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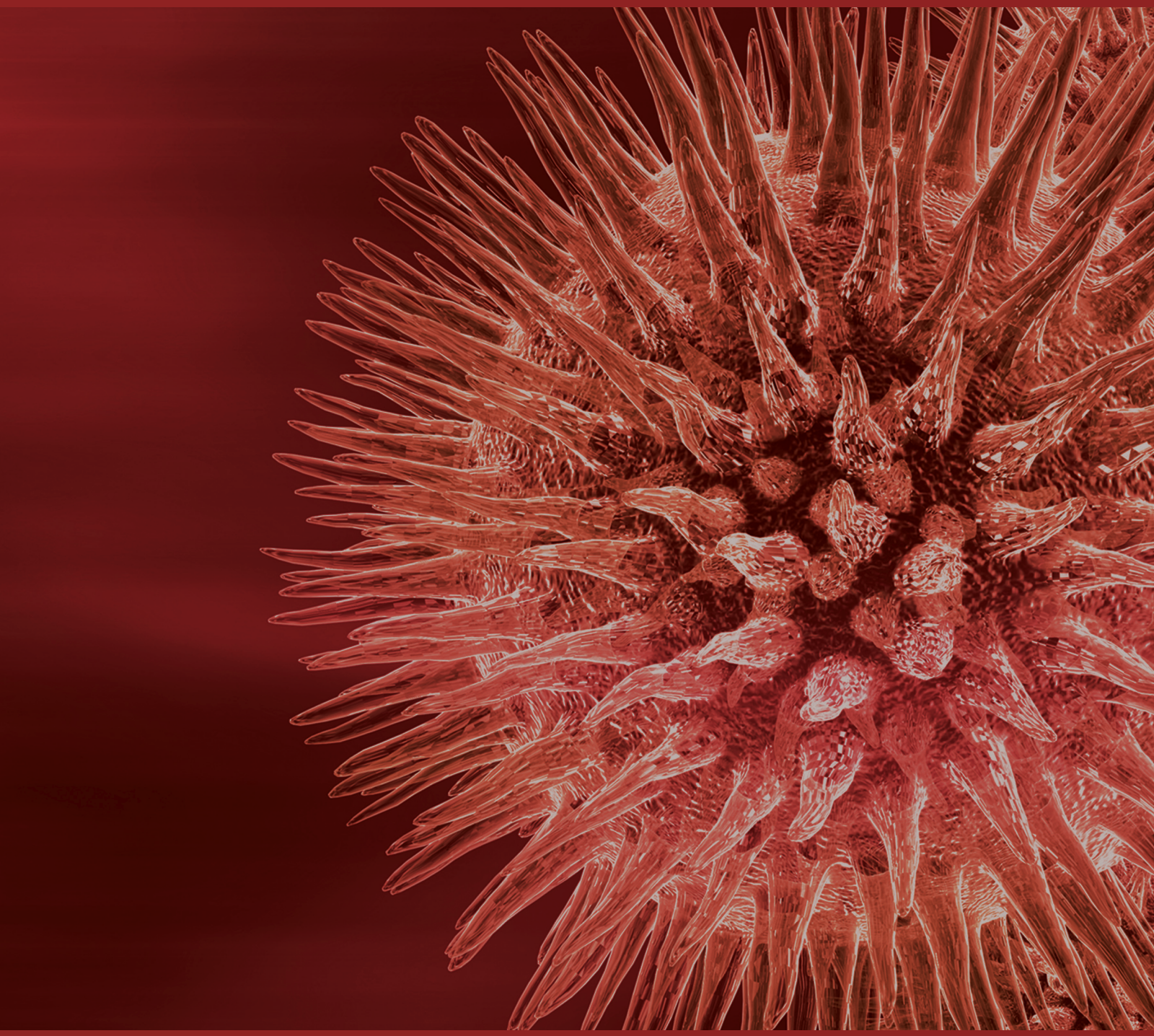
Novel Medicines and Strategies in Cancer Treatment and Prevention

Guest Editors: Chih-Hsin Tang, Gautam Sethi, and Po-Lin Kuo



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Editorial

Novel Medicines and Strategies in Cancer Treatment and Prevention

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Cancer is one of the leading causes of death around the world [1]. There are a number of challenges that the limitation of new drugs to potentiate antitumor therapies [2]. Furthermore, despite a positive response to initial treatment, many patients eventually develop a local recurrence and spread of the primary tumor [3–5]. Additional prognostic biomarkers are urgently needed to improve decision making regarding adjuvant therapy for these patients.

In response to the call for papers, we received many submissions from all over the world. After an initial screening, we selected eight articles that are proper in this special issue. All manuscripts underwent a very rigorous peer-review process. The full papers in this issue can be broadly organized into three main categories: (i) new anticancer agents, (ii) new antimetastasis agents, and (iii) new strategies in cancer prevention.

(i) New Anticancer Agents. Y. Ishima et al. have prepared *S*-nitrosated human serum albumin (SNO-HSA) with many conjugated SNO groups (poly-SNO-HSA) using chemical modification with 2-iminothiolane, which can be a reliable and safe NO donor. The poly-SNO-HSA can be used as an effective multifunctional antitumor agent because NO release from Poly-SNO-HAS is able to inhibit tumor cell growth and induce apoptosis "*Poly-s-nitrosated albumin as a safe and effective multifunctional antitumor agent: characterization, biochemistry and possible future therapeutic applications*."

C-Y. Tu et al. found that histone deacetylases (HDACs) inhibitor, trichostatin A (TSA), is able to attenuate EGFR expression through induction of microRNA-7 expression, which is an off-target activity of TSA to improve the anticancer efficiency of lapatinib-based therapy via HDAC-independent manner "*Trichostatin A suppresses EGFR expression through induction of microRNA-7 in an HDAC-independent manner in lapatinib-treated cells*."

(ii) New Antimetastasis Agents. Clinical reports have indicated that HER2 is frequently overexpressed in HBV-encoded X protein (HBx)-expressing HCC patients and is associated with their poor prognosis. In consistence to these findings, C.-M. Hung et al. showed that HBx is able to upregulate HER2 expression via HuR-dependent mRNA stabilization in HCC cells, which subsequently rendered HCC cells more metastatic. Therefore, in addition to targeting to HER2, RNA-binding protein, HuR, is as a new target for therapy in those patients "*Hepatitis B virus X upregulates HuR protein level to stabilize HER2 expression in hepatocellular carcinoma cells*."

P.-C. Chen et al. describe the important roles of CCN family proteins in skeletal development, and abnormal expression of CCN proteins is related to the tumorigenesis of primary bone tumors such as osteosarcoma, Ewing sarcoma, and chondrosarcoma. CCN proteins could therefore serve as potential therapeutic targets for drug development against primary and metastatic bone tumors "*The CCN family*

proteins: modulators of bone development and novel targets in bone-associated tumors."

J.-C. Chen et al. also discussed various mechanisms and mediators that regulate the expression of integrins and integrin-mediated signaling, contributing to increased cell migration. Therefore, the development of new drugs that can selectively target regulators of integrin gene expression and ligand-integrin signaling might hold great promise for the treatment of chondrosarcomas "*Novel strategies for the treatment of chondrosarcomas: targeting integrins*."

In addition, J.-Y. Kan et al. reported that even antimicrobial drug, gemifloxacin, could be a novel anticancer agent for the treatment of metastasis in colon cancer, which suppresses the activation of NF-κB, leading to a decrease in snail expression "*Gemifloxacin, a fluoroquinolone antimicrobial drug, inhibits migration and invasion of human colon cancer cells*."

(iii) New Strategies in Cancer Prevention. H. Orita et al. performed retrospective analysis of the connection between pfetin expression, clinical-pathological data and incidences of recurrence in the patients with gastrointestinal stromal tumor (GIST) that is the most common mesenchymal tumor of the digestive tract. The results demonstrate that lack of pfetin expression is an additional predictor of recurrence in resected GIST.

M. Wang et al. first studied the mutations of JAK2 V617F, FLT3-ITD, NPM1, and DNMT3A in Chinese patients with myeloproliferative neoplasms (MPN). The results indicated that patients bearing different forms of mutations may contribute to the pathogenesis.

All together, we hope this special issue will provide new inputs for those who are interested in the development of novel cancer prevention and treatment strategies.

Chih-Hsin Tang
Gautam Sethi
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Research Article

Pfetin as a Risk Factor of Recurrence in Gastrointestinal Stromal Tumors

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Background. Despite complete resection of gastrointestinal stromal tumors (GIST), recurrent and/or metastatic disease occurs, often depending on the grade of malignancy. As such, markers are needed that accurately predict patients at high risk for recurrence. Previously our group reported Pfetin as a prognostic biomarker for GIST. In order to create an approach for predicting risk of recurrence, we incorporated Pfetin expression with clinicopathological data to produce a predictive model. *Object*. Forty-five patients with localized primary GIST were treated with complete gross surgical resection surgically at our institution between 1995 and 2010 were included. The majority of tumors originated in the stomach (38 cases), as well as small intestine (6 cases) and rectum (1 case). *Method*. (1) We performed retrospective analysis of the connection between Pfetin expression, clinicopathological data, and incidences of recurrence, using bivariate and multivariate analyses. (2) The reactivity of the monoclonal antibody against Pfetin was examined by immunohistochemistry. *Pfetin*. We have reported Pfetin, identified microarray technology, and compared between statistically different GISTs for good and poor prognoses and for prognostic marker. *Results*. There were 7 cases of recurrences. (1) By univariate analysis, tumor size, mitoses, exposure to abdominal cavity, and complete tumor removal predicted risk of recurrence. (2) Pfetin-negative cases were significantly related to recurrence ($P = 0.002$). *Conclusions*. This analysis demonstrates that lack of Pfetin expression is an additional predictor of recurrence in resected GIST. Further study may determine the role of this variable added to the current predictive model for selection of adjuvant therapy.

1. Background

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the digestive tract. The precise cellular origin of GIST recently has been proposed to be the interstitial cell of Cajal, an intestinal pacemaker cell [1–3].

Although it comprises a low incidence (2/100,000 people/year) in Japan, their rates of recurrence and mortality remain high (30–40%) [4, 5].

These tumors are almost 90% associated with mutations of c-KIT [6, 7]. And recently c-kit negative cases have been identified as harbor activating mutations of platelet-derived growth factor receptor alpha (PDGFRA).

Most frequently, GISTs are located in the stomach (more than 60%) and the small bowel (30%) but can arise anywhere from the esophagus to the rectum and in the omentum and peritoneum [8, 9].

Before imatinib mesylate (inhibitor of tyrosine kinase) was developed, there were no effective treatments. GISTs uniquely metastasize by hematogenous spread and peritoneal seeding. It is most frequently recurrent in the liver, omentum, and peritoneum [8, 10]. The 5-year survival rate was 11–30%. In 2001, the first reported case of imatinib mesylate yielding striking effects in the treatment of GISTs was reported [11]. Imatinib dramatically changed the mean survival time of GIST (from 32–39 to 58 months) [8].

Despite complete resection, GISTs sometimes recur or metastasize, according to the degree of the malignancy. Viewed in this light, there are many arguments for adjuvant therapy. Treatment for recurrent and/or metastatic GIST with

imatinib has shown effectiveness. The recent Z9001 trial in the USA that tested 1 year of adjuvant treatment by imatinib reported reduction of the recurrences and metastases (Lancet) [8]. As such, the standard of care for high-risk patients after complete resection is one year of adjuvant therapy.

There are also risks associated with imatinib treatment which include costs and drug resistant from long time treatment by imatinib. One of the imatinib induced complications is the GI bleeding, such as GAVE (define). It can be due to the presence of residual tumor or to other less common etiologies [12, 13]. A multitude of dermatological toxicities also occurs, from various acute rashes to Steven-Johnson syndrome [14].

As with any therapy, balancing risks and benefits is paramount. Thus, it is necessary to diagnose the high-risk group for recurrences and/or metastases accurately. A sensitive and specific marker is sought to select patients at high risk for recurrence. Previously our group has reported Pfetin as a prognostic biomarker for GIST, identified using a proteomic approach [15]. Pfetin was originally cloned as a gene highly expressed in the fetal cochlea and brain [16]. Pfetin is also a member of KCTD family [16] and contributes to carcinogenesis and cancer progression.

The aim of this study was to determine the role of Pfetin expression in predicting the incidence of recurrence, in particular related to incorporation of this marker into the current model.

2. Patients

We examined the primary tumor tissues of 45 GIST patients who underwent surgery with complete (R0) resection at our institution between 1995 and 2011. Tumors originated in the stomach (37 cases), duodenum (1 case), small intestine (6 cases), and rectum (1 case). There were 7 recurrences. Diagnosis was based on the World Health Organization classification system for soft-tissue tumors: tumor size, presence of necrosis, differentiation, mitotic rate, MIB-1 index, and presence of epithelioid cells. C-kit expression in all GIST samples was confirmed using immunohistochemical staining (CD117 antibody, DAKO Japan Corp., Tokyo, Japan). Clinicopathological features of the 45 GIST patients are listed in Table 1.

3. Pathologic Analysis

All tumors included in the study were rereviewed by one pathologist, and the diagnosis of GIST was confirmed by positive staining for KIT (CD117) protein, as previously described. Tumor morphology was classified as predominantly epithelioid or spindle-shaped. Mitotic rate was determined by counting the number of mitotic figures per 50 high power fields (HPF) and categorized as <5, 5–10, or _10. Mib-1.

4. Immunohistochemistry

Pfetin expression was examined immunohistochemically using paraffin-embedded tissues. 4-mm-thick tissue sections were autoclaved in 10 mmol/L citrate buffer (pH 6.0) at 1218°C for 30 min and incubated with the antibody against Pfetin (1 : 1000 dilution). Immunostaining was carried out according to the streptavidin-biotin peroxidase method using the Strept ABC complex/horseradish peroxidase kit (DAKO). More than 20% of tumor cells were stained with the anti-Pfetin antibody which was considered to be Pfetin positive.

In most cases, the difference was quite obvious and the two reviewers concurred with the results.

Table 1: Clinicopathological features of the 45 gastrointestinal stromal tumors (GIST) cases.

		Cases	Recurrence	(%)	P value
Sex	Male	24	3	12.5	0.613
	Female	21	4	19.0	
Age	38~87 years old (mean 66.2)				0.771
Location	Stomach	37	5	14.0	0.635
	Intestine	6	2	25	
	Duodenum	1	0	0	
	Rectum	1	0	0	
Tumor size	<5 cm	25	0	0	$P < 0.001$
	5~10 cm	10	4	40	
	>10 cm	10	3	30	
Mitosis	<5/50 HPF	32	2	6.3	$P = 0.035$
	>5/50 HPF	13	5	38.5	
MIB-1 index	≧10%	6	1	16.7	0.963
	<10%	39	6	15.4	

Tumor size and mitosis have significant correlations with recurrence. These factors are also Fletcher's agents. These data lead Fletcher's classification to be good collation to recurrences.

5. Statistical Analysis

(1) We performed retrospective analysis of the connection between clinicopathological data and incidences of recurrent, using bivariate and multivariate analyses.

(2) All statistical analyses were carried out using the Chi2 test to assess the relationships between Pfetin expression and clinicopathological factors. The tumor-specific and disease-free survivals were calculated from the initial resection of the primary tumor until first evidence of metastasis and recurrence, respectively. All time-to-event endpoints were computed by the Kaplan-Meier method. Calculations were carried out using the SPSS software statistical package (SPSS Japan, Inc., Tokyo, Japan).

6. Results

6.1. Clinical Features. The median age of the population was 66.2 years (range: 38–87) and there were 24 (53.3%) males. Tumor locations included the stomach in 42 (82%), small bowel in 6 (13.3%), duodenum in 1 (2%), and rectum in 1 (2%) (Table 1). The median tumor size was 5.76 (0.6–15) cm.

Table 2: Clinical malignant factors of the 45 gastrointestinal stromal tumors (GIST) cases.

		Cases	Recurrence	(%)	P value
Dissemination (tumor explosion)	Positive	9	4	44.4	0.088
	Negative	36	3	8.3	
Invasion (other organs)	Positive	4	3	75	0.094
	Negative	41	4	9.8	
Damage on operation	Positive	2	2	100	0.287
	Negative	43	5	11.6	
Tumor necrosis	Positive	8	1	12.5	0.866
	Negative	37	6	16.2	

In clinical malignant factors, dissemination and invasion have a tendency for recurrence.

All patients had complete resections. Seven cases had recurrence and/or metastasis after resection of the primary tumor. Six cases were gastric cases (14%) and 2 cases were intestinal cases (25%).

(1) Tumor Size and Mitosis are High-Risk Markers for GIST. On univariate analysis, tumor size and mitoses were significantly correlated with recurrence (Table 1). These factors were a significant correlation with Fletcher's risk classification. These data led Fletcher's classification to be a good correlation with recurrences. All 7 cases of recurrence were grouped as high risk according to Fletcher's classification.

This demonstrated high accuracy. Dissemination and invasion also correlated with recurrence (Table 2).

(2) Pfetin-Negative Cases Were Significantly Related to Recurrence. Thirteen cases were Pfetin negative and 5/13 of these cases recurred (see Table 3). ($P = 0.002$). Clinicopathologically, Pfetin correlated with mitoses ($P = 0.024$).

These data were consistent with those in our previous study in which Pfetin expression was strongly correlated with recurrence and/or metastasis of GIST patients. Pfetin expression was strongly correlated with the prognostic value of GIST patients.

The Kaplan-Meier estimated disease-free survival curves revealed Pfetin expression to correlate significantly and inversely with recurrence. (Figure 1). Disease-free survival was dramatically longer in Pfetin-positive than in Pfetin-negative cases; Pfetin expression was strongly correlated with the prognostic value of GIST patients.

7. Discussion

Imatinib remains the standard of care for adjuvant therapy [10, 17, 18] after surgical resection for primary, localized GIST. Given significant recurrence, despite adjuvant imatinib, we need accurate predictive models for recurrence to guide adjuvant treatment. Current risk classification systems assist in determining the risk of disease recurrence in individual patients with GIST, so disease management can be personalized. However, risks of imatinib including cost, severe side effects, and inconvenience must be considered. Additional

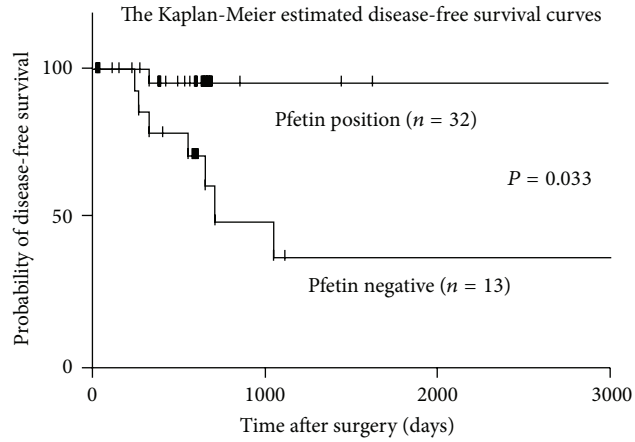

Figure 1: The Kaplan-Meier estimated disease-free survival curves. Pfetin expression was strongly correlated with the prognostic value of GIST patients.

