	0.034
Sample size < 200	10	[18, 19, 21–23, 25, 27–29, 31]	2.15 (2.00–2.31)	<0.001	2.15 (2.00–2.31)	<0.001	0%	0.43	
Subgroup: cutoff value									
(1) Cutoff ≤ 2.2	5	[13, 24, 28, 30, 33]	1.80 (1.43–2.26)	<0.001	1.80 (1.43–2.26)	<0.001	0%	0.43	
(2) 2.2 < cutoff ≤ 3	7	[19, 20, 22, 23, 25, 29, 34]	1.96 (1.84–2.08)	<0.001	1.88 (1.56–2.26)	<0.001	0%	0.47	0.112
(3) 3 < cutoff ≤ 4	3	[18, 26, 32]	2.32 (1.85–2.89)	<0.001	2.31 (1.81–2.94)	<0.001	41%	0.13	
(4) 4 < cutoff ≤ 5	4	[14, 21, 27, 31]	2.27 (1.59–3.26)	<0.001	2.36 (1.38–4.03)	0.002	54%	0.09	
Subgroup: stage IV									
Resection	2	[30, 32]	1.75 (1.30–2.36)	<0.001	1.73 (0.85–3.54)	0.13	83%	0.02	
Chemotherapy	3	[18, 20, 26, 29, 34]	1.94 (1.81–2.07)	<0.001	1.83 (1.49–2.24)	<0.001	90%	<0.001	
Clinicopathological parameters			OR (95% CI)	p	OR (95% CI)	p	I^2	p	
TNM stage (I + II vs. III + IV)	4	[13, 19, 24, 31]	2.59 (1.91–3.50)	<0.001	2.76 (1.36–5.61)	0.005	80%	0.002	
Tumor differentiation (well versus poor)	3	[13, 20, 30]	1.05 (0.77–1.43)	0.75	1.05 (0.77–1.44)	0.74	0%	0.38	
CEA (<5 ng mL^{-1} versus ≥5 ng mL^{-1})	2	[23, 24]	1.43 (0.64–3.21)	0.38	1.31 (0.77–2.25)	0.32	52%	0.15	



(a) Begg's funnel plot with pseudo 95% confidence limits

(b) Egger's publication bias plot

Figure 4: (a) Begg's test. (b) Egger's test.

demonstrated that there was no publication bias for OS (p = 0.141, Figure 4(a)). Egger's test also showed that there was no publication bias for OS (p = 0.628, Figure 4(b)).

4. Discussion

Several studies have suggested that elevated NLR, an inflammation-based prognostic score, is correlated with the poor survival of many types of cancers. The mechanism of NLR responses to tumors may be explained as an increase in neutrophils or decrease in lymphocytes that may restrain lymphokine-activated killer cells and increase metastasis [35]. However, some other studies have reported negative results for the NLR for prognosis and clinicopathologic characteristics. At the same time, the optimal cutoff value for the NLR is uncertain. For gastric cancer in particular—a disease which has been proved to be associated with chronic inflammation—the conclusions remain controversial. To address the questions above, we performed this study using meta-analysis.

We included 19 articles with 5431 patients with gastric cancer to evaluate the prognostic role of NLR. We found that pretreatment NLR can predict OS and PFS for patients with gastric cancer. We also investigated the relationship between the cutoff values and predictive function of NLR in gastric cancer and found a trend that the NLR might influence prognosis along with the increase of cutoff value. Moreover, we used subgroup and meta-regression analysis to establish the source of heterogeneity, and subgroup analysis found lower heterogeneity in each group, as expected. The results indicated that elevated NLR was associated with late stages of gastric cancer, and elevated NLR predicted poor prognosis for patients who received palliative chemotherapy for stage IV gastric cancer.

In recent decades, our understanding of the inflammatory microenvironment of cancer has improved, and research has focused on the association between cancer and inflammation. Inflammation plays an important role in the development and progression of several cancers by suppressing or stimulating tumor cells [36]. Therefore, many inflammatory indicators, including NLR, platelet to lymphocyte ratio, or CRP, are diagnostic and prognostic biomarkers for various cancers [37]. NLR, in particular, is a prognostic indicator for several other solid cancers such as urinary [38] and colorectal [39, 40] cancer. Chronic inflammation may be caused by *Helicobacter pylori,* and it is an important risk factor for stomach neoplasms [41]. In our meta-analysis, we demonstrated that the prognosis of patients with high NLRs was worse than that for patients with a normal NLR amongst early-stage gastric cancers. Furthermore, we found that high NLRs are associated with late-stage gastric cancer. However, the mechanisms involved in the association of elevated NLR and poor outcome for patients with gastric cancer remain unclear. There are several explanations for the correlation between poorer prognosis and elevated NLR in gastric cancer. A high NLR reflects a decrease in the number of lymphocytes and/or an elevated number of neutrophils. Neutrophils may play an important role in cancer development and progression by offering a suitable microenvironment for their growth. Circulating neutrophils may contain and secrete the majority of circulating vascular endothelial growth factor, interleukin-18, and matrix metalloproteinase, which are thought to be closely associated with tumorigenesis, development, and metastasis [42–44]. Furthermore, the antitumor immune responses of activated T cells and natural killer cells may be inhibited by an elevated number of neutrophils surrounding tumor tissues. Therefore, a high level of circulating neutrophils may have a negative effect on patients with gastric cancer and lead to poor outcome. At the same time, lymphocytes play an important role in cellular adaptive immunity against cancer by attacking and clearing tumor cells at the outset of tumorigenesis [45]. Patients who have lymphocyte infiltration surrounding their tumors may have a better prognosis than those with less or no infiltration [46]. In addition, lymphocytes may be suppressed by large numbers of neutrophils when two cells are cocultured [47]. Our results indicate that an elevated NLR denotes a pretreatment inflammatory condition that is correlated with poor prognosis for patients with gastric cancer. Although the NLRs were tested before treatment and status of patients was favorable, NRL still might be influenced by a number of

confounding factors in peripheral blood. So the control of confounding factors in studies about the association between NLR and gastric cancer may be an important research point in the future.

For most gastric cancer patients, recurrence and metastasis remain the main factors that may cause death and influence survival, even after curative resection [48]. The identification of sensitive markers that can predict prognosis and help select patients who may receive different treatments is needed. TNM staging is a good indicator for gastric cancer patients who undergo surgery [21]. Inflammation-based prognostic scores such as NLR could predict the prognosis of patients before they receive treatment. In this study, we analyzed the relationship between the NLR level and TNM stage in gastric cancer. Elevated NLR was associated with late-stage gastric cancer and indicated that elevated NLR indicates worse prognosis. We analyzed the predictive role of NLR for patients with stage III/IV gastric cancer. Elevated NLR predicts poor outcome for patients with stage III/IV gastric cancer. Furthermore, immunosuppression induced by surgery is associated with delaying postoperative recovery time, increasing the cancer recurrence rate, and reducing the survival time [49]. We analyzed NLR in stage IV gastric cancer to establish whether pretreatment NLR values indicate prognosis for patients who have received surgery. Elevated NLR indicated poor outcome for patients with stage IV gastric cancer. Nevertheless, subgroup showed that elevated NLR was associated with poor outcome in stage IV gastric cancer patients who received palliative chemotherapy and the surgery subgroup did not significantly differ. The pretreatment NLR was not predictive of prognosis when stage IV gastric cancer patients received palliative surgery. However, there were only two studies in the surgery group and three in the palliative chemotherapy group, and fewer included articles might have caused heterogeneity when we pooled the effect sizes. Hence, more attention should be focused on the predictive role of the NLR for late-stage gastric cancer in evaluating the prognosis of different treatments.

Studies of other tumors together with our study demonstrate that an elevated NLR plays an important role in predicting prognosis before treatment. However, the optimal cutoff value for NLR in predicting the prognosis of gastric cancer remains unclear. The cutoff values in our analysis ranged from 1.44 to 5.00, and they were determined by receiver operating characteristic curves, by the median value of all patients, or on the basis of previous studies, such as a score of 5.00. To establish a suitable cutoff value, we performed meta-regression and subgroup analyses with quartiles of the cutoff values (2.20, 3.00, and 4.00). The role of elevated NLR in predicting prognosis differed significantly among the four subgroups. In Subgroups 1 and 2 and Subgroups 3 and 4, the pooled HR was similar, which suggests that the HRs were almost the same when the cutoff values were set as the first two subgroups and the last two subgroups. The pooled HRs in Subgroups 3 and 4 were higher than those in Subgroups 1 and 2. From the results above, we thought that the predictive prognosis ability of the NLR might be slightly influenced by cutoff values when the range was from 1.44 to 5.00. We also found that when the cutoff value was set at 3.00, the results from original articles that used 3.00 as a cutoff value might be more stable and close to each other. However, in a study of 1028 patients, Shimada et al. [32] reported that an NLR of 4.00 appeared to be more useful than a cutoff value of 3.00, which was similar to our study. However, in our Subgroup 4, there were two studies that reported no significant difference with a cutoff value of 5 in multivariate analysis. The negative results of included articles in Subgroup 4 that may lead to the pooled result trend to be close to the result of Subgroup 3. Hence, we thought it may be a key point for performing a study of the NLR to define or help clarify an appropriate cutoff when the variation is wide. More attention should focus on the choice and comparison of cutoff values during analysis of the NLR in the future studies.

A previous meta-analysis evaluated the predictive role of the NLR for OS and DFS for gastric cancer [50]. Our study differed in several ways. Firstly, this study included eight more articles, which makes the results more powerful and robust. With the larger sample size, elevated NLR may reflect poor outcome in western and eastern countries. Secondly, we found that the NLR was higher in late-stage compared with early-stage gastric cancer. We discussed the predictive role of NLR in stage III and IV gastric cancer using rational and robust subgroups. Finally, this study explored suitable cutoff values for NLR for evaluating the prognosis of gastric cancer.

There were some limitations to our meta-analysis. First, all included articles were retrospective studies, and the level of evidence was not high enough. In addition, original articles supplied only summarized but not individualized data, which may have increased the heterogeneity of the articles. Second, not all studies supplied data for all analyses; thus, the results may be slightly influenced due to the limited number of included articles, particularly for the analysis of tumor differentiation and CEA. Third, sample size was analyzed as a potential source of heterogeneity. In the subgroup with fewer samples, heterogeneity was not significant. However, in the subgroup with more samples, significant heterogeneity was observed. Although the subgroup with fewer samples had no significant heterogeneity, studies including more samples might provide more robust results. For heterogeneity, sensitivity analysis could not provide additional information to address this limitation. Finally, several articles reported HRs, which, from the multivariate analysis and results, demonstrated no significant difference. These results might have been caused by other markers such as Glasgow score and CRP which may have a similar function as the NLR and influenced the analysis. We also aimed to address the confounding factors by sensitivity analysis, but we could not find a statistically significant result. More well-designed and high-quality multicenter clinical trials are required.

5. Conclusions

The presented meta-analysis demonstrated that pretreatment NLRs play a significant role in predicting the prognosis of gastric cancer, particularly for late-stage gastric cancer. Increased cutoff values of NLR may reflect prognosis as a biomarker better than the decreased values in gastric cancer. The ability of NLR to evaluate the prognosis of patients may

be used in the future. Whether these findings can be used to adjust treatment decisions remains uncertain and is an area for further research.

Conflict of Interests

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

Authors' Contribution

Jingxu Sun and Xiaowan Chen contributed equally to this work.

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Research Article

Increased Avidity of the *Sambucus nigra* Lectin-Reactive Antibodies to the Thomsen-Friedenreich Antigen as a Potential Biomarker for Gastric Cancer

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Aim. To determine whether the naturally occurring Thomsen-Friedenreich (TF) antigen-specific antibodies differ in avidity between cancer patients and controls to find a novel biomarker for stomach cancer. *Methods*. Serum samples were taken from patients with cancer and controls. The level of TF-specific antibodies and their sialylation were determined using ELISA with synthetic TF-polyacrylamide conjugate as antigen and sialic acid-specific *Sambucus nigra* agglutinin (SNA). The avidity was determined using ammonium thiocyanate as a chaotrope. *Results*. A significantly higher SNA lectin binding to anti-TF antibodies was found in cancer patients irrespective of disease stage. The avidity of only IgM TF-specific antibodies was significantly higher in cancer patients compared to controls. The SNA-positive anti-TF antibodies of cancer patients showed a significantly higher avidity, $P < 0.001$. The sensitivity and specificity of this increase for gastric cancer were 73.53% and 73.08%, respectively, with a 73.2% diagnostic accuracy. The higher avidity of SNA-reactive anti-TF antibodies was associated with a benefit in survival of stage 3 cancer patients. *Conclusion*. The SNA-reactive TF-specific antibodies display a significantly higher avidity in gastric cancer patients compared to controls, which can be used as a potential serologic biomarker for gastric cancer. It appears that IgM is the main target responsible for the above changes.

1. Introduction

Over the past two decades protein posttranslational modifications have attracted ever-increasing attention in medical research. The altered immature O-glycophenotype often observed in cancer cells leads to the expression of modified glycopeptide epitopes and tumor-associated glycans (TAGs) that may be autoimmunogenic and recognized by autoantibodies [1–9]. In cancer patients, an abnormal glycosylation pattern has also been observed for many circulating glycoconjugates, including immunoglobulins [10–15].

The O-linked tumor-associated glycans such as the Thomsen-Friedenreich (TF) antigen (Galβ1-3GalNAcα/β-O-Ser/Thr, TF, CD176) and Tn antigen (GalNAcα1-O-Ser/Thr, CD175) are expressed in the majority of human carcinomas [1, 16–18], including cancer-initiating cells [19]. TAGs are considered as a promising target for cancer immunotherapy [20–23]. The overexpression of these commonly hidden glycotopes and the reduced level of naturally occurring anti-TF or anti-Tn antibodies are associated with tumor progression and aggressiveness and a patients survival rate [16, 24–29]. The TF antigen seems to play a crucial role in the adhesion of cancer cells to the endothelium through the interaction with galectin-3, thereby promoting metastases [30, 31].

The presence of autoantibodies (AAbs) against antigens expressed by tumors, including TAGs, is a well-established fact [4]. However, a majority of AAbs to tumor-assocoated antigens (especially anti-peptide Abs) is revealed only in a minority of cancer patients, thus limiting the clinical potential of the approach. An appreciable amount of TF- and Tn-specific IgM and IgG antibodies is present in normal human serum, being decreased in patients with cancer though there are large interindividual variations [17, 25–27]. Moreover,

the anti-TF and -Tn IgG level is rather stable over time at an individual level in both patients and controls [25, 32]. However, the antitumor potential of tumor-specific Abs remains to be further elucidated because the latter may actually have various effects [8, 21, 33, 34], suggesting that these antibodies are heterogeneous functionally and structurally. Of note is that up to now there are very limited data available on the glycosylation of naturally occurring human TAG-specific Abs [15, 35–37] and, to our knowledge, no data about the avidity profile of these antibodies in cancer or other pathologies have been reported either. Gastric cancer is the second leading cause of cancer deaths worldwide. Yet there are no reliable serum biomarkers for gastric cancer diagnostics and prognostics.

We showed recently that patients with gastric cancer demonstrated an increased sialylation of TF-specific Abs irrespective of disease stage, tumor morphology, and gender [36]. Moreover, we found that similar changes in anti-TF Ab sialylation were also observed in patients with breast cancer (unpublished), suggesting that it may be a common cancer-related phenomenon. In the present study we show, for the first time, that gastric cancer is associated with a significantly higher avidity ($P < 0.001$) of SNA-positive TF-specific antibodies that may be used as a serologic biomarker for gastric cancer.

2. Material and Methods

2.1. Subjects and Samples. Serum samples were obtained from healthy blood donors ($n = 34$), patients with nonmalignant gastric diseases ($n = 15$), and patients with histologically verified gastric carcinoma ($n = 104$) (Table 1). Tumor staging and morphology were based on the histopathological (pTNM) classification of malignant tumors. The distribution of cancer patients by stage is presented in Figure 1. The investigation was carried out in accordance with the ICH GCP Standards and approved by the Tallinn Medical Research Ethics Committee. A written informed consent was obtained from each subject. The serum samples were stored in aliquots at −20°C until used.

2.2. The Anti-TF Antibody Assay. The anti-TF IgG, IgM, and a pool of IgG+IgM+IgA antibody levels were determined by enzyme-linked immunosorbent assay (ELISA) as described elsewere [36]. The plates (Maxisorp, NUNC, Denmark) were coated with synthetic TF-polyacrylamide conjugate (10 mol% of carbohydrate; 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 30 min at 25°C, the serum samples (diluted 1 : 25 in PBS-0.05% Tween) were applied for 1.5 hr at 25°C. After subsequent washing with PBS-Tw, the bound anti-TF antibodies were detected using alkaline phosphatase conjugated goat anti-human IgG, IgM (Sigma, USA), IgA (Dako, Denmark), or rabbit anti-IgG+IgM+IgA (Dako) and developed with p-nitrophenylphosphate (Sigma, USA). The absorbance values were read at 405 nm (Tecan Reader, Austria) and each sample was analysed in duplicate.

Table 1: Characteristics of the subjects tested.

Group	n	Males	Females	Median age (range)
Donors	34	9	25	63 (24–73)
Benign group[x]	15	9	6	62 (27–72)
Gastric cancer	104	59	45	66 (28–80)

[x]Nonmalignant chronic gastric diseases: peptic ulcer disease ($n = 9$); chronic gastritis ($n = 6$).

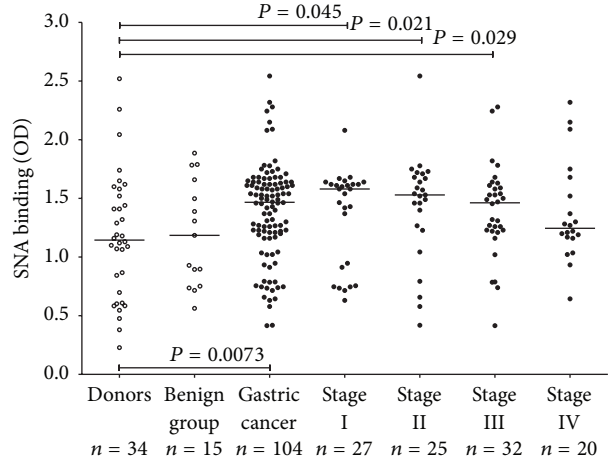

Figure 1: The binding of *Sambucus nigra* agglutinin (SNA) to serum TF-specific antibodies (all isotypes) in controls and gastric cancer patients by stage of disease. Each dot represents one individual and group median is indicated by horizontal lines. P values were calculated by the Mann-Whitney U test and are shown for significant differences.

2.3. The Reactivity of Anti-TF Antibodies to Sambucus nigra Agglutinin (SNA). The SNA lectin-reactivity of TF-glycotope specific antibodies was measured in a similar way.

The plates (Maxisorp, NUNC, Denmark) were coated with synthetic TF polyacrylamide conjugate (10 mol% of carbohydrate; 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 30 min at 25°C, the serum samples (diluted 1 : 25 in PBS-0.05% Tween) were applied for 1.5 hr at 25°C. After subsequent washing with PBS-Tw, the biotinylated SNA (Vector Laboratories Inc., USA) in 10 mmol/L Hepes, 0.15 mol/L NaCl, 0.1 mmol/L $CaCl_2$, and pH 7.5 was applied at a concentration of 5 μg/mL for 1.5 hr at 25°C. The bound lectin was detected with a streptavidin-alkaline phosphatase conjugate (Dako, Denmark) and p-nitrophenylphosphate (Sigma, USA). The optical density value (OD) of control wells (no sample) was subtracted from the Ab coated wells. Each sample was analysed in duplicate.

2.4. The Avidity of TF-Specific Antibodies. The avidity of anti-TF IgG, IgM, or a pool of IgG+IgM+IgA antibodies was determined by ELISA. The plates were coated with synthetic TF-polyacrylamide conjugate in carbonate buffer, pH 9.6,

5 μg per well. After overnight incubation at +4°C, washing and blocking with Superblock solution as above, the serum (diluted 1 : 25 in PBS-0.05% Tween) was applied for 1.5 hr at 25°C. After subsequent washing ammonium thiocyanate (NH_4SCN) as a dissociating agent was added at a concentration of 1.25 mol/L for 15 min at +25°C. The bound antibodies were detected with alkaline phosphatase conjugated goat anti-human IgG, IgM or anti-(IgG+IgM+IgA) Igs, and p-nitrophenylphosphate. The absorbance values were read at 405 nm.

A relative avidity index (AI) was calculated for each sample and was expressed as the percentage of reactivity remaining in the thiocyanate-treated wells in relation to untreated wells (PBS-Tw instead of the chaotrope).

2.5. The Avidity of Sambucus nigra Agglutinin- (SNA-) Reactive Anti-TF Antibodies. The avidity of SNA-reactive anti-TF antibodies (a pool of all isotypes) was determined by ELISA in a similar way. The plates (Maxisorp, NUNC, Denmark) were coated with synthetic TF polyacrylamide conjugate as above. After overnight incubation at +4°C, triple washing and blocking with Superblock solution for 30 min at 25°C, the serum samples (diluted 1 : 25 in PBS-0.05% Tween) were applied for 1.5 hr at 25°C. After subsequent washing ammonium thiocyanate (NH_4SCN) as a dissociating agent was added at a concentration of 1.25 mol/L for 15 min at +25°C. To detect the lectin reactive antibodies, the biotinylated SNA (Vector Laboratories Inc., USA) in 10 mmol/L Hepes, 0.15 mol/L NaCl, 0.1 mmol/L $CaCl_2$, and at pH 7.5 was applied at a concentration of 5 μg/mL for 1.5 hr at 25°C. The bound lectin was detected with a streptavidin-alkaline phosphatase conjugate and p-nitrophenylphosphate. The proportion of TF-specific antibody SNA reactivity remaining after treatment with chaotrope was defined as the avidity index of SNA-reactive anti-TF antibodies.

2.6. Statistical Analysis. The results were analysed using the nonparametric Mann-Whitney U test due to the abnormal distribution of values. The difference between the groups was considered to be significant when $P \leq 0.05$. The sensitivity and specificity of the differences between cancer patients and controls were evaluated by the receiver operator characteristic (ROC) curve analysis. Overall survival was analyzed by the Kaplan-Meier method. All calculations and comparisons were performed using GraphPad Prism 5 and SPSS 15.0 software.

3. Results

The levels of anti-TF IgG in cancer patients and both controls were very similar and were decreased only in patients with advanced cancer: mean O.D. = 0.50 ± 0.05 (SE) and 0.36 ± 0.05 (stage 4), $P = 0.018$ compared to healthy donors.

In a parallel testing of several anti-TF Ab isotypes (IgG, IgM, IgA, and a pool of all isotypes) only IgM showed a clear trend to a lower level in cancer ($n = 36$) compared to healthy donors (0.22 ± 0.07 (SD) and 0.31 ± 0.2, resp., $P = 0.08$) and a significant decrease compared to the benign group (0.37 ± 0.12, $n = 15$, $P < 0.001$).

Table 2: The avidity of anti-TF IgG, IgM, and a pool of all anti-TF Ab isotypes in gastric cancer patients and controls. The mean ± SD and P values are presented.