Table 3: Pfetin expression.

		Recurrence (+)	Recurrence (−)	P value
Pfetin	Positive	2	30	0.002
	Negative	5	8	

Pfetin negative cases were significantly related to recurrence ($P = 0.002$).

prognostic markers are urgently needed to improve decision-making regarding adjuvant therapy for these patients.

The current standard, NIH consensus classification system for GIST, classified patients into risk groups on the basis of tumor size and mitotic index. Subsequently, Mietteinen modified this into the pathology risk stratification system by including tumor location and histology. Gold et al. reported the importance of intestinal location [19, 20] showing that intestinal GISTs are more likely to recur those gastric locations.

Nomograms created by DeMatteo presented risk of recurrence as percentages on a continuous scale. The goal of our study was to consider the impact of the addition of Pfetin level on these models.

In this study, Pfetin expression also correlated with outcome. This protein was discovered by microarray analysis using proteomic technology in Professor Kondo's lab. Pfetin's exact role remains to be determined.

Regardless, in this study test set, we showed an inverse relationship between Pfetin expression and risk of recurrence. Future testing in a validation set is planned. If this predictive relationship is confirmed, one might consider including it in a revised model.

8. Conclusion

Pfetin is an independent predictor of recurrence/metastasis for completely resected primary, localized GIST. Further investigation of this role is planned.

Conflict of Interests

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

Acknowledgments

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Research Article

Mutation Analysis of JAK2V617F, FLT3-ITD, NPM1, and DNMT3A in Chinese Patients with Myeloproliferative Neoplasms

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Since the discovery of JAK2V617F tyrosine kinase-activating mutation, several genes have been found mutated in myeloproliferative neoplasms (MPNs). FLT3-ITD, NPM1, and DNMT3A mutations frequently occurred in AML patients and have been found conferred with myeloproliferative neoplasms in mouse model. Therefore, we sought to search for mutations in JAK2V617F, FLT3-ITD, NPM1, and DNMT3A in 129 cases including 120 classic MPN cases and 9 MDS/MPN cases. JAK2V617F mutation was found in 60% of the 120 classic MPNs. However, none of the patients displayed FLT3-ITD and NPM1 mutations; only 2 patients harbored DNMT3A R882 mutation. Further studies including whole-genome sequence will be conducted to investigate the possible involvement of these genes in MPN.

1. Introduction

Myeloproliferative neoplasms (MPNs) are a class of stem cell-derived myeloid hematologic malignancies, characterized by expansion of one or more hematopoietic cell lineages with resulting bone marrow hypercellularity, a trend of transformation to myelofibrosis or acute leukemia. Mature and immature marrow elements readily traffic into the peripheral blood, as evidenced by increased white blood cells count, hemoglobin, or platelet count [1, 2]. Chronic myeloid leukemia (CML) and the three nonleukemic forms (polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF)) comprise the majority of MPNs and are commonly referred to as the classical forms [1].

The underlying causes of MPN are largely unknown. Genetic studies have identified that recurrent somatic and germline alterations may be responsible for the pathogenesis of MPN [3]. Since the discovery of the JAK2V617F mutation in 2005, an increasing number of novel somatic and germline mutations have been described in MPN in recent years, including myeloproliferative leukemia virus (MPL), TET oncogene family member 2 (TET2), additional sex combs-like 1 (ASXL1), casitas B-lineage lymphoma proto-oncogene (CBL), isocitrate dehydrogenase (IDH), and IKAROS family zinc finger 1 (IKZF1). However, none of these mutations was MPN specific, displayed mutual exclusivity, or could be traced back to a common ancestral clone [4]. Several lines of evidence suggest that mutations in genes other than these mentioned above must be present in MPN patients, and the initiating genetic events responsible for the development of MPN are still not totally understood [3].

FMS-like tyrosine kinase 3 (FLT3), a member of the class III receptor tyrosine kinase family that is expressed by early hematopoietic progenitors, plays a key role in growth regulation of hematopoietic progenitor cells [5]. Some studies have reported that FLT3 is also expressed on AML leukemic cells and stimulates survival and proliferation of leukemic blasts [6, 7]. FLT3-ITD (internal tandem duplication of FLT3) is the most prevalent mutation found in AML and has been identified in 20–30% of all AML patients [8]. Studies suggest that AML patients with FLT3-ITD have significantly elevated peripheral white blood cell counts and increased bone marrow blasts at diagnosis [9]. Moreover, Li et al. recently showed that knock-in of an ITD mutation into murine FLT3 conferred myeloproliferative disease in a mouse model [10], which indicated the potential involvement of

FLT3-ITD in MPN. However, to date, the data about FLT3-ITD mutation in human MPN remain poorly defined.

The nucleophosmin 1 (NPM1, localized on 5q35) gene encodes for a multifunctional phosphoprotein located primarily in the nucleolus. NPM1 mutations are known to be common in AML and are commonly associated with a diploid karyotype [11]. Sportoletti et al. demonstrated that NPM1 acts indeed as a haploinsufficient tumor suppressor gene *in vivo* [12], while some findings suggested that NPM1 mutation in AML is likely a gain-of-function one rather than simple haploinsufficiency [13]. The most common NPM1 mutation in AML is a duplication of a TCTG tetra-nucleotide at positions 956 to 959 of the reference sequence (GenBank accession number NM_002520) and accounts for 75% to 80% of cases [14]. One "conventional" knock-in model of NPM1 mutation demonstrated that NPM1 mutation can result in myeloproliferative disease but is insufficient for leukemogenesis [13]. Nevertheless, the frequency of NPM1 mutation and its possible pathogenetic role in MPNs are rarely investigated until now.

Alterations of epigenetic markers are thought to play an important role in myeloid malignancies. In particular, aberrant DNA methylation is a hallmark of these diseases [15]. DNA methyltransferases (DNMTs) catalyze the methylation of cytosine residues of CpG dinucleotides in DNA and are encoded by the human genes DNMT1, DNMT3A, and DNMT3B [16]. More recently, a whole-genome sequencing study in AML uncovered recurrent mutations of DNMT3A in 22% of AML patients and DNMT3A mutations were associated with poor outcome [17]. Many of the reported DNMT3A mutations mainly occurred at codon R882 in exon 23, but the occurrence of DNMT3A mutations in MPN patients is not well clarified.

In the last few years, the prevalence of DNMT3A, NPM1, or FLT3-ITD has been individually studied in some myeloid neoplasms mainly including AML or MDS. Furthermore, some studies indicate that the mutagenesis of these genes may differ within different races and be associated with patient's age. Therefore, in this study, we put the three genes together, along with JAK2V617F, to determine their mutational status in a series of well-defined adult Chinese patients with MPN and explored their clinical significance; also, the association between JAK2V617F mutation and DNMT3A, NPM1, or FLT3-ITD mutation was also investigated.

2. Materials and Methods

2.1. Patients and Samples. A total of 120 newly diagnosed classic MPN patients and 9 MDS/MPN patients according to the World Health Organization (WHO) criteria [18] were included in this study. Enrollment took place between January 2011 and December 2012 in Department of Hematology, QiLu Hospital of Shandong University, China. Bone marrow or peripheral blood samples were collected at diagnosis. The study was approved by the Institutional Review Boards of QiLu Hospital of Shandong University. Informed consent was obtained from each patient before being included in this study. The clinical characteristics of these subjects were summarized in Table 1.

2.2. DNA Extraction. Leukocytes were separated from bone marrow or peripheral blood samples using erythrocyte lysing solution. Genomic DNA was isolated using the TIANGEN DNA isolation kit (TIANGEN, China) according to the manufacturer's protocol. Store the DNA samples at −80°C for the following polymerase chain reaction (PCR) amplification.

2.3. PCR and Sequencing for DNMT3A, NPM1, and FLT3. As the recurrent mutation points, exon 23 of DNMT3A and exon 12 of NPM1 as well as FLT3 exons 11 and 12 were amplified by PCR; then the PCR products were sequenced. Briefly, PCR was performed in 25 μL volume containing 12.5 μL 2x Taq PCR MasterMix (TIANGEN, China), 7.5 μL RNase-free ddH2O, 1 μL forward and reverse primer, respectively, and 3 μL genomic DNA. The primers are shown as follows: DNMT3A exon 23 forward: 5′-TCC TGC TGT GTG GTT AGA CG-3′, reverse: 5′-TAT TTC CGC CTC TGT GGT TT-3′; NPM1 exon 12 forward: 5′-GGT CTC TGT TCT TTC TGT TGA TTT CC-3′, reverse: 5′-CAA CAC ATT CTT GGC AAT AGA ACC T-3′; FLT3 exon 11 forward: 5′-GCA ATT TAG GTA TGA AAG CCA GC-3′, exon 12 reverse: 5′-CTT TCA GCA TTT TGA CGG CAA CC-3′. The amplification was done with a DNA thermal cycler (BIO-RAD S1000 Thermal Cycler). After denaturing at 94°C for 3 min, the amplification was conducted for 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by reextension for 5 min at 72°C.

The PCR products were loaded onto a 2% agarose gel containing ethidium bromide and electrophoretically separated. After being purified, the PCR products were directly sequenced on both directions using the ABI PRISM 3730xl analyzer (Applied Biosystems Inc., Foster City, CA, USA) to screen for the presence of mutations. The samples with mutated FLT3-ITD, mutated NPM1, or mutated DNMT3A were used as positive controls.

2.4. JAK2V617F Mutation Analysis. We used the TaqMan MGB probe joint real-time PCR to detect JAK2V617F mutation. The TaqMan MGB probes and Mix were purchased from Applied Biosystems. PCR amplifications of DNA were done in a total volume of 10 μL PCR mixture containing 5 μL TaqMan Universal PCR Master Mix, 0.4 μL forward and reverse primer, respectively, 0.2 μL FAM and VIC fluorophore, respectively, 2.8 μL RNase-free ddH2O, and 1 μL genomic DNA. The primer and TaqMan MGB probe sequences are as follows, forward primer: 5′-AAG CTT TCT CAC AAG CAT TTG GTT G-3′, reverse primer: 5′-AGA AAG GCA TTA GAA AGC CTG TAG TT-3′, probe1: FAM-TCC ACA GAA ACA TAC-MGB, probe2: VIC-TCT CCA CAG ACA TAC-MGB. PCR amplification conditions were 50°C 10 min, 95°C 30 s, 95°C 15 s, and 62°C 1 min, 45 cycles. PCR was performed with ABI 7500 Real-Time PCR system (Applied Biosystems).

2.5. Statistical Analysis. Statistical analysis was performed using the SPSS Statistical Analysis Software. Differences in JAK2V617F percentage, age, and peripheral blood cells counts were accessed by Chi-squared tests, Fisher's exact tests,

Table 1: Basic characteristic of JAK2V617F-positive and negative MPN patients.

	All patients	JAK2V617F mutation		P value
		Negative	Positive	
Number (%)				
All patients	120	48 (40%)	72 (60%)	
PV patients	25	6 (24%)	19 (76%)	
ET patients	55	22 (40%)	33 (60%)	
MF patients	24	15 (62.5%)	9 (37.5%)	
MPN-u patients	15	5 (33.3%)	10 (66.7%)	
CNL patients	1	0 (0%)	1 (100%)	
Age (years), $\overline{x} \pm s$				
All patient	55 ± 16	48 ± 16	60 ± 11	0.000
PV patients	53 ± 23	40 ± 15	63 ± 8	0.011
ET patients	53 ± 3	47 ± 17	57 ± 12	0.016
MF patients	58 ± 12	56 ± 14	62 ± 4	0.184
MPN-u patients	53 ± 0	43 ± 20	58 ± 12	0.183
CNL patients	68	—	68	
Gender (Male/Female)				
All patients	56/64	21/27	35/37	0.709
PV patients	15/10	5/1	10/9	0.345
ET patients	20/35	8/18	12/17	0.575
MF patients	15/9	6/5	9/4	0.675
MPN-u patients	6/9	2/3	4/6	1.000
CNL patients	0/1	—	0/1	

P value refers to the comparison of JAK2V617F-positive versus -negative subjects.
MPN: myeloproliferative neoplasms; PV: polycythemia vera; ET: essential thrombocythemia; MF: myelofibrosis; MPN-u: MPN-unclassifiable; CNL: chronic neutrophilic leukemia.

and t-tests, respectively. P values less than 0.05 (two tailed) are considered significantly different.

3. Results

3.1. JAK2V617F Mutation in MPN

3.1.1. JAK2V617F Mutation. In the 120 classic MPNs studied, JAK2V617F mutation was found in 72/120 (60%). The frequency of JAK2V617F mutation was 76% among patients with PV (19 of 25), 60% among patients with ET (33 of 55), 37.5% among patients with MF (9 of 24), and 66.7% among MPN-u patients (10 of 15). There was significant difference between these four groups ($P = 0.046$, $P < 0.05$), and the positive incidence in PV group was remarkably higher than that in the other three groups. In addition, the only one chronic neutrophilic leukemia (CNL) patient was also observed with JAK2V617F mutation. Among the 9 MDS/MPN patients, only one had JAK2V617F mutation.

3.1.2. The Association of JAK2V617F Mutation with Clinical Characteristics. As shown in Table 1, the patients with JAK2V617F mutation were much older than those without mutation ($P = 0.000$). In PV and ET group, the JAK2V617F mutant patients were older than those JAK2V617F-negative patients, while no significant difference was found in MF and MPN-u group. As shown in Table 2, compared with younger patients aged <60 years, 46.9% (30 of 64), the frequency of JAK2V617F mutation was significantly higher in older patients aged ⩾60 years, 73.1% (41 of 56) ($P = 0.003$). JAK2V617F mutation rate was higher in older patients with PV, while not in ET, MF, and MPN-u patients. In older patients, JAK2V617F mutation rate in PV patients was higher than in ET, MF, and MPN-u patients; however, this phenomenon was not seen in younger patients. As for gender, there was no statistical significance of JAK2V617F mutation incidence between male 62.5% (35 of 56) and female 57.8% (37 of 64) patients ($P = 0.709$).

We analyzed the peripheral hemogram of the 85 hospitalized patients. The number of white blood cells (WBCs), red blood cells (RBCs) and hemoglobin (HB) of JAK2V617F-positive patients was significantly higher than those of JAK2V617F-negative patients; in contrast, no significant difference was found when comparing platelet (PLT) count, while in PV patients with JAK2V617F mutation, the PLT count was higher than those without mutation. In ET and MF patients, the number of WBCs of JAK2V617F-mutated patients was much higher; besides, the JAK2V617F-mutated ET patients also had higher HB counts (as shown in Table 3).

3.2. DNMT3A Mutation in MPN. By sequence analysis of the DNMT3A gene, we found DNMT3A mutations in 2 patients. The two mutations were heterozygous and missense: one was a MF patient (c.2644C>T, p.R882C; JAK2V617F-positive); the other one was a MDS/MPN patient (c.2645G>A,

Table 2: The relationship between JAK2V617F mutation and age at diagnosis.

	≥60 years n = (56)		<60 years n = (64)		P value
	Positive	Negative	Positive	Negative	
PV patients	14 (100%)	0	5 (45%)	6	0.003
ET patients	17 (74%)	6	16 (50%)	16	0.098
MF patients	6 (46%)	7	3 (27%)	8	0.423
MPN-u patients	4 (80%)	1	6 (60%)	4	0.6
*P value	0.016		0.471		
All patients	41	14	30	34	0.003

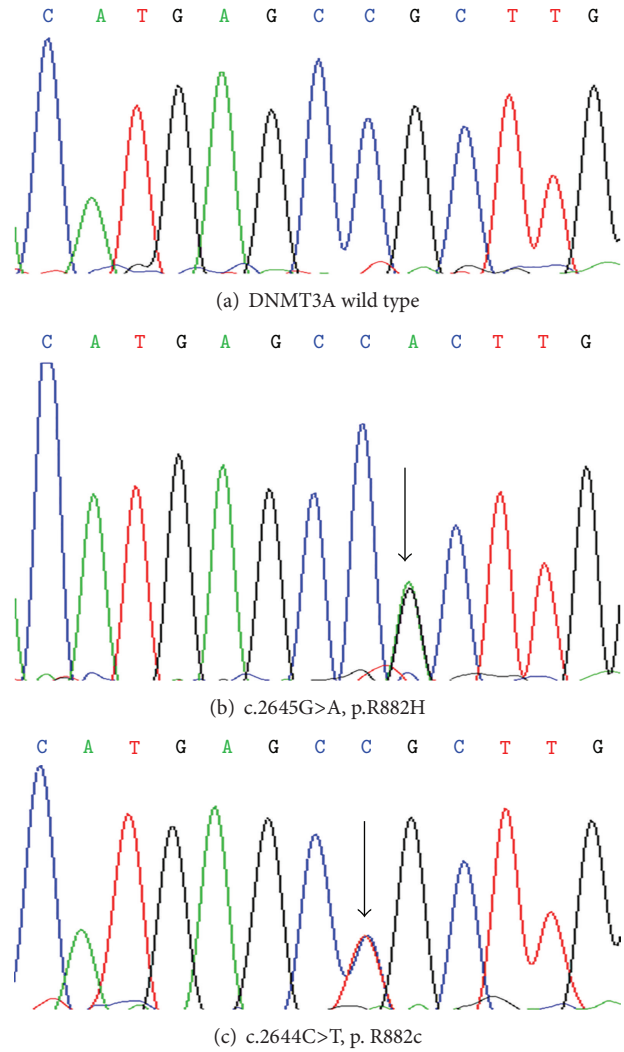
P value refers to the comparison of JAK2V617F mutation rate between patients >60 years old and <60 years old within all patients and every subgroup.
*P value refers to the JAK2V617F mutation rate difference between four subgroups in patients >60 years old and patients <60 years old.
MPN: myeloproliferative neoplasms; PV: polycythemia vera; ET: essential thrombocythemia; MF: myelofibrosis; MPN-u: MPN-unclassifiable; CNL: chronic neutrophilic leukemia.

p.R882H; JAK2V617F-negative) (Figure 1). Hence, the frequency of DNMT3A exon 23 mutation in MF was 4% (1/24), and the overall frequency in our MPN patients was close to 1% (1/120). Both the 2 DNMT3A-mutant patients were wild type for FLT3-ITD and NPM1. Moreover, the DMNT3A-mutated MF patient also had an abnormal karyotype: 46, *XY*,−3,+mar. This patient was a 60-year-old man who was diagnosed with MPN 9 years ago and later developed into postpolycythemia vera MF. And the patient also had spleen infarct complications and died ultimately with serious infections and systemic organ failure.

3.3. FLT3-ITD and NPM1 Mutation in MPN. We analyzed the mutations of FLT3-ITD and NPM1 by PCR followed by sequencing. However, our present study did not reveal any sequence variation in the 120 MPN patients and 9 MDS/MPN patients we studied, as shown in Figures 2(a) and 2(c), while Figures 2(b) and 2(d) showed the chromatograms of positive controls (samples from AML patients with FLT3-ITD or NPM1 mutation; Figure 2(b) showed the FLT3 internal tandem duplications mutation in exons 11 and 12; Figure 2(d) showed the insertion of a TCTG tetra nucleotide at positions 956 to 959 of the reference sequence). All these results suggested that the studied FLT3-ITD or NPM1 mutation points are unlikely the candidate factors for human MPN development.

4. Discussion

In this current study, we have mainly screened for the three putative candidate genes along with well-determined JAK2V617F in a cohort of Chinese MPN patients. The frequency of JAK2V617F was 60% in MPN patients. However, none of the 120 MPN patients and 9 MDS/MPN patients displayed known FLT3-ITD mutation at the juxtamembrane (JM) coding sequences and NPM1 mutation. Two kinds of mutations of DNMT3A were observed.



(a) DNMT3A wild type

(b) c.2645G>A, p.R882H

(c) c.2644C>T, p. R882c

Figure 1: The DNMT3A mutation and wild type. (a) The DNMT3A wild type, (b) the MF patient who has a DNMT3A mutation (c.2644C>T, p.R882C), and (c) the MDS/MPN patient who has a DNMT3A mutation (c.2645G>A, p.R882H).

Amounts of molecular and clinical evidences have shown that JAK2V617F mutation has a direct causal role in the pathogenesis of MPNs. JAK2V617F mutations were found in approximately 70%–90% of patients with PV, in 35%–70% with ET, and in 30%–50% with MF, which was in line with our results. Studies have suggested that JAK2V617F mutation is more common in old than in young patients with MPN [19]. In our study, the presence of JAK2V617F was found to be significantly correlative with advanced age (≥60 years) at diagnosis. Besides, JAK2V617F mutation was common in old patients with PV. Complete blood cell count (CBC) is essential in diagnosis of MPN; several studies have showed that hemogram is altered in patients with JAK2V617F mutation. However, thus far, the relationship between JAK2V617F mutation and blood cell counts is controversial and the impact of JAK2V617F mutation on the patients' hemogram variation remains not very clear [20].

Table 3: JAK2V617F mutation and peripheral hemogram.

JAK2V617F	Number	WBC (10^9/L)	RBC (10^{12}/L)	HB (g/L)	PLT (10^9/L)
PV	21				
Mutation	16	11.2 ± 4.5	6.8 ± 1.1	185.6 ± 32.4	362.2 ± 222.3
Wild type	5	9 ± 5.7	6.2 ± 1.7	190.2 ± 37.4	185.7 ± 71.2
P value		0.383	0.543	0.813	0.015
ET	35				
Mutation	20	19.4 ± 15.5	4.4 ± 0.8	133.2 ± 24.7	1046.8 ± 608.3
Wild type	15	9.3 ± 5	3.8 ± 0.8	110.1 ± 21.9	871.8 ± 296.9
P value		0.012	0.052	0.013	0.272
MF	22				
Mutation	8	18.7 ± 12.8	3.4 ± 1.3	83.5 ± 21.4	188 ± 51.2
Wild type	14	8.1 ± 9.2	2.6 ± 1.0	74 ± 26.5	99.9 ± 89.5
P value		0.035	0.179	0.372	0.135
MPN-u	6				
Mutation	4	23.8 ± 6.0	5.7 ± 2	141 ± 43.8	549.8 ± 221.5
Wild type	2	27.4 ± 17.8	3.5 ± 1.5	95.4 ± 43.3	1125.5 ± 839.3
P value		0.821	0.251	0.293	0.507
CNL	1				
Mutation	1	26.77 ± 0	4.22 ± 0	138.5 ± 0	233 ± 0
Total	85				
Mutation	49	17.3 ± 12.2	5.2 ± 1.7	143 ± 45.1	639.8 ± 554.4
Wild type	36	9.8 ± 8.5	3.7 ± 1.6	106.2 ± 47.2	490.4 ± 467.6
P value		0.002	0.000	0.001	0.197

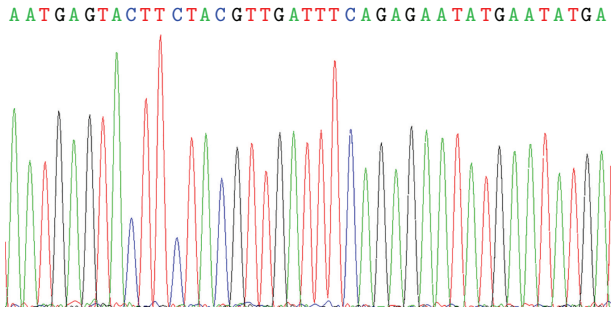
P value refers to the comparison of JAK2V617F-positive versus -negative subjects.
MPN: myeloproliferative neoplasms; PV: polycythemia vera; ET: essential thrombocythemia; MF: myelofibrosis; MPN-u: MPN-unclassifiable; CNL: chronic neutrophilic leukemia; WBC: white blood cell; RBC: red blood cell; HB: hemoglobin; PLT: platelet.

So we determined the relationship between the JAK2V617F mutation rate and hemogram in adult Chinese classic MPNs; our data demonstrated that patients harboring JAK2V617F mutation had higher leukocyte counts, red blood cell counts, and hemoglobin levels. Our JAK2V617F-mutated ET patients were found with advanced age and remarkably higher leukocytes, which was consistent with previous studies [21], while in PV and MF group, the association of JAK2V617F mutation with hemogram variations was relatively not well determined. As far as we know, our research is the first one to study the relationship between JAK2V617F mutation and hemogram as well as age factor in adult Chinese classic MPNs.

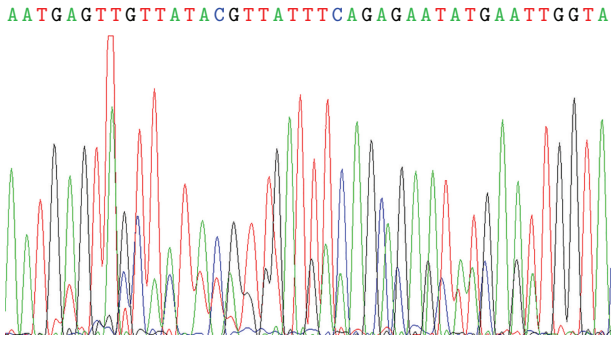
The majority of FLT3 mutations are ITDs in the JM domain encoded by exons 11 and 12 and were first reported in patients with AML in 1996 [6]. Xu et al. detected the patients with various malignant hematologic diseases and found that FLT3-ITD mutation mainly occurred in AML patients and might be a strong prognostic factor [22]. In another study, FLT3 mutations were also observed in patients with MDS or CMML, but at a much lower frequency than AML, and did not predict poor outcome [9]. However, the data about FLT3 mutations in MPN patients and their relationship with JAK2V617F mutations were limited.

Several studies in animal models uncovered the importance of FLT3-ITD in MPN; four studies have indicated that FLT3-ITD could induce myeloproliferative disease using transgenic mouse models, respectively [23–26]. Because of the putative involvement of FLT3-ITD in MPN development, we detected the total 129 MPN and MDS/MPN cases using PCR followed by sequencing method. However, no FLT3-ITD patients were found. Our negative result is similar to Pardanani et al. [27] report that no FLT3 mutations were found in a cohort of patients with chronic myeloid disorders, while being in contrast to Lin's study that FLT3 mutations occur in approximately 10% of Philadelphia (Ph) chromosome-CMPD and CMPD/MDS [5]. In a recent article, FLT3 mutation analysis was performed on 90 cases of JAK2-negative MPNs or MDS/MPNs and 62 cases of JAK2V617F-positive MPNs. One FLT3-ITD mutation was identified in the JAK2V617F-negative group (1.1%), and none were identified in the JAK2V617F-positive group, confirming the absence of FLT3 mutations in JAK2V617F-positive specimens [28], which is basically the same as our results that FLT3 mutation was rare in the usual types of MPN and the two mutations are mutually exclusive. These differences may be due to the diversity of the studied diseases or population.

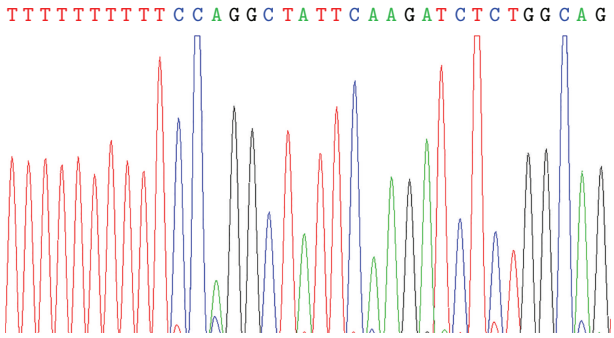
NPM1 mutations, first identified by the aberrant cytoplasmic localization of NPM1 protein, were found to be frequent events in AML [14]. Some animal models bearing enforced human NPM1-mutant expression showed an expansion of hematopoietic cells and developed myeloproliferation, indicating a pathogenic role of mutant NPM1 protein in myeloid disorders [29, 30]. Some studies showed that NPM1 mutation occurred with low frequencies in patients with MDS [11, 31],



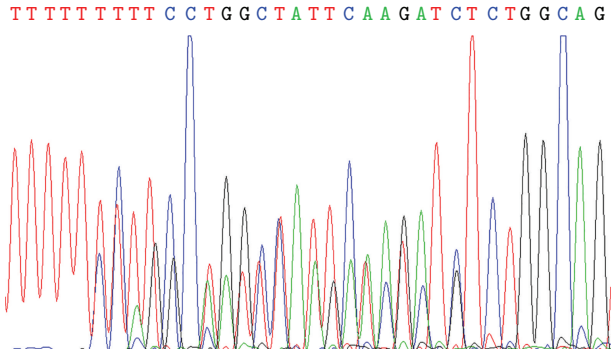
(a) FLT3 wild type



(b) FLT3-ITD mutation



(c) NPM1 wild type



(d) NPM1 mutation

Figure 2: FLT3 and NPM1 wild type and mutation. The sequencing chromatograms of FLT3-ITD and NPM1. (a) FLT3 wild type, (b) the FLT3-ITD mutation in exon 11 and 12, (c) the NPM1 wild type, and (d) the NPM1 mutation in exon 12.

while others found no mutations in MDS [32, 33]. Ernst et al. showed that NPM1 mutations occurred in 6/187 (3%) MDS/MPN patients and the 6 patients were all CMML patients, indicating that NPM1 mutation may be associated with a poor prognosis [34]. Schnittger et al. found that NPM1 mutation was observed in 6/67 secondary AML (s-AML) patients with a history of MPN and concluded that the NPM1 mutations are not only a key factor in the initiation of *de novo* AML but may contribute to s-AML following MPN [35]. However, relatively few data regarding the presence of NPM1 mutations are available for classic MPN cases. One study in a small cohort of classic MPNs (14 PV, 7 ET, and 9 MF) reported that NPM1 mutations were not observed in these patients [32]. In our relative larger cohort of Chinese MPN patients, no NPM1 mutation was found, indicating that NPM1 mutation might not be prevalent in MPN.

Mutations in DNMT3A were shown to be one of the early initiating events in AML pathogenesis [36]. DNMT3A mutations were also noted in patients with MDS and s-AML. Walter et al. found DNMT3A mutations in 8% of MDS patients, similar to the trend noted in AML. The major mutant point was at amino acid R882 and has a significantly poorer outcome [37]. The studies about DNMT3A mutation in MPN were limited and inconsistent. Stegelmann et al. reported DNMT3A mutations in 7% PV, 15% MF, and 14.3% s-AML and indicated DNMT3A alterations occurred concurrently with JAK2 [38]. Abdel-Wahab et al. delineated that total 3 DNMT3A-positive cases in 46 primary MF patients were also found to have cooccurring JAK2V617F mutation [39]. However, other studies identified that DNMT3A mutations were rare or absent [15, 40, 41]. We explored the mutation frequency of DNMT3A in 120 Chinese MPN patients, and mutation was only observed in a MF patient concurrently with JAK2V617F mutation. Moreover, this positive patient had an abnormal karyotype and spleen infarct complications and died ultimately with serious infections and systemic organ failure. Therefore, DNMT3A mutation may not be a frequent characteristic of MPN, but could be a poor prognostic indicator, always concurrently with JAK2V617F mutation. However, larger cohort of patients are needed to determine the exact frequency of DNMT3A mutations in Chinese MPN patients and to clarify its role in the molecular pathogenesis of MPN.

So far, more and more genetic events which may contribute to the pathogenesis of MPN have been elucidated. However, despite significant insight into the role of specific mutations, including the JAK2V617F mutation, the precise mechanisms in MPN remain elusive. It is very likely that additional mutations in MPN will be described soon, but practical relevance in terms of either disease prognostication or value as drug targets has so far been limited [42].

MPN genes belong to two major pathways: intracellular metabolism and epigenetic regulation [43]. The molecular pathogeneses of these three candidate genes mutations we studied here are different. (1) DNMT3A alterations are involved in epigenetic regulation of gene transcription—aberrant DNA methylation. (2) FLT3 mutation is associated with signaling pathways and proliferation. FLT3 as well as JAK2V617F abnormity can activate tyrosine kinase and result

in aberrant activation of tyrosine kinase signaling [34]. (3) NPM1 mutation in exon 12 results in loss of its nuclear localization signal; the altered protein concentrates in the cytoplasm, where it dimerizes to wild-type NPM1, blocking its activity in the nucleus [43]. So burgeoning insight into the role of these genes mutations in the pathogenesis of myeloid malignancies has prompted increased interest in development of novel targeted therapies. Methyltransferase inhibitors and JAK2 inhibitors are commonly used in clinical trials; FLT3 kinase inhibitors and NMP1 targeted therapy are reported to have made exciting progress recently [44]. However, further studies that explore their precise roles in hematopoiesis and in the pathogenesis of MPN as well as their prognostic impact and potential as a therapeutic target are needed.

5. Conclusion

In conclusion, we first studied the mutations of JAK2V617F, FLT3-ITD, NPM1, and DNMT3A together in Chinese adult MPN patients. However, the DNMT3A R882 amino acid residue, which is a mutation hotspot in AML, was only mutated once in our series of MPNs (in a MF patient) and the other 2 mutations were not found, suggesting that these genes may be not involved in the pathogenesis of MPN, and of course this hypothesis should be further validated in the future researches, while hotspot mutations in the DNMT3A, FLT3, and NPM1 genes are not common in MPN patients maybe due to that these selected genes harbor activating mutations in other regions which were not examined. This is a limitation in our study. So further studies including whole-genome sequence will be conducted to clarify the comprehensive mutational status as well as possible involvement of these genes in Chinese MPNs.

Conflict of Interests

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

Authors' Contribution

Min Wang and Na He contributed equally to this paper.

Acknowledgments

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Research Article

Hepatitis B Virus X Upregulates HuR Protein Level to Stabilize HER2 Expression in Hepatocellular Carcinoma Cells

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Hepatitis B virus- (HBV-) associated hepatocellular carcinoma (HCC) is the most common type of liver cancer. However, the underlying mechanism of HCC tumorigenesis is very complicated and HBV-encoded X protein (HBx) has been reported to play the most important role in this process. Activation of downstream signal pathways of epidermal growth factor receptor (EGFR) family is known to mediate HBx-dependent HCC tumor progression. Interestingly, HER2 (also known as ErbB2/Neu/EGFR2) is frequently overexpressed in HBx-expressing HCC patients and is associated with their poor prognosis. However, it remains unclear whether and how HBx regulates HER2 expression. In this study, our data showed that HBx expression increased HER2 protein level via enhancing its mRNA stability. The induction of RNA-binding protein HuR expression by HBx mediated the HER2 mRNA stabilization. Finally, the upregulated HER2 expression promoted the migration ability of HBx-expressing HCC cells. These findings deciphered the molecular mechanism of HBx-mediated HER2 upregulation in HBV-associated HCC.

1. Introduction

Hepatocellular carcinoma (HCC) accounts for the majority of liver cancer. The mechanism underlying HCC tumorigenesis involves several etiological factors, and chronic viral infection is the most critical mediator [1, 2]. Hepatitis B virus (HBV) infection is of particular importance for HCC development since the occurrence of over half of HCC cases is associated with its chronic infection [3, 4]. So far, the mechanism of HBV-associated HCC is still not understood completely yet. HBV may mediate HCC formation directly due to the viral inflammation process. But accumulating evidence shows that HBV-encoded regulatory proteins directly contribute to the HCC tumor progression [5]. HBV-encoded X protein (HBx), one of these regulatory proteins, has been reported to play the most significant role in this regulation [6, 7]. Although HBx is a relatively small protein with 154 amino acids, it has diverse functions in both the cytoplasm and nucleus. In the nucleus,

it can turn on gene expressions that are important to tumor progression by interacting with transcription factors. In the cytoplasm, it works through activation of RAF/MEK/ERK and PI3K-Akt signaling pathways [6, 8, 9] which are critical downstream effectors of HER receptor tyrosine kinases (RTKs) family [10]. Furthermore, HBx can regulate protein stability via interacting with proteasome subunits [11, 12]. More recently, it is reported to fine-tune gene levels by regulating microRNA (miRNA/miR) expressions [13].

HER family (also known as EGFR/ErbB family) comprises HER1-4 proteins and its activation plays pivotal roles in the regulation of cell growth and survival. Under normal condition, the activation of HER family proteins is strictly controlled by ligand-mediated endocytic degradation. However, once its expression is dysregulated, tumorigenesis may occur. Therefore, overexpression of HER family proteins is frequently observed in many solid tumors, including HCC [14]. Notably, upregulation of HER2 protein (also known as Neu/ErbB2/EGFR2) in HCC has been reported to be associated with HBV infection [15]. More importantly, elevated HER2 protein expression is also found in HCC tumors with HBx expression and is associated with the poor prognosis of HCC patients [16]. However, it remains unclear whether and how HBx regulates HER2 protein expression. As for the regulation of HER2 expression in tumors, several models have been proposed. The HER2 mRNA may be upregulated either by gene amplification or by promoter activation [17, 18]. On the other hand, regulations by RNA-binding protein HuR (also known as Elavl1) or by microRNAs have been reported to contribute to the stabilization of HER2 mRNA [19, 20]. Moreover, the stability of HER2 protein can also be enhanced at posttranslational level [21, 22].

In this study, we demonstrated that HBx increased HER2 protein expression via enhancing its mRNA stability. The induction of HuR expression by HBx contributed to the elevation of HER2 expression, which subsequently rendered HCC cells more metastatic. Our data provided the plausible molecular mechanism of HER2 upregulation by HBx in HBV-associated HCC tumors.