Groups	n	IgG	IgM	IgG/M/A
Donors	16	56.7 ± 14.8	44.9 ± 14.0	55.1 ± 7.1
Benign group	15	59.1 ± 11.2	39.9 ± 6.0	47.7 ± 6.7
Gastric cancer	36	59.9 ± 10.3	59.2 ± 12.2	54.4 ± 5.9
		P values		
Donors versus benign group		0.61	0.22	0.01
Donors versus gastric cancer		0.43	0.002	0.75
Benign group versus gastric cancer		0.8	<0.0001	0.003

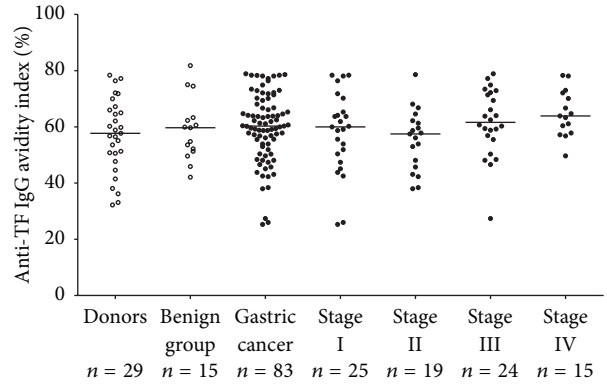

Figure 2: The avidity of anti-TF IgG in controls and patients with gastric cancer by stage.

The SNA lectin binding to serum TF-specific antibodies (all isotypes) was significantly higher in cancer patients compared to controls ($P = 0.0073$) (Figure 1). The stage distribution had no impact on this increase, except in stage 4 patients that showed no significant changes in SNA binding compared to controls.

The avidity of anti-TF IgG reveals no significant differences between cancer patients and controls (59.7 ± 12.2 (SD) and 57.1 ± 13.1, resp.) with no significant changes by stage of the disease (Figure 2), being in the range of 56–64%.

A group of cancer patients, blood donors, and patients from the benign group were tested in parallel for the avidity of anti-TF IgG, IgM, and a pool of IgG+IgM+IgA anti-TF Abs (Table 2). The avidity of only IgM TF-specific Abs was significantly higher in cancer compared to both controls ($P = 0.002$ and $P < 0.0001$ for donors and the benign group, resp.), suggesting that the anti-TF IgM is the main target

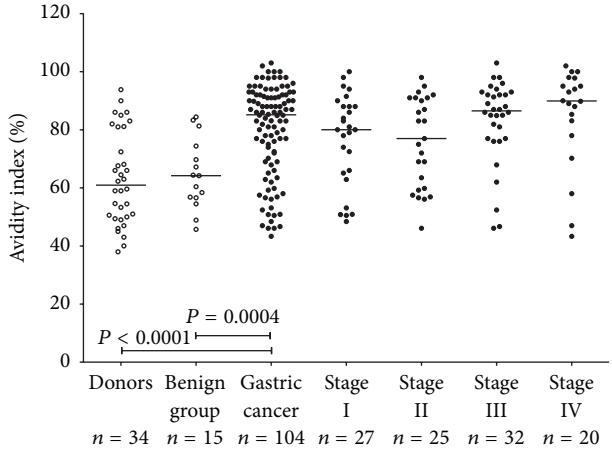

Figure 3: The avidity of SNA-positive anti-TF antibodies in controls and patients with gastric cancer by stage. *P* values were calculated by the Mann-Whitney *U* test and are shown for significant differences.

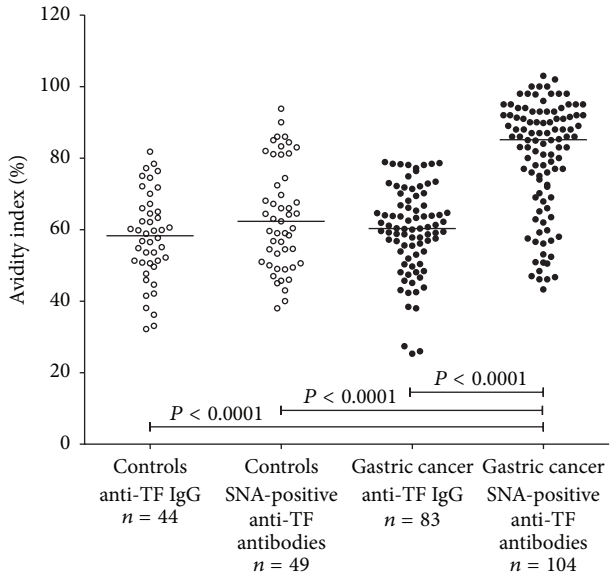

Figure 4: Comparison of the avidity of anti-TF IgG and SNA-positive TF-specific serum antibodies in cancer patients and controls. *P* values are shown for significant differences.

for changes in the TF-specific Ab avidity found in cancer patients. Interestingly, patients with nonmalignant gastric diseases showed an even lower level of Ab avidity than healthy blood donors.

The avidity of SNA-positive anti-TF antibody (all isotypes) was significantly higher in patients with cancer compared to both control groups ($P < 0.0001$ and $P < 0.0004$ for donors and the benign group, resp.) with slightly higher avidity index values in advanced cancer (Figure 3). It is notable that in controls the avidity of anti-TF IgG was very similar to that of anti-TF IgG in cancer patients, whereas the avidity of SNA-positive TF-specific antibodies in cancer patients was significantly higher compared with that of the other groups of patients and controls (Figure 4). The higher

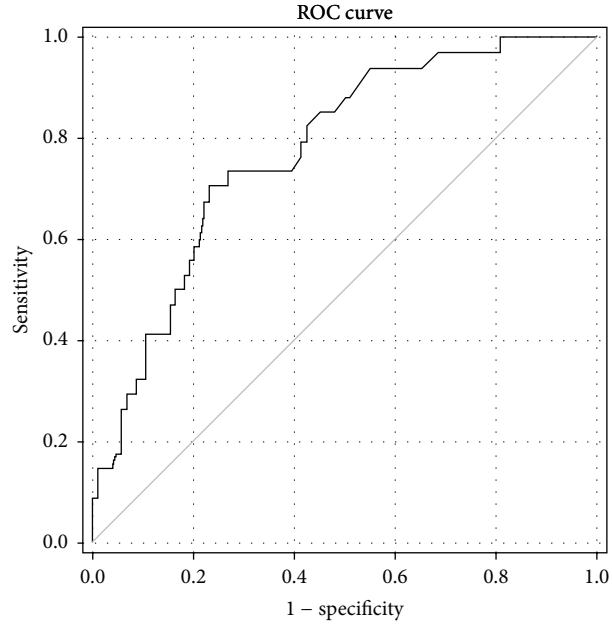

Figure 5: The sensitivity and specificity of serum anti-TF antibody avidity changes for gastric cancer by receiver operator characteristic (ROC) curve analysis.

avidity of SNA-positive anti-TF antibodies in cancer patients was not dependent on gender and/or age (data not shown).

Using the SNA-positive TF-specific antibody avidity value equal to 72.45% as a cut-off limit, which allows the best discrimination between cancer patients and controls (calculated by ROC curve analysis), the sensitivity and specificity for gastric cancer were 73.53% and 73.08%, respectively, with a 73.2% accuracy of diagnostics (ROC statistics: the area under a curve 0.776, $P < 0.0001$) (Figure 5). The sensitivity of the test was 70.37%, 60%, 84.37%, and 80% for stages 1, 2, 3, and 4, respectively. At a specificity of 90% the sensitivity was 47.9%.

For the whole group of cancer patients (all stages), no significant association of the avidity of anti-TF IgG antibodies with survival was found (HR = 0.72 (95% CI 0.38–1.37), $P = 0.32$). However, the higher avidity of SNA-reactive serum anti-TF antibodies was associated with a benefit in survival of stage 3 cancer patients (HR = 2.4 (0.86–6.36), $P = 0.09$) (Figure 6).

4. Discussion

An aberrant glycosylation of glycoconjugates, including immunoglobulins, is a common phenomenon in cancer [3, 5, 32, 38]. It has been shown that various Ab glycoforms display different effector functions and determine the activity of antibodies against tumors [5, 39–41]. Previous studies have demonstrated that naturally occurring antibodies to tumor associated glycans are involved in natural tumor immunity, being associated with tumor progression and cancer patients survival [18, 26, 42, 43]. Natural anti-TF antibodies (Abs) of different isotypes (IgG, IgM, and IgA) are present in each

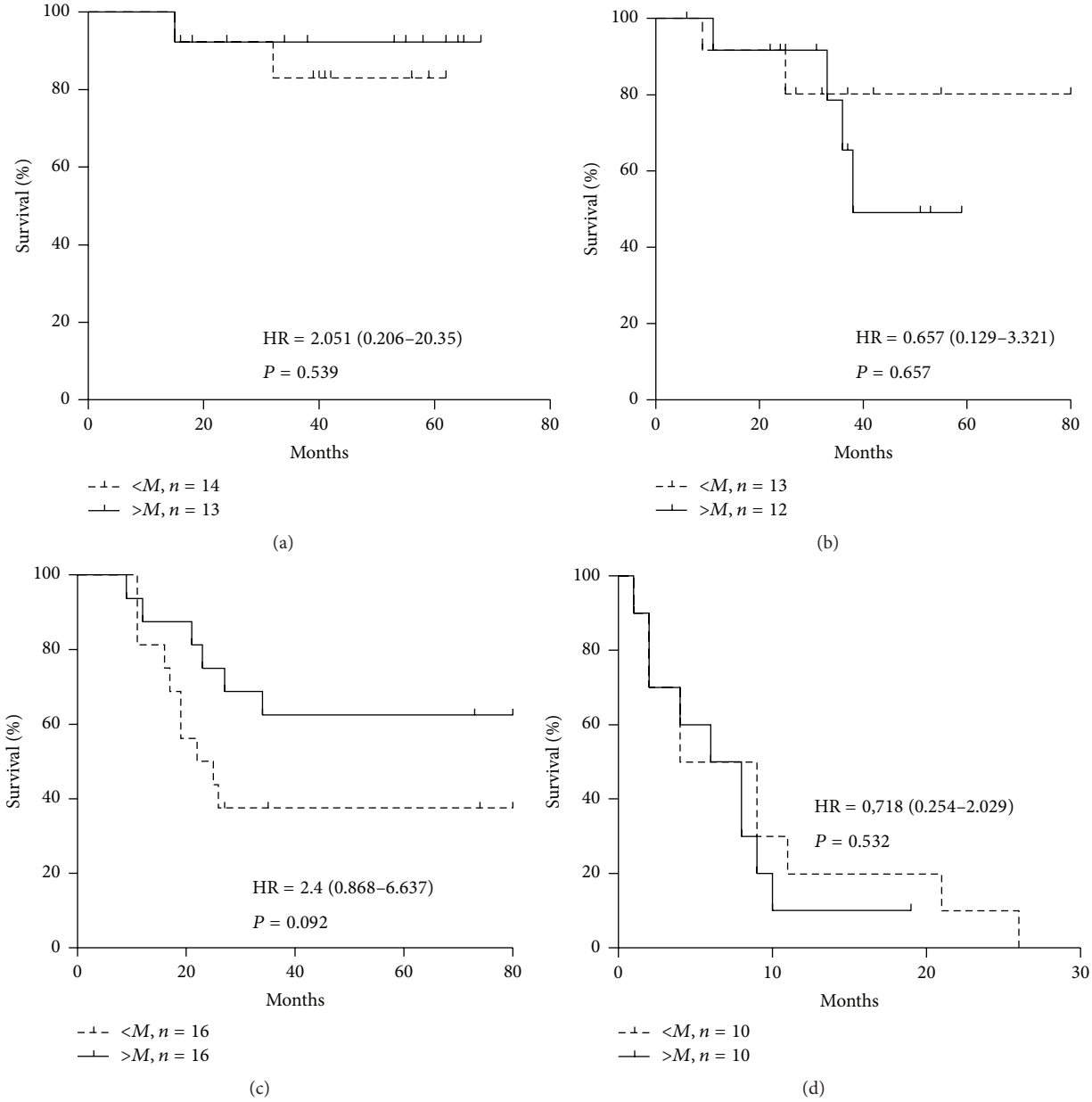

Figure 6: The probability of survival of gastric cancer patients in relation to the avidity of SNA-positive anti-TF antibodies. Patients with avidity index values lower than, equal to (a dashed line), or higher than median (a solid line) are compared using the Kaplan-Meier method. The hazard ratio (HR) with a 95% confidence interval and P values are shown. Patients: (a) stage 1; (b) stage 2; (c) stage 3; (d) stage 4.

individual thus making these antibodies a convenient target for analysis, in contrast to tumor-derived products that may be detected in a minority of patients due to their extreme dilution in the circulation and rapid degradation or clearance. Although the levels of TF-specific antibodies demonstrate some decrease in cancer patients [7, 17, 26], these changes did not show sufficient sensitivity and specificity for gastric cancer [36].

We analysed the TF-specific Abs present in the whole untreated serum, using TF-PAA as a catcher and the *Sambucus nigra* agglutinin (SNA) in the lectin-ELISA assay, thus excluding possible structural or conformational modifications of Igs during their purification or the presence of so called "hidden" Abs that may remain undetectable due to being in complexes with some TF-positive ligands such as aberrantly glycosylated MUC1, for instance. We showed recently that the increase of SNA binding to TF-specific Abs in cancer patients was not dependent on the stage of disease, histological type of tumor growth (diffuse, intestinal), or gender [36]. In contrast, the SNA reactivity of anti-TF IgG in purified IgG samples was even decreased in patients with gastric cancer [15]. The higher level of the fully sialylated IgG glycoform, as defined by LC-ESI-MS, may predict a better survival of patients with gastric cancer [14].

In the present study we show that the avidity of serum anti-TF IgG is not increased in cancer, in contrast to that of

IgM (Figure 2, Table 2), indicating that IgG is not involved in cancer-related changes of TF-specific antibody avidity. Preliminary data show that the IgG depletion does not influence significantly the SNA binding to all TF-specific Abs and the avidity index of SNA-reactive Abs in both donors and cancer patients (unpublished). It appears that the anti-TF IgM remain to be the main target responsible for that [36].

An increase in avidity of SNA-reactive anti-TF antibodies showed a rather good diagnostic accuracy (73.2%). Note that already stage 1 patients showed a relatively high diagnostic sensitivity (70.37%). At present, we can give no explanation for why the SNA-positive anti-TF Abs have a relatively high avidity only in cancer patients and not in donors. The increased SNA reactivity of anti-TF Abs in cancer indicates that they are more sialylated than those in donors. It has been shown that the SNA mostly bound the IgG Fc glycan at Asn297, which has two sialic acids if both glycan branches terminated with sialic acid [44]. The pentameric IgM is more glycosylated and has five N-glycosylation sites (four of which on Fab) on each of its heavy chains [5]. There are still no data about the sialylation diversity of pentameric IgM Fc and Fab in health and disease, and its influence on Ab avidity. It is possible that different proportions of IgM and/or IgA fully sialylated anti-TF Abs may be present in patients and controls. The IgM polyvalency and possible variations in the glycosylation of different monomers may also be infuencing factors. Another reason might be the increased activity of sialyltransferases that orchestrate the diversity of glycan structures and are frequently overregulated in cancer cells [45–47] and tumor microenvironment where TF-specific antibodies may interact with TF-positive tumor cells and undergo further sialylation *in situ*. But the question remains unanswered yet.

In any case, the higher avidity of serum SNA-reactive TF-specific Abs we observed in patients with gastric cancer needs further investigation from several points of view: (i) specifying the Ig isotype responsible for these changes, especially the site-specific glycosylation patterns; (ii) studying how this alteration in Ab sialylation influences the functional (antitumor) Ab activity; (iii) exploiting a combination of Ab avidity testing with other Ab-based parameters such as Ab levels, the diversity of other glycoforms, the presence of hidden Abs, and putative hidden antigens (ligands) in the circulation; (iv) further stratifying the patients on the basis of additional parameters, as has been done for SNA binding and anti-TF IgM level patterns in patients with gastric cancer [36].

Given that the expression of TF antigen on tumor cells promotes metastasis by interaction with galectin-3 on the endothelial cells [30], it is logical to assume that the circulating TF-specific antibody may modulate this interaction *via* binding with TF antigen-positive circulating tumor cells. It is not known yet whether the aberrantly glycosylated (sialylated) anti-TF antibodies interact differently with tumor cells *in situ* or in the circulation. Since the sialylated (anti-inflammatory) Abs display immunosuppressive or tolerogenic effects [37, 48], they may eliminate undesirable inflammatory reactions in tumor tissue that may promote tumor growth [8, 49]. Alternatively, a benefit in survival we found in stage 3 cancer patients with a higher avidity of TF-specific Abs might be the result of a more efficient blockade of TF antigen on circulating tumor cells, thus protecting against metastases through the inhibition of the TF/galectin-3 pathway. The other functional activities of aberrantly sialylated TF-specific antibodies remain to be determined.

In conclusion, we provide evidence that the aberrant sialylation of TF-specific antibodies is associated with gastric cancer-specific changes in Ab avidity, which may be used as a potential serological biomarker for gastric cancer detection and prognosis. It is to be noted that a rather high diagnostic sensitivity (70.37%) was observed already in stage 1 patients. Our data suggest that the evaluation of not just the level of antibodies to tumor-associated antigens but rather their structural and functional diversity might improve the clinical potential of antibody signatures in cancer diagnostics and prognostics. Since the glycosylation of Abs specific to various antigens may considerably differ from that of total Ab isotypes in the circulation, the focus of further studies should be shifted to the glycoprofiling of Abs specific to antigens directly involved in the pathogenesis of the disease under study. Such a noninvasive approach which is not yet widely used in practical medicine may be a good prerequisite for the improvement of the clinical utility of antibody-based biomarkers.

Conflict of Interests

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

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Research Article

Clinicopathological Significance of MicroRNA-20b Expression in Hepatocellular Carcinoma and Regulation of HIF-1α and VEGF Effect on Cell Biological Behaviour

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miRNA-20b has been shown to be aberrantly expressed in several tumor types. However, the clinical significance of miRNA-20b in the prognosis of patients with hepatocellular carcinoma (HCC) is poorly understood, and the exact role of miRNA-20b in HCC remains unclear. The aim of the present study was to investigate the association of the expression of miR-20b with clinicopathological characteristics and overall survival of HCC patients analyzed by Kaplan-Meier analysis and Cox proportional hazards regression models. Meanwhile, the HIF-1α and VEGF targets of miR-20b have been confirmed. We found not only miR-20b regulation of HIF-1α and VEGF in normal but also regulation of miR-20b in hypoxia. This mechanism would help the tumor cells adapt to the different environments thus promoting the tumor invasion and development. The whole study suggests that miR-20b, HIF-1α, and VEGF serve as a potential therapeutic agent for hepatocellular carcinoma.

1. Introduction

Hepatocellular carcinoma (HCC) represents an extremely poor prognostic cancer that remains one of the third causes of cancer-related death and aggressive human malignancies represented worldwide [1, 2]. The dismal outcome has been attributed to the major hallmarks of HCC, intrahepatic metastases or postsurgical recurrence [3]. With much progress made in surgery and other treatments, the prognosis of HCC patients is still unsatisfactory due to the high rate of recurrence and metastasis. Thus, it is crucial to improve our understanding of the molecular mechanisms underlying HCC which will be critical for the improvement of therapeutic strategies for HCC patients [4]. However, tumor metastasis is considered to be one of the most complex cell activities because it is a multistep process of cascade involving cell invasion and intramedullary pin into the blood and lymph vessels, survival and arrests in the vascular system, the proliferation of extravasation.

MicroRNAs (miRNAs) are a class of small noncoding RNA molecules that regulate gene expression by binding to partially complementary recognition sequences of target mRNAs, either repressing miRNA translation or cleaving target miRNA and RNAs that are highly conserved between species [5–8]. In recent years, increasing studies indicate that miRNAs have crucial functions in specific cellular processes such as differentiation, morphogenesis, and tumorigenesis and they are also considered as oncogenes and tumor suppressors [9–12]. Recently, it has been manifested that the deregulation or dysfunction of miRNAs is involved in cancer development and related to clinical outcomes of cancer patients including HCC [13]. In HCC, miRNAs have been discovered to be aberrantly expressed and some of them are functionally involved in HCC carcinogenesis, progression, and metastasis [14, 15]. However, the roles of a large number of miRNAs are still unexplored in HCC [4].

Hypoxia inducible factor-1 (HIF-1) is the first identified mediator of cell response to hypoxia in mammalian cells

cultured under reduced oxygen tension [16]. The transcription factor HIF-1 consists of HIF-1α and HIF-1β and is a key regulator responsible for the induction of genes that facilitate adaptation and survival of tumor cells from hypoxic microenvironment and confer on the tumor a worse malignant phenotype [17, 18]. The overexpression of HIF-1α was found in various types of cancers of both human and mouse [17, 19]. The HIF-1α complex acts as a transcription factor for many target genes in several aspects of cancer progression including angiogenesis, glucose metabolism, cell proliferation, and apoptosis [20, 21]. Vascular endothelial growth factor (VEGF) is one of the major target genes for HIF-1α that directly participates in angiogenesis and a recognized therapeutic target [22, 23]. VEGF, the most potent angiogenic molecule, participates specifically in promoting vascular endothelial cell division, proliferation, and migration [24].

Some studies have shown that miRNA-20b is deregulated in several types of cancers [25–29] and upregulation of miRNA-20b correlates with worse prognosis; all these studies indicated that miRNA-20b acts as a tumor promoter [28, 30–34]. Some studies have shown that miRNA-20b modulates HIF-1α and VEGF to keep tumor adapting to different environment and promoting cell division, proliferation, and migration [27, 33, 34]. At present, the clinical significance of miRNA-20b in the prognosis of patients with HCC is poorly understood, and the exact role of miRNA-20b in HCC remains unclear. Here, we investigated the association between miRNA-20b expression and clinicopathological parameters and assessed the effect of miRNA-20b modulating HIF-1α and VEGF on biological behaviours including cell proliferation, apoptosis, and migration of HepG2 cells.

Table 1: Association between miR-20b expression and clinicopathological features of HCC.

Variables	miRNA-20b expression high ($n = 45$)	miRNA-20b expression low ($n = 31$)	P
Age (years)			
≤57	20	18	0.243
>57	25	13	
Gender			
Male	32	25	0.356
Female	13	6	
Tumor size (cm)			
≥5	27	15	0.317
<5	18	16	
AFP (ng/mL)			
≥400	19	17	0.279
<400	26	14	
Metastasis			
Yes	37	10	**0.000***
No	8	21	
Tumor grade			
G1	12	6	
G2	17	7	0.147
G3	16	18	
TNM stage			
I-II	14	26	**0.000***
III-IV	31	5	
Tumor recurrence			
Yes	32	14	**0.023***
No	13	17	
Microvascular invasion			
Yes	29	5	**0.016***
No	16	26	

*$P < 0.05$.

2. Materials and Methods

2.1. Tissue Samples. A total of 76 cases of HCCs tissues were obtained from patients collected by the Institute of General Surgical Research, Second Affiliated Hospital, Yangzhou University. In addition, 76 normal liver tissues were used as controls. Clinicopathological characteristics parameters are shown in Table 1. All of the HCC patients have not received previous treatments like local ablation, radiation therapy, chemoembolization, or chemotherapy.

2.2. Cell Culture. The human HCC-derived cell lines HepG2 were provided from the Medical Academy of Yangzhou University and cells were cultured in Dulbecco's modified essential medium (DMEM, Invitrogen Corp., USA). Both media were supplemented with 10% heat-inactivated fetal bovine serum (Zhejiang Tianhang Biotechnology Co., Ltd., China), penicillin, and streptomycin, at 37°C in a humidified incubator with 5%CO_2. Cellular hypoxia environments were stimulated with 300 mM $CoCl_2$ (Sigma-Aldrich Co. LLC., USA) for 24 h.

2.3. RT-qPCR. Total RNA from cell lines or tissue samples was extracted using a mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. The purity and concentration of RNA samples were assessed by standard spectrophotometric methods 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Briefly, 5 ng of RNA was added to RT reaction, and then the cDNA served as the template for amplification of PCR with sequence-specific primers (Sangon Biotech, Shanghai, China) using SYBR PrimeScript miRNA RT-PCR kit (Takara Biotechnology Co. Ltd., Dalian, China). All reactions were run in triplicate on the iCycler iQ Multicolor Real-Time PCR Detection System (BioRad, Hercules, CA, USA). Small nucleolar RNA GAPDH was used as an internal standard for normalization. Each sample was run in duplicate for analysis. The change for mRNA in HCC tissues relative to the matched normal liver tissues was calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = \Delta Ct$ HCC/normal liver tissues and $\Delta Ct = Ct_{miR\text{-}20b} - Ct_{GAPDH}$.

For miR-20b, the primers were as follows: forward, 5′-TGTCAACGATACGCTACGA-3′ and reverse, 5′-GCTCATAGTGCAGGTAGA-3′; GAPDH forward, 5′-GTGGTCCAGGGTTTCTTACT-3′ and reverse, 5′-GTTGTCTCCTGCGACTTCA-3′; HIF-1α forward, 5′-AACGACAAGAAAAAGATAAGTTCT-3′ and reverse, 5′-GTTTGGTGTGGTTACATA-3′; VEGF forward, 5′-CAGGAACAAGGGCCTCTGTCT-3′ and reverse, 5′-TGTCCCTCTGACAATGTGCCATC-3′.

2.4. Transfection of miRNA. Transfection was performed using Lipofectamine 2000 (Invitrogen Life Technologies), in accordance with the manufacturer's instructions. For miRNA-20b functional analysis, the HepG2 cells were transfected with the scrambled miRNA as a negative control, miRNA-20b mimics, or miRNA-20b inhibitor (Ambion, Life Technologies, Grand Island, USA). For HIF-1α or VEGF functional analysis, the HepG2 cells were transfected with HIF-1α or VEGF-specific small interfering (si)RNA or pcDNA3.1-HIF-1α plasmid (Sangon Biotech, Shanghai, China). The transfection assay was performed as described in study [27].

2.5. Luciferase Reporter Gene Assay. The mRNA sequence targeted by the miRNA was predicted using TargetScan, miRanda, and NBmiRTar. The fragment was designated as HIF-1α 3′-UTR and inserted into pMIR-REPORTTM luciferase reporter vector (Sac I and Hind III restriction enzyme sites; Ambion, Life Technologies, Grand Island, USA). Another expressing vector was also constructed by the insertion of a mutated HIF-1α 3′-UTR using QuikChangeH Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA). Then, the recombinant reporter vectors with normal and mutated HIF-1α 3′-UTR were cotransfected with miR-20b into HepG2 cells, respectively, using TransMessenger Transfection Reagent (Tiangen Biochemical Technology (Beijing) Co., Ltd., Beijing, China). The luciferase assay was performed according to the manufacturer's instructions.

2.6. Western Blot Analysis. Protein concentration was determined by BCA Protein Assay Kit (Santa Cruz, USA). About 30 μg protein extracts were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), transferred onto PVDF membranes (BioRad Laboratories, Hercules, CA, USA), and incubated for 1 h in TBS containing 5% nonfat milk and 0.1% Tween-20 incubated overnight at 4°C with the following primary antibodies: anti-HIF-1α (1 : 1000, BD Transduction Laboratories, USA). After washing in TBS with 0.1% Tween-20, they were incubated for 1 h at room temperature with HRP-conjugated anti-rabbit antibody (1 : 1,000, Santa Cruz Biotechnology Inc., USA). Immunoreactivity was detected by enhanced chemiluminescence (ECL kit, Santa Cruz Biotechnology Inc., USA) and visualized by autoradiography. The level of β-actin (1 : 1,000, Santa Cruz Biotechnology Inc., USA) was used as a control of the amount of protein loaded into each lane and the optical density of each band was measured using ImageJ.

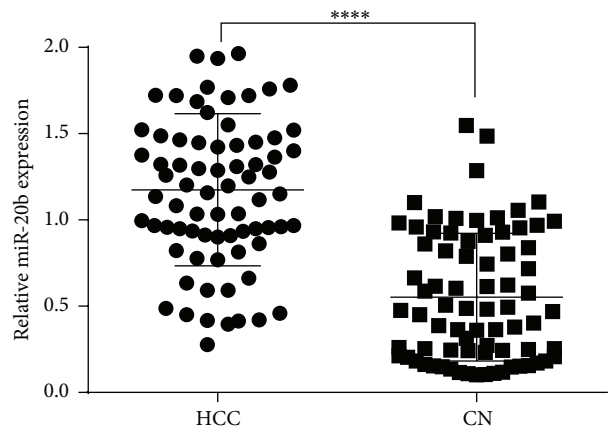

Figure 1: Comparison of miR-20b expression levels between HCC tissues and normal tissues ($P = 0.000$).

2.7. Viability Assay. For the cell viability assay, cells were seeded into a 96-well plate in quintuplicate; the cell growth was measured by CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS; Promega (Beijing) Biotechnology Co., Ltd., Beijing, China) after the indicated periods. Absorbance was measured at 540 nm using Versamax microplate reader (Molecular Devices, Sunnyvale, CA).

2.8. Annexin V-FITC/PI Determination of Apoptosis. Add precooled 70% ethanol to fixed cells. Take 1 mL of the cell suspension and add it to a centrifuge tube, centrifuge (4°C, 1000 r/min, 5 min). Use 1x binding buffer to adjust 1×10^6/mL; take 100 μL cell suspension to detect cell apoptosis, according to the Annexin V-FITC/PI kit (Nanjing Kaiji Biological Technology Development Co., Ltd., Nanjing, China) specification method steps.

2.9. Statistical Analysis. Data are presented as the mean ± standard deviation (SD). The significance of differences was evaluated by t-test. Differences with P values < 0.05 were considered statistically significant. The postoperative survival rate was analyzed with the Kaplan-Meier method, and differences in survival rates were assessed with the log-rank test. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA).

3. Result

3.1. miR-20b Is Upregulated in HCC Tissues. From the qRT-PCR result, we found that miR-20b expression levels were significantly upregulated in HCC cancer tissues compared to those in the normal control ($P = 0.000$, Figure 1).

Correlation between levels of miR-20b expression and clinicopathological characteristics of HCC patients was observed.

The miR-20b expression levels were classified as high or low in relation to the median value. Meanwhile, we found the expression levels of miR-20b in HCC patients

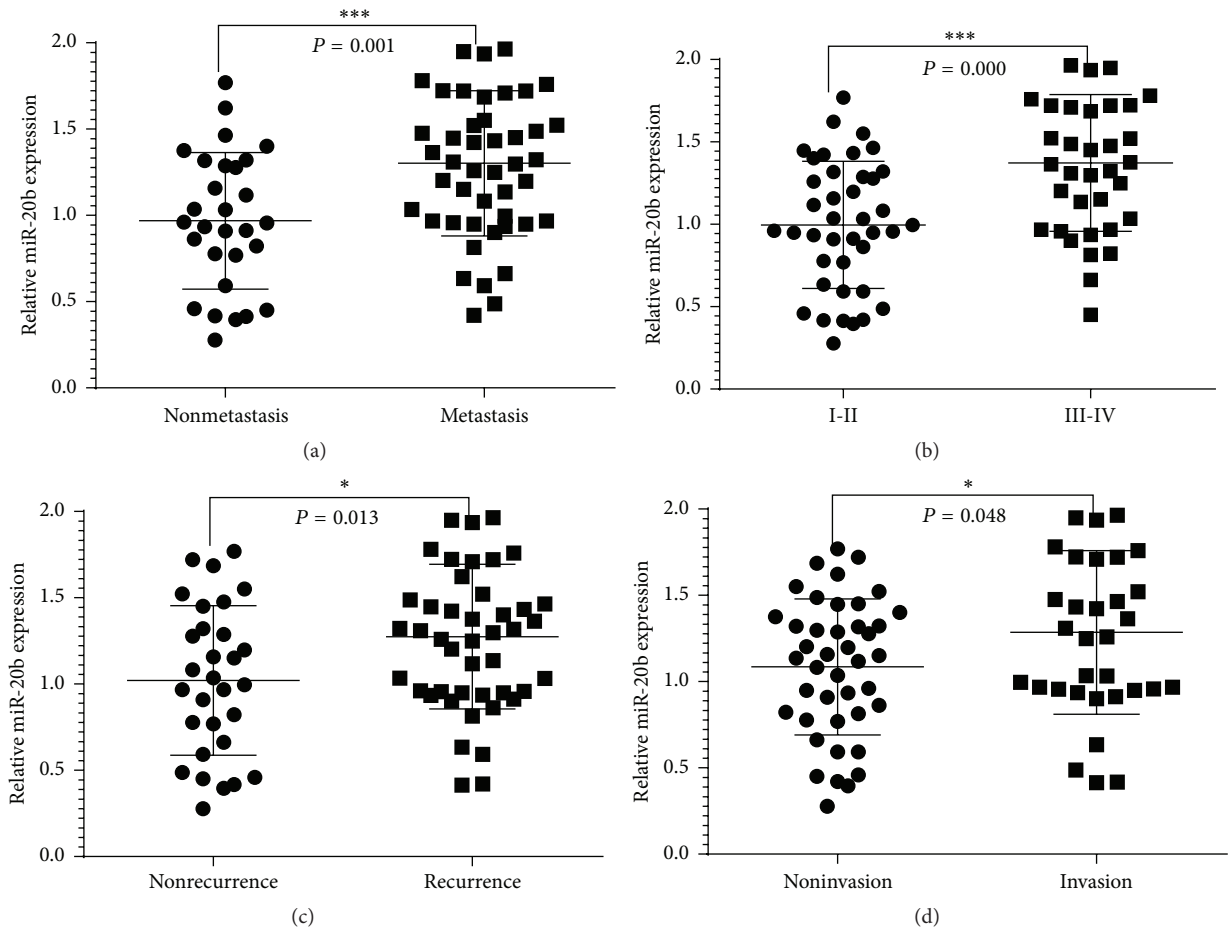

Figure 2: The correlation between levels of miR-20b and tumor metastasis, TNM stage, tumor recurrence, and microvascular invasion. (a) Tumor metastasis ($P = 0.001$), TNM stage ($P = 0.000$), tumor recurrence ($P = 0.013$), and microvascular invasion ($P = 0.048$).

had no significant correlation with gender, age, tumor size, AFP (ng/mL), and tumor grade ($P > 0.05$; Table 1). But when comparing miR-20b expression in tumor metastasis, TNM stage, tumor recurrence, and microvascular invasion we found a significant difference (Figure 2).

3.2. miR-20b Expression and Postoperative Survival. Kaplan-Meier survival curve and log-rank test for 76 patients with HCC with high expression or low expression of miRNA-20b in tumor tissue were analyzed. The overall survival (OS) rate of HCC patients was significantly lower with high miR-20b mRNA expression than that in those with low expression; it means that high miR-20b expression might be correlated with poor prognosis of HCC patients ($P = 0.01$; Figure 3). We also use univariate and multivariate Cox model analyses which were performed to determine the correlation of miR-20b expression with overall survival of HCC patients. In univariate analysis the found metastasis, TNM stage, tumor recurrence, microvascular invasion, and miR-20b were statistically significant prognosis factors (Table 2). A multivariate analysis confirmed that metastasis, TNM stage, microvascular invasion, and miR-20b expression were significant independent predictors of poor survival of HCC (Table 3).

3.3. Inverse Level of miR-20b and HIF-1α, VEGF in HepG2 Cells under Normal or Hypoxia-Mimetic Conditions. To further investigate the role of miR-20b in the regulation of HIF-1α, VEGF expression in normal and hypoxia-mimetic conditions was analyzed. We used $CoCl_2$ to mimic hypoxia conditions, the cells treatment of $CoCl_2$ for 24 h. The levels of miR-20b and HIF-1α, VEGF expression in normal and hypoxic conditions were analyzed (Figure 4). Then the HepG2 cells were transfected with scrambled miRNA, miR-20b mimics, and miR-20b inhibitor, respectively. The transfection efficiency is as shown in Figure 5.

3.4. HIF-1α, VEGF Are Direct Targets of miR-20b. Bioinformatical predication was performed using TargetScan, miRanda, and NBmiRTar to predict targets of miR-20b. We found miR-20b at the 3′-UTR of HIF-1α, VEGF which were highly conserved (Figure 6). To verify whether HIF-1α, VEGF were direct targets of miR-20b, the wild and mutant types of HIF-1α, VEGF 3′-UTR were generated.

Table 2: Univariate analysis of clinicopathological factors for overall survival.

Variable	n	Hazard ratio	95% CI	P value
Age (years)				
≤57	38	1	0.866–1.417	0.350
>57	28	0.728		
Gender				
Male	57	1	0.912–3.749	0.088
Female	19	1.849		
Tumor size (cm)				
≥5	42	1	0.264–1.116	0.097
<5	34	0.543		
AFP (ng/mL)				
≥400	36	1	0.387–1.506	0.436
<400	40	0.434		
Metastasis				
Yes	47	1	1.396–5.647	*0.004**
No	29	2.808		
Tumor grade				
G1	18	1		
G2	24	1.398	0.710–2.751	0.333
G3	34			
TNM stage				
I-II	40	1	1.626–6.946	*0.001**
III-IV	36	3.360		
Tumor recurrence				
Yes	46	1	0.171–0.657	*0.007**
No	30	0.360		
Microvascular invasion				
Yes	34	1	1.187–5.931	*0.017**
No	42	2.654		
miR-20b				
High	45	1	0.129–0.528	*0.000**
Low	31	0.261		

* $P < 0.05$.

Table 3: Multivariate analysis of clinicopathological factors for overall survival.

Variable	n	Hazard ratio	95% CI	P value
Metastasis				
Yes	47	0.295	0.124–0.696	*0.005**
No	29			
TNM stage				
I-II	40	5.031	1.627–15.558	*0.005**
III-IV	36			
Tumor recurrence				
Yes	46	1.418	0.522–3.854	0.493
No	30			
Microvascular invasion				
Yes	34	0.215	0.088–0.527	*0.001**
No	42			
miR-20b				
High	45	5.018	2.325–11.223	*0.000**
Low	31			

* $P < 0.05$.

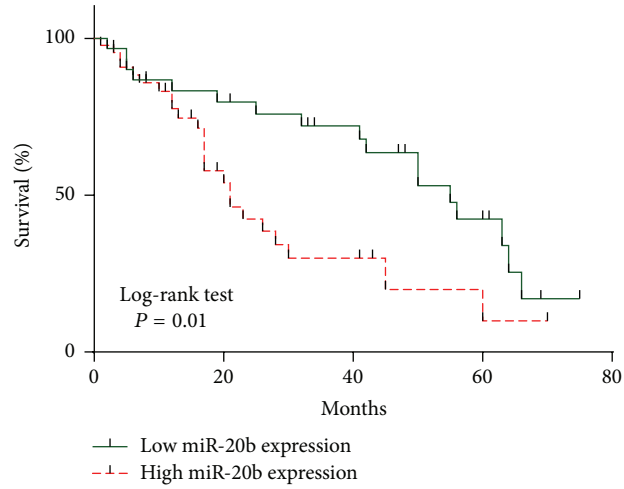

Figure 3: Kaplan-Meier survival curve of HCC patients. Patients in the high expression group had significantly poorer prognosis than those in low expression group, analyzed by log-rank tests which are indicated ($P = 0.01$).

The dual-luciferase reporter assay was subsequently performed in hepatocellular carcinoma HepG2 cells. As shown in Figures 7(a) and 7(b), the luciferase activity was significantly reduced in HepG2 cells cotransfected with the wild-type 3′-UTR of HIF-1α, VEGF and miR-20b mimics but unchanged in HepG2 cells cotransfected with the mutant HIF-1α, VEGF 3′-UTR and miR-20b mimics, indicating that miR-20b directly binds to the 3′-UTR of HIF-1α, VEGF in HepG2 cells. We transfected miR-20b mimics and miR-20b inhibitor to HepG2 cells. The miR-20b inhibitor was transfected into normoxic cells; miR-20b mimics were transfected into hypoxic cells and the HIF-1α, VEGF expression detected by Western blot (Figure 8).

3.5. miR-20b Enhances HepG2 Cell Proliferation. The MTS assay was used to investigate the miR-20b effects cell viability. We transfected miR-20b mimics into cells; the cell viabilities significantly increased after transfected miR-20b mimics for 24 h in normoxia comparison with that of negative control (miR-NC) (Figure 9; $^{**}P < 0.01$). We also transfected miR-20b inhibitor into cells in normoxia; the cell viabilities were dramatically restrained compared with that of negative control (inhibitor-NC) transfected cells (Figure 9; $^{**}P < 0.01$), to verify the ways miR-20b affects tumor cells growth. Furthermore we also transfected inhibitor + si-HIF-1α or inhibitor + si-VEGF into cells. When the cells were cotransfected with miR-20b inhibitor and HIF-1α-siRNA or VEGF-siRNA the cell viabilities significantly increased compared

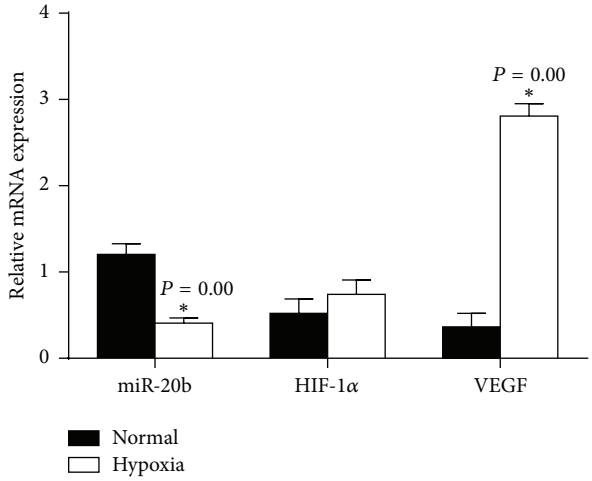

Figure 4: The levels of miR-20b and HIF-1α, VEGF expression after treatment of $CoCl_2$ for 24 h. $^{**}P < 0.01$ compared with normal group.

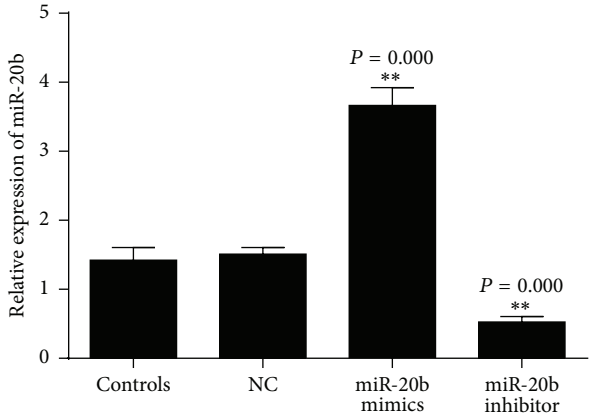

Figure 5: The transcription efficiency was performed to determine the levels of miR-20b transfected with scrambled miRNA (NC), miR-20b mimics, and miR-20b inhibitor. $^{**}P < 0.05$ compared with control.

with only transfected miR-20b inhibitor (Figure 9; $^{**}P < 0.01$).

3.6. Downregulation of miR-20b Enhanced the Resistance to Apoptosis. The Annexin V-FITC/PI assay was used to investigate the cell apoptosis effects of miR-20b. We transfected miR-20b inhibitor into cells; the cell apoptosis significantly decreased in comparison with that of control in normal environment (Figure 10(a); $^{*}P < 0.05$). Furthermore we also transfected inhibitor + si-HIF-1α or inhibitor + si-VEGF into cells for 24 h in normoxia. When the cells were cotransfected with miR-20b inhibitor and HIF-1α-siRNA or VEGF-siRNA the cell apoptosis significantly increased compared with only transfected control group (Figure 10(a); $^{**}P < 0.01$). By the way, we transfected miR-20b mimics for 24 h and used $CoCl_2$ to mimetic hypoxia conditions; the cells treatment of $CoCl_2$ for 24 h was then detected by flow cytometry. Comparison with control group transfected miR-20b mimics the cells apoptosis significantly increased (Figure 10(b); $^{*}P < 0.05$).

4. Discussion

In the present study, many miRNAs have confirmed contribution to the initiation and progression of HCC. With further in-depth studies more and more miRNAs have shown that take part in the regulation of hepatocellular carcinoma development. Furthermore, it has been shown that miRNAs can function as tumor suppressors or oncogenes and repress the expression of important cancer-related genes and might prove useful biomarkers in the diagnosis and treatment of cancers [35]. The important role in hepatocellular carcinoma tells us that understanding of miRNA function will provide us with broad prospects to understand and overcome tumor in the future.

miR-20b belongs to the miR-106a-363 cluster, which together with miR-17-92 and miR-106b-25 clusters forms a large family of highly similar miRNAs called the miR-17 family [28]. In the present study, the high expression levels of miR-20b often promote tumor development so the miR-20b suggested can serve as a potential oncogene. In our study we found that miR-20b expression levels were significantly upregulated in HCC cancer tissues compared to those in the normal control ($P = 0.000$, Figure 1). Furthermore, miR-20b expression showed significant association with tumor metastasis, TNM stage, tumor recurrence, and microvascular invasion comparison of clinicopathological factors which is an important clinical determinant for the prognosis of HCC patients. In Kaplan-Meier survival curve analysis, OS rates of HCC patients rates indicated that with high miR-20b expression there was significantly poorer survival in comparison with low miR-20b expression. In a multivariate Cox model, we found that high miR-20b expression was an independent factor for predicting the 5-year OS of HCC patients. From clinicopathological characteristics study we found that the expression of miR-20b was upregulated in HCC. High expression of miR-20b was significantly associated with tumor progression and decreased OS in patients of HCC indicating that it might play critical roles in HCC progression and development.

Based on our clinicopathological characteristics study, we further investigated the function and possible mechanisms of miR-20b in regulating some biological properties of HepG2 cells. We confirmed that miR-20b binds with the HIF-1α and VEGF 3′-UTR by luciferase reporter assay. Under normoxic conditions, posttranslational HIF-1α is rapidly degraded by the proteasome and usually not detectable and HIF-1α is a mediator of cell response to hypoxia in mammalian cells cultured under reduced oxygen tension [24]. Among the target genes of HIF-1α, vascular endothelial growth factor (VEGF) is one of the major target genes for HIF-1α that directly participates in angiogenesis [36]. We used $CoCl_2$ in mimetic hypoxia conditions and found inverse level of miR-20b and HIF-1α, VEGF in HepG2 cells under normal or hypoxia-mimetic conditions. From the result we found that in hypoxia group miR-20b expression significantly decreased and VEGF

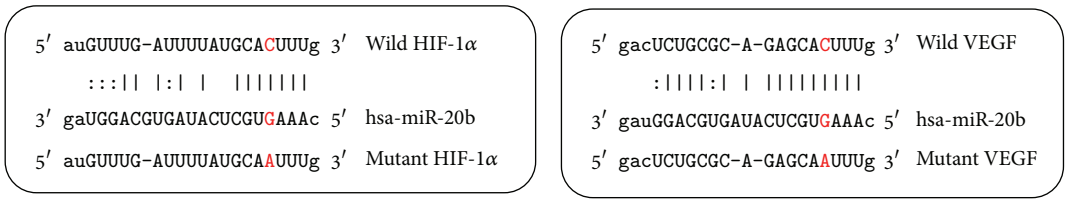

Figure 6: miR-20b targeted 3′-UTR of HIF-1α, VEGF mRNA.