2. Materials and Methods

2.1. Cell Culture and Reagents. The human hepatocellular carcinoma Hep3B, HepG2, and their HBx-expressing derivatives were cultured and maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum. We purchased antibodies against HER2, EGFR, and HuR as well as bortezomib from Santa Cruz (Santa Cruz, CA). The antibody against HBx was from either Abcam (Cambridge, UK) or GeneTex (Irvine, CA). The antibodies against myc-tag and Tubulin, MG132, Actinomycin D as well as the validated siRNAs for negative control, HBx, HER2, and HuR were all purchased from Sigma-Aldrich (St. Louis, MO). Transfection reagents of DharmaFECT1 and TransIT-2020 were from Dharmacon (Lafayette, CO) and Mirus Bio LLC (Madison, WI), respectively. The QuickGene RNA cultured cell kit was from Kurabo (Osaka, JP). The RevertAid H Minus First Strand cDNA synthesis kit was purchased from Thermo Fisher Scientific (Waltham, MA). The VeriQuest Fast SYBR Green qPCR Master Mix was from Affymetrix (Cleveland, OH). Transwell chambers (24-well insert; pore size, 8 μm) were purchased from Costar Corp. (Cambridge, MA).

2.2. Transfection Assay. For plasmid transfection, cells with 60–80% of confluence in a 3.5 cm dish were transfected with 1 μg of myc-HBx expression vector by using 1 μL of TransIT-2020 transfection reagent according to the manufacturer's instruction. Forty-eight hours later, whole cells lysates or mRNAs were harvested and subjected to indicated experiments. For siRNA transfection, cells with 60–80% of confluence in a 3.5 cm dish were transfected with siRNA at final concentration of 100 nM by using 3 μL of DharmaFECT 1 or 1 μL of TransIT-2020 transfection reagent according to the manufacturer's instruction. After 4 days, whole cells lysates or mRNAs were harvested and subjected to further experiments.

2.3. Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR). The QuickGene RNA cultured cell kit was used for total RNA extraction and the procedure was performed according to manufacturer's instruction. One μg of RNA was applied to reverse transcription by using the RevertAid H Minus First Strand cDNA synthesis kit. The qPCR analysis of HER2 mRNA expressions was performed on Illumina Eco system (Bio-genesis Technologies Inc.) by using VeriQuest Fast SYBR Green qPCR Master Mix and was normalized to actin expression. Student's t-test was used to assess the statistical significance.

2.4. mRNA Stability Assay. For examination of mRNA stability, cells were first treated with 5 μM Actinomycin D, followed by extraction of total RNA at indicated time point. The extracted RNA was subjected to RT-qPCR and HER2 mRNA stability was in turn determined and quantified. Student's t-test was used to assess the statistical significance.

2.5. Cell Growth Assay. Cell growth was measured by crystal violet staining assay. Cells with previous treatment were seeded in a density of 1×10^4 and 5×10^4 in each group and allowed to grow. Five days later, relative cell amounts were determined by crystal violet staining. Briefly, cells were washed with 1X PBS once, followed by fixation and staining with 1% crystal violet in a solvent of 30% ethanol for 15–30 minutes at room temperature. Then, cells were washed with tape water till complete elimination of the background interference.

2.6. Cell Migration Assay. Cell migration ability was determined by Transwell migration assay with using Transwell chambers. Cells (5×10^4 per well) with previous experimental conditions were seeded on the noncoated membrane of the upper chamber [23]. After 48-hour incubation, cells were washed with 1X PBS once and fixed by 4% formaldehyde for 30 minutes. Then, cells were washed with 1X PBS once again and stained with 1% crystal violet in a solvent of 30% ethanol for 15–30 minutes at room temperature. Cells remaining on

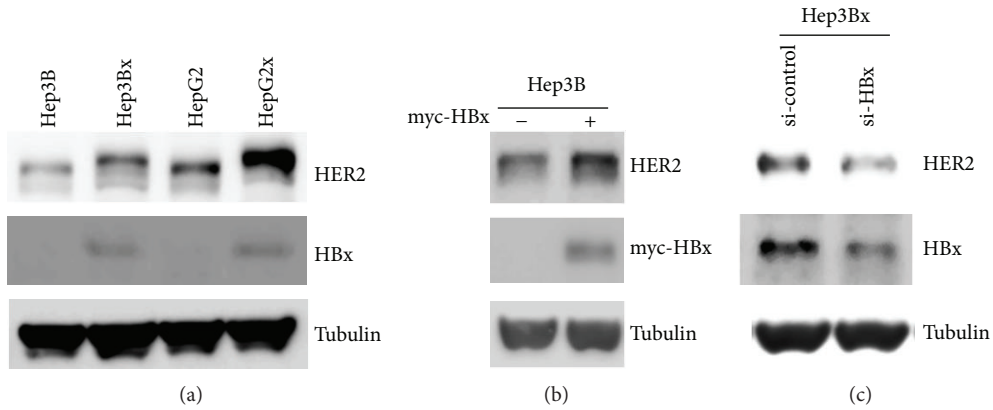

Figure 1: HBx induced HER2 protein expression in HCC cells. (a) The protein expressions of HER2, HBx, and Tubulin in two HBx-paired HCC cells were examined by Western blot ($N = 4$). (b) Myc-HBx expression vector was transiently transfected into Hep3B HCC cells for 48 hrs. The HER2 and myc-HBx protein expressions were analyzed by Western blot ($N = 3$). (c) Transient transfection of HBx siRNA was performed in Hep3Bx cells for 4 days. The HER2 protein expression and gene silencing of HBx protein expression were examined by Western blot ($N = 3$).

the upper chamber were removed by using cotton swab. The number of cells migrating through the pores to the opposite side of the membrane was shown under microscope. Then, the membrane stained by crystal violet was torn out and dissolved in 33% glacial acetic acid overnight. The migrated cell number was quantified by determining the absorbance of OD570. Student's t-test was used to assess the statistical significance.

3. Results

3.1. HBx Expression Was Responsible for the Increase of HER2 Protein Level in HCC Cells. To study the association between HBx and HER2 expressions in HCC, two HCC cell lines and their derivatives with stable HBx expression were employed to examine the expression patterns of HER2 in these cells. Consistent with the previous observation in human HCC specimens [16], HER2 protein expression was higher in HBx-expressing Hep3Bx and HepG2x HCC cells than in their Hep3B and HepG2 counterparts (Figure 1(a)). To further demonstrate that the increase in HER2 protein level was caused by HBx expression, the effect of HBx overexpression and gene silence on HER2 expression was examined. We found that enforced expression of HBx into Hep3B cells resulted in the significant increase of endogenous HER2 protein expression (Figure 1(b)). On the contrary, the endogenous HER2 protein expression in Hep3Bx cells was decreased when the HBx expression was silenced (Figure 1(c)). Taken together, these results suggest that HBx expression is responsible for the increase of HER2 protein level in HCC cells.

3.2. HBx Increased HER2 Protein Expression by Stabilizing HER2 mRNA in HCC Cells. Next, we explored how HBx regulates HER2 expression. Since it has been reported that prolyl isomerase Pin1 is able to maintain the protein stability of HER2 by attenuating its ubiquitin-dependent degradation [24], we first examined whether HBx increased HER2 expression through regulation of its protein stability. To this end, proteasomal inhibitors, including MG132 and bortezomib, were used. As shown in Figure 2(a), HER2 protein expression was not changed in response to treatments with DMSO or proteasomal inhibitors in both parental (Hep3B and HepG2) and HBx-expressing HCC (Hep3Bx and HepG2x) cells, indicating that HBx does not regulate HER2 expression at posttranslational level. We further examined the HER2 mRNA level in these two pairs of HCC cells. We observed that the mRNA expression of HER2 was higher in both Hep3Bx and HepG2x cells as compared to their respective parental cells (Figure 2(b)). It is known that both synthesis and stability contribute to the mRNA expression. We thus clarified whether HBx affects the mRNA stability of HER2 in HCC cells. HepG2 and HepG2x cells were treated with a transcriptional inhibitor Actinomycin D to block the mRNA biosynthesis, and then HER2 mRNA levels were examined after 3 and 6 hrs of treatments. Interestingly, the HER2 mRNA in HepG2x cells was more stable than in HepG2 counterpart (Figure 2(c)), implying that HBx may stabilize the mRNA expression of HER2. To confirm this hypothesis, HCC cells were enforced with HBx expression, followed by Actinomycin D treatment. As shown in Figure 2(d), when HBx was expressed in Hep3B cells (lower panel), HER2 mRNA was present in a more stable state (upper panel). Altogether, these results suggest that HBx increases HER2 protein expression by stabilizing its mRNA in HCC cells.

3.3. HBx Upregulated RNA-Binding Protein HuR to Increase HER2 mRNA Stability in HCC Cells. Next, the molecular mechanism underlying the regulation of HER2 mRNA stability by HBx was further pursued. It is well known that HuR is a ubiquitously expressed RNA-binding protein and is responsible for the mRNA stabilization of many genes, including HER2 and cyclooxygenase-2 (COX2) [25, 26]. Furthermore, it is reported to be involved in the human

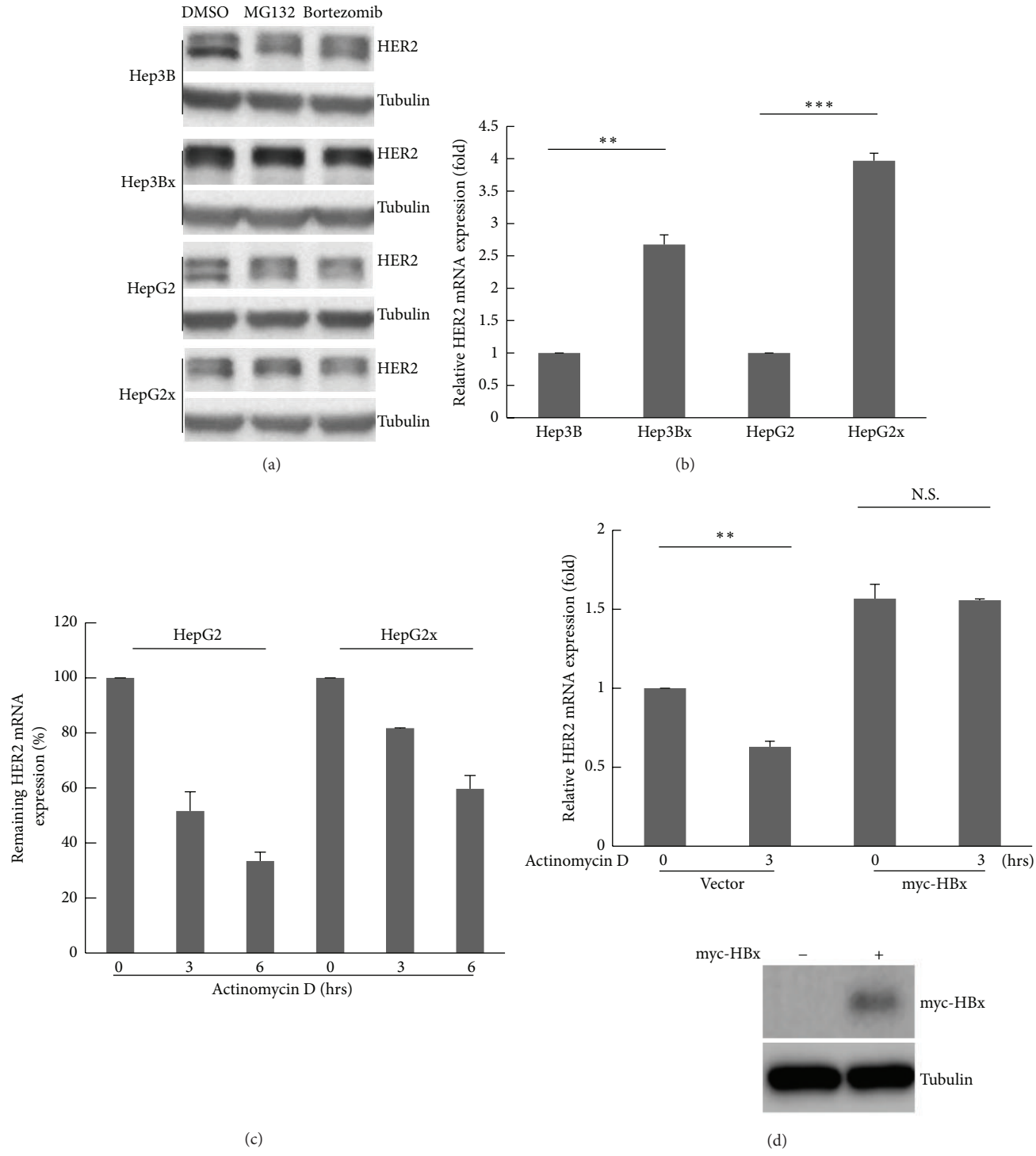

Figure 2: The HER2 mRNA expression was stabilized in HBx-expressing HCC cells. (a) The two HBx-paired HCC cells were treated with proteasomal inhibitors (MG132 and bortezomib) for 24 hrs. The HER2 protein expression was analyzed by Western blot ($N = 3$). (b) The HER2 mRNA expression in two HBx-paired HCC cells was examined by RT-qPCR. The HER2 mRNA expression was normalized to actin expression. Statistical analysis was performed by Student's t-test. $^{**}P < 0.01$; $^{***}P < 0.001$ as compared to each control group ($N = 3$). (c) The HepG2 and HepG2x HCC cells were treated with 5 μM Actinomycin D for indicated time periods. The relative remaining HER2 mRNA expression in each of the HCC cells was determined by RT-qPCR. The HER2 mRNA expression was normalized to actin expression ($N = 4$). (d) Hep3B HCC cells were transiently transfected with myc-HBx expression vector for 48 hrs, followed by treatment of 5 μM Actinomycin D. The relative remaining HER2 mRNA expression in each group was determined by RT-qPCR. The HER2 mRNA expression was normalized to actin expression. The protein expression of myc-HBx was confirmed by Western blot. Statistical analysis was performed by Student's t-test. $^{**}P < 0.01$ as compared to each control group. N.S. denoted "not significant" ($N = 4$).

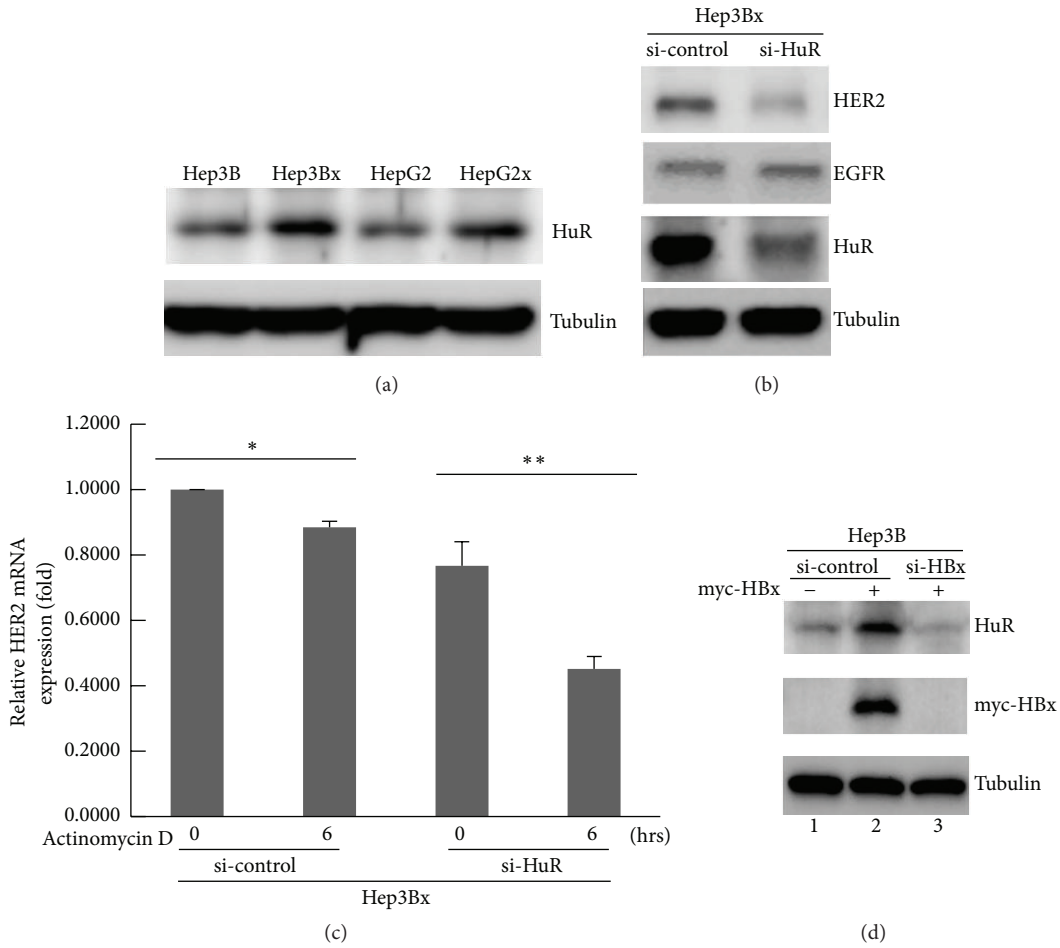

Figure 3: HBx increased HER2 protein expression by HuR-dependent mRNA stabilization in HCC cells. (a) The HuR protein expression in HBx-paired HCC cells was examined by Western blot ($N = 4$). (b) Hep3Bx cells were transiently transfected with either si-control or si-HuR for 4 days. The protein expressions of HER2, EGFR, and HuR were analyzed by Western blot ($N = 3$). (c) Hep3Bx cells were transiently transfected with either si-control or si-HuR for 4 days, followed by the treatment of 5 μM Actinomycin D. The relative remaining HER2 mRNA expression in each group was determined by RT-qPCR. The HER2 mRNA expression was normalized to actin expression. Statistical analysis was performed by Student's t-test. $^{*}P < 0.05$; $^{**}P < 0.01$ as compared to each control group ($N = 3$). (d) Transient transfection of HBx siRNA was performed in Hep3B cells for 48 hrs, followed by overexpression of myc-HBx expression vector for another 48 hrs. Whole cell lysates were harvested for the examination of HuR and myc-HBx protein expressions by Western blot ($N = 4$).

diseases, including cancers [19, 27]. Here, we investigated whether HuR plays a role in the upregulation of HER2 expression by HBx. The results showed that HuR protein expression was significantly increased in Hep3Bx cells as compared to the Hep3B counterpart. Similar result was also observed in HepG2 and HepG2x cells (Figure 3(a)). Accordingly, HuR siRNA was used to further address whether the upregulated HuR protein expression contributes to the stabilization of HER2 mRNA expression by HBx. As shown in Figure 3(b), when HuR protein expression in Hep3Bx cells was silenced by HuR siRNA, HER2 but not EGFR (epidermal growth factor receptor) protein expression was decreased in parallel, suggesting that the HuR-mediated regulation by HBx is specific for HER2 expression. In consistence with this result, the HER2 mRNA in Hep3Bx cells was degraded faster when cells were transfected with HuR siRNA (Figure 3(c)), indicating that HuR plays a critical role in the stabilization of HER2 mRNA expression. To establish the causal relationship between HuR-mediated mRNA stabilization and HBx-enhanced HER2 expression, the effects of both overexpression and silencing of HBx on HuR expression were examined. We observed that HuR protein expression was significantly induced in Hep3B cells in response to myc-HBx enforced expression (Figure 3(d), compared *lane 2* with *lane 1*). However, the upregulated HuR protein expression was further attenuated when myc-HBx expression was depleted by siRNA (Figure 3(d), compared *lane 3* with *lane 2*). Collectively, these results indicate that HBx protein upregulates HuR expression to stabilize HER2 mRNA, in turn leading to the increase of HER2 protein expression.