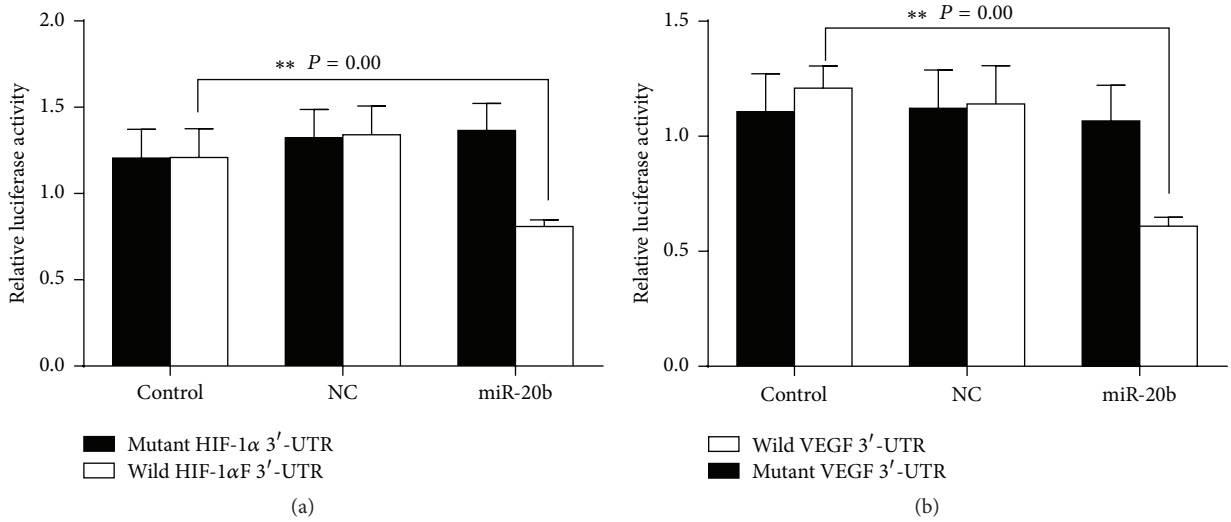

Figure 7: Luciferase reporter assay in HepG2 cells. Cells were cotransfection of HepG2 cells with miR-20b mimics and wild-type HIF-1α, VEGF 3′-UTR led to a marked decrease in luciferase activity and cotransfection with miR-20b and mutant HIF-1α, VEGF 3′-UTR had no effect on luciferase activity, and cotransfection with NC miRNA and wild-type HIF-1α, VEGF 3′-UTR or mutant HIF-1α, VEGF 3′-UTR also showed no difference, $^{**}P < 0.05$ compared with negative control.

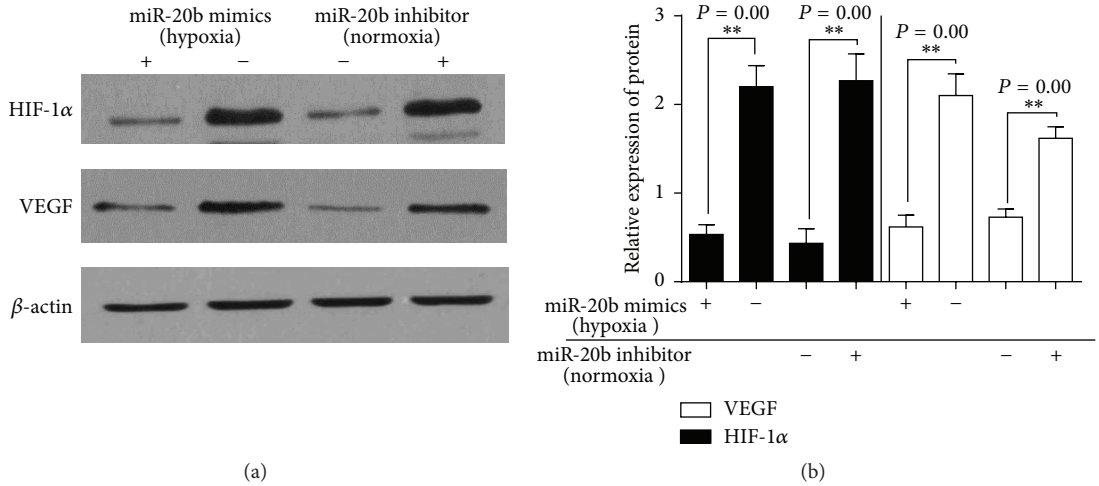

Figure 8: In hypoxia group showing HIF-1α, VEGF decreased after miR-20b treated transfected. In normal group miR-20b inhibitor increased HIF-1α, VEGF protein by Western blot (a). Use ImageJ to analyze relative expression of proteins (b). $^{**}P < 0.05$ compared with each control group.

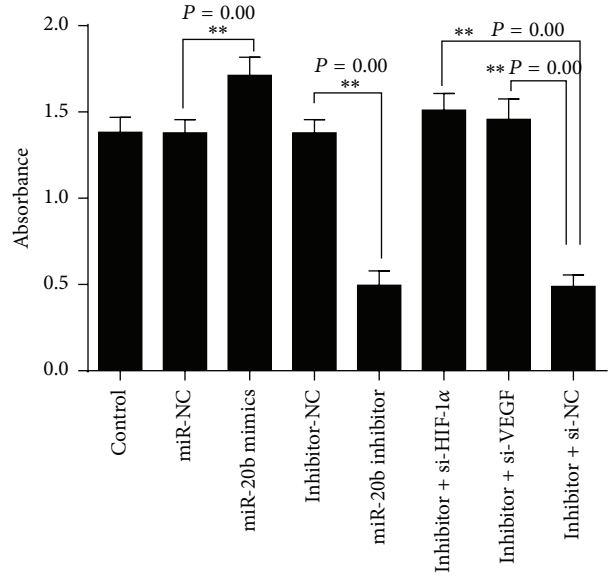

Figure 9: The cells viability detected by MTS assays. HepG2 cells were transfected with miR-20b mimics, miR-20b inhibitor, inhibitor + si-HIF-1α, and inhibitor + si-VEGF. Inhibitor + si-HIF-1α: miR-20b inhibitor + HIF-1α-siRNA; inhibitor + si-VEGF: miR-20b inhibitor + VEGF-siRNA. $^{**}P < 0.01$ compared with control.

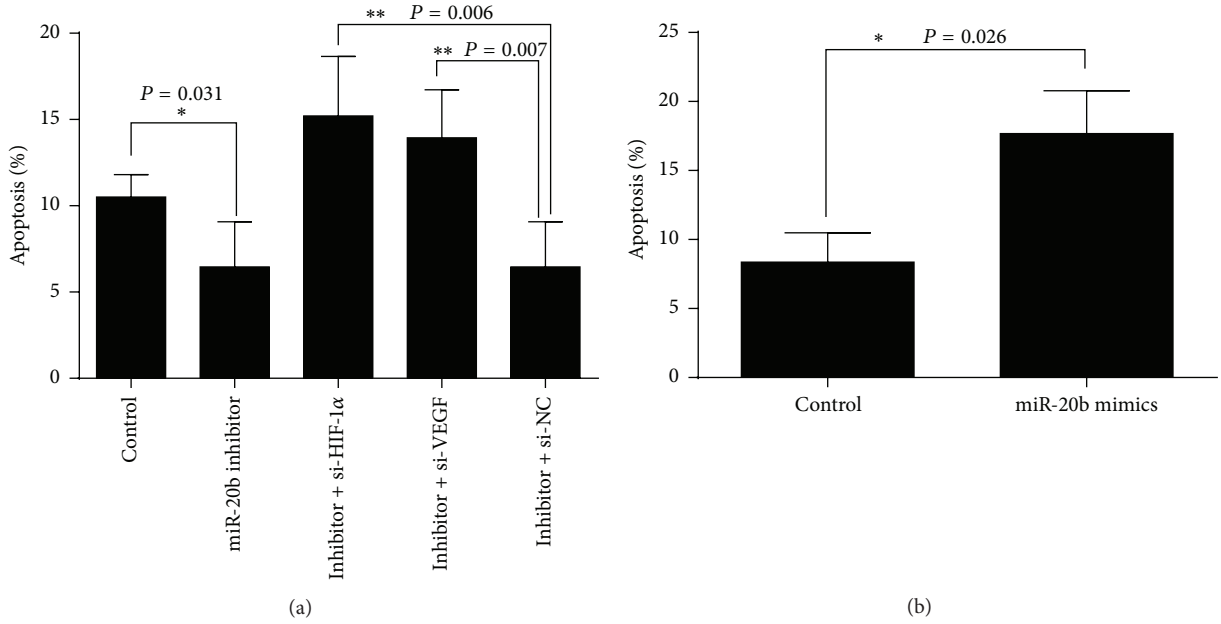

Figure 10: The cells apoptosis detected by Annexin V-FITC/PI assay. HepG2 cells were transfected with miR-20b inhibitor, inhibitor + si-HIF-1α, and inhibitor + si-VEGF. Inhibitor + si-HIF-1α: miR-20b inhibitor + HIF-1α-siRNA; inhibitor + si-VEGF: miR-20b inhibitor + VEGF-siRNA. $^{**}P < 0.01$ compared with control in normoxia (a). Transfected miR-20b mimics for 24 h in mimetic hypoxia conditions; apoptosis significantly increased in hypoxia conditions (b).

mRNA significantly increased; the hypoxia conditions are not to change HIF-1α mRNA expression but significantly increase HIF-1α protein. To verify relationship between miR-20b and HIF-1α, VEGF expression, we also transfected miR-20b mimics and miR-20b inhibitor to HepG2 cells. The miR-20b inhibitor was transfected into normoxic cells; miR-20b mimics were transfected into hypoxic cells and the HIF-1α, VEGF expression detected by Western blot. As a result we found HIF-1α and VEGF are targets by miR-20b. In normal condition HepG2 highly expresses miR-20b but reduces HIF-1α, VEGF. Interestingly, once we put the cells in hypoxia-mimetic conditions the cell response to hypoxia would highly express HIF-1α, VEGF and then reduced levels of miR-20b.

In present studies reported, miR-20b expression could affect cell biological behaviour [28, 31, 32, 34, 37]. We used the loss-of-function and gain-of-function approaches which

showed that miR-20b could impact the cell viability and apoptosis. The miR-20b mimics and miR-20b inhibitor were transfected into HepG2 cells in normoxic condition. In miR-20b mimics group, the cells viability significant increased compared with control group. While in miR-20b inhibitor group the cells viability decreased significantly, at some time we cotransfected miR-20b inhibitor and HIF-1α-siRNA or VEGF-siRNA; the cells proliferative capacity has been enhanced. The result tells us that miR-20b maintains tumor cell growth through its regulation of HIF-1α and VEGF in normal condition. The cells apoptosis result also suggests that high expression of HIF-1α and VEGF in normal condition would help cells to resist apoptosis. Considering the regulation of apoptosis genes by HIF-1α and VEGF [38, 39], when in hypoxia-mimetic conditions we transfected miR-20b mimics into cells; it is means that we also decreased levels of HIF-1α and VEGF which lead to cells apoptosis raised.

In conclusion, in the present study we report that miR-20b is associated with poor overall survival of HCC patients, suggesting its potential prognostic values in this disease type, and identified HIF-1α and VEGF as direct targets of miR20b in hepatocellular carcinoma cells. From our cells studies we found not only miR-20b regulation of HIF-1α and VEGF in normal but also HIF-1α and VEGF regulation of miR-20b in hypoxia. This mechanism would help the tumor cells adapt to the different environments thus promoting the tumor invasion and development.

Ethical Approval

The study was approved by the Institute of General Surgical Research, Second Affiliated Hospital, Yangzhou University, Jiangsu, China. All specimens were handled and made anonymous according to the ethical and legal standards.

Consent

The authors obtained written informed consent from all participants involved in their study.

Disclosure

Tong-min Xue, Li-de Tao, and Miao Zhang are listed as co-first authors with equal contribution to the paper.

Conflict of Interests

The authors have no conflict of interests to disclose.

Acknowledgments

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Research Article

Bone Marrow Stromal Antigen 2 Is a Novel Plasma Biomarker and Prognosticator for Colorectal Carcinoma: A Secretome-Based Verification Study

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Background. The cancer cell secretome has been recognized as a valuable reservoir for identifying novel serum/plasma biomarkers for different cancers, including colorectal cancer (CRC). This study aimed to verify four CRC cell-secreted proteins (tumor-associated calcium signal transducer 2/trophoblast cell surface antigen 2 (TACSTD2/TROP2), tetraspanin-6 (TSPAN6), bone marrow stromal antigen 2 (BST2), and tumor necrosis factor receptor superfamily member 16 (NGFR)) as potential plasma CRC biomarkers. *Methods*. The study population comprises 152 CRC patients and 152 controls. Target protein levels in plasma and tissue samples were assessed by ELISA and immunohistochemistry, respectively. *Results*. Among the four candidate proteins examined by ELISA in a small sample set, only BST2 showed significantly elevated plasma levels in CRC patients versus controls. Immunohistochemical analysis revealed the overexpression of BST2 in CRC tissues, and higher BST2 expression levels correlated with poorer 5-year survival (46.47% versus 65.57%; $p = 0.044$). Further verification confirmed the elevated plasma BST2 levels in CRC patients (2.35 ± 0.13 ng/mL) versus controls (1.04 ± 0.03 ng/mL) ($p < 0.01$), with an area under the ROC curve (AUC) being 0.858 comparable to that of CEA (0.867). *Conclusion*. BST2, a membrane protein selectively detected in CRC cell secretome, may be a novel plasma biomarker and prognosticator for CRC.

1. Introduction

Colorectal cancer (CRC) is the third leading cause of cancer deaths in the United States [1]. In Taiwan, the incidence of CRC increased promptly (up to 70%) during 1991 to 2001. Moreover, CRC had become the third leading cause of cancer death since 1996 and became the first most common malignancy since 2009 in Taiwan. In 2011, there were 14,087 CRC patients diagnosed, and approximately 5,000 patients died of CRC each year. Among them, patients who are younger than 65 years old contributed to about one-third of the CRC death, while the potential life lost was 13.3 years for each patient [2]. Therefore, CRC is an important public health issue and an important socioeconomic problem in Taiwan.

As other malignancies, CRC patients with earlier stage disease have better outcome. In Taiwan, the 5-year survival rate is 81%, 72%, 57%, and 12% for stages I, II, III, and IV diseases, respectively [3]. Furthermore, earlier detection of CRC makes the management more effective and easier. It means earlier diagnosis of CRC can achieve simpler and more

straightforward surgery, more cost-effective treatment, better outcome, and better quality of life for patients and somehow avoid post-operative chemotherapy [4–7], although part of stage II patients still suffers disease recurrence [8]. At present, however, definite diagnosis of CRC still depends on colonoscopy. Colonoscopy which is the golden standard of diagnosis of CRC has some disadvantages such as suffering during procedures, risk of colonic perforation and bleeding, and risk of electrolytes imbalance during colon preparation [9–11]. To aid detection and/or monitoring of CRC, carcinoembryonic antigen (CEA), a blood-based tumor marker, has been used extensively in clinic, but it lacks satisfying sensitivity for early tumor [12, 13].

In this "omics" era, the application of high-throughput genomic and proteomic technologies has enabled the discovery of hundreds to thousands of biomarker candidates. However, only very few biomarkers have been brought to clinical settings, and "personalized medicine" is still difficult to achieve [14–16]. The gap between benches to clinics persists. Most previously discovered candidate biomarkers in bench are still lacking rigorous validation, and only few biomarkers had gotten FDA approval in the US. To solve this problem, some researchers questioned sample handling and suggested better study design to decrease selection bias in discovery phase [17–19]. In recent years, more and more strategies have been tried to reduce sample complexity. One of them is proteomic analysis of conditioned media from cancer cell, the so-called cancer cell secretome. The cancer cell secretome-based strategy seems promising in CRC biomarkers discovery. Secreted proteins are easier to be analyzed in cancer cell lines, through which the influence of abundant plasma proteins can be largely reduced. Moreover, cancer cell lines represent a more homogenous cell population than human tissues [20–22]. Comparative analysis of secretomes from different cancer cell lines has been reported. We previously established a 4,584 protein-containing secretomes' dataset of 23 human cancer cell lines from 11 cancer types, in which 109 proteins were selectively identified from three CRC cell lines: Colo205, SW480, and SW620 [21]. These 109 proteins represent a valuable reservoir for further verification study to find novel blood markers for CRC. We integrated these 109 proteins with the Human Protein Atlas (HPA) [23] and Human Plasma Proteome Project (HPPP) [24] datasets and then applied stringent literature search to narrow down candidate list. We have selected four candidates (TACSTD2/TROP2, TM9SF2, TSPAN6, and NGFR) and preliminarily verified their overexpression at protein levels in 30 CRC patients' tissue samples by immunohistochemistry [25]. In the present study, we extended prior work and examined the plasma levels of four targets (TROP2, TSPAN6, NGFR, and BST2) by ELISA of a small sample set of CRC patients and controls and further selected BST2 for detailed analysis. BST2, a type II transmembrane protein also known as HM1.24/CD317, has been identified to be overexpressed in a variety of cell lines from different cancer types, including multiple myeloma, breast, lung, and kidney cancers [26–29]. However, there are still few studies about BST2 expression in human cancers and its potential as a cancer biomarker. Our present study showed that BST2 levels were significantly elevated in both CRC tissues and plasma specimens, implicating the potential of BST2 as a novel CRC biomarker.

2. Materials and Methods

2.1. Datasets and Criteria for Prioritization of Candidate Biomarkers. Our main dataset comes from secretome of 23 human cancer cell lines derived from 11 cancer cell types, including three CRC cells (Colo 205, SW 480, and SW 620). Among the 4,584 nonredundant proteins identified, there are 109 proteins selectively detected in the CRC cell secretome [21]. These 109 candidates were further prioritized by examining if they (1) are identified in HPPP, (2) are identified to be upregulated over 50% in HPA, (3) are identified to be upregulated in CRC in published references using different laboratory methods, such as microarray, immunohistochemistry (IHC), tissue array, and reverse transcription polymerase chain reaction (RT-PCR), and (4) are secreted proteins or are involved in apoptosis or signal transduction. One exclusion criterion is applied to those which have published ELISA data in CRC research. According to this criterion, 66 proteins were sorted out from the aforementioned 109 CRC-unique candidates into three categories: A, B, and C (see Supplemental Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/874054). Verifying candidates of categories A and B as clinically useful blood biomarkers for CRC are our prior concern. One protein in category A (TACSTD2/TROP2) and three proteins in category B (TSPAN6, NGFR, and BST2) were selected and verified in the present study owing to available commercial antibodies and more interesting biological functions. TSPAN6 has been reported to be involved in invasive microdomains in cancer cells, which has been found to be involved in tumorigenesis [30, 31]. NGFR has been studied in neurologic malignancy, which is also involved in cell growth control [32–34]. BST2 is a membrane protein which exists as a dimer or a polymer. It was found to stabilize membrane microdomains, to participate in cell adhesion and cell migration, and to block virus budding [35–37].

2.2. Patient Population and Clinical Specimens. All clinical samples were collected at Chang Gung Memorial Hospital (Taoyuan, Taiwan). Tissue samples were collected from surgical CRC patients in 1995. Ten CRC tissue specimens were used for initially checking the BST2 protein levels in tumor cells, followed by a large sample set (132 tissue blocks) for more detailed immunohistochemical analysis. Plasma samples were collected from 152 CRC patients before surgery and 152 controls without CRC between 2010 and 2013 according to the protocol as described previously [21]. Briefly, plasma samples were prepared by collecting blood in EDTA tubes (10 mL from each subject) and left at room temperature (for a maximum of 30 min) until centrifugation. Plasma samples were centrifuged at 2,000 ×g for 10 min at room temperature to pellet the cells. After centrifugation, samples were divided into 1.0 mL aliquots in sterile cryotubes and immediately frozen at −80°C for storage until use. All CRC patients had histologically verified adenocarcinoma. Patients'

characteristics were obtained from clinical and pathology records including gender, age, tumor location, histological grade, tumor stage, CEA level, preoperative laboratory data, operation date, operation method, tumor recurrence, follow-up date, and follow-up status. All patients were subjected to a follow-up strategy that included regular outpatient visits, CEA test every 3 to 6 months, regular colonoscopy every 1 year to 2 years, and regular image studies (chest X-ray and liver sonography or computed tomography) every year. The characteristics of all study subjects are summarized in Supplemental Table 2. This study was approved by the Institutional Review Board at Chang Gung Memorial Hospital (IRB numbers 99-0515B, 101-0712B, and 102-1446C).

2.3. Immunohistochemistry. The tumor tissue blocks used for immunohistochemical staining were first fixed in 4% paraformaldehyde and then embedded in paraffin. The paraffin embedded tumor sections (5 μm) were deparaffinized with xylene, dehydrated with ethanol, heated in citrate buffer, and then exposed to 3% H_2O_2 at room temperature for 30 min before heating in a microwave oven for antigen retrieval (10 mm citrate buffer, pH 6.0; 20 min, 700 W). The sections were blocked with 10% nonimmune goat serum at 37°C for 30 min. Slides were then incubated with rabbit anti-human BST2 antibody (catalog number HPA017060; Sigma-Aldrich, St. Louis, MO) for 30 min at room temperature. Following washing with PBS (pH 7.4), slides were incubated with HRP-conjugated anti-rabbit IgG antibody (1 : 2000 dilution; Abcam, Inc., Cambridge, UK) for 30 min at room temperature and then developed using 3,3′-diaminobenzidine (Sigma, St. Louis, MO). Sections were counterstained with hematoxylin, washed in running tap water, dehydrated, and mounted in Neo-Mount (Merck, Darmstadt, Germany). Immunostaining was evaluated and scored by two experienced pathologists who were blinded to any knowledge of clinical or pathological parameters and clinical outcome. The percentage of antigen-positive tumor cells was determined semiquantitatively by assessing the entire tumor section. Expression of these protein was categorized as positive or negative and was evaluated according to the percentage of cells stained (0–100%) and the intensity of cell staining (3: strong; 2: moderate; 1: weak; or 0: no cell staining). The two scores are multiplied to obtain the final score.

2.4. ELISA of TSPAN6, BST2, NGFR, and CEA. Commercial ELISA kits were used for three candidates (Human TSPAN6 ELISA kit, Cusabio Cat#: CSB-EL025164HU; Human BST2 ELISA kit, Cusabio Cat#: CSB-EL002837HU; Human NGFR ELISA kit, RayBio Cat#: ELH-NGFR-001) as below. Briefly, 100 μL per well of standard and sample was added on 96-well coated plates and was incubated at 37°C for 2 hours (overnight at 4°C for NGFR). After removing the liquid, 100 μL of biotin-antibody was added to each well and incubated at 37°C for 1 hour. After adequate aspiration and washing, 100 μL of HRP-avidin was added to each well and incubated at 37°C for 1 hour. Repeat the aspiration/wash process, and add 90 μL of TMB substrate. Incubate for 15–30 minutes under light protection at 37°C. Finally, add 50 μL of stop solution. Then, determine the optical density within 5 minutes. Subtract readings at 540 nm or 570 nm from the readings at 450 nm. The procedures all followed manufacturers' protocol. The CEA concentrations in plasma samples were measured using a commercial ELISA kit of CEA (Carcinoembryonic Antigen ELISA, BQ Kits Cat#: BQ062T). Fifty microliters per well of standard and sample was added on 96-well coated plates. Then, 100 μL of CEA enzyme conjugate was added to all wells and incubated at room temperature for 1 hour. After the aspiration/wash process, 100 μL of TMB substrate was added to each well and incubated at room temperature for 10 minutes. After adding 50 μL of stop solution to each well, the absorbance at 450 nm was recorded on ELISA Reader within 15 minutes.

2.5. Sandwich ELISA of TROP2 (TACSTD2). In-house ELISA of TROP2 was developed as previously described [38]. White polystyrene 96-well microtiter plates (Corning, NY, USA) were coated with goat anti-TROP2 antibodies (AF650, R&D Systems, Minneapolis, MN) by incubation at 4000 ng/mL in PBS (50 μL in each well) for 2 hours at room temperature. After washing, the plates were blocked by the addition of 200 μL per well of 1% BSA (Sigma)/PBS and incubated overnight at 4°C. After washing with PBS, 50 μL of plasma sample diluted 1 : 10 in blocking buffer was added and incubated at room temperature for 1 hour. Recombinant TROP2 protein (650-T2, R&D) was used as a standard. Biotinylated anti-human TROP2 (BAF650, R&D) antibodies (1 : 50 dilution in PBS containing 1% BSA) were applied and the plates were incubated at room temperature for 1 hour. Then, the streptavidin-alkaline phosphatase (RPN1234, Amersham Bioscience, UK) (50 μL, diluted 3000-fold in PBS containing 1% BSA) was added and incubated at room temperature for 40 min. One hundred microliters of substrate 4-methylumbelliferyl phosphate (Molecular Probes, Eugene, OR) (diluted to 100 μM with alkaline phosphatase buffer) was added to each well. The fluorescence was measured with a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) with excitation and emission wavelength set at 355 and 460 nm, respectively.

2.6. Statistical Analysis. The relationship between clinicopathologic features and BST2 protein expression levels was assessed by the chi-square method. Mean values of BST2 protein expression in different groups were compared by independent t-test or ANOVA method. Overall survival and time-to-event probabilities were computed using univariate analysis by the Kaplan-Meier method. Differences were estimated by log-rank test. Receiver operating characteristic (ROC) analysis was performed for plasma BST2 and CEA in discriminating CRC patients from controls. Multivariate analysis was done using Cox proportional hazard models. Statistical significance was set at $p < 0.05$. All analyses were performed using the statistical software, Statistical Package for the Social Sciences (Version 13.0, SPSS Inc., Chicago, IL).

3. Results

3.1. Initial Measurement of Plasma Levels of Four Candidates (TROP2, TSPAN6, BST2, and NGFR) in a Small Sample Set. Four candidate proteins, including TROP2, TSPAN6, BST2, and NGFR, were selected for initial verification by ELISA in plasma samples from 32 CRC patients and 32 healthy controls. The plasma levels of TROP2, TSPAN6, BST2, and NGFR in CRC patients and healthy controls were determined to be 48.88 ± 3.00 ng/mL versus 63.05 ± 5.61 ng/mL ($p = 0.02$), 68.15 ± 1.02 pg/mL versus 65.02 ± 0.01 pg/mL ($p < 0.01$), 2.23 ± 0.20 ng/mL versus 1.13 ± 0.06 ng/mL ($p < 0.01$), and 140.00 ± 3.85 pg/mL versus 314.40 ± 137.00 pg/mL ($p = 0.20$), respectively (Figure 1(a)). This analysis suggests that the plasma levels of TROP2 and BST2 might have been significantly altered in CRC patients, which deserves further verification in a large sample set.

3.2. Extended Verification of Plasma TROP2 and BST2 Levels in a Large Sample Set. We then performed extended verification of TROP2 and BST2 in another independent plasma sample set, consisting of 120 CRC patients and 120 controls. In agreement with the previous result, the plasma BST2 levels still showed a significant increase in CRC patients as compared to the controls in this independent sample set (2.35 ± 0.13 ng/mL versus 1.04 ± 0.03 ng/mL, $p < 0.01$, independent t-test; Figure 1(b), *left panel*). Under the same assay condition, however, plasma TROP2 levels did not maintain significant difference between CRC patients and controls (53.37 ± 12.27 ng/mL versus 57.11 ± 15.02 ng/mL; $p = 0.31$, independent t-test; Figure 1(b), *right panel*). We further examined the relationship between plasma BST2 levels and different clinicopathologic characteristics of these 120 CRC patients. Although plasma BST2 levels did not significantly differ in CRC patients at different stages (Supplemental Figure 1), higher plasma BST2 levels were observed in older patients (2.61 ± 1.34 versus 2.03 ± 1.63; $p = 0.03$), mucinous carcinoma (4.63 ± 0.45 versus 2.28 ± 1.48; $p = 0.05$), and CRC patients with hypoalbuminemia (4.14 ± 2.46 versus 2.12 ± 1.14; $p < 0.01$) (Supplemental Table 3).

3.3. Overexpression of BST2 in Tumor Cells of CRC Tissues. Since we have observed the significant elevation of BST2 plasma levels in CRC patients, we then turned to examine the expression levels of BST2 in CRC tissue specimens by immunohistochemistry. Although BST2 has been identified to be overexpressed in a variety of cell lines from different cancer types [26–29], to our knowledge, there were no studies reporting BST2 protein expression in CRC tissue specimens. In the majority of 132 CRC tissue specimens examined and evaluated, the BST2 antibody strongly stained the cytoplasm of tumor cells but stained weakly or not at all the adjacent nontumor epithelial cells (see Figure 2(a) for representative images). The immunohistochemical staining (IHC) scores of tumor parts were found to be significantly higher than those of adjacent nontumor counterparts (141.60 ± 45.14 versus 13.14 ± 3.00, $p < 0.01$, independent t-test; Figure 2(b)). We further examined the relationship between BST2 tissue expression levels and different clinicopathologic characteristics of these 132 CRC patients. We found that higher BST2 tissue expression levels were associated with higher TNM stages and worse 5-year survival, respectively (Supplemental Table 4). The BST2 tissue expression levels were not significantly associated with other clinicopathologic characteristics, such as gender, age, tumor location, histological grade, and distant metastasis.

3.4. Tissue BST2 Levels and Overall Survival. The 132 CRC patients were stratified into two groups representing high versus low BST2 expression. An IHC score of 150 was selected as cutoff value because (i) this score is the median of the IHC scores of these 132 CRC tissue sections, which can divide these CRC cases into subgroups with comparable sizes, and (ii) most of the adjacent nontumor counterparts have IHC scores below 150. Overall survival of these CRC patients and their time-to-event probabilities were computed using univariate analysis by the Kaplan-Meier method. The result showed that CRC patients of group 1 (IHC score 0–149, $n = 61$) had 5-year survival rate of 65.57%, better than 46.47% of group 2 (IHC score 150–300, $n = 71$; $p = 0.044$, log-rank test; Figure 3), indicating BST2 tissue expression level as a potential prognostic factor of CRC patients.

3.5. Multivariate Analysis. In multivariate analysis, BST2 tissue expression (IHC score) still showed marginal effect on 5-year survival (Table 1). The hazard ratios of high protein expression compared to low expression were 1.64 (95% CI, 0.98–2.74, $p = 0.05$). BST2 showed significance with marginal p value in multivariate analysis.

3.6. ROC Analysis of BST2 and CEA. We performed ROC analysis to evaluate the efficacy of plasma BST2 and CEA levels for discriminating CRC patients ($n = 120$) and controls ($n = 120$). The area under the ROC curve (AUC) was 0.858 (95% CI, 0.811–0.904) for BST2, 0.867 (95% CI, 0.821–0.912) for CEA, and 0.872 (95% CI, 0.828–0.916) for combination of BST2 and CEA (Figure 4(a)). We did the same analysis for early stage (stage 1 to stage 2) CRC patients ($n = 61$) and controls. The AUC was determined to be 0.818 (95% CI, 0.751–0.886) for BST2, 0.853 (95% CI, 0.792–0.914) for CEA, and 0.871 (95% CI, 0.813–0.929) for combination of BST2 and CEA (Figure 4(b)). Furthermore, when a cutoff value of 5.0 ng/mL was chosen for CEA as clinical practice and applied to the sample set used here (120 CRC patients and 120 controls), the sensitivity was 23.5% and the specificity was 100.0%. Notably, when a cutoff value of 1.20 ng/mL was chosen for BST2 (with 81.7% sensitivity and 64.2% specificity), 74 of 93 CRC patients with CEA level lower than 5.0 ng/mL could be further distinguished from healthy controls (Figure 4(c)). Taken together, these results indicate that BST2 represents a potential, novel plasma biomarker for CRC, especially when used together with CEA.

4. Discussion

CRC is an important public health issue and a socioeconomic problem in Taiwan. Earlier detection of CRC makes better

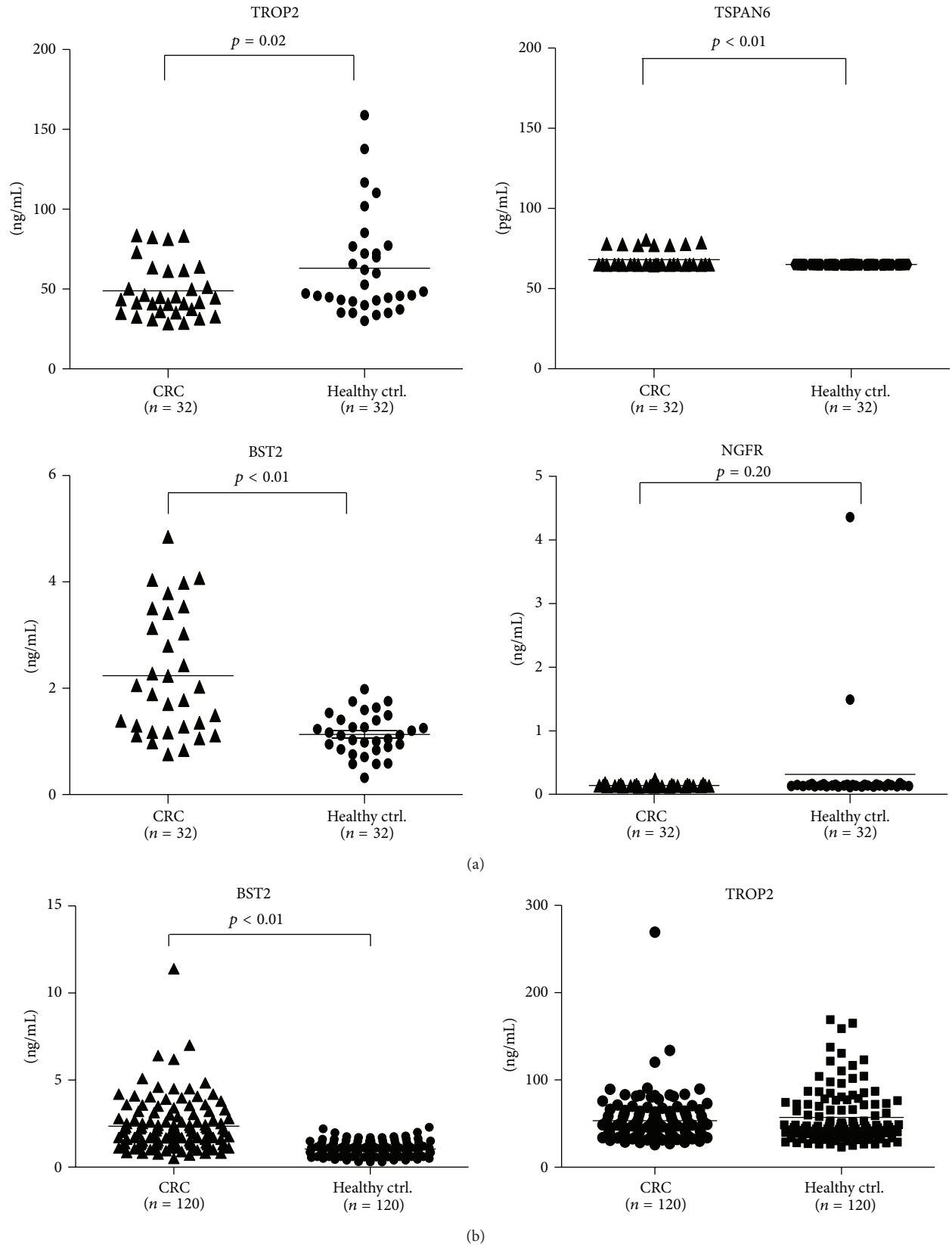

Figure 1: ELISA for four candidate proteins in plasma samples from CRC patients and healthy controls. (a) Plasma samples from 32 CRC patients and 32 healthy controls were used in this study. All p values are shown on figures. (b) Extended verification of ELISA of BST2 and TROP2 in another plasma sample set containing 120 CRC patients and 120 healthy controls.

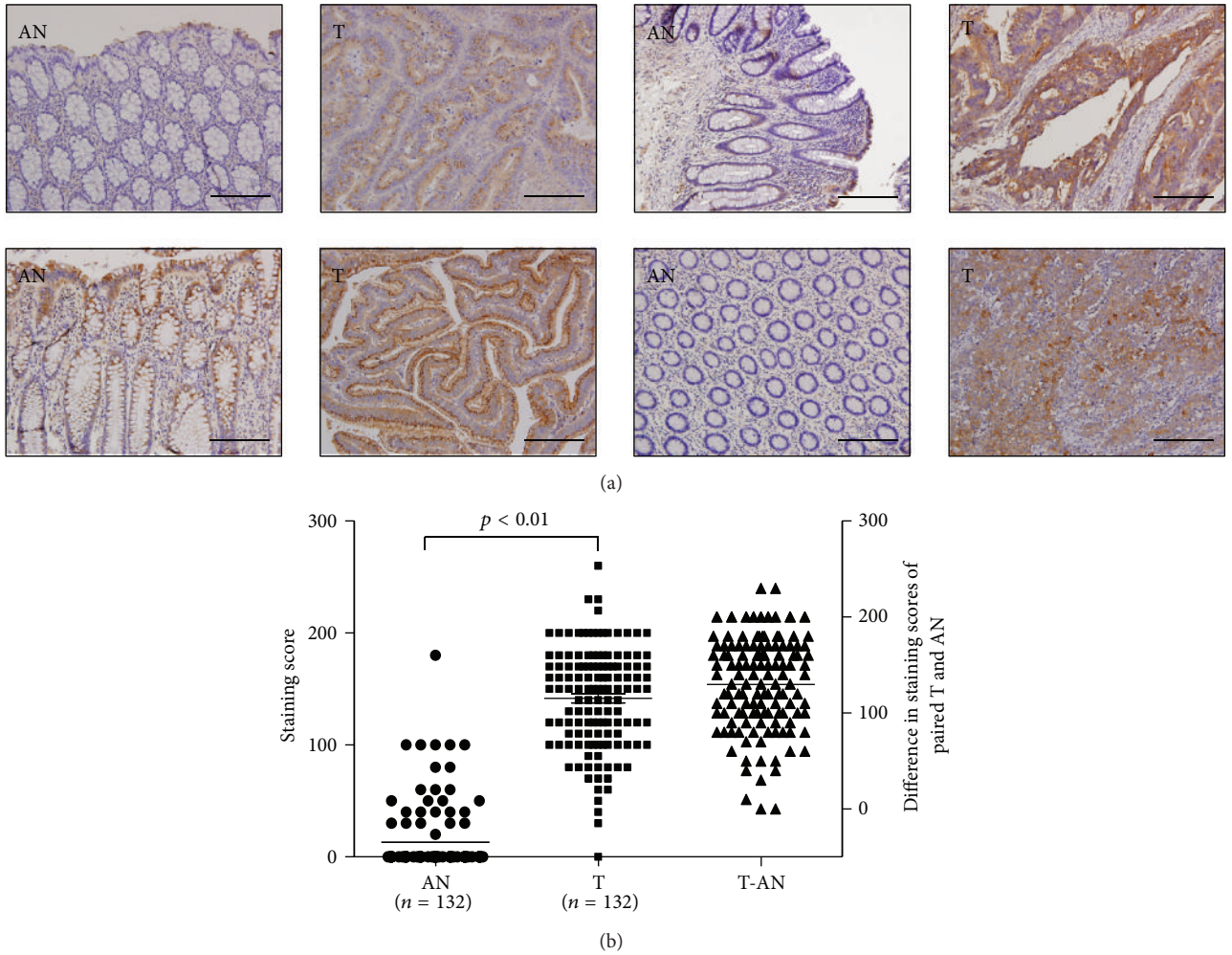

Figure 2: Overexpression of BST2 in CRC tissues. (a) The representative pictures of immunohistochemical staining patterns of BST2 in four pairs of tumor (T) and adjacent normal counterpart (AN) sections (scale bar = 200 μm). (b) Analysis of the IHC scores of BST2 in 132 CRC tissue specimens harboring both tumor and adjacent normal counterpart. T-AN: the difference of IHC score between paired T and AN.

outcome and simpler treatment. Metastasized disease has much worse outcome than localized disease [39]. At present, colonoscopy is still the golden standard of diagnosis of CRC. However, the potential severe complication of colonoscopy should be warranted. The rate of major complications has been reported at 0.12% for perforation and 0.2% for bleeding [40, 41]. Carcinoembryonic antigen (CEA), discovered by Gold and Freedman in the 1960s, is now the only biomarker used for CRC clinically [42]. As a blood biomarker of CRC, the specificity and sensitivity of CEA are around 70~80% [12, 43]. The sensitivity of CEA for early colon cancer patients is low and increases with an increasing stage of the disease. The sensitivity in stages I~II, stage III, and stage IV CRC is 36%, 74%, and 83%, respectively [44]. Therefore, the false negative rates are relatively high for both diagnosis and detection of metastasis.

Although hundreds to thousands of biomarker candidates had been found through the applications of advanced high-throughput genomic and/or proteomic technologies, only very few among them have been verified and brought to clinical settings [15, 16, 45]. In contrast to the extensive survey on genomics and transcriptomes of human CRC, the proteomics survey in CRC is still limited. Currently, only few CRC biomarkers had been approved by FDA. They are CEA, fibrin/fibrinogen degradation product (DR-70), and human hemoglobin (fecal occult blood) [46]. Until now, there is still no single CRC biomarker comparable to CEA.

The present study applying secretome-based strategy has successfully verified BST2 as a potential CRC plasma biomarker for the first time. BST2 (also known as tetherin/CD137/HM1.24 antigen) is a transmembrane glycoprotein with a molecular weight of 19.7 kDa. The gene encoding BST2 was initially cloned in 1995 and reported to be involved in pre-B cell growth via cell-cell interaction [25, 47]. In 2008, BST2 was identified as a restrictive factor of HIV-1 replication [48]. BST2 functions as the physical link between HIV-1 particles and retains virion particles in restrictive cells, and it is responsive to interferon [49]. In recent years, several studies reported the connection between BST2 and cancer. In 2009, Cai et al. reported that BST2 protein expression

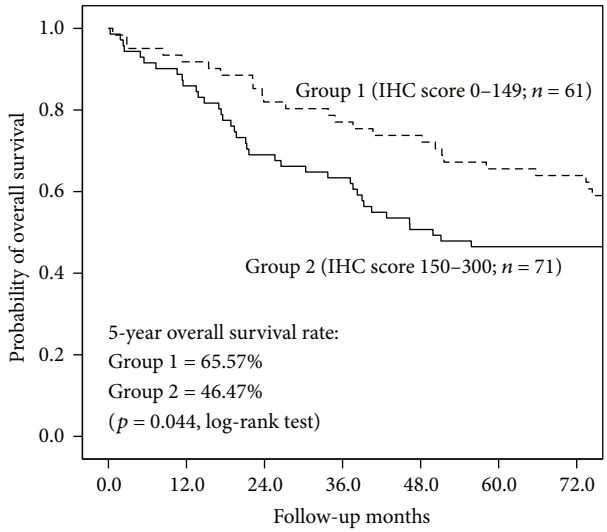

Figure 3: Association of BST2 tissue expression levels with survival among CRC patients used in this study. The IHC score of 150 of BST2 was used as cutoff value for survival analysis of 132 CRC patients.

Table 1: Multivariate analysis of BST2 tissue expression levels and clinicopathologic factors of 132 CRC patients.

Variable	HR*	95% CI**	*p* value
Gender			
Male	1		
Female	0.70	0.42~1.17	0.17
Age (years)			
<65	1		
≧65	1.91	1.18~3.11	<0.01
TNM stage			
Early (I~II)	1		
Late (III~IV)	3.29	1.94~5.58	<0.01
Differentiation			
Well	1		
Moderate	1.25	0.66~2.36	0.01
Poor	1.77	0.55~5.69	
Histological type			
Adenocarcinoma	1		
Mucinous carcinoma	1.82	0.65~5.14	0.25
Chemotherapy			
Yes	1		
No	1.40	0.80~2.46	0.23
CEA (ng/mL)			
<5	1		
≧5	2.02	1.21~3.37	<0.01
BST2 (IHC score)			
Low (0–149)	1		
High (150–300)	1.64	0.98~2.74	0.05

*HR: hazard ratio.
**CI: confidence interval.

is associated with bone metastasis in human breast cancer. They found overexpression of BST2 in the bone metastatic breast cancer tissues (compared to nonbone metastatic breast cancer tissues), as well as elevated BST2 levels in breast cancer patients with bone metastasis (compared to breast cancer patients without bone metastasis) [27]. In 2013, Yokoyama et al. reported the overexpression of BST2 in endometrial cancer and found the cytotoxic effect of anti-BST2 antibody on endometrial cancer cells in vitro and in an in vivo xenograft model [50]. More recently, Fang et al. showed that overexpression of BST2 is associated with nodal metastasis and poorer prognosis in oral cavity cancer [51]. Our present study demonstrated the overexpression of BST2 in tumor cells of CRC tissues, which is correlated with poor prognosis of CRC patients. We also showed the significant elevation of plasma BST2 levels in CRC patients compared to normal controls and showed that BST2 may increase AUC after combining CEA for CRC detection, especially for stage 1-2 CRC detection (Figure 4). However, unlike CEA, plasma BST2 levels did not correlate with the disease progression of CRC in the sample set used in this study (Supplemental Figure 1). As an unusual type II transmembrane protein found in lipid rafts, the mechanistic involvement of BST2 in malignancies is not clear yet.

Although our data showed the significant elevation of tissue and plasma BST2 levels in CRC patients compared to normal controls, how BST2, a type II transmembrane protein, can be released from tumor tissue into the blood circulation remains unclear at present. According to the information from Ensembl database, three BST2 transcripts have been identified to date; only one of them represents protein coding transcript and the other two are processed, nonprotein coding transcripts [52]. As a type II transmembrane protein, BST2 has been shown to be involved in microdomains of cell membrane [35, 53]. Several previous studies regarding the identification of proteins in exosomes derived from a variety of cell/tissue types provided important clue about the potential mechanism for shedding of BST2 into blood circulation. Using mass spectrometry-based proteomics approach, BST2 has been repeatedly detected in the exosomal fractions purified from B cells [54], ovarian cancer cells [55], thymic tissues [56], and urine [57]. Taken together, these observations suggest that the exosome-based secretion pathway may represent one of the potential mechanisms for shedding of BST2 into blood circulation from CRC tumor cells. This obviously represents an intriguing question that deserves further investigation.