3.4. The Enhanced Migration Ability of HBx-Expressing HCC Cells Was Attributed to Upregulated HER2 Expression. We next investigated the functional roles of increased HER2

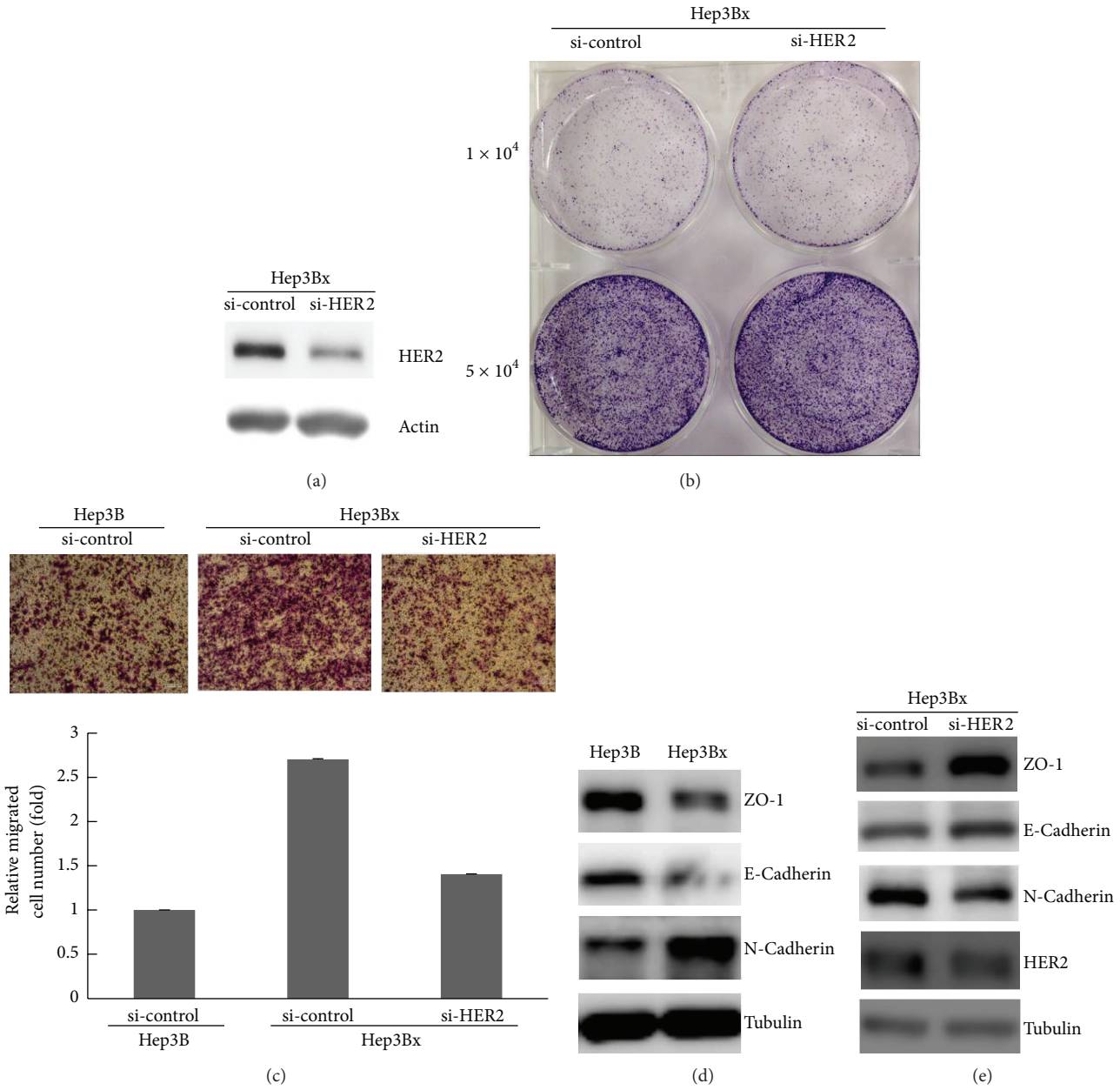

Figure 4: The increased HER2 protein expression was responsible for the migration ability of HBx-expressing HCC cells. ((a), (b), (c), and (e)) Hep3Bx cells were transiently transfected with either si-control or si-HER2 for 4 days. Then, cells were either harvested or reseeded for further experiments. Gene silence of HER2 expression was confirmed by Western blot (a) ($N = 3$). The relative growth rate was determined by crystal violet staining (b) ($N = 3$). The migration of Hep3Bx cells was examined by Transwell migration assay for 48 hrs. The representative pictures of migrated cells were visualized and quantified (c) ($N = 3$). The expressions of metastatic factors were examined by Western blot (e) ($N = 3$). (d) The expressions of metastatic factors in both Hep3B and Hep3Bx cells were examined by Western blot ($N = 3$).

protein level in response to HBx expression. Since HER2 is an important oncogene in regulating tumor progression, including tumor growth and metastasis [28], the effect of HBx-increased HER2 expression on cell growth was examined. To this end, deprivation of HER2 expression by siRNA was performed and confirmed (Figure 4(a)). Regardless of the cell density of seeding, silence of HER2 by siRNA did not significantly affect the cell number of HBx-expressing Hep3Bx cells (Figure 4(b)), implying that the cell growth of HBx-expressing cells was not driven mainly by HER2. Our previous study indicated that HBx expression renders HCC cells more metastatic in an Akt/nuclear IKK-α-dependent manner [23]. Since HER2 is also known to induce Akt activation and cell metastasis [29], we next investigated whether the increased HER2 expression mediated HBx-enhanced cell migration. As shown in Figure 4(c), the level of

cell migration of Hep3Bx cells was obviously less when HER2 expression was silenced by siRNA. To further support this observation, we examined whether this regulation involves an EMT process. It is known that EMT (epithelial-to-mesenchymal transition) is characterized by loss of ZO-1 and E-cadherin and increase of N-cadherin [30]. As shown in Figure 4(d), we found that the expressions of ZO-1 and E-cadherin were decreased whereas N-cadherin expression was increased in Hep3Bx cells, which is correlated to the increased migration ability of Hep3Bx cells. However, this effect was reversed when HER2 protein was silenced (Figure 4(e)). Taken together, these results suggest that HBx protein enhances the migration of HCC cells at least in part through increasing HER2 protein expression.

4. Discussion

Gene amplification of the pivotal oncogene HER2 is frequently observed in 20–30% of breast cancer patients and is associated with the disease aggressiveness and poor prognosis [31]. Therefore, HER2 is a rationale target for cancer therapy in those patients. Indeed, HER2 targeted therapies, including monoclonal antibody (trastuzumab) and tyrosine kinase inhibitor (lapatinib), bring promising benefits to breast cancer patients and prolong their overall survival [32–34]. Since there is no effective strategy for HCC therapy so far, many efforts are made to identify the potential oncogenic drivers in HCC and HER2 is one of such potential candidates. However, the results are controversial. Some reports show that HER2 overexpression is uncommon in HCC [35, 36]. In contrast, although HER2 gene amplification is less observed in HCC, several lines of evidence indicate that HER2 protein is overexpressed and plays roles in some HCC cases [37–40]. Notably, it is reported that upregulation of HER2 protein in HCC is found in HCC with HBx expression and is associated with poor prognosis of HCC patients [15, 16]. In consistence with these findings, our data also provided the evidence that HBx is indeed responsible for the upregulation of HER2 protein expression in this study (Figure 1). The investigation of molecular mechanism revealed that HBx increased HuR protein expression to stabilize the mRNA stability of HER2 (Figures 2-3). Furthermore, our unpublished results showed that HBx-expressing HCC cells exhibit higher level of Ser10 phosphorylation of histone H3, an indicator for the transcriptional activity, in HER2 promoter regions. Therefore, the possibility of HER2 promoter activation by HBx still cannot be excluded and needs further investigation. The increased HER2 protein expression rendered HBx-expressing HCC cells more metastatic without affecting their cell growth rate (Figure 4), which may provide a plausible explanation for the poor prognosis of HCC patients with HBx expression [16]. Based on these studies, upregulation of HER2 protein was observed in HCC with HBV infection, especially with detectable HBx expression. Therefore, targeting HER2 in such subgroup of HCC patients may be an appropriate and effective strategy for HCC therapy, which awaits further studies to approve.

In our previous study, HBx-expressing HCC cells were shown to have higher migration ability. The nuclear translocation of IKK-α by Akt-dependent ubiquitination to mediate gene expression accounts for the underlying molecular mechanism [23, 41]. HER2 has also been found to increase IKK-α nuclear translocation in our previous study [23]. Therefore, it is possible that HBx-increased HER2 expression may enhance cell migration of HCC cells via increasing Akt activity and subsequent nuclear translocation of IKK-α. In addition to IKK-α, HBx could activate IKK-β/TSC-1/mTOR signaling to enhance HCC progression [42]. Furthermore, it is also reported that HBx increases β-catenin expression through ERK-dependent GSK-3β inactivation [43]. Therefore, further investigations are required to examine whether HER2 also regulates the HBx-dependent HCC progression through these pathways.

In this study, we identified RNA-binding protein HuR as a new target of HBx and also uncovered another pleiotropic role of HBx in the mRNA stabilization (Figures 2-3). The mechanism underlying HuR regulation by HBx is still largely unknown, which needs further studies for clarification. In fact, growing evidence recently indicates that HBx could regulate gene expressions in a microRNA-dependent manner [13, 44–46]. It is known that microRNAs inhibit protein translation by targeting on the 3′ untranslated region (3′UTR) of mRNA. It seems that HBx could simultaneously regulate expressions of both HuR and microRNAs with opposing functions. It will be interesting to investigate how HBx fine-tunes the gene expression by integrating the effects of HuR and microRNAs [47, 48]. It is worth mentioning that our previous report indicates that HBx downregulates EGFR (also known as (HER1/ErbB1) expression in a miR-7-dependent manner [45], whereas the current study shows that HBx upregulates HER2 expression in a HuR-dependent manner. The possibility that HBx exerts such effects to render HCC cells more addicted to HER2 signaling is being investigated.

5. Conclusion

This study provides the evidence that HBx protein upregulates HER2 expression through HuR-dependent mRNA stabilization. Thus, HBx-expressing HCC cells exhibit the higher migration ability. Our findings not only clarified the mechanism underlying the HER2 upregulation by HBx, but also demonstrated HuR as a novel target of HBx, implying another pleiotropic function of HBx in the regulation of mRNA stability.

List of Abbreviations

HBV: Hepatitis B virus
HCC: Hepatocellular carcinoma
HBx: HBV-encoded X protein
EGFR: Epidermal growth factor receptor
RTKs: Receptor tyrosine kinases
miR/miRNA: MicroRNA
COX-2: Cyclooxygenase-2.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Chao-Ming Hung and Wei-Chien Huang contributed equally to this work.

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Research Article

Trichostatin A Suppresses EGFR Expression through Induction of MicroRNA-7 in an HDAC-Independent Manner in Lapatinib-Treated Cells

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Lapatinib, a dual EGFR/HER2 tyrosine kinase inhibitor, has been shown to improve the survival rate of patients with advanced HER2-positive breast cancers. However, the off-target activity of lapatinib in inducing EGFR expression without tyrosine kinase activity was demonstrated to render HER2-negative breast cancer cells more metastatic, suggesting a limitation to the therapeutic effectiveness of this dual inhibitor in HER2-heterogeneous tumors. Therefore, targeting EGFR expression may be a feasible approach to improve the anticancer efficiency of lapatinib-based therapy. Inhibition of HDAC has been previously reported to epigenetically suppress EGFR protein expression. In this study, however, our data indicated that treatment with HDAC inhibitors trichostatin A (TSA), but not suberoylanilide hydroxamic acid (SAHA) or HDAC siRNA, can attenuate both protein and mRNA expressions of EGFR in lapatinib-treated triple-negative breast cancer cells, suggesting that TSA may suppress EGFR expression independently of HDAC inhibition. Nevertheless, TSA reduced EGFR 3′UTR activity and induced the gene expression of microRNA-7, a known EGFR-targeting microRNA. Furthermore, treatment with microRNA-7 inhibitor attenuated TSA-mediated EGFR suppression. These results suggest that TSA induced microRNA-7 expression to downregulate EGFR expression in an HDAC-independent manner.

1. Introduction

Amplification and overexpression of HER2 (also named ErbB2) receptor tyrosine kinase, detected in 20–30% of breast cancer, are associated with a poor clinical patient outcome, including lymph node metastasis, shorter survival, and shorter time to recurrence [1, 2]. Activation of HER2 initiates a cascade of signal transduction, including PI3K/Akt and MAPK pathways, to mediate cell growth and survival [3]. The dysregulation of these signal pathways from the overexpressed HER2 elicits multiple gene transcriptions associated with neoplastic transformation, initiation, cellular immortalization, and tumor progression [4]. Thus, targeting the tyrosine kinase activity of this receptor is viewed as promising therapeutic strategy to treat breast cancer patients with HER2 overexpression [3, 5].

Lapatinib (Tykerb, GW-572016), a dual tyrosine kinase inhibitor of epidermal growth factor receptor (EGFR) and HER2 receptors, has been used for advanced HER2-positive breast cancer patients who failed to chemotherapy or HER2-targeted therapy with monoclonal antibody trastuzumab [6, 7]. Although the majority of clinical benefits from lapatinib-based treatment were observed in patients with HER2-positive breast cancers, there are still several clinical trials of lapatinib in HER2-negative patients due to its EGFR inhibition activity [8–16]. Expression of EGFR has been found in up to 80% of triple-negative (HER2/ER/PgR-negative) breast cancers, and targeting EGFR thus has also been viewed as a potential therapeutic strategy for such disease [17–20]. When used as a monotherapy or in combination with chemotherapies, the clinical benefits of lapatinib in triple-negative or HER2-negative breast cancers have been tested in phase II trials [21, 22]. However, no significant benefit derived from the addition of lapatinib to paclitaxel was found in overall HER2-negative diseases, and surprisingly a worse clinical outcome with shorter median even-free survival was even found in breast cancer patients with triple-negative or HER2-negative/PgR-negative tumors [14]. Our previous study further uncovered an off-target activity of lapatinib in promoting the aggressiveness of triple-negative cell lines to axillary lymph node and lung in orthotopic tumor-xenograft mice [23]. Elevation of EGFR through downregulation of microRNA-7 [24] has been demonstrated to contribute to the lapatinib-increased cell motility. Therefore, targeting EGFR protein expression would be an effective strategy to prevent the lapatinib-elicited cell metastasis.

Histone deacetylases (HDACs), which regulate gene transcriptions by removing the acetyl groups from lysine residues of histones or transcription factor proteins, were frequently overexpressed in a variety of cancer types [25]. Higher expression of several HDAC subtypes was associated with enhanced migration and invasion of breast cancer cells [26–28]. The prometastatic effects of HDACs are connected to the transcriptional regulation of EGFR [29]. By suppressing EGFR expression, HDAC inhibitors were also shown to possess antitumor [30] and antidiabetes-associated kidney growth [31] activities and to synergize the anticancer activity of EGFR tyrosine kinase inhibitor gefitinib [29]. But the molecular mechanisms of HDAC inhibitor-reduced EGFR expression remain largely unknown. Thus, these open questions prompted us to investigate whether and how HDAC inhibitors suppress the lapatinib-induced EGFR expression.

In this study, we unexpectedly found that HDAC inhibitor trichostatin A (TSA), but not suberoylanilide hydroxamic acid (SAHA), represses EGFR protein level independently of HDAC inhibition in the lapatinib-treated breast cancer cells. Regardless of its HDAC inhibition activity, TSA induced microRNA-7 to target EGFR 3′UTR. These results discovered an off-target activity of TSA in regulating microRNA expression.

2. Materials and Methods

2.1. Cell Lines, Constructs, Antibodies, and Reagents. Human breast cancer cell lines MDA-MB-231 and their derivatives were cultured in DMEM/F-12 with 10% fetal bovine serum. Lapatinib-selected cancer cells were established by selection with gradually increasing concentrations of lapatinib for over two months. Established resistant cancer cell lines were tested for their insensitivity to the corresponding drug and were cultured in the presence of 1 μM lapatinib. HDAC siRNA clones were purchased from Dharmacon. Cells were transfected with siRNA oligo (5′-GAUGCUGAACCAUGCACCUTT-3′) and (5′-CACCAUGCAGAUCAUUCAATT-3′) to target HDAC3 and HDAC7, respectively, or with nontargeting control siRNA (5′-UGGUUUACAUGUCGACUAA-3′) with DharmaFECT 1 (Dharmacon) for 72 hrs for further experiments. Anti-EGFR (SC-03), anti-HDAC3, anti-HDAC7, and antiactin antibodies from Santa Cruz were used for Western blot analysis. HDAC inhibitors (TSA and SAHA), proteasomal inhibitors, and lysosomal inhibitors were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Western Blot Analysis. Total cell lysates were prepared and subjected to SDS-PAGE using 7.5% running gels. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, which was then incubated at room temperature for 1 h with 0.1% milk in TTBS (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.05% Tween-20), for 1 h with specific primary antibodies and for 30 min with HRP-labeled anti-rabbit antibody. After each incubation, the membrane was washed extensively with TTBS. The immunoreactive bands are detected using ECL detection reagent and Hyperfilm ECL (Amersham International).