In conclusion, we found a marked difference of BST2, a membrane protein selectively detected in CRC cell secretome, between CRC patients and healthy controls. The combination of BST2 and CEA outperformed each marker alone in distinguishing CRC patients from healthy individuals. The plasma level of BST2 may be a potential novel CRC biomarker.

Conflict of Interests

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

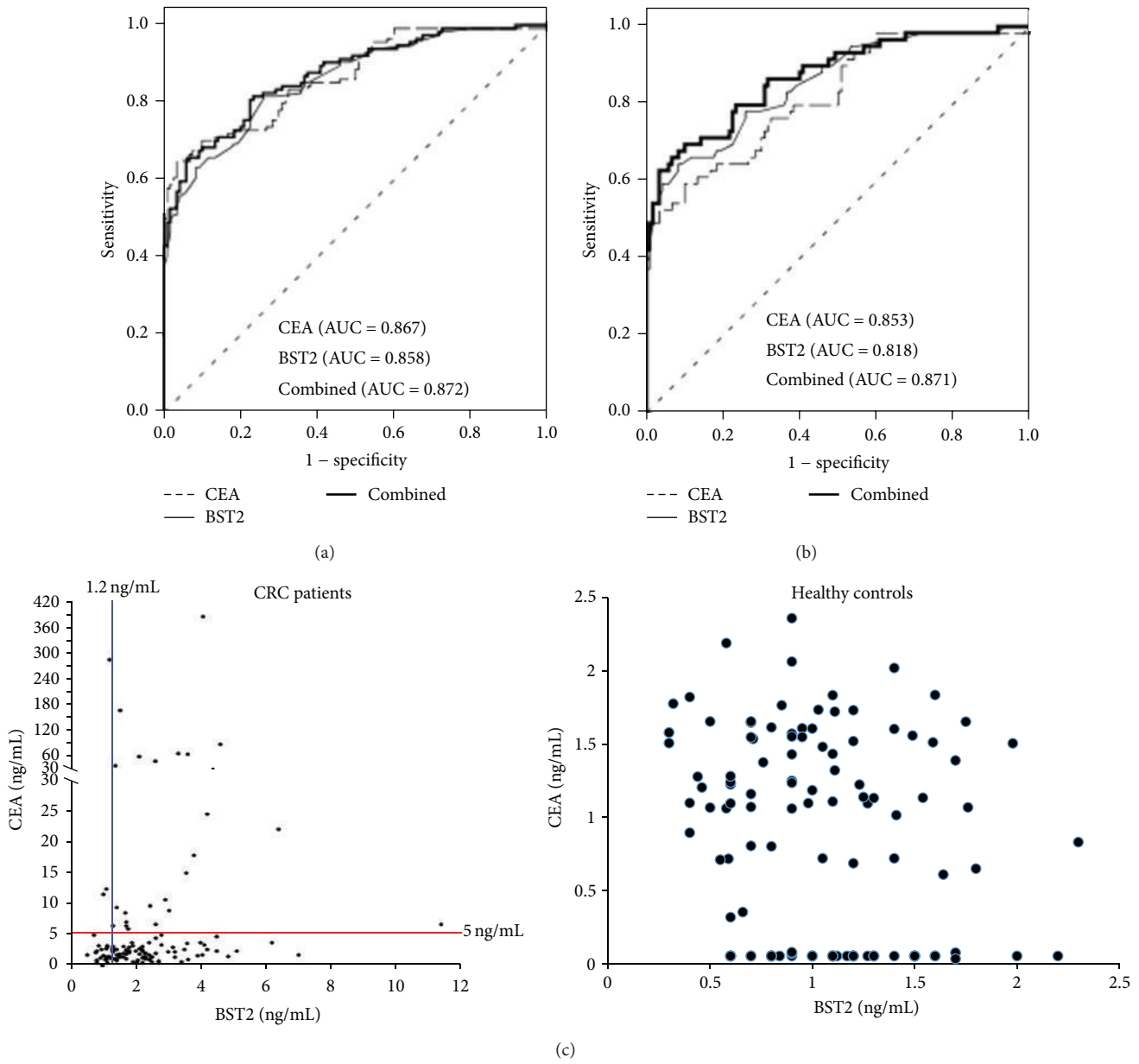

Figure 4: ROC curve analyses for the use of BST2, CEA, and the two-marker panel in discriminating CRC patients from healthy controls. (a) Analysis using all stage CRC patients (n = 120) and healthy controls (n = 120). (b) Analysis using stages I-II CRC patients (n = 61) from healthy controls (n = 120). (c) The distributions of plasma levels of BST2 and CEA among the 120 CRC patients and 120 healthy controls (CEA-BST2 plot).

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Review Article

HER2 Status in Premalignant, Early, and Advanced Neoplastic Lesions of the Stomach

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Objectives. HER2 expression in gastric cancer (GC) has received attention as a potential target for therapy with Trastuzumab. We reviewed the current knowledge on HER2 status in premalignant gastric lesions and in early (EGC) and advanced (AGC) GC to discuss the possible pathogenetic and prognostic roles of HER2 overexpression in GC. *Results*. HER2 overexpression was documented in gastric low-grade (LG) and high-grade intraepithelial neoplasia (HG-IEN), with higher frequency in gastric type dysplasia. HER2 overexpression was significantly associated with disease recurrence and poor prognosis in EGC representing an independent risk factor for lymph node metastases. HER2 overexpression was more frequent in AGC characterized by high grade, advanced stage, and high Ki-67 labeling index. The discordance in HER2 status was evidenced between primitive GC and synchronous or metachronous metastases. *Conclusions*. HER2 overexpression in premalignant gastric lesions suggests its potential involvement in the early steps of gastric carcinogenesis. The assessment of HER2 status in EGC may be helpful for the identification of patients who are at low risk for developing nodal metastases. Finally, the possible discordance in HER2 status between primary GC and its synchronous metastases support routine assessment of HER2 both in the primary GC and in its metastatic lesions.

1. Introduction

Although the incidence and mortality from gastric carcinoma (GC) significantly decreased over the last fifty years, this tumor still represents the third most common malignancy and the second leading cause of cancer death worldwide [1]. The high mortality rate from GC is mainly related to late diagnosis and to the lack of programs for early detection of this tumor [2–4]. The EUROCARE-5 results show that the 5-year survival rate to GC is 25.1%, with a significant difference recorded between men and women [1]. Of note, survival with GC varies depending upon the geographic area, with the highest survival rate observed in southern and central Europe and the lowest in Eastern Europe, United Kingdom, and Ireland [1]. Among the European countries, a high incidence of mortality from GC is encountered in Italy [2, 5, 6]; interestingly, a remarkable peculiar geographic variation was reported in this country [2, 5, 6] with the highest death rate in central and northern regions and the lowest in southern Italy [2, 6, 7].

Although the infection from *Helicobacter pylori* (*H. pylori*) is a known trigger of gastric carcinogenesis, many other external and internal events play a role in the development of this neoplasia [8]. Microscopically, GC is preceded by several precancerous lesions, including atrophic gastritis, hyperplasia, intestinal metaplasia, and dysplasia [8–14]. Those conditions are characterized by the accumulation of multiple genetic abnormalities, such as oncogene activation, tumor suppressor gene inactivation, and telomerase reactivation [15, 16], which may originate in part from chromosomal instability (CIN) [16, 17]. The latter consists in the loss or gain of whole chromosomes with aneuploidy and altered DNA copy number or in the partial alteration of chromosomes due to translocation, amplification, or deletion [17, 18]. Hence CIN may lead to the loss or gain of oncogenes, tumor suppressor genes, or genes involved in DNA repair or cell cycle

checkpoints [17, 18]. Recently, the Cancer Genome Atlas (TCGA) project classified tumors with CIN as a distinct biomolecular subgroup of GC characterized by the frequent amplification of genes such as HER2, EGFR, MET, FGFR2, and RAS genes (KRAS/NRAS) which are all related to the receptor tyrosine kinase RTK/RAS signaling [19]. In particular, HER2 gene encodes for HER2/erbB2 protein which belongs to the epidermal growth factor receptor family that comprises three other proteins with a similar structure, namely, HER1/erbB1, HER3/erbB3, and HER4/erbB4. HER2 plays an important role in the proliferation and differentiation of normal cells [20] and binding to its ligand gives rise to the creation of homodimers and heterodimers and activation of downstream signaling pathways [20]. Any aberrations in the structure or function of this receptor may lead to uncontrolled cell proliferation, neoplastic development, and progression [20]. Trastuzumab is a humanized monoclonal antibody that selectively targets HER2 receptor and inhibits its downstream signaling pathways in cells with HER2 overexpression [21]. A recent phase III randomized study (ToGA) demonstrated a significant survival benefit in patients affected by advanced GC with HER2 overexpression and treated with combined Trastuzumab and chemotherapy [22]. Hence, in recent years, the evaluation of HER2 overexpression has received attention as a target for novel therapeutic strategies aimed at increasing the survival to GC. In addition, assessment of HER2 status in all GCs at the time of diagnosis has been recommended in order to establish patient eligibility for treatment with Trastuzumab.

In this paper we review the controversial role of HER2 in gastric cancerogenesis and focus on the prevalence and potential prognostic significance of HER2 expression in preneoplastic lesions as well as in early and advanced GC.

2. HER2 in Premalignant Gastric Lesions

Although chronic atrophic gastritis and intestinal metaplasia of the stomach are considered to be preneoplastic lesions of GC, some Japanese studies do not clearly indicate a role in gastric carcinogenesis [23, 24]. Therefore dysplasia of the gastric mucosa represents the only universally accepted precancerous lesion of GC. Dysplasia is characterized by a wide range of cellular and structural atypia and it is defined as intraepithelial neoplasia (IEN), a pathological condition which lies between atrophic gastritis and GC [25]. IEN may develop in the gastric epithelium affected or not by intestinal metaplasia and it can be classified into four categories: indefinite for intraepithelial neoplasia, low-grade intraepithelial neoplasia (LG-IEN), high-grade intraepithelial neoplasia (HG-IEN), and suspicious for invasive cancer [26, 27]. The histological distinction between LG and HG IEN relies on the severity of architectural and cytological atypia. In detail, in LG-IEN the mucosa maintains tubular differentiation and the proliferative zone is limited to the outward portion, while in HG-IEN mucosal architecture is distorted and shows crowded irregular glands with marked cellular atypia and diffuse proliferative activity [28]. HG-IEN is associated with increased risk of GC [28–31]. Compared to LG-IEN, it is characterized by higher frequency of genetic abnormalities, including 8q gain, p53 overexpression, e-cadherin loss, and HER2 amplification, which are also present in invasive GC [32–36].

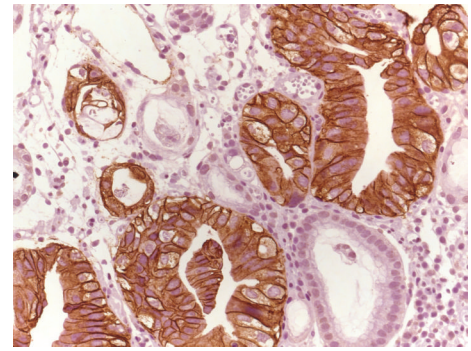


Figure 1: 3+ intense HER2 immunoreactivity in gastric HG-IEN. Note absence of staining in normal glands (original magnification, ×400: Mayer's Haemalum nuclear counterstain).

The possible occurrence of HER2 amplification in precancerous lesions was previously investigated in bronchial and breast epithelia [37–40]. HER2 amplification was evidenced in bronchial dysplasia with a role in cellular proliferation, but not in the progression to invasive carcinoma [37, 38]. In addition, HER2 overexpression was documented in breast ductal carcinoma in situ with negative prognostic significance, but not in benign and atypical proliferative lesions [39, 40].

Only few studies investigated HER2 overexpression in gastric dysplasia [36, 41–46]. In a series of surgical and bioptic samples, HER2 immunostaining with 2+/3+ score was evidenced in 12.6% of HG-IEN (Figure 1, authors' collection). Benign gastric mucosa did not show HER2 positivity in any of the specimens, although weak membranous reaction in the foveolae and cytoplasmic staining in specialized glands were observed, as elsewhere previously reported. The comparison of HER2 status between dysplasia and invasive GC showed six cases with concordant 3+ HER2 reactivity and seven with discordant HER2 status; in detail, three cases showed HER2 positivity in the dysplastic epithelium but not in the invasive GC, four cases displayed HER2 overexpression in GC but not in dysplasia [46]. It may be argued that the possible discordant HER2 status between paired dysplasia and GC should indicate that extrapolation of HER2 status of invasive carcinoma based on that observed in dysplasia is not reliable. Moreover, it may pose practical difficulties in assessing HER2 expression in biopsies with high-grade dysplasia transiting to carcinoma, determining false positive results in biopsies, due to the misinterpretation of HER2-positive dysplasia as invasive carcinoma [46].

HER2 overexpression has been also documented in LG-IEN, although with significantly lower frequency (4–8%) compared to that found in HG-IEN (16–20%) [41–43]. On the whole, these findings suggest that HER2 overexpression characterizes the early steps of gastric carcinogenesis [41–43]. However, the absence of HER2 overexpression in invasive GC matching HER2-positive dysplasia indicates that this

molecular deregulation may involve only a subset of cells in the intraepithelial neoplastic population [42].

By using immunohistochemistry, gastric dysplasia has been also classified into adenomatous/type I (intestinal phenotype), which is characterized by immunostaining for CD10 and CDX2; foveolar or pyloric/type II (gastric phenotype), which shows staining for MUC5AC and MUC6 and absence of CD10 expression; hybrid, which displays a mixed phenotype; null, when none of the aforementioned markers is expressed [47–50]. HER2 amplification was observed in cases classified as gastric or hybrid, which suggests that this type of dysplasia may represent the precursor of gastric type adenocarcinoma originating de novo from gastric mucosa [50]. An extensive analysis of HER2 status in immunoclassified gastric dysplasia may help to identify those patients at higher risk to develop a specific type of cancer, although the relationship between HER2 overexpression and progression of dysplasia to GC still requires further investigation.

3. HER2 in Early Gastric Cancer

There is some evidence that the identification of precursor lesions may be helpful for the early diagnosis of GC [51]. In Japan and Korea, endoscopy-based population screening allows frequent detection of early gastric cancer (EGC), which can be a suitable candidate for conservative treatments such as endoscopic submucosal dissection [51]. EGC is defined, irrespectively of the tumor size, as a carcinoma invading the mucosa and/or submucosa with or without lymph node metastases [52]. The incidence of nodal metastases in EGC depends upon the size, depth of invasion in the gastric wall, and histological differentiation of the tumor [53–55]. In detail, the incidence of nodal involvement is 0% for well-differentiated tumors of less than 2 cm in size and restricted to gastric mucosa, while it is higher than 30% for tumors showing infiltration in the submucosa, poor differentiation, and size larger than 2 cm [53–55].

According to the macroscopic classification of Japanese Endoscopic Society, EGC is divided into Type I, which includes tumors with polypoid growth, Type II which comprises tumors with superficial growth, Type III which describes tumors with excavating growth, and Type IV which refers to tumors with infiltrative growth and lateral spreading. Then, Type II EGC is further subdivided into IIa (elevated), IIb (flat), and IIc (depressed) and, on microscopic viewpoint, the most common histological architecture found in EGC is well differentiated, tubular, and/or papillary pattern [56]. For this reason, it may be challenging at times to discriminate between well-differentiated adenocarcinoma and high grade dysplasia, especially in superficial specimens of gastric mucosa [56]. EGC has good prognosis, with 5-year survival rate around 90% for N0 tumors [57] and around 70–75% for N+ carcinomas [57].

The presence of lymph node metastases is the main factor conditioning the surgical procedure for the resection of EGC. Indeed, according to the National Comprehensive Cancer Network guidelines [58], EGC without lymph node metastases can be a suitable candidate to endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD)

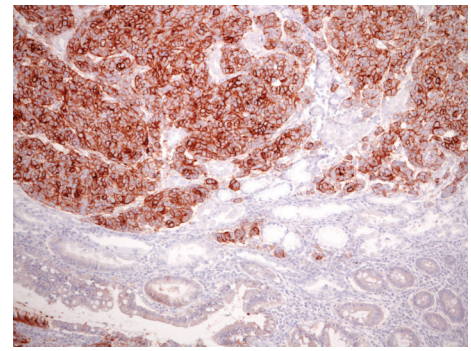


Figure 2: Intramucosal early gastric cancer with 3+ HER2 positivity; adjacent intestinal metaplasia present was unstained (original magnification, ×160; Mayer's Haemalum nuclear counterstain).

[55, 59, 60]. The size, histological type, depth of invasion, and lymphatic or venous invasion of the primary tumor were evidenced as factors predictive of nodal metastases in EGC [61–65]. With reference to molecular alterations, microsatellite instability (MSI), mutations in the p53 gene and overexpression of the epidermal growth factor receptor (EGFR) and HER2 genes seem to have a prognostic role in EGC. In detail, high microsatellite instability (MSI-H), a form of genomic instability associated with defective DNA mismatch repair, was demonstrated in EGC with a frequency ranging between 8.2% and 37% [65–67] and it was shown as an independent predictor of low frequency of lymph node metastases and long survival in this subset of tumors [65, 68]. On the other hand, mutation in the *p53* gene, which is one of the most frequent genetic abnormalities observed in GC, was associated with nodal metastases in EGC [65]. Finally, overexpression of EGFR and HER2 genes was significantly correlated with disease recurrence and poor prognosis in patients affected by EGC [65, 69, 70]. As a matter of fact, patients with HER2-negative pN0 EGC have significantly higher 5-year overall survival (91.1%) compared to patients with HER2-positive (Figure 2, authors' collection) pN0 EGC (81.8%) [60]. In addition, HER2 immunoexpression appears to be significantly associated with development of micrometastases in pN0 EGC [60, 71].

4. HER2 in Advanced Gastric Cancer

According to the published literature, HER2 overexpression/amplification, assessed by immunohistochemistry and/or in situ hybridization, ranges between 7% and 34% in advanced GC [72–78]. Of note, based on the results of an international randomized controlled trial (ToGA), patients with advanced gastric adenocarcinoma overexpressing HER2 are eligible for target treatment with Trastuzumab [22, 79]. Indeed a significant reduction of mortality rate was observed in patients with HER2 overexpressing advanced GC treated with combined chemotherapy and Trastuzumab [22, 79]. On the whole, HER2 positivity is significantly more frequent in gastroesophageal junction cancer (24–35%) compared to GC (9.5–21%) [73, 78, 80, 81]. Moreover, the rate of HER2 overexpression varies according to the histotype of GC [73, 75–77, 80],

with higher frequency evidenced in the intestinal histotype (81.6%–91%) compared to the diffuse or mixed (4%–7.9%) [77, 82–85]. Of note, the pattern of HER2 immunoreactivity is frequently heterogeneous in intestinal GC, which showed intermingled HER2-positive and HER2-negative areas. On the other hand, a more uniform unreactive HER2 pattern was encountered in diffuse histotype. Interestingly, HER2 overexpression rate progressively increases moving from the poorly cohesive WHO histotype to the mitochondrion-rich adenocarcinoma (MRC), tubular adenocarcinoma, and hepatoid carcinoma (HAS) [74, 76] which has the highest frequency of HER2 positivity and the worst prognosis [74, 76]. HER2 overexpression is also significantly associated with high histological grade, high Ki-67 labeling index (LI), and advanced stage [78]; thus it represents an additional morphological parameter reflecting aggressiveness of GC [78]. The biological reasons for the peculiar association between HER2 overexpression and the histotype of GC have not been yet fully elucidated and additional investigation is required. However, a possible explanation for this phenomenon may reside in the relationship between e-cadherin and HER2 expression. Indeed HER2 amplification is inversely associated with e-cadherin mutations [75, 86], and e-cadherin mutations are frequent in diffuse gastric and lobular breast carcinoma and rare in intestinal and ductal breast cancer [73, 75].

HER2 overexpression/amplification is frequently heterogeneous in GC [46, 87, 88] compared to breast cancer, in which HER2 heterogeneity is uncommon [89, 90]. For this reason, several recommendations on methodology, interpretation, and quality control for HER2 testing in GC have been proposed, especially with regard to the assessment in bioptic specimens of surgically unresectable cases. In addition, criteria for the assessment of HER2 amplification in bioptic and surgical specimens of GC have been significantly modified from those routinely applied to breast carcinoma [91]. In particular, the guidelines for the assessment of HER2 status in GC state that the staining intensity (light, moderate, and strong) and distribution (complete, lateral, and basolateral) at cell membrane should be evaluated in at least 10% of neoplastic cells in surgical specimens and in a cluster of at least 5 tumor cells in the biopsy [77, 82, 87] (Table 1). This HER2 scoring system represents a reliable tool for the evaluation of HER2 status in GC biopsy and surgical specimen, and it results in good concordance between paired biopsy and surgical specimen of advanced GC, mainly if all the available specimens are tested [46, 77, 92–96]. Nonetheless, a low rate of HER2 discordance has been reported between paired biotic and surgical samples of GC [97].

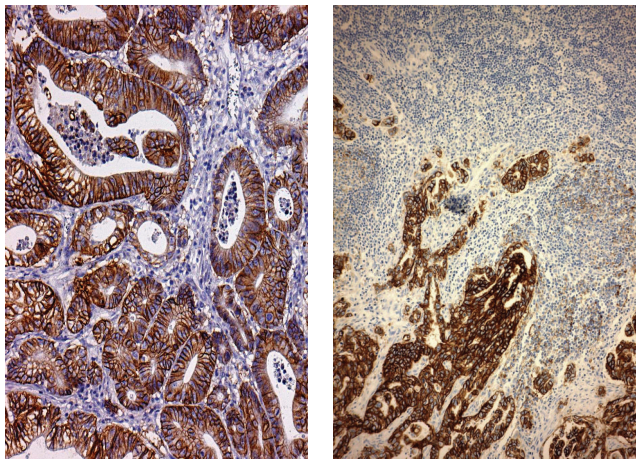
No guidelines are currently available on the number of tumor blocks to be tested for HER2 expression. However it was proposed that more than one (at least three) representative tumor blocks, obtained from different neoplastic areas, should be analyzed in order to overcome HER2 heterogeneity [82, 92, 98]. Moreover, it was suggested that at least 6 to 8 tumor fragments are required for adequate assessment in biopsies, mainly in patients who have low chance of being submitted to surgery [46, 77].

Recently, several studies addressed the issue of HER2 concordance between primary carcinoma and its metastases

Table 1: Immunohistochemical criteria for HER2 scoring in neoplastic specimens of the stomach.