2.3. RNA Isolation, Reverse Transcription (RT), and Real-Time Polymerase Chain Reaction (PCR). Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions as described previously [32]. For microRNA, each RT reaction contained 2 μg of RNA, 50 nmol/L of the stem-loop RT primer, 0.25 mmol/L of each deoxynucleotide triphosphate, 50 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen), 1 × RT buffer, 10 mmol/L DTT, and 4 units of RNase inhibitor. The stem-loop RT primer for hsa-miR-7 was designed according to mature miRNA sequence (Sanger Center miRNA Registry, http://www.mirbase.org/). The sequences of the RT primers

are as follows: hsa-miR-7 RT primer, 5′-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACACAACA-3′; U48 RT primer, 5′-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACTCAGCG-3′. Real-time PCR reaction contained 0.5 μmol/L of each forward and reverse primer, 0.1 μmol/L of the Universal ProbeLibrary Probe #21 (Roche), the 1 × LightCycler TaqMan Master, and 2 μL of cDNA using a Roche LightCycler 480 Real-Time PCR system. U48 small nuclear RNA was used as an internal control. The sequences of the forward primers were as follows: hsa-miR-7, 5′-GCGGCGTGGAAGACTAGTGAT-3′; U48, 5′-CGGCGGTAACTCTGAGTGTGT-3′. The reverse primer for all of the above sets of genes was 5′-GTGCAGGGTCCGAGGT-3′. For *EGFR* and *Actin* mRNA, 1 μg of total RNA was subjected to RT with an oligo-dT primer using a reverse transcriptase kit (Invitrogen). Equal amounts of cDNA (2 μL) were subjected to PCR and amplified with 30 cycles using the following primers: *EGFR*, forward 5′-GTTGATATCATGCGACCCTCCGGGACG-3′ and reverse 5′-GGTTCTAGATCATGCTCCAATAAATTC-3′; HDAC3, forward 5′-ATGAAGTCGGGGCAGAGAGTG-3′ and reverse 5′-CACAATGCACGTGGGTTGG-3′; HDAC7, forward 5′-TCTGTCCCGGGCTCAGTCTT-3′; *Actin*, forward 5′-CTGGAACGGTGAAGGTGACA-3′ and reverse 5′-AAGGGACTTCCTGTAACAATGCA-3′. The PCR products were subjected to 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining. Real-time PCR reactions containing 0.3 μL of cDNA, 0.3 μL of the forward and reverse primers, 5 μL of 2X SYBR Green (Roche), and 1.4 μL of distilled water were performed with a Roche LightCycler 480 Real-Time PCR system.

2.4. Histone Deacetylase Activity Assay. The assay was performed according to the manufactures instruction (Enzo Life Sciences, Farmingdale, NY, USA). Nuclear extracts were prepared and subjected to immunoprecipitation with specific HDAC antibodies. A reaction of substrate deacetylation was initiated by mixing fluorescence-labeled acetylated peptide with HDAC-containing nuclear extract or immunoprecipitates within a set time period. The developer was then added to the reaction to cleave the resultant deacetylated fluorescence-labeled peptide and to stop HDAC activity, resulting in the production of the chemiluminescent compound. The enhancer was then added to make the visualization of the chemiluminescent product.

2.5. Transfection and Reporter Gene Assay. The luciferase reporter gene containing full-length 3′ untranslated region (UTR) of human *EGFR* gene was a gift from Dr. Keith Giles (Western Australian Institute for Medical Research). Cells with 60–80% of confluence were transfected with 0.5 μg of EGFR-3′UTR luciferase plasmid by using TransIT-2020 transfection reagent according to the manufacturer's instruction. After 24 hrs of transfection, cells were treated with TSA for another 24 hrs and total lysates were harvested and subjected to luciferase activity assays. Luciferase activity was normalized to β-gal. For siRNA/microRNA transfection, cells with 60–80% of confluence were transfected with various siRNA/microRNA by using DharmaFECT 1 transfection reagent. Cells were harvested at indicated time points and subjected to further experiment.

2.6. Statistical Analysis. In vitro experiments are repeated thrice and statistical analysis is done using Student's t-test. Data are presented as mean ± SE. A probability level of a P value of <0.05 is considered significant.

3. Results

3.1. TSA Suppressed EGFR Expression Independently of HDAC Inhibition. Our previous study showed that chronic treatment of triple-negative MDA-MB-231 breast cancer cells with lapatinib dramatically increased EGFR expression, which contributed to cell migration and invasion [23]. It led us to further study whether HDAC inhibitors possess the suppressive effect on EGFR expression in the lapatinib-treated MDA-MB-231 (231/Lap#6) cells. The IC50 of TSA or SAHA for HDAC inhibition is 0.01-0.02 μM [33, 34], and treatments with TSA and SAHA at 1 μM can completely abolish the total HDAC activity (Figure 1(a)). Treatment of both parental and 231/Lap#6 cells with pan-HDAC inhibitor TSA up to 1 μM for 24 hours dramatically inhibited EGFR expression in a dose-dependent manner (Figure 1(b)). However, treatment with another pan-HDAC inhibitor SAHA up to 1 μM did not affect the EGFR expression (Figure 1(c)). Moreover, treatment with TSA but not SAHA also time-dependently suppressed EGFR expression in 231/Lap#6 cells (Figure 1(d)). In parallel to the protein level, the mRNA level of EGFR in 231/Lap#6 cells was also reduced by treatment with TSA but not SAHA for 24 hours (Figure 1(e)). Since TSA and SAHA at 1 μM showed the different effects on EGFR expression even though both of them can completely suppress the total HDAC activity at the same concentration, we next addressed whether inhibition of HDAC is involved in the suppression of EGFR by TSA in the lapatinib-treated cells.

In response to lapatinib treatment, the acetylations of histone H2B at K5 and H3 at K9 were suppressed in various lapatinib-treated clones of MDA-MB-231 cells (Figure 2(a)). In parallel to the downregulation of histone acetylation, our data also showed that the protein levels of HDAC3 and HDAC7 but not HDAC1, HDAC2, HDAC4, and HDAC5 were significantly increased in 231/Lap#6 cells (Figure 2(b)). Furthermore, silence of both HDAC3 and HDAC7 by siRNA can significantly restore the acetylation of histone H3 K9 (Figure 2(c)). These results suggest that the elevated HDAC3 and HDAC7 play a major role in lapatinib-mediated histone hypoacetylation at these residues. Therefore, we examine the regulation of EGFR expression by HDAC with focus on the elevated HDAC3 and HDAC7. However, silence of HDAC3 or HDAC7 did not affect the protein (Figures 3(a) and 3(b)) and mRNA (Figure 3(c)) levels of EGFR in 231/Lap#6 cells. Similar to SAHA, however, siRNA-mediated silence of HDAC3, which has been reported to contribute to EGFR transcription, did not change EGFR protein (Figure 3(a)) and mRNA (Figure 3(c)) expressions in 231/Lap#6 cells. Silence of HDAC7, a member of class IIa HDAC, also did not affect

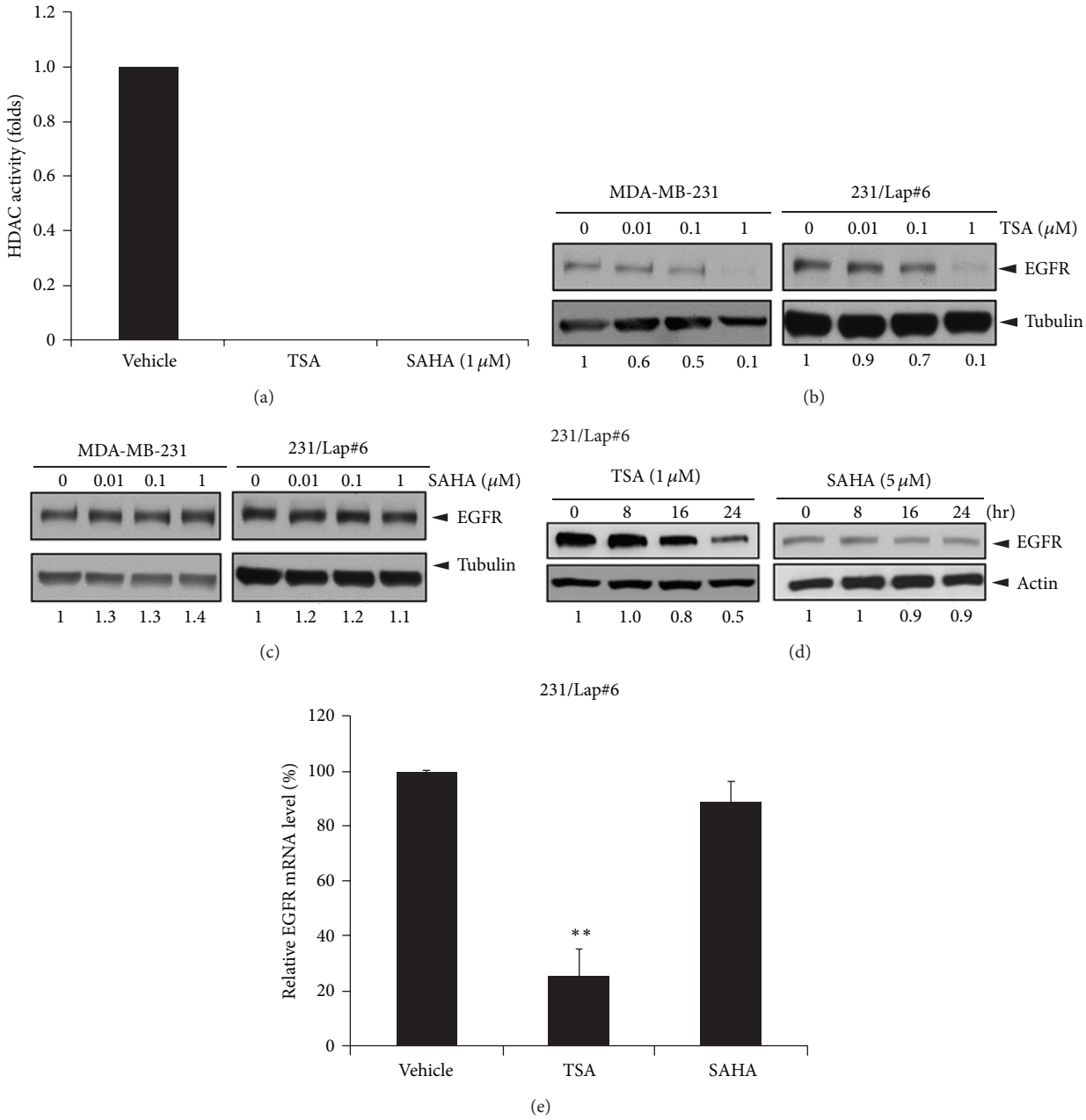

Figure 1: TSA but not SAHA suppressed lapatinib-induced EGFR expression. (a) Nuclear extract of HeLa cells was added with 1 mM TSA or SAHA for 30 min and then subjected to HDAC activity assays. (b) and (c) MDA-MB-231 and 231/Lap#6 cells were treated with indicated concentration of TSA (b) or SAHA (c) for 24 hours. Total lysates were prepared and subjected to Western blot analysis with indicated antibodies. (d) 231/Lap#6 cells were treated with 1 μM TSA or 5 μM SAHA for 8, 16, or 24 hours. Total lysates extracted from these cells were subjected to Western blot analysis with anti-EGFR and anti-actin antibodies. (e) 231/Lap#6 cells were treated with 1 μM TSA or 5 μM SAHA for 24 hours. Total RNA extracted from these cells was subjected to RT-qPCR with EGFR-specific primers. The induction of EGFR mRNA was normalized to GAPDH expression.

the protein and mRNA levels of EGFR (Figures 3(b) and 3(c)). To further confirm that these HDAC isoforms were not involved in the TSA-mediated EGFR suppression, HDAC3 and HDAC7 were ectopically overexpressed followed by treatment with TSA. Treatment of 231/Lap#6 cells with 1 μM TSA suppressed the histone deacetylase activities of HDAC3 and HDAC7 (Figure 3(d)), but overexpression of myc-HDAC3 (Figure 3(e)) or myc-HDAC7 (Figure 3(f)) still did not restore the TSA-mediated EGFR suppression in 231/Lap#6 cells. These results suggest that TSA suppressed EGFR expression through an HDAC3/7-independent manner in 231/Lap#6 cells regardless of the changes in HDAC protein expression and histone acetylation in response to lapatinib.

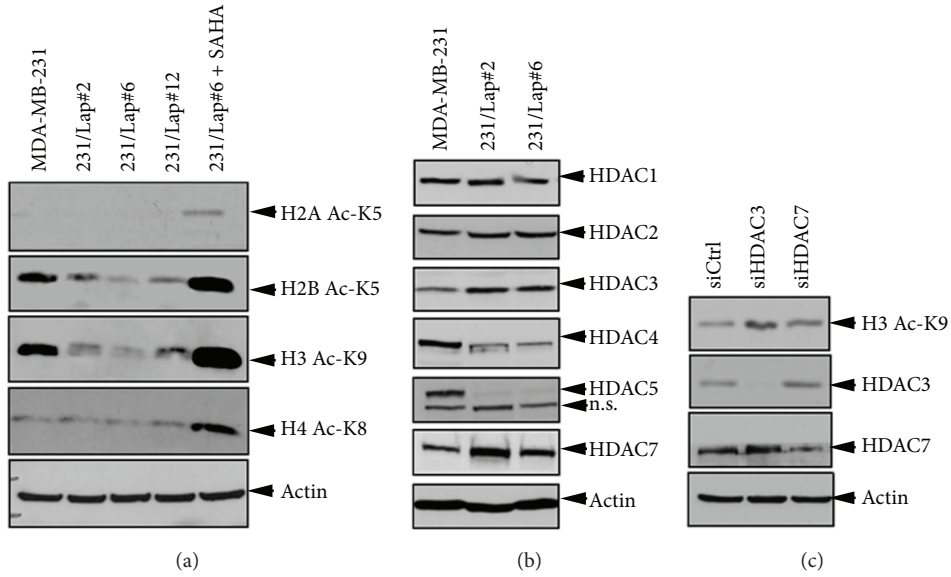

Figure 2: HDAC3 and HDAC7 expressions were elevated to cause histone hypoacetylations in lapatinib-treated MDA-MB-231 cell. (a) and (b) Total lysates from parental and various lapatinib-treated MDA-MB-231 cells were subjected to Western blot analysis with indicated antibodies. (c) Total lysates from 231/Lap#6 cells transfected with specific HDAC3 or HDAC7 siRNA for 72 hours were subjected to Western blot analysis.

3.2. TSA-Induced EGFR Suppression Did Not Involve Proteasomal and Lysosomal Degradation. Treatment with HDAC inhibitors has been shown to induce ubiquitination of proteasomal degradation of erbB family to potentiate the antitumor activity of EGFR tyrosine kinase inhibitor in head and neck squamous tumors [29]. To test whether TSA suppressed EGFR expression in a proteasomal pathway, 231/Lap#6 cells were pretreated with proteasome inhibitors MG132, PSI, and lactacystin. However, the protein level of EGFR remains suppressed by TSA in the presence of these proteasomal inhibitors (Figure 4(a)), indicating that proteasomal degradation was not involved in the TSA-induced EGFR turnover. HDAC6, a cytoplasmic lysine deacetylase, was found to negatively regulate EGFR endocytosis and degradation by controlling the acetylation status of α-tubulin and subsequent EGFR trafficking along microtubules [35]. Therefore, loss of the microtubule-associated HDAC6 activity resulted in the EGFR lysosomal degradation through accelerating EGFR segregation from early endosomes to late endosomal and lysosomal compartments [35]. Next, we examined whether HDAC6 inhibition and proteasomal degradation are involved in TSA-mediated EGFR suppression. Both treatments with TSA or SAHA induced tubulin acetylation (Figure 4(b)), supporting their inhibitory effect on HDAC6. However, pretreatment with two lysosomal inhibitors, NH_4Cl and chloroquine (CQ), still cannot prevent the TSA-mediated EGFR downregulation (Figure 4(c)), ruling out the possibility that TSA decreases EGFR expression through HDAC6-dependent lysosomal degradation.

3.3. TSA Attenuated EGFR Expression through Induction of miR-7 Expression. MicroRNAs (miRNAs), a class of endogenous 17–24 base-long single-stranded, noncoding RNAs, widely regulate gene expression via targeting the 3′ untranslated region (UTR) in a sequence-specific manner. MicroRNA-7 (miR-7) has been reported to target the 3′UTR of *EGFR* mRNA and cause its degradation [36–38]. Our previous study also demonstrated that the elevation of EGFR expression is due to the downregulation of miR-7 in 231/Lap clones as compared with MDA-MB-231 cells. Thus, the possibility that TSA reduced EGFR expression in 231/Lap cells through induction of miR-7 was further addressed. To this end, the inhibitory effect of TSA on EGFR 3′UTR activity was examined in both parental and lapatinib-treated clones of MDA-MB-231 cells. In consistent with our previous results, the 3′UTR activity of EGFR was higher in 231/Lap#6 cells than in the parental cells (Figure 5(a)). Treatment with TSA 1 μM for 24 hours can significantly suppress the EGFR 3′UTR activity in both cells (Figure 5(a)). However, the EGFR 3′UTR activity in 231/Lap#6 cells was not suppressed by 1 μM SAHA (Figure 5(b)). Furthermore, the increase in miR-7 expression in both parental and lapatinib-treated MDA-MB-231 cells was also observed after treatment with TSA for 8 hours in quantitative RT-PCR assays (Figure 6(a)). However, the induction of miR-7 was not observed in SAHA-treated cells (Figure 6(b)). MicroRNA-7 is an intronic miRNA encoded in the host genes, including heterogeneous nuclear ribonucleoprotein K (HNRNPK) (for miR-7-1) and pituitary gland specific factor 1 (PGSF1) (for miR-7-3). Our data further showed that treatment with TSA dramatically induces the mRNA level of PGSF1 but not HNRNPK (Figure 6(c)), suggesting that TSA may induce miR-7-3 level through transcriptionally upregulating PGSF1 expression to target EGFR 3′UTR activity. Indeed, transfection of 231/Lap#6 cells with miR-7 inhibitor can dose-dependently reverse TSA-reduced EGFR expression (Figure 6(d)), demonstrating that TSA may induce miR-7 expression to target EGFR protein expression.