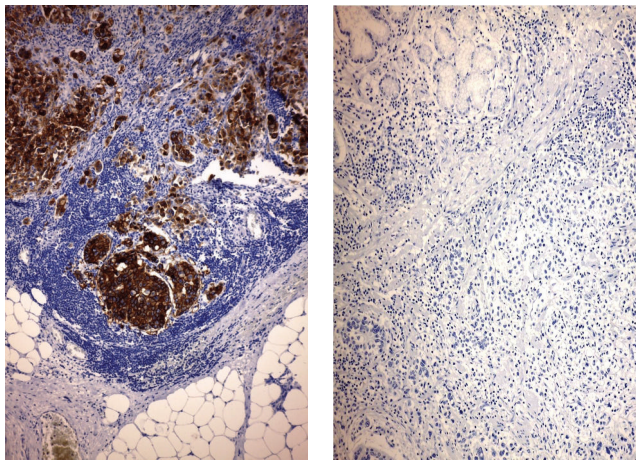
Surgery	Biopsy	HER2 score
No reactivity or membranous reactivity in <10% of tumor cells	No reactivity in any tumor cell	Negative (0)
Faint or barely detected membranous reactivity in ≥10% tumor cells	Tumor cell cluster of ≥5 cells with faint or barely detected membranous reactivity irrespective of percentage of tumor cells stained	Negative (1+)
Weak to moderate complete, basolateral, or lateral membranous reactivity in ≥10% tumor cells	Tumor cell cluster of ≥5 cells with weak to moderate complete, basolateral, or lateral membranous reactivity irrespective of percentage of tumor cells stained	Equivocal (2+)
Strong complete, basolateral, or lateral membranous reactivity in 10% or more of tumor cells	Tumor cell cluster of ≥5 cells with strong complete, basolateral, or lateral membranous reactivity irrespective of percentage of tumor cells stained	Positive (3+)

(Figures 3 and 4, authors' collection). Indeed it was shown that HER2 status may differ between primary tumor and matched metastases in both breast and stomach cancers [99–108]. Although a preliminary report did not show any significant changes in HER2 status in metastatic lesions compared to primary GC [84], more recent data demonstrated discordant HER2 status between primary carcinoma and synchronous or metachronous locoregional/distant metastases, with a mean rate of 7% and either positive or negative conversion [41, 99, 101, 104, 109–113]. In addition, changes in HER2 status, consisting in either positive or negative conversion, were evidenced in a comparative analysis between paired primary GC and corresponding synchronous metastatic lymph nodes in patients who did not receive adjuvant chemotherapy [101, 104, 108]. This latter fining may have relevant clinical impact [108]. Indeed, if HER2 expression is tested only in the primary GC, a percentage of patients with HER2-positive conversion in lymph node metastases may be excluded from targeted therapy [108]. Positive conversion may be related to the development of a HER2-positive subclone in metastatic lymph nodes as a result of disease progression [108]. On the other hand, negative conversion observed in metastatic deposits of patients who had not received any neoadjuvant treatments [108] cannot develop as the result of resistance to Trastuzumab therapy. Of note, discrepancy in HER2 status between primary tumor and paired nodal metastases was already highlighted in breast cancer [106, 107]. Although at



(a) (b)

Figure 3: (a) Concordant HER2 status in primary GC (original magnification, ×320; Mayer's Haemalum nuclear counterstain) and (b) corresponding metastatic lymph node (original magnification, ×160; Mayer's Haemalum nuclear counterstain).



(a) (b)

Figure 4: (a) Positive HER2 conversion in metastasis (original magnification, ×160; Mayer's Haemalum nuclear counterstain, ×160) in comparison to (b) negative primary GC (original magnification, ×120; Mayer's Haemalum nuclear counterstain).

present there is no indication of testing HER2 status in synchronous nodal metastases from GC, possible discordance in HER2 expression in metastatic tumors compared to primitive cancer is relevant for the therapeutic management and prognosis of the patients. Indeed further patients eligible for Trastuzumab-based therapy may be identified by assessing HER2 status in synchronous metastases from patients with HER2-negative primary GC.

5. Conclusions

HER2 putative role in gastric carcinogenesis still needs investigation. The evidence of a higher rate of HER2 overexpression in gastric HG-IEN compared to LG-IEN suggests that HER2 may be involved in the early steps of gastric carcinogenesis. In accordance, GC showing CIN, frequent amplification of genes related to receptor tyrosine kinase RTK/RAS signaling such as HER2, and Lauren's intestinal type has been recognized as a distinct molecular subtype of GC [19, 114].

Although HER2 has emerged as a new therapeutic target in GC, its role as a prognostic marker in this tumor is still controversial [115–121]. Indeed, some studies demonstrated that HER2 overexpression is a poor prognostic factor in GC [122, 123], while others showed that it may be favorable or irrelevant for prognosis [85, 123, 124]. In view of the correlation between HER2 overexpression and the immunohistochemical subtype of gastric dysplasia, HER2 assessment in gastric dysplasia may be helpful in order to identify patients at increased risk of developing a specific type of cancer. In addition, in our opinion, HER2 testing can be used as a prognostic factor to predict the risk of poor outcome in EGC,

since patients with HER2-negative pN0 EGC have significantly higher 5-year overall survival compared to patients with HER2-positive pN0 EGC [60].

In advanced GC, HER2 overexpression is significantly more frequent in tumors showing tubular histotype, high histological grade, advanced stage, and high Ki-67 LI, which suggests that it may represent an additional prognostic negative parameter. Finally, in view of the possible difference in HER2 status between primary GC and synchronous lymph node metastases, we suggest that HER2 status is routinely assessed not only in primary GC, but also in nodal and distant metastases, in order to identify possible candidates eligible for targeted Trastuzumab therapy.

Conflict of Interests

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

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Review Article

Helicobacter pylori Antibody Titer and Gastric Cancer Screening

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The "ABC method" is a serum gastric cancer screening method, and the subjects were divided based on *H. pylori* serology and atrophic gastritis as detected by serum pepsinogen (PG): Group A [*H. pylori* (−) PG (−)], Group B [*H. pylori* (+) PG (−)], Group C [*H. pylori* (+) PG (+)], and Group D [*H. pylori* (−) PG (+)]. The risk of gastric cancer is highest in Group D, followed by Groups C, B, and A. Groups B, C, and D are advised to undergo endoscopy, and the recommended surveillance is every three years, every two years, and annually, respectively. In this report, the reported results with respect to further risk stratification by anti-*H. pylori* antibody titer in each subgroup are reviewed: (1) high-negative antibody titer subjects in Group A, representing posteradicated individuals with high risk for intestinal-type cancer; (2) high-positive antibody titer subjects in Group B, representing active inflammation with high risk for diffuse-type cancer; and (3) low-positive antibody titer subjects in Group C, representing advanced atrophy with increased risk for intestinal-type cancer. In these subjects, careful follow-up with intervals of surveillance of every three years in (1), every two years in (2), and annually in (3) should be considered.

1. Introduction

Clinicians usually regard the results of *H. pylori* serology as a categorical variable (i.e., positive or negative), while not considering the actual titer of anti-*H. pylori* antibodies. Although the antibody titer itself suggests little clinically useful information in individual cases, subjects at high risk for gastric cancer can be detected effectively by evaluating antibody titer results in populations stratified by the degree of gastric mucosal atrophy. Many investigators have used the "ABC method," combining *H. pylori* antibody titers and serum pepsinogen (PG) concentrations, to evaluate the individual grade of atrophy and cancer risk [1–4]. Typically, the significance of a serum screen for the measurement of *H. pylori* antibody titer has been discussed in the context of the ABC method. For example, "high-positive antibody titer" subjects exhibit increased risk of diffuse-type gastric cancer compared to populations without gastric atrophy, and "low-positive antibody titer" subjects exhibit increased risk of differentiated adenocarcinoma in populations with gastric mucosal atrophy. These seemingly contradictory results have been confirmed by several investigators, and the scientific basis of these results has also been analyzed in detail [5–8]. However, these data have been known only to a limited number of investigators and clinicians and have not been widely disseminated.

In the present review, we first describe the characteristics of *H. pylori* antibody titers in the context of screening for *H. pylori* infection, including consideration of the biological meaning of the serum anti-*H. pylori* antibody titer. We then discuss several reported results concerning anti-*H. pylori* antibody titers. These data suggest the use (in daily clinical practice) of an expanded ABC method to detect patients with elevated risk for gastric cancer.

2. Characteristics of the *H. pylori* Antibody Titer as a Screening Method for *H. pylori* Infection

Measurement of the serum anti-*H. pylori* antibody titer is a noninvasive, inexpensive, and readily available method for

detection of *H. pylori* infection. Histology, culture, polymerase chain reaction (PCR), and the rapid urease test all require biopsy and/or collection of specimens by endoscopy, an invasive technique that is not suitable for mass screening [9, 10]. The urea breath test and stool antigen test are regarded as noninvasive tests, but the results of both methods are significantly affected by proton pump inhibitor therapy [11–13]. However, validated serology tests can be used even in patients being treated with proton pump inhibitors.

H. pylori strains possessing the cytotoxin-associated gene A (CagA) protein, a well-known virulence factor, cause more extensive inflammation and severe atrophy in gastric mucosa than nonproducers [14, 15]. However, there is still controversy regarding the significance of CagA serology, especially in East Asia, where most strains of *H. pylori* are CagA producers [16–19]. Therefore, gastric cancer screening is usually performed using the *H. pylori* antibody titer alone, except in limited areas [20].

Burucoa et al. [21] investigated the accuracy of 29 different serological tests and reported positive and negative predictive values of 70% and 100%, respectively. In general, better performance in serological screening depends on the use of the appropriate antigens and adjustment of cut-off values [22]. These considerations are among the disadvantages of using serum *H. pylori* antibody as a screening test for gastric cancer. Another disadvantage of using *H. pylori* antibody is that serology alone presents a challenge in distinguishing past and current infections [23]. The use of serology to identify posteradicated cases is considered later in this review.

3. The ABC Method: Gastric Cancer Screening Using *H. pylori* Antibody Titer and Pepsinogen Levels

High seropositive rates for *H. pylori* antibody are observed in East Asia, Eastern Europe, and parts of Central and South America, all areas with populations that have high levels of gastric cancer. Thus, in these regions, the anti-*H. pylori* antibody titer alone is insufficient for use as a screening tool for gastric cancer risk [24, 25]. For example, the frequency of anti-*H. pylori* seropositivity in subjects born before 1950 is reported to be over 70% in north Japan [26].

PG protein is classified into two types (PGI and PGII) based on biochemistry and immunology. PGI is produced by the chief cells in the gastric body; PGI levels decrease proportionally with the progression of atrophy of the gastric body. PGII is secreted by most parts of the gastric mucosa, as well as by parts of the duodenum; PGII levels increase in association with gastric mucosal inflammation regardless of the degree of atrophy [27–29]. As a result, atrophic gastritis can be diagnosed serologically by assaying the serum levels of the two pepsinogen isozymes. Reductions in serum PGI levels and in the serum PGI/II ratio are reliable markers for atrophic gastritis; cut-off values of PGI ≤70 ng/mL and PGI/II ratio ≤3 have frequently been applied [30–32].

The European Society of Gastrointestinal Endoscopy has proposed PG levels as a predictor of extensive atrophic gastritis [33]. Miki reported that the odds ratio (OR) for death from gastric cancer within 3 years after screening by the PG method was 0.290 [1]. Several other well designed, large-cohort studies have suggested an association between PG levels and dysplasia/gastric cancer. Watabe et al. found that *H. pylori*-positive patients with PGI <70 and PGI/II ratio <3 had a hazard ratio (HR) for gastric cancer of 6.0 [2]; under the same conditions, Yamaji et al. reported an HR of 6.2, and Yanaoka et al. reported an HR of 2.77 [34, 35]. The preceding studies corresponded to Japanese populations, but a report from Portugal showed consistent results in Western individuals [36]. However, gastric cancers did develop in a fraction (approximately 40%) of individuals who were atrophy-negative as defined by PG levels; the pathological characteristics of such cases revealed an unexpectedly high percentage of diffuse cancer [35].

About half of the cases of diffuse cancer, of which the prognosis is sometimes poor, cannot be detected by PG methods. This observation represents a critical weak point in mass screening by the PG method alone. Thus, a combined analysis (the "ABC method") incorporating the anti-*H. pylori* antibody assay and mucosal atrophy (as determined by serum PG) has been proposed to stratify the risk for the development of gastric cancer [1–4, 37]. Notably, the ABC method permits detection of gastric cancer in PG-negative subjects, addressing the fact that most diffuse cancer is *H. pylori*-positive but PG-negative. According to the original ABC method (as proposed by Miki [1]), PG was defined as "atrophic" when the criteria of both PGI ≤ 70 ng/mL and PGI/II ≤3 were fulfilled. Under the ABC method, the subjects were divided into the following 4 groups according to the presence of atrophic gastritis identified by serum PG and *H. pylori* seropositivity: Group A [*H. pylori* (−) PG (−)], Group B [*H. pylori* (+) PG (−)], Group C [*H. pylori* (+) PG (+)], and Group D [*H. pylori* (−) PG (+)]. Subjects in Groups B, C, and D were advised to undergo *H. pylori* eradication and endoscopic screening. The recommended endoscopy intervals in Groups B, C, and D are every three years, every two years, and every year, respectively, because the risk of gastric cancer was highest in Group D, followed by the risks in Groups C, B, and A [1, 4, 35]. Mizuno et al. reported (using Group A as a reference) that the HR of gastric cancer in Group B was 4.2, and those of Groups C and D were 11.23 and 14.81, respectively [4]. The ABC method is a screening strategy of "risk stratification" to identify high-risk subjects. Using the ABC method, we can advise high-risk subjects to undergo (1) regular endoscopic surveillance and (2) eradication of *H. pylori* infection. Subjects with a past history of *H. pylori* eradication were strictly excluded from screening by the ABC method; specifically, a subset of cases with successful eradication showed negative antibody titers in combination with increased PGI/II ratios, thus rendering "false-negative" results [HP (−) PG (−)] in most eradication cases. Kudo et al. reported that only 10.6% of all gastric cancer patients would have been classified into Group A, indicating that approximately 90% of cancer subjects would have been regarded as positive by the ABC method [3].

Despite the apparent relationship between serum PG and extensive atrophic gastritis, the association between endoscopic atrophic grade and histological diagnosis of atrophic

gastritis remains controversial. Several investigators have concluded that conventional white-light endoscopy cannot accurately differentiate and diagnose atrophic gastritis [38, 39]. This inconsistency suggests that endoscopic findings are insufficient for the accurate prediction of the grade of atrophy.

The essential weakness of the ABC method is the fact that remarkably high percentages of subjects are classified into Groups B–D, for whom endoscopy is indicated. One study suggested that only 45–60% of Japanese subjects in their 50s and 60s would be classified as Group A [26]. This limitation should be recognized when using this method for gastric cancer screening, especially in populations with high endemic rates of *H. pylori* infection.

The other limitation of the ABC method is that heterogeneity of risk in each subgroup has been reported by several investigators. All subjects in Group A do not show the same risk for gastric cancer; the high-risk subjects in Group A can be detected by stratification using antibody titers, and the same heterogeneity is seen in Groups B and C. We will refer to the details of the association between antibody titer and risk stratification of each subgroup in the ABC method in the following section.

4. Significance of the *H. pylori* Antibody Titer in the ABC Method

4.1. Associations between Anti-H. pylori Antibody Titer and the Degree of H. pylori Colonization. The immunopathological response induced by *H. pylori* infection is hypothesized to be dose-dependent. Higher bacterial counts induce intense immune response, resulting in subsequent higher antibody titers. However, genetic differences of each human host may affect antibody levels to pathogens [40]. Immune suppression by advanced cancer is another conceivable factor affecting the antibody titer of *H. pylori*, although direct proof of immune suppression in relation to the progression of gastric cancer has not yet been demonstrated [41]. Nonetheless, several investigators have inferred significant associations between the anti-*H. pylori* antibody titer and *H. pylori* density. Several investigators have found that the serological absorbance index of IgG antibodies against *H. pylori* is related to the density of antral *H. pylori* colonization and polymorphonuclear cell infiltration [42, 43]. The observation of significant decreases in *H. pylori* antibody titers following successful eradication implies the existence of a quantitative relationship [44]. Marchildon et al. reported that the mean decrease in IgG titer of *H. pylori* antibody at 6 months is approximately 40% [45]. Considered together, these findings indicate that the *H. pylori* antibody titer might be used to estimate the density of *H. pylori* in whole gastric mucosa.

4.2. Significance of "Low" Antibody Titer of H. pylori in Gastric Cancer Screening

4.2.1. Spontaneous Disappearance of H. pylori after Progression of Atrophic Gastritis in ABC Method-Defined Groups C and D. Chronic infection by *H. pylori* leads to *H. pylori*-related gastritis, which starts in the antrum and expands proximally towards the gastric body [46, 47]. Several investigators analyzing the association between gastric cancer development and *H. pylori* antibody titer demonstrated that this association is not proportional [35, 48, 49]. Multiple studies have reported that gastric cancer risk is elevated in subjects who display low-titer seropositivity for *H. pylori* antibody in combination with advanced mucosal atrophy (as determined by the PG level) (i.e., Group C in the ABC method) compared to high-titer subjects [5, 35, 50, 51]. Yanaoka et al. first referred to the significance of a "low-positive titer" in screening for gastric cancer. Those authors reported that the hazard ratio (HR) of overall cancer incidence in low-positive antibody titer subjects with serological atrophy was significantly higher (HR = 11.4) than in those with high-positive antibody titers (HR = 6.7), suggesting an increased risk in "low titer" patients, especially atrophic subjects [35]. Based on a case-controlled study, Fujioka et al. reported that the median serum antibody titer was lower in cancer cases than in control cases, such that high-positive titer patients have a reduced risk for gastric cancer (OR = 0.39) [50]. Note, however, that that study population was not confined to subjects with atrophy (i.e., not limited to Group C only). In a large study (with >36,000 participants), Tatemichi et al. clearly demonstrated an association between a low-seropositive *H. pylori* antibody titer and a high incidence of gastric cancer among PG-positive subjects [5]. That paper reported that, among the severely atrophic subjects in Group C, the ORs of cancer incidence (using Group A as reference) in low-positive titer and high-positive titer subjects were 14.9 and 8.3, respectively, suggesting that the risk in low-positive titer cases was almost double that in high-positive titer cases. That paper also analyzed the risk of intestinal- and diffuse-type cancers in "low-positive antibody titer" patients, demonstrating a significant association between the risk of cancer and "low-positive antibody titer" in intestinal-type cancer, but not in diffuse-type cancer.

To make sense of these results, we need to understand the environment suitable for survival of *H. pylori* in gastric mucosa. *H. pylori* can survive in gastric epithelial cells; thus, the loss of gastric epithelial cells after the progression to advanced atrophic gastritis with intestinal metaplasia can lead to spontaneous elimination of *H. pylori* [52, 53]. Thus, after infection with *H. pylori* in childhood, the bacterial density is expected to increase; presumably during this period, atrophy is limited to the antrum. Subsequently, a subset of these cases will progress to further atrophy of the gastric corpus; in these individuals, *H. pylori* numbers will gradually decrease, with some of these cases developing intestinal-type cancer in accordance with Correa's hypothesis [46]. In our experience, approximately 20% of subjects older than 60 years who are classified as Group C (by the ABC method) show low-positive *H. pylori* antibody titers (data not shown). This observation suggests that most subjects in Group C do not show progressive atrophy. Thus, for Group C patients, an elevated risk of gastric cancer development is indicated by the combination of both low *H. pylori* antibody titer and mucosal atrophy.

We have also focused on the role of antibody titer, with data suggesting the significance of "low-positive titer" in

subjects with atrophy. Specifically, our investigation of the association between the *H. pylori* antibody titer and fasting gastric pH revealed that impairment of acid secretion (a functional indicator of gastric atrophy) was most severe in patients with atrophic mucosa [PG(+)] and low-positive *H. pylori* antibody titer (i.e., Group C as defined by the ABC method) [54]. Our findings suggest that these subjects have an extremely high risk for gastric cancer, an interpretation that is consistent with previous reports, including Tatemichi et al. [5]. These results may also explain the occurrence of gastric cancer in ABC-defined Group D [PG(+), HP (−)]: *H. pylori* may be "disappearing" from the mucosa in a state of advanced atrophy [5]. Presumably, when the antibody titer in Group C fell below the cut-off value, those cases were classified as Group D (seronegative). Comparison of severely atrophic *H. pylori*-negative cases (Group D) to less atrophic *H. pylori*-positive cases (Group C) yielded respective HR values in gastric cancer of 131.98 versus 2.77 in Yanaoka et al. [35] and of 61.85 versus 14.85 in Ohata et al. [55]. The European Society of Gastrointestinal Endoscopy also noted that the spontaneous disappearance of *H. pylori* antibody was associated with the progression to severe gastric mucosal atrophy [33].

Consequently, the consensus of opinion now holds that subjects harboring the combination of low-positive *H. pylori* antibody titer and atrophic gastric mucosa are at higher risk for gastric cancer than subjects with a high-positive antibody titer, especially intestinal-type cancer. We recommend the following two management steps for these subjects: eradication of *H. pylori* and short-interval endoscopic surveillance. Eradication of *H. pylori* is usually advised in the ABC method; however, we recommend reconfirming it, especially in subjects with a low-positive antibody titer in Group C. In Group D, infection of *H. pylori* should be strictly investigated using other methods (urea breath test, stool antigen, etc.), and it should be eradicated if positive. Based on the ABC method, the interval for endoscopy is every two years in Group C and every year in Group D; however, we consider it desirable for patients with a low-positive antibody titer in Group C to undergo endoscopy every year, based on the result of Tatemichi et al. that the HR of gastric cancer in subjects with a low-positive antibody titer is twice that of those with a high-positive antibody titer.

Because the normal value of *H. pylori* antibody titer is different with each EIA kit, optimization of the cutoff value used to divide the "low-positive" and "high positive" subjects should be considered. Previous reports by Tatemichi et al. [5] and our own [54] set the cutoff point to 57.7 U/mL and 50 U/mL for the low-positive and high-positive antibody titer groups using the EIA kit of E plate, Eiken, ensuring the ratio of both group becomes about half of all *H. pylori*-seropositive subjects. Thus, we advocate that *H. pylori*-seropositive cases should be subdivided into two groups, low-positive and high-positive groups, and the cutoff value should be set to the points at which about one half of *H. pylori*-seropositive subjects are classified into each group.

4.2.2. Disappearance of H. pylori after Unexpected Eradication by Antibiotics and Its Significance in Group A. Dissemination of antimicrobial resistance induced by inappropriate use or abuse of antibiotics is a global public health problem [56]. The reported rate of inappropriate antibiotic use in hospitals ranges from 26% to 57% [57, 58], and the adherence rates for drug selection and treatment duration are relatively low, with rates as low as 38% even in Japan [59]. The failure of conventional triple therapies to eradicate *H. pylori* has been attributed primarily to bacterial resistance to one of the most commonly used antibiotics, clarithromycin [60–62]. The prevalence of clarithromycin resistance in the USA has increased from 9.1% in 2009-2010 to 24.2% in 2011–2013 [57]. In Italy, the prevalence of clarithromycin resistance has increased rapidly, doubling from 10.2% to 21.3% between 1990 and 2005 [62]. The rate of clarithromycin resistance at younger ages (in individuals under 30 years) was reported to be as high as 57% in Japan and 7.4% in USA [63, 64].

These findings suggest the possibility that a large fraction of *H. pylori* subjects achieved unexpected and complete *H. pylori* eradication after exposure to antibiotics for the treatment of other infectious diseases. The ABC method specifies the exclusion of subjects with a past history (as determined by medical interview) of eradication of *H. pylori* infection. However, subjects with unexpected eradication cannot be excluded completely, and such subjects are at risk for developing cancer as a result of a previous *H. pylori* infection.