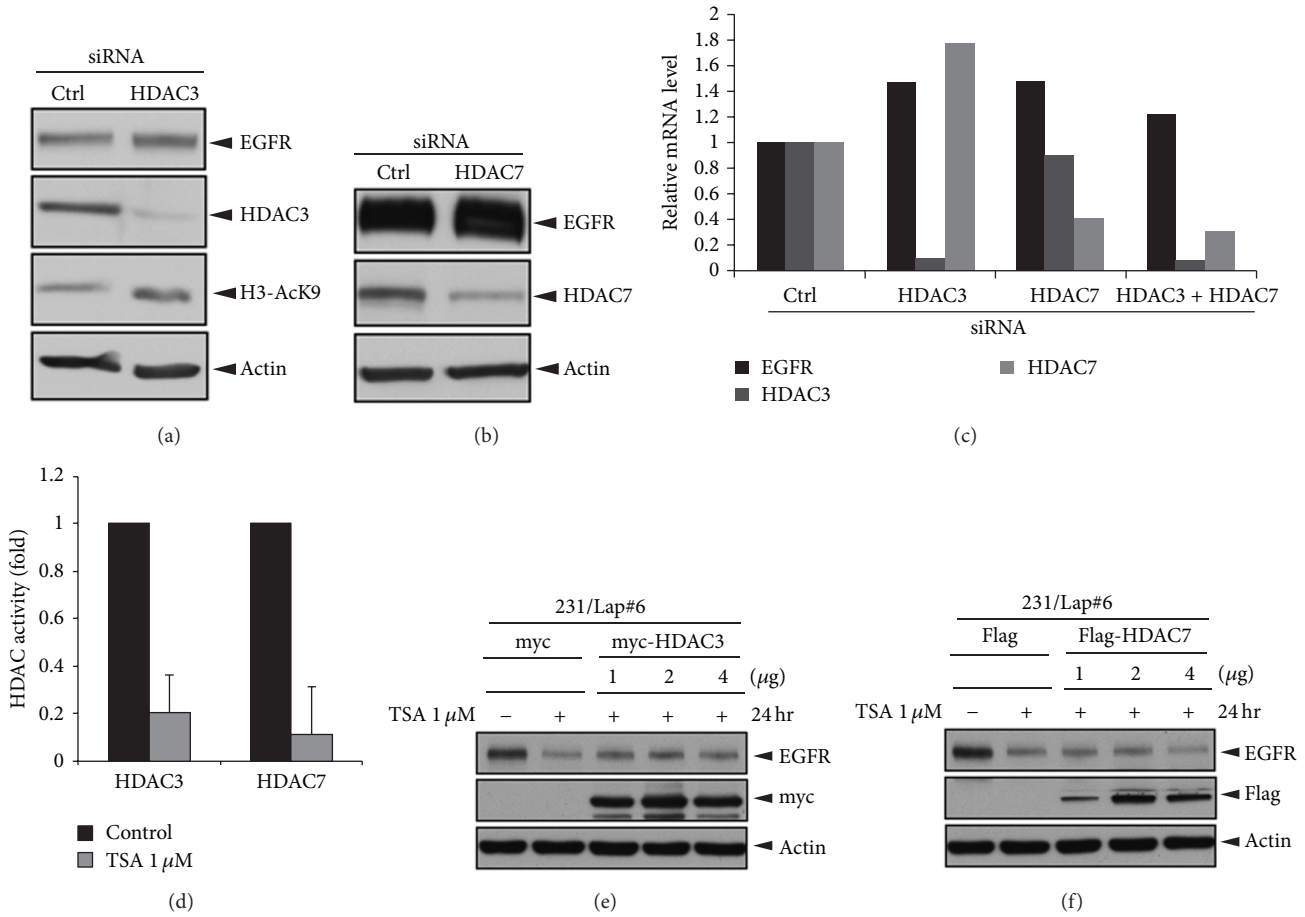

Figure 3: Silence of HDACs did not affect EGFR expression in lapatinib-treated MDA-MB-231 cells. (a)–(c) 231/Lap#6 cells were transfected with control siRNA or specific siRNA against HDAC3 ((a) and (c)) or HDAC7 ((b) and (c)) for 3 days. Total protein lysates prepared from these cells were subjected to Western blot analysis with indicated antibodies ((a) and (c)). Total RNA extracted from these cells was subjected to RT-qPCR. The relative mRNA levels of EGFR, HDAC3, and HDAC7 were normalized to GAPDH expression. (d) Total lysates of 231/Lap# cells transfected with myc-HDAC3 or myc-HDAC7 were subjected to immunoprecipitation with anti-myc antibody. The HDAC activities in the immunoprecipitates were measured in the HDAC activity assays. (e) and (f) 231/Lap#6 cells were transfected with increasing doses of myc-HDAC3 or myc-HDAC7 followed by treatment with TSA. Total lysates were prepared and subjected to Western blot.

4. Discussion

In addition to the promising efficacy in HER2-positive breast cancer, use of lapatinib in HER2-negative diseases, especially in triple-negative cancers due to its frequent EGFR overexpression, is of interest and being tested currently [21, 22] but has been found to elicit diverse effects in different subgroups [8, 14, 39, 40]. Our previous findings indicated that treatment with lapatinib enhanced EGFR protein level through downregulation of miR-7 without affecting EGFR promoter activity in MDA-MB-231 cells. The lapatinib-induced EGFR subsequently maintained the NF-κB-mediated COX-2 expression through HuR-dependent mRNA stabilization. These events rendered the triple-negative breast cancer cells showing more aggressive and higher metastasis rate to lymph node and lung [23]. These results provided a possible molecular mechanism explaining how addition of lapatinib to chemotherapy worsens the clinical outcome in breast cancer patients with triple-negative and HER2/PgR-negative tumors [14]. In this current study, we further explored that the pan-HDAC inhibitor TSA but not SAHA suppressed lapatinib-induced EGFR expression in an HDAC3/7-independent manner in 231/Lap cells.

HDACs are associated with the progression of cancer and have been demonstrated to mediate migration and invasion of cancer cells through different mechanisms [41–43]. Although the different roles of HDAC isoforms in the tumor development and progression of various cancer types have been characterized, the modulation of HDAC expression in response to anticancer treatments and their involvement in the metastatic relapse after the treatment is less understood. Our results showed that treatment with lapatinib increased HDAC3 and HDAC7 expressions accompanied with hypoacetylations of histone H3 K9 and histone H2B K5 (Figure 2). Suppression of these elevated HDACs by TSA and SAHA can reduce the motility of the lapatinib-treated cells (unpublished data). Despite their common effect on COX-2 suppression, TSA and SAHA were surprisingly found to have

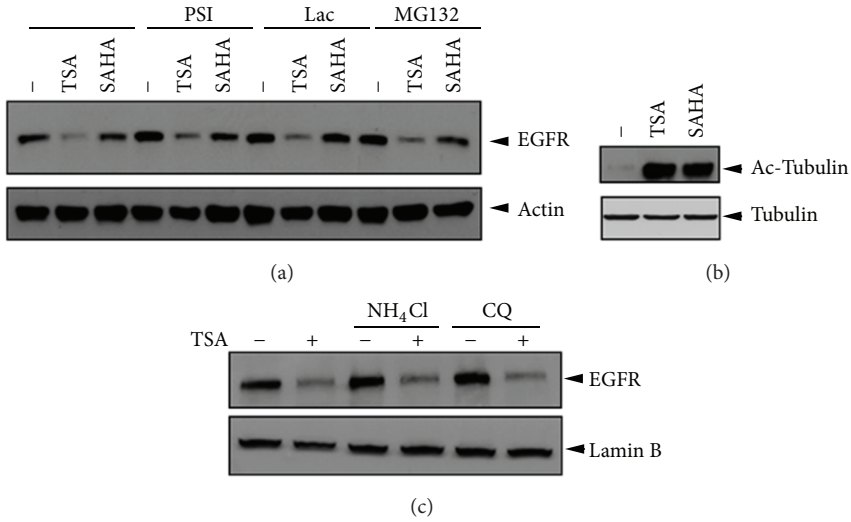

Figure 4: Proteasomal or lysosomal protein degradations were not involved in TSA-induced EGFR downregulation. 231/Lap#6 cells were pretreated with proteasomal inhibitor 5 μM PSI, 5 μM lac, and 1 μM MG132 for 2 hrs (a) or with lysosomal inhibitor 1 μM NH_4Cl and 25 μM CQ for 2 hrs (b) followed by treatment with 1 μM TSA or 5 μM SAHA for 24 hours. Total lysates were then extracted and subjected to Western blot analysis with indicated antibodies.

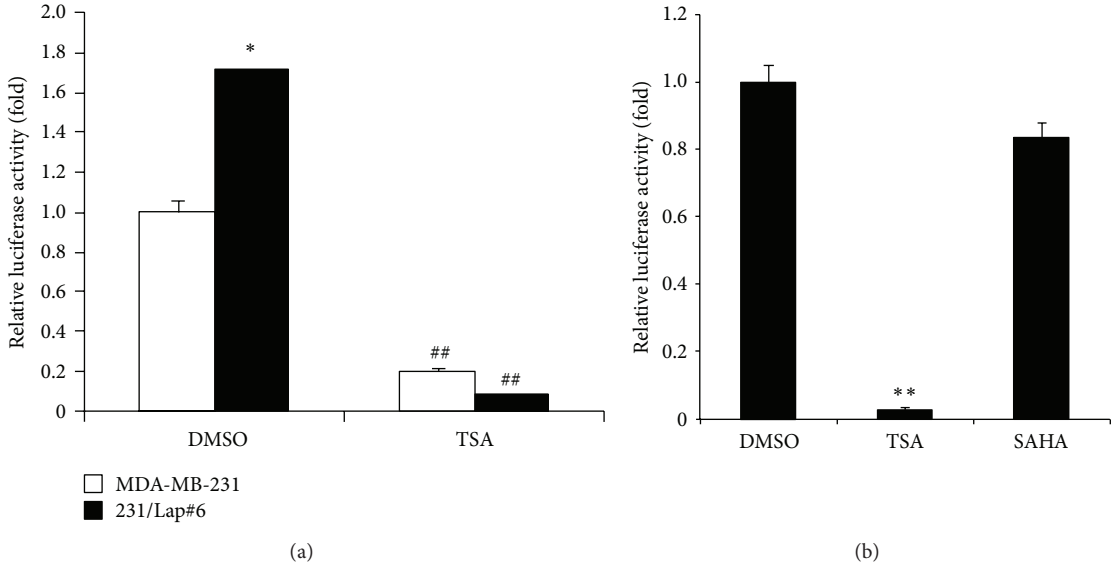

Figure 5: TSA suppressed the 3′UTR activity of *EGFR mRNA*. MDA-MB-231 and 231/Lap#6 cells were cotransfected with EGFR 3′UTR-luciferase plasmid and β-galactosidase for 24 hrs followed by treatment with 1 μM TSA or SAHA for another 24 hrs. Total lysates were then prepared and subjected to luciferase and β-galactosidase activity assays. The relative luciferase activity was normalized to β-galactosidase.

different activity in suppressing EGFR expression (Figure 1), suggesting a unique mechanism underlying the TSA-induced EGFR suppression. Although treatment with SAHA at 5–15 μM has been reported to suppress EGFR expression in MDA-MB-231 cells [44], the concentration of SAHA for EGFR suppression used in their study is far higher than the IC50 of SAHA for HDAC inhibition (0.01-0.02 μM) [33, 34]. Several lines of evidences provided from this study further support that TSA suppressed EGFR expression in an HDAC-independent manner in both parental and lapatinib-treated cells. (1) While treatments with TSA and SAHA at 1 μM can completely abolish the total HDAC activity (Figure 1(a)), only TSA but not SAHA at the same concentration repressed EGFR expression in both parental and lapatinib-treated cells (Figures 1(b) and 1(c)). (2) Silence of HDAC3 or HDAC7 did not affect the protein (Figures 3(a) and 3(b)) and mRNA (Figure 3(c)) levels of EGFR in 231/Lap#6 cells. (3) Treatment of 231/Lap#6 cells with 1 μM TSA suppressed the histone deacetylase activities of HDAC3 and HDAC7 (Figure 3(d)), but overexpression of myc-HDAC3 (Figure 3(e)) or myc-HDAC7 (Figure 3(f)) did not restore the EGFR expression in 231/Lap#6 cells. (4) HDAC6, a cytoplasmic class

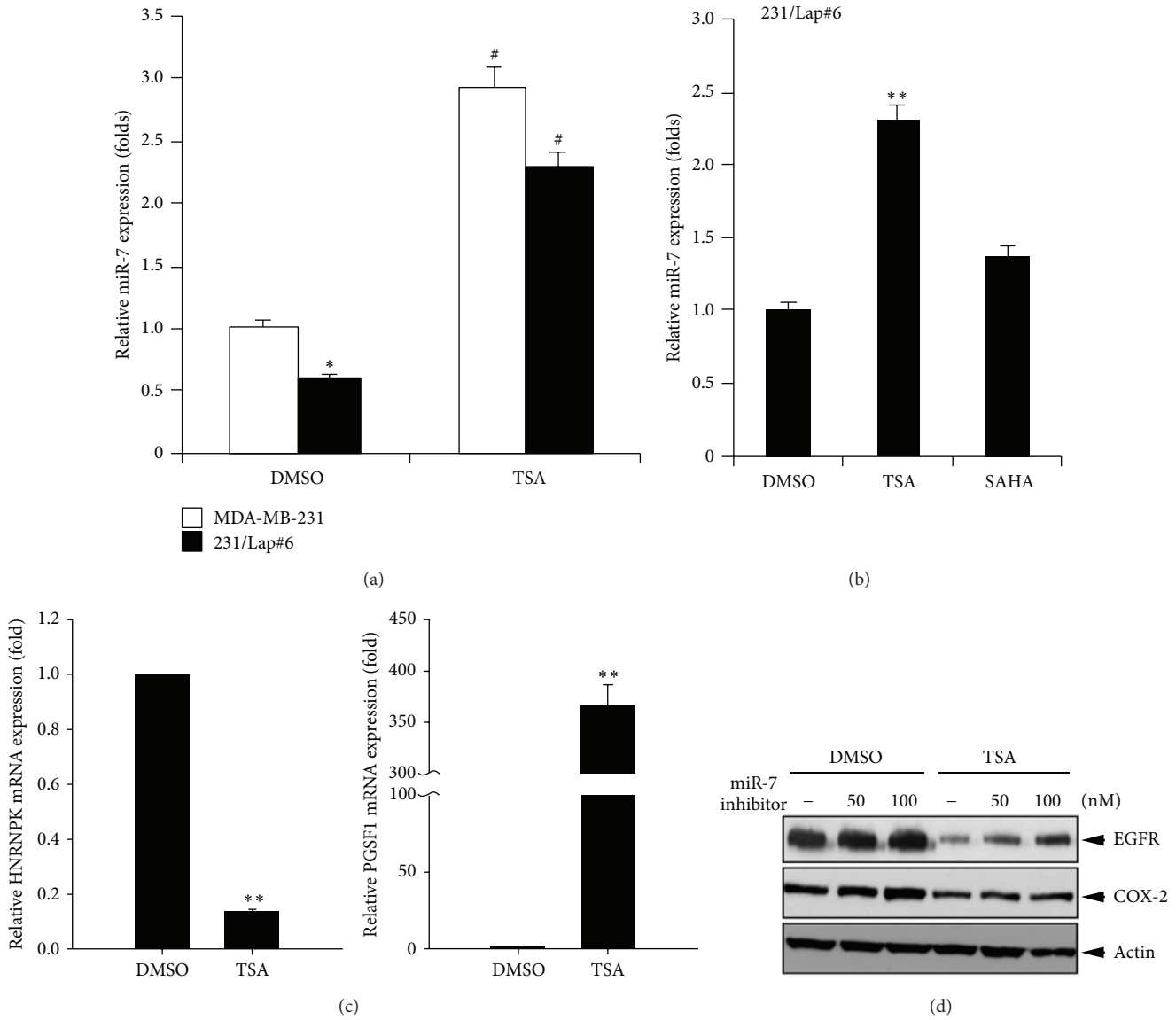

Figure 6: TSA induced miR-7 to suppress EGFR expression. (a)–(c) MDA-MB-231 and 231/Lap#6 cells were treated with 1 μM TSA or SAHA for 24 hrs and then subjected to total RNA extraction. The levels of miR-7 and its host genes were measured by RT-qPCR analysis. (d) 231/Lap#6 cells were transfected with increasing doses of miR-7 inhibitor followed by treatment with TSA. Total lysates were prepared and subjected to Western blot.

IIb deacetylase, was found to negatively regulate EGFR endocytosis and lysosomal degradation by controlling the acetylation status of α-tubulin [45]. When 231/Lap cells were treated with lysosomal and proteasomal inhibitors, the EGFR protein level was slightly enhanced, indicating that the lysosomal and proteasomal degradations indeed were involved in the turnover of EGFR. Although TSA induced tubulin acetylation, however, pretreatment with these lysosome or proteasome inhibitors did not prevent the TSA-reduced EGFR expression (Figure 4), ruling out the possibility that TSA decreases EGFR expression through targeting HDAC6-reduced EGFR endocytosis in 231/Lap#6 cells. These observations indicate that TSA may suppress EGFR independent of its HDAC inhibition activity.

In our previous study, treatment with lapatinib can down-regulate miR-7 to target EGFR mRNA 3′UTR and thereby result in the derepression of EGFR expression in the 231/Lap cells. Our current data further revealed that treatment with TSA increased the expression of miR-7 in both parental and lapatinib-treated MDA-MB-231 cells. Transfection of miR-7 inhibitor dose-dependently prevented TSA-induced EGFR suppression (Figure 6(d)). However, treatment with SAHA or silence of HDAC3 and HDAC7 by siRNA did not affect the miR-7 expression (Figure 6(b) and data not shown), further suggesting that TSA may induce miR-7 expression through an HDAC-independent manner. Microarray results revealed that TSA altered expressions of many microRNAs involving tumor suppression, antimetastasis, and

antiepithelial-mesenchymal transition (EMT) in apoptosis resistant MCF-7TN-R cells [46] or that involving general metabolisms in primary rat hepatocytes [47]. In contrast to inducing microRNA expression, TSA was also found to suppress miR-106b-93-25 cluster expression to inhibit proliferation and induce apoptosis in human endometrial cancer [48]. These results revealed that TSA may directly or indirectly regulate microRNA expressions through HDAC inhibition. However, the alteration of miR-7 by TSA was not found in these literatures, suggesting that cell contents may be critical for the regulation of microRNAs by TSA. In most cases, TSA and SAHA have similar effects on the regulation of gene and microRNA expressions [49]. Although both SAHA and TSA are derivatives of the hydroxamic acid and are structurally related to each other, acquisition of resistance to TSA or SAHA showed different dependence on MLH expression status [50]. Our data also showed that TSA but not SAHA suppressed EGFR expression through induction of miR-7. These observations suggest that actions of SAHA and TSA are different in some areas. Although our data suggest that miR-7 plays a critical role in TSA-mediated EGFR suppression, miR-7 inhibitor cannot totally restore EGFR expression in lapatinib-treated cells in response to TSA treatment, implying that, in addition to microRNA-7, other mechanisms underlying the TSA-mediated EGFR suppression cannot be ruled out. Treatment with HDAC inhibitor has been previously found to decrease EGFR mRNA and promoter activity by dissociation of transcription factor SP1 from the EGFR promoter around the transcription start site of EGFR gene in colorectal cancer cells [30]. Therefore, the Sp1 suppression and the HDAC-independent microRNA-7 induction may be both required for the TSA-mediated EGFR inhibition.

In conclusion, our data uncovered a unique activity of TSA in inducing miR-7 expression. In distinction to its structural relative SAHA, TSA suppressed EGFR 3′UTR activity to attenuate its protein expression independently of HDAC inhibition in lapatinib-treated breast cancer cells. These results suggest a possible off-target activity of TSA in suppressing EGFR expression.

Abbreviations

TSA:	Trichostatin A
SAHA:	Suberoylanilide hydroxamic acid
EGFR:	Epidermal growth factor receptor
RTKs:	Receptor tyrosine kinases
miR/miRNA:	MicroRNA
HDAC:	Histone deacetylase.

Conflict of Interests

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

Authors' Contribution

Chih-Yen Tu and Chia-Hung Chen contributed to this study equally.