The Shandong Intervention Trial is the first single trial indicating that the incidence of gastric cancer was significantly reduced in *H. pylori*-eradicated cases, with an OR of 0.6 after a follow-up period of 14.3 years [65]. Additional reports, including meta-analyses, have demonstrated that the eradication of *H. pylori* decreases the possibility of future cancer development by 30% [66–68]. These findings, however, suggest that the future cancer risk is not completely eliminated. Although several investigators have reported a low incidence of gastric cancer in subjects classified into Group A [55, 69], other investigators recently reported that approximately 2–10% of gastric cancer patients were serologically classified into "Group A." Most of these subjects were hypothesized to be "unexpectedly and successfully eradicated cases," given that subjects with a past history of eradication are excluded from screening by the ABC method but nonetheless have a risk of later development of gastric cancer [70–73].

The pathological characteristics of cancer in patients classified in Group A are controversial. Boda et al. reported that 92.6% of gastric cancer cases classified in Group A are intestinal-type; however, those authors investigated a limited number of early-stage subjects treated by endoscopy [70]. We also found that 90% of cancer in the subjects in Group A was the intestinal-type. Meanwhile, Kato et al. reported that only 33% of cancer cases in Group A were intestinal-type [72]. It is speculated that diffuse-type cancer arises from truly negative cases, and intestinal-type cancer arises from posteradicated cases. However, the details of the pathological characteristics of cancer in Group A will need to be investigated further. In this context, "successfully eradicated cases" are misclassified as Group A in the ABC method, and so these patients are not regarded as candidates for endoscopy, despite an elevated risk for subsequent transformation. Histological damage from

undiagnosed but resolved *H. pylori* infections is hypothesized to be the main source of gastric neoplasia among members of Group A.

We have recently been investigating predictive factors associated with gastric cancer in subjects who are negative for serum *H. pylori* antibody with normal PG status (i.e., Group A). We found that high-negative antibody titer (≥3 U/mL and <10 U/mL) is the significant factor for the prediction of gastric cancer in Group A (unpublished data). Although significant decreases in antibody titer after successful eradication have been reported [44, 45], complete seronegativity (antibody titer of zero) is typically not observed. We speculate that this persistence of a nonzero titer after eradication is the reason why lowering the cut-off value of the antibody titer is effective for the detection of neoplasia in Group A. Clinicians should be aware that a subset of serologically *H. pylori*-negative subjects are not true-negative cases but eradicated cases, even in patients who deny a past history of eradication treatment. It should also be noted that there is a possibility that *H. pylori* is not completely eradicated in serologically *H. pylori*-negative subjects; eradication is necessary in such cases.

Patients with unexpected eradication can also be detected by a characteristic change in serum PG. These individuals correspond to cases previously reported to possess "increased PGI/II ratios and decreased PGI levels," who were regarded as "normal" by the PG test [74, 75].

Based on the ABC method, subjects in Group A are not usually advised to undergo second-level diagnostic assessment using endoscopy; however, we consider that Group A subjects with a high-negative antibody titer should undergo endoscopy at least every three years, as in Group B. We also recommend investigating the *H. pylori* infection status in these subjects, and if *H. pylori* is detected by methods other than serology (urea breath test or stool antigen), we consider that it should be eradicated.

We advocate that *H. pylori* seronegative cases should be divided into two subgroups, low-negative and high-negative groups, with the ABC method. Optimization of the cut-off value should be considered to identify subjects with a "high-negative" *H. pylori* antibody titer, which corresponds to 3 U/mL (3 U/mL is the minimum determination limit and 10 U/mL is the recommended cutoff point) when using E plate, Eiken in our examination. We recommend resetting the cut-off value to the lower limit of each EIA kit and to evaluate the ratio of neoplasia cases in group A defined by strict criteria.

4.2.3. Classification of Subjects with Negative H. pylori Antibody Titers May Be Assisted by Serological Characterization of the Titer. Given the influence on the *H. pylori* antibody titer of the above-mentioned mechanisms, we propose classifying subjects negative for *H. pylori* antibody titer into the following three categories: (1) truly infection-negative; (2) infection-positive with deep progression of atrophic gastritis (in which antibody titer was normalized following spontaneous disappearance of *H. pylori* from the gastric mucosa); and (3) infection-positive, in which infection has been successfully eradicated. The eradicated cases (Class 3) are further subdivided into two categories: subjects in whom infection is eradicated by conventional triple therapy and subjects in whom infection is unexpectedly eradicated without a past history of eradication therapy.

Class 1 (infection-negative) individuals are "true-negative" cases. Most infection-negative cases will exhibit normal PG levels, and so would be categorized as Group A [*H. pylori* (−) PG (−)] according to the ABC method. Members of Class 1 typically will exhibit high PGI levels and are at extremely low risk for future gastric carcinogenesis.

Class 2 (infection-positive with deep progression of atrophic gastritis) cases will exhibit normalized antibody titers and PG-positive status, and so would be categorized as Group D [*H. pylori* (−) PG(+)] according to the ABC method. Members of Class 2 are at extremely high risk for intestinal-type gastric carcinogenesis.

Class 3 (infection-positive but successfully eradicated) cases will yield normal PG levels, and so would be categorized as Group A [*H. pylori* (−) PG (−)] according to the ABC method. Members of Class 3 have a chance of developing intestinal-type gastric cancer.

4.3. Significance of "High" Antibody Titer of H. pylori in Gastric Cancer Screening. About 20–30% of stomach cancers in Western countries develop from nonatrophic stomach, and the major histopathological type of these cancer cases has been reported to be "diffuse-type" [76, 77]. The most significant characteristic of diffuse-type cancer is a higher malignant potential than the intestinal type. The inflammation of gastric mucosa induced by *H. pylori* infection is postulated to directly induce this diffuse-type cancer without passing through the well-known sequence of atrophy to metaplasia to dysplasia to cancer. Inflammation-induced DNA methylation has been implicated in the induction of diffuse-type gastric cancer [78]. Consequently, diffuse-type cancer typically does not show advanced atrophy, and this is the reason why PG level, a reliable marker of gastric atrophy, cannot detect this type of cancer effectively. Thus, the risk factors for developing cancer among nonatrophic stomach cases include high PGII level and high *H. pylori* antibody titer, both of which reflect active gastric mucosal inflammation [79].

The association between diffuse-type cancer and a "high-positive" titer *H. pylori* antibody has been reported by several investigators. Tatemichi et al. conducted a case-controlled study using gastric cancer cases and matched control subjects, and they demonstrated that high-titer patients have the highest risk for diffuse cancer development [80]. Watanabe et al. recently elucidated, for the first time, the risk for diffuse-type gastric cancer in PG-negative individuals with high-titer *H. pylori* antibody (ABC method Group B). By performing annual endoscopies in *H. pylori*-positive subjects who did not exhibit chronic atrophic gastritis (i.e., Group-B subjects), Watanabe et al. demonstrated that cancer incidence was significantly higher in the *H. pylori* high-titer group than in the low-titer group, obtaining an HR of 6.51 [7]. That study also reported a marked increase in cancer development (at 1524/100,000 person-years) in Group B subjects harboring

high-titer antibody and high PGII levels (PGI >50 ng/mL and PGI/II ratio ≤3). Other investigators have reported similar results [35]. Yoshida et al. recently reported that the HR values of diffuse-type cancer in high-titer subjects stratified by PGII (<30 ng/mL and >30 ng/mL) were 3.8 and 8.5, respectively, when cancer incidence in low-titer subjects with PGII <30 ng/mL was used as in [8]. In our own work, we have observed that approximately 15% of Group B subjects over the age of 60 years show high *H. pylori* antibody titers (data not shown), suggesting that most subjects in Group B do not show progressive mucosal inflammation.

The high-positive anti-*H. pylori* antibody titer in Group B represents a risk factor for future gastric carcinogenesis, especially for diffuse-type cancer. Considering the high prevalence of diffuse-type cancer in Western countries, the combination of increased *H. pylori* antibody titer and high PGII levels is expected to be of use as a serum (noninvasive) screen for gastric cancer among Western populations. Because *H. pylori* eradication reduces the cancer risk more effectively in subjects without extensive chronic atrophic gastritis [81], eradication of *H. pylori* infection in these subjects is strongly recommended for prevention of gastric cancer in these patients in Group B. Although eradication is usually advised after conducting the ABC method, we recommend reconfirming eradication in these subjects. Based on the ABC method, endoscopy every three years is recommended for the subjects in Group B; however, the HR of subjects with a high-positive antibody titer in Group B is two to three times higher than of those with high-positive titers [8], and thus the HR of cancer in these high-positive subjects is estimated to be similar to that of Group C. Thus, meticulous endoscopic surveillance should be performed every two years in Group B subjects with a high-positive antibody titer.

The cutoff value should be set so that one half of *H. pylori*-seropositive subjects are classified to each of the "low-positive" and "high-positive" groups, as referred to in Section 4.2.1. This value is 50 U/mL when using E plate, Eiken based on our investigations (10 U/mL is the recommended cutoff point).

5. Natural History of *H. pylori*-Positive Subjects and the Relationship with the *H. pylori* Antibody Titer

The natural history of subjects with *H. pylori* infection has usually been discussed in the context of Correa's hypothesis [46], which postulates that gastric carcinogenesis occurs as a continuous process beginning with superficial gastritis and proceeding through metaplasia and dysplasia before reaching adenocarcinoma. However, few investigators have reviewed the natural history of *H. pylori*-infected subjects from the standpoint of the *H. pylori* antibody titer profile.

Here, we present the "typical" natural history of *H. pylori*-positive subjects and the associations with *H. pylori* antibody titer. We believe that readers will gain an overview of the significance of the antibody titer in each pathophysiology by reading this section.

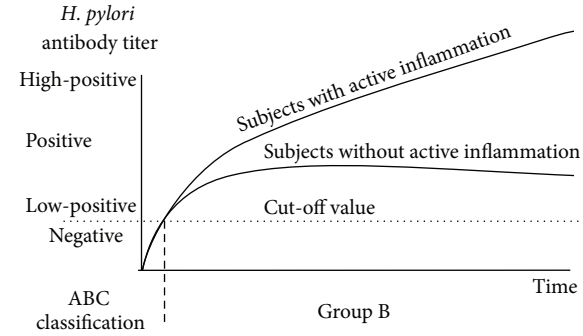

Figure 1: Time course of changes in the *H. pylori* antibody titer in *H. pylori*-infected subjects without significant progression of mucosal atrophy (Grade B in the ABC method). The antibody titer does not show significant change in most Group B cases. An increase in the *H. pylori* antibody titer is observed in a subset of these patients with progressive mucosal inflammation; such cases have an increased risk for diffuse-type cancer. Thus, subjects with a high-positive antibody titer should be considered at high risk for future development of diffuse-type gastric cancer.

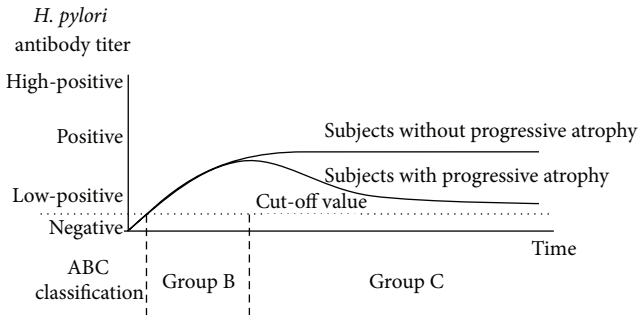

Figure 2: Time course of changes of the *H. pylori* antibody titer in *H. pylori*-infected subjects with progression of mucosal atrophy (Grade C in the ABC method). In Group C, the antibody titer does not change significantly (in contrast to the pattern in most Group B cases). However, in a subset of Group C cases, the antibody titer of *H. pylori* decreases gradually due to shrinkage of the effective area of the gastric mucosa as a result of the progressive atrophy induced by *H. pylori* infection. Thus, Group C subjects with low-positive *H. pylori* IgG titers exhibit an elevated risk for intestinal-type gastric cancer.

Figures 1–4 show a schematic view of the four patterns of the serial change of *H. pylori* antibody titer.

Figure 1 shows the typical pattern of *H. pylori* antibody titer in subjects who do not show significant progression of damage to the gastric mucosa despite infection with *H. pylori*. This subset of patients is classified as Group B in the ABC method. In most of these cases, antibody titer does not show significant change. However, an increase in *H. pylori* antibody titer is observed in a subset of these cases, specifically those with progressive mucosal inflammation, who have an increased risk for diffuse-type cancer. Thus, the subjects in Group B are subdivided into the following two subgroups: subjects with a low antibody titer (at low risk for future gastric cancer) and subjects with a high antibody

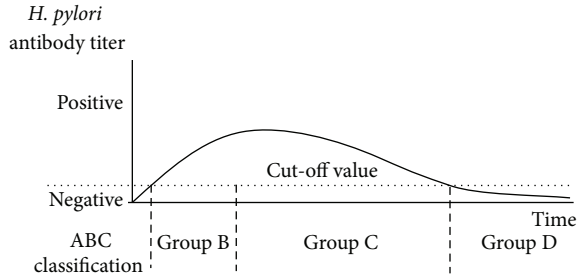

Figure 3: Time course of changes of the *H. pylori* antibody titer in *H. pylori*-infected subjects with severe progression of mucosal atrophy (Grade D in the ABC method). Subjects in Group D show significant "advanced" progression of atrophy; members of this group become seronegative for *H. pylori* antibody titer following the spontaneous disappearance of *H. pylori* from gastric mucosa. This subset of patients would be classified originally as Group B and then as Group C as atrophy progresses and finally as Group D when the antibody titer falls below the cut-off value (converting to antibody-negative status, as indicated by the arrow). Approximately 1% of all subjects in Japan will be classified as Group D, suggesting that only a limited number of cases show "advanced" progression of atrophy.

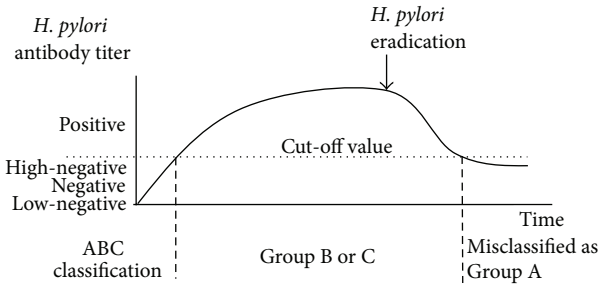

Figure 4: Time course of changes of the *H. pylori* antibody titer in successfully eradicated cases (classified as Grade A, although these cases should be excluded by medical interview in the ABC method). After successful eradication of *H. pylori*, the antibody titer decreases due to disappearance of *H. pylori* from the gastric mucosa, and from that point, the degree of atrophic grade progresses no further. Thus, a defined risk for gastric cancer persists throughout the lifetime of these patients, because a certain degree of gastric mucosal atrophy exists even after infection eradication. Therefore, eradicated cases should not be included as candidates in the ABC method, and they will require surveillance by endoscopy.

titer due to advanced mucosal inflammation (at high risk for future gastric cancer) (Figure 1).

Figure 2 shows the typical pattern of *H. pylori* antibody titer in subjects who show significant progressive atrophy of the gastric mucosa. This subset of patients is classified as Group C in the ABC method. In some cases, the antibody titer will not change significantly (unlike the situation in Group B); however, in other cases, the *H. pylori* antibody titer will decrease gradually due to shrinkage of the effective area of gastric mucosa by *H. pylori* infection, although the degree of atrophy will differ depending on the pathogenicity of specific *H. pylori* strains and the immunological response of each human host. Subjects with a lower *H. pylori* IgG titer have a higher risk for gastric cancer than high-positive titer subjects in Group C, as reported by Tatemichi et al. [5]. We have confirmed this pattern in our own work investigating the association between gastric acidity and *H. pylori* antibody titer [54].

Figure 3 shows the typical pattern of the *H. pylori* antibody titer in subjects who show significant "advanced" progression of atrophy of gastric mucosa, with the *H. pylori* antibody titer becoming negative following the spontaneous disappearance of *H. pylori* from the gastric mucosa. This subset of patients is classified as Group D in the ABC method. About 99% of *H. pylori*-infected patients are classified into Groups A, B, and C; the remaining approximately 1% of *H. pylori*-infected cases are defined as belonging to Group D. Specifically, separate reports by Miki and Yoshida et al. reported the incidence of Group D as 0.66% (33 of 5209 subjects) and 0.71% (33 of 4655 subjects), respectively [1, 8]. A limited number of *H. pylori*-infected patients show extremely deep progression of atrophy with negative conversion of *H. pylori* antibody titer; most of these cases are hypothesized to originate as low antibody titer Group C subjects. Members of this class show high risk for gastric carcinogenesis, especially differentiated adenocarcinoma.

Figure 4 shows the typical pattern of the *H. pylori* antibody titer in subjects from whom *H. pylori* has been eradicated, irrespective of whether elimination resulted from treatment with conventional triple therapy or by "unexpected" eradication. This subset of patients is sometimes misclassified as Group A in the ABC method; however, the risk for the development of gastric cancer is comparable to that in Group B. These subjects are not true-negatives for *H. pylori* and thus should be distinguished from Group A. We have demonstrated [54] that a high-negative *H. pylori* antibody titer is a significant predictor of gastric neoplasia in Group A cases. We advocate lowering the cut-off value for the *H. pylori* antibody titer, a step that would permit detection of such "eradicated" cases.

6. Modified Schematic Diagram of the ABC Method Considering the Significance of the *H. pylori* Antibody Titer

Figure 5 shows the generally accepted classification of the ABC method, in which the cases are divided into the following four groups: Group A [*H. pylori* (−) PG (−)], Group B [*H. pylori* (+) PG (−)], Group C [*H. pylori* (+) PG (+)], and Group D [*H. pylori* (−) PG (+)]. The incidence of gastric cancer increases in a stepwise and significant manner from A to D, as the color gradation shows the increasing risk for gastric cancer.

Figure 6 shows a modified schematic diagram of the ABC method incorporating the antibody titer as a continuous variable, as proposed in this review. The color gradation shows the risk for gastric cancer. The horizontal axis showing *H. pylori* serology is transformed from a categorical variable (i.e., positive or negative) to a continuous variable of antibody titer. For Group A, we demonstrated that the incidence of intestinal-type gastric neoplasia in the high-negative antibody subset was higher than in the low-negative antibody

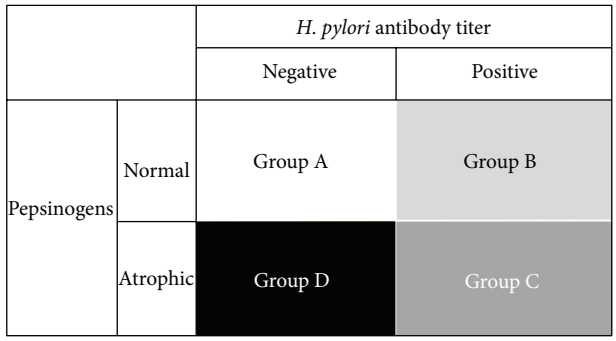

Figure 5: ABC method classification by *H. pylori* serology and pepsinogen status. Subjects are divided into four groups according to the serology of *H. pylori* antibody and pepsinogen (PG) status: Group A [*H. pylori* (−) PG (−)], Group B [*H. pylori* (+) PG (−)], Group C [*H. pylori* (+) PG (+)], and Group D [*H. pylori* (−) PG (+)]. The incidence of gastric cancer increases in a stepwise and significant manner from A to D. The color gradation shows the risk for gastric cancer.

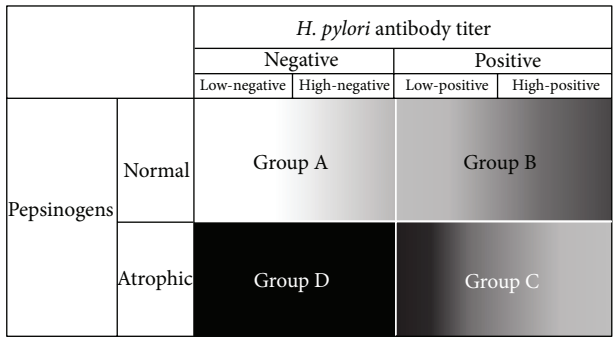

Figure 6: Modified schematic diagram of the ABC method considering the antibody titer as a continuous variable. Groups A, B, and C can each be divided into two subgroups based on antibody titer. The color gradation shows schematically the risk for gastric cancer. In Group A, the incidence of gastric cancer in the high-negative antibody subset is higher than that in the low-negative antibody titer subset. In Group B, the incidence of gastric cancer (typically diffuse-type cancer) in the high-positive titer antibody subset is higher than that in the low-positive titer antibody subset. In Group C, the HR of gastric cancer (typically intestinal-type cancer) is higher in low-positive titer subjects than in high-positive titer subjects.

titer subset [54]. For Group B, the incidence of diffuse-type gastric cancer in the high-titer antibody subset was elevated compared to the cases that were *H. pylori*-positive but possessed low antibody titer [7]. For Group C, the HR of intestinal-type gastric cancer was higher in low-titer subjects than in high-titer subjects.

7. Conclusions

Clinicians should consider the meaning of the antibody titer when interpreting a patient's status as defined by the ABC method. The risk for gastric cancer in subjects classified into each group is not the same, but clinicians should understand the fact that some high-risk populations detected by antibody titer stratification are included. We recommend dividing the cases by antibody titer into four subgroups, not two (negative and positive) subgroups: low-negative, high-negative, low-positive, and high-positive. One example of the range of each subgroup is <3 U/mL, 3–10 U/mL, 10–50 U/mL, and >50 U/mL, respectively. We especially advocate attention to the antibody titer itself in the following three clinical conditions.

The first condition occurs when a high-negative antibody titer is observed in PG-negative cases (Group A). Most of these subjects are posteradicated cases who have an elevated risk for developing gastric cancer. Lowering the cut-off value of the *H. pylori* antibody titer to the lower limit of each EIA kit will be important for the detection of these high-risk eradicated cases.

The second condition occurs when a high-positive antibody titer is observed in PG-negative cases (Group B). These subjects exhibit active mucosal inflammation and have an elevated risk for developing diffuse-type cancer.

The third condition occurs when positive but low-positive antibody titer is observed in subjects with mucosal atrophy (as defined by PG levels) (Group C). These subjects have severe progressive atrophy with a high risk for intestinal-type gastric cancer. The cutoff values of low and high-positive antibody titers should be set at values that put about half of the seropositive subjects into each subgroup.

In these cases, we consider that the interval of endoscopic surveillance should be shorter than the interval recommended by the ABC method: every three years for subjects with a high-negative antibody titer in Group A; every two years for subjects with a high-positive antibody titer in Group B; and every year for subjects with a low-positive antibody titer in Group C. Moreover, eradication of *H. pylori* is also strongly recommended to prevent future development of gastric cancer. Even in subjects who are serologically negative but have a high-negative antibody titer (Group A), *H. pylori* should be evaluated by other methods, and it should be eradicated if the results of the other evaluation methods are positive.

There is a possibility that gastric cancer screening solely by the ABC method is insufficient for the above-mentioned limited number of cases detected only by considering antibody titer. We believe that focusing on the serum *H. pylori* antibody titer as a quantitative (rather than qualitative/categorical) parameter will enhance the power of the ABC method as a screen for future risk for gastric cancer.

Conflict of Interests

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

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