Acknowledgments

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Review Article

The CCN Family Proteins: Modulators of Bone Development and Novel Targets in Bone-Associated Tumors

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The CCN family of proteins is composed of six extracellular matrix-associated proteins that play crucial roles in skeletal development, wound healing, fibrosis, and cancer. Members of the CCN family share four conserved cysteine-rich modular domains that trigger signal transduction in cell adhesion, migration, proliferation, differentiation, and survival through direct binding to specific integrin receptors and heparan sulfate proteoglycans. In the present review, we discuss the roles of the CCN family proteins in regulating resident cells of the bone microenvironment. In vertebrate development, the CCN family plays a critical role in osteo/chondrogenesis and vasculo/angiogenesis. These effects are regulated through signaling via integrins, bone morphogenetic protein, vascular endothelial growth factor, Wnt, and Notch via direct binding to CCN family proteins. Due to the important roles of CCN family proteins in skeletal development, abnormal expression of CCN proteins is related to the tumorigenesis of primary bone tumors such as osteosarcoma, Ewing sarcoma, and chondrosarcoma. Additionally, emerging studies have suggested that CCN proteins may affect progression of secondary metastatic bone tumors by moderating the bone microenvironment. CCN proteins could therefore serve as potential therapeutic targets for drug development against primary and metastatic bone tumors.

1. Introduction

The extracellular matrix (ECM) primarily serves as a scaffold for the organization of cells into tissues. However, it has also been recognized as a multifunctional modulator of cellular behavior [1, 2]. Through direct interaction, ECM proteins could modulate activities of many growth factors, cytokines, chemokines, and extracellular proteins or elicit signal transduction cascades, thus regulating diverse cellular functions. Recently, many studies have focused on a group of matrix proteins known as "matricellular" proteins for their function in extracellular signal modulation and coordination [3]. The CCN family, a small group of such matricellular proteins, is composed of six structurally conserved secreted proteins that have been identified in several biological studies [4–6].

The CCN family is named after its three initially discovered members: cysteine rich 61 (Cyr61, CCN1), connective tissue growth factor (CTGF, CCN2), and nephroblastoma overexpressed (Nov, CCN3) [7]. The CCN family includes three other members, Wnt induced secreted proteins 1–3 also known as CCN4, CCN5, and CCN6. The CCN members

share approximately 40% to 60% amino acid homology and comprise a signal peptide followed by 4 functional domains with 38 conserved cysteine residues [8]. In general, the common structure consists of an N-terminal signal peptide followed by an insulin-like growth factor binding protein domain (IGFBP), a von Willebrand type C repeat (VWC), a thrombospondin type I domain (TSP-1), and a cysteine knot carboxyl terminal (CT) [9]. The CCN proteins regulate cell adhesion, migration, proliferation, and differentiation to modulate variant biological functions including tumorigenesis, chondrogenesis, osteogenesis, angiogenesis, apoptosis, and hematopoiesis [5]. Numerous studies have shown that the biological functions of CCN proteins are mediated through interactions with cell surface receptors such as integrins, heparan sulfate proteoglycans (HSPGs), Notch1, neurotrophic tyrosine kinase receptor type 1 (TrkA), and low-density lipoprotein receptor-related proteins (LRPs). Moreover, CCN proteins could interact with other components outside of the cells such as ECM proteins, including fibronectin and fibulin 1C, and growth factors, including bone morphogenetic proteins (BMPs), tumor growth factor beta (TGF-β), and vascular endothelial growth factor [4].

The CCN family proteins were initially classified as growth factors. However, later studies showed that CCN proteins are matricellular proteins that modify cellular responses to extracellular factors via direct binding to cell surface receptors [5, 10–14]. Importantly, *in vivo* studies have indicated that aberrant expression of CCN proteins is involved in many diseases, including arthritis, atherosclerosis, fibrosis, diabetic nephropathy, retinopathy, and cancer [15]. Although the CCN proteins were discovered a decade ago, their mechanisms of action remain ambiguous. In the present report, we summarize recent literature that focuses on the regulation and function of CCN proteins in various bone tumors, discuss their potential as diagnostic markers and therapeutic targets, and review the recent therapeutic strategies targeting these proteins.

2. Receptors of CCN Family Proteins

CCN proteins were shown in previous studies to exert their function through direct binding to integrins or HSPGs. The interaction between CCN proteins and integrins was first discovered in 1998 by Kireeva et al. [16]. To date, at least 8 integrins have been demonstrated to interact with CCN proteins [4], which, however, do not possess the typical integrin binding sequence "RGD." Therefore, the interaction is thought to occur through nontypical binding sites, which is confirmed by site-directed mutagenesis that inhibits the biological activities induced by integrin binding. For example, a GVCTDGR sequence in CT domain of CCN2 interacts with integrin $\alpha5\beta1$ binding site and regulates CCN2-stimulated functions [17, 18]. In addition, CCN3 has been shown to bind to Notch and regulate myoblast and osteoblast functions [19, 20].

Other coreceptors are also involved in CCN protein signaling regulation. Cell surface HSPGs such as syndecan-4, perlecan, decorin, and biglycan have been reported to regulate CCN protein function in human fibroblasts [21–25]. CCN2 could also bind to coreceptors of the Wnt receptor LDL-receptor related protein 6 (LRP6) and LRP1 through variant modules [26, 27]. Moreover, Edwards et al. reported that CCN2 binds to TrkA (also known as neurotrophic tyrosine kinase receptor type 1) (NTRK1) in human mesangial and glioma cells [28, 29] and that TrkA serves as a co-receptor with integrins in this interaction. In summary, the complexity of receptors and coreceptors contributes to the unique activities and functions of CCN proteins in various cell types.

3. Functions of CCN Family Proteins

3.1. Adhesion and Migration. As expected from matricellular proteins modulating ECM signaling, the most familiar functions of CCN proteins are their roles in cell adhesion and migration. For example, CCN1 and CCN2 regulate adhesion in several types of cells [30]. In human skin fibroblasts, CCN1- and CCN2-regulated cell attachment is mediated through integrin $\alpha6\beta1$ and HSPGs [31]. In vascular smooth muscle cells, endothelial cells, and fibroblasts, CCN3 promotes cell adhesion through integrins and HSPGs [5]. Despite the lack of the RGD motif, the canonical binding motif for integrins, CCN3 could interact with many integrin receptors such as integrin $\alpha6\beta1$, $\alpha5\beta1$, $\alpha v\beta3$, $\alpha v\beta5$, $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha7\beta1$. CCN proteins can mediate cell migration through interaction with cell surface receptors. Previous studies have shown that all CCN proteins could regulate cell migration in many cell types. CCN1, CCN2, and CCN3 proteins promote cell migration in different types of cells [4]. CCN4 is involved in the migration and proliferation of vascular smooth muscle cells [32]. CCN5, however, is an important negative regulator of motility through matrix metalloproteinase (MMP)-2 gene expression modulation [33]. Finally, CCN6 stimulates migration of undifferentiated mesenchymal stroma cells [34].

3.2. Cell Survival and Apoptosis. Adhesion to the ECM is a crucial process to promote cell survival, whereas detachment from the ECM induces rapid cell death. The mechanism of CCN proteins regulating cell fates varies in different cell types. For example, CCN1 can promote cell survival in human umbilical vein endothelial cells through integrin $\alpha v\beta3$ [35] but induces fibroblast apoptosis through integrin $\alpha6\beta1$-HSPG syndecan-4 interaction [24]. These results suggest that specific CCN matricellular protein can either induce or suppress apoptosis via variant receptor interaction in a cell type-specific manner.

3.3. Proliferation. The first discovered CCN protein CCN1, also known as Cyr61, was believed to be a classic growth factor. However, later efforts established that CCN1, instead of being a growth factor itself, enhanced the activity of some growth factors such as fibroblast growth factor and platelet-derived growth factor [36]. CCN2 has been demonstrated to induce proliferation of chondrocytes through the MAPK/ERK signaling pathway [37], and knockdown of CCN2 expression inhibits cell proliferation and increases

apoptosis [38]. CCN3, however, has been reported to have negative regulatory properties, and its abnormal expression is associated with cancer progression [39]. Other studies have otherwise indicated that high CCN3 expression is associated with increased proliferation rates or tumor promoting potential in many cancer types [40]. The ambiguous effects of CCN3 therefore require further investigation.

3.4. Angiogenesis. CCN proteins have been suggested as potent angiogenic modulators, with activity mediated by interactions with different integrins and growth factors [41]. Treatments with recombinant CCN1, CCN2, and CCN3 increase angiogenesis *in vivo*, demonstrated via subcutaneous injection into corneas and the chick chorioallantoic membrane assay [5]. CCN1 and CCN2 play crucial roles in embryonic angiogenesis. Knockdown of CCN1 expression induces cardiovascular defects and is associated with embryonic lethality due to placental vascular inefficiency and compromised blood vessels [42]. CCN2, however, regulates angiogenesis via a different developmental process. *In vivo* results show that CCN2-null mutant mice show angiogenic deficiency in the growth plates during endochondral bone formation [43]. In addition, CCN3 has been demonstrated as a novel angiogenic regulator acting directly on endothelial cells to stimulate proangiogenic activities and as an angiogenesis inducer *in vivo* [44]. However, the roles of CCN4, CCN5, and CCN6 in angiogenesis remain poorly understood.

3.5. Inflammation. Abundant evidences indicate that CCN proteins are involved in inflammatory responses [45]. CCN protein expression is tightly regulated by different inflammatory mediators, including cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, and TGF-β, or by small factors such as prostaglandins, nitric oxide, histamine, and serotonin. Moreover, viral or bacterial infection also induces CCN proteins expression. Recognized as being encoded by an immediate early gene induced by environmental changes, CCN proteins subsequently regulate activity and expression of inflammatory cytokines and chemokines. For example, a recently published study showed that CCN1 promoted a proinflammatory program in murine macrophages. Bai et al. reported that CCN1 induced the expression of proinflammatory cytokines such as TNF-α, IL1-α, IL1-β, and IL-6; chemokines; and regulators of oxidative stress and inhibited the expression of anti-inflammatory factors such as TGF-β [46]. In addition, numerous studies have demonstrated a pivotal role of CCN proteins in chronic inflammatory diseases such as atherosclerosis, rheumatoid arthritis, inflammatory kidney diseases, and Alzheimer disease [45]. Therefore, CCN proteins may be classified as a new class of inflammatory regulators.

4. The Role of CCN Proteins in Bone

Bone is a complex tissue composed of two major cell types, bone resorption osteoclasts and bone formation osteoblasts, responsible for bone remodeling. Another cell population resident in the cartilage is chondrocytes. Abundant evidence suggests that CCN proteins regulate the differentiation of these cells (osteoblasts, osteoclast, and chondrocyte) [47]. In addition, CCN proteins are highly regulated during chondrogenic and osteogenic differentiation in mesenchymal stem cells [48–50]. However, CCN proteins can play either a positive or a negative regulatory role in skeletogenesis, which has been demonstrated both *in vitro* and *in vivo* [5, 51].

CCN1 has been detected in mouse limb bud mesenchymal cells during chondrogenesis and has been shown to promote chondrogenic differentiation through expression of type II collagen [52]. In another study, the tightly regulated CCN1 expression was shown to be involved in Wnt3A-induced osteoblast differentiation of mesenchymal stem cells [50]. Moreover, CCN1 has been shown to promote osteogenesis by increasing osteoblast differentiation while inhibiting osteoclast formation [53].

CCN2 is the most discussed member of the CCN protein family, accounting for approximately 50% of all reports published on the subject [54], most of which focus on the role of CCN2 in fibrosis and osteo/chondrogenesis. These reports indicate that CCN2 plays a crucial role in embryogenesis and skeletogenesis. For example, CCN2 has been shown to promote proliferation, chondrogenic differentiation, and chondrocyte maturation [55, 56]. CCN2 expression is high in the vascular tissue and maturing chondrocytes of the embryo and is important for cell proliferation and matrix remodeling during chondrogenesis [43]. In addition, CCN2 could also interact with many BMPs, important bone formation regulators, to regulate chondrocyte proliferation and differentiation [57, 58]. Finally, *in vivo* results have shown that CCN2 deficiency leads to skeletal dysmorphisms caused by impaired chondrocyte proliferation and reduced ECM composition in the growth plate [43].

Reported results on CCN3, however, are contradicting. CCN3 has been found to inhibit osteoblastogenesis and cause osteopenia, through antagonizing BMP-2, and Wnt activity in mice [59]. CCN3 has also been shown to inhibit osteoblast differentiation by neutralizing BMP2, a well-known enhancer of osteoblastogenesis in MC3T3 osteoblast precursor cells, in *in vivo* studies [20]. In another study, CCN3 showed antagonistic properties, inhibiting osteoblastogenesis and osteoblastic function through BMP2 neutralization and impairment of Wnt3 signaling [60]. In summary, these results suggest CCN3 as a negative regulator of osteoblastogenesis through multiple mechanisms including BMP2 and Wnt signaling or via activation of the Notch1 pathway. In contrast, a recent study has indicated that CCN3 promotes osteoblast differentiation and bone mineralization by upregulation of BMP-4, a well-known inducer of osteoblast differentiation [61]. In that study, a lower dose CCN3 (30 ng/mL) increased osteoblast differentiation whereas a higher dose (600 ng/mL) exhibited an opposite phenomenon, suggesting concentration-dependent mechanisms of action for CCN3.

Studies on the other three members of the CCN family are scarce, except for a report on CCN6. In that study, point mutations in CCN6 were shown to relate to the autosomal recessive skeletal disease progressive pseudorheumatoid dysplasia, a human disease resulting in progressive degeneration of articular cartilage [54].

Table 1: CCN proteins in primary bone cancers.

Cancer	CCN proteins	Expression level	Experimental observation	References
Osteosarcoma	CCN1	Higher	CCN1 associates with poor prognosis, tumour stage, metastasis and mortality	[62]
			CCN1 knockdown inhibits osteosarcoma cell invasion, migration, and lung metastases	[63]
	CCN3	Higher	CCN3 expression level is associated with higher risk to develop lung metastases	[64]
	CCN4	Higher	CCN4 associates with tumor stage and enhances the migration of osteosarcoma cells by increasing MMP-2 and MMP-9 expression	[65]
Ewing's sarcoma	CCN3	Higher	CCN3 is expressed in approximately 30% of Ewing's sarcomas and associated with lower survival rate	[64]
			High expression of CCN3 is detected in recurrences and metastases when compared to the primary tumor.	[66]
		N/A	Forced expression of CCN3 shows decreased cell proliferation while increased migration and invasion	[67]
Chondrosarcoma	CCN1	N/A	CCN1 enhances the migration of chondrosarcoma cells by increasing MMP-13 expression	[68]
	CCN2	N/A	CCN2 increases the migration through upregulating MMP-13 expression	[69]
	CCN3	N/A	CCN3 increases the migration and expression of matrix metalloproteinase MMP-13	[70]
	CCN4	N/A	CCN4 enhances the migration of chondrosarcoma cells by increasing MMP-2 expression	[71]
	CCN6	N/A	CCN6 enhances the migration of chondrosarcoma cells by increasing ICAM-1 expression	[72]

5. The Role of CCN Family Proteins in Primary Bone Cancers

CCN proteins are tightly regulated in osteo/chondrogenic cell lineages and are involved in skeletogenesis. Abnormal levels or altered forms of CCN proteins are associated with tumor progression. We hereby discuss the correlation between CCN proteins and primary bone cancers (Table 1).

5.1. Osteosarcoma. Osteosarcoma is the most common primary bone tumor found in children and young adults. The existing literature suggests that osteosarcoma might originate from mesenchymal cells with osteoblastic features [73–75]. The CCN1 expression level in osteosarcoma biopsies has been shown to correlate with poor prognosis, regardless of metastatic or nonmetastatic disease. Moreover, an *in vivo* murine model showed that overexpression of CCN1 in the low-metastatic human SaOS-2 osteosarcoma cell line increased cell proliferation and promoted lung metastasis [62]. Fromigue et al. also demonstrated that CCN1 protein expression was higher in human osteosarcoma than in normal bone tissue and was most highly expressed in metastatic tissues. They also found that CCN1 knockdown inhibited *in vitro* osteosarcoma cell invasion and migration as well as *in vivo* lung metastases in mice [63]. Therefore, these results demonstrate great potential for CCN1 as a novel prognosis marker and therapeutic target in osteosarcoma. In addition, another study showed that CCN3 was expressed in primary tumors of osteosarcoma patients and that a high CCN3 expression level was associated with an increased risk of developing lung metastases [64]. CCN4 also showed similar correlation like CCN1 and CCN3 in osteosarcoma. In our previous work, we show that the expression of CCN4 in osteosarcoma patients was significantly higher than that in normal bone and corrected with tumor stage. CCN4 increases cell motility through upregulating matrix metalloproteinase (MMP)-2 and MMP-9 expression [65].

5.2. Ewing Sarcoma. Ewing sarcoma is the second most common malignant bone tumor that mainly occurs in children. CCN3 is expressed in approximately 30% of all Ewing sarcoma cases, and its expression is associated with a lower survival rate [64]. In a study by Benini et al., overexpression of CCN3 led to decreased *in vitro* cell proliferation and soft-agar growth in Ewing sarcoma cells and *in vivo* tumorigenicity in nude mice. However, these Ewing sarcoma cells showed increased migration and invasion in Matrigel [67]. Finally, an immunohistochemistry study on 170 human Ewing sarcoma specimens showed that the expression of CCN3 was higher in recurrences and metastases than in primary tumors. The same study also suggested that a low level of CCN3 expression was associated with better patient prognosis [66].

5.3. Chondrosarcoma. Chondrosarcoma is the second most common malignancy of the bone, associated with a poor response to currently used chemotherapy and radiation treatment, making chondrosarcoma management a complicated challenge [76, 77]. All CCN proteins have been

Table 2: CCN proteins in metastatic bone cancers.

Cancer	CCN proteins	Expression level	Expreimental observation	References
Breast cancer metastasize to bone	CCN1	Higher	CCN1 associates with poor prognosis, nodal involvement, and metastatic disease	[79]
		N/A	Zoledronic acid downregulates CCN1, thus inhibits tumor growth	[80]
		N/A	Anti-CCN1 neutralizing antibody suppresses primary tumor growth and spontaneous lymph node metastasis *in vivo*	[81]
	CCN2	Higher	The expression of CCN2 is higher in breast cancer bone metastases when compared to normal breast tissue	[82]
		Higher	CCN2 is significantly overexpressed in metastatic tumor cells as compared to disseminated tumor cells	[83]
		N/A	CCN2 is crucial for osteolytic metastasis and is induced by PKA- and PKC-dependent activation of ERK1/2 signaling by PTHrP	[84]
		Higher	CCN2 expression is further increased by the prometastatic cytokine TGFβ	[85]
	CCN3	Lower	Expression of CCN3 is lower in tumor when compared to normal specimens	[79]
		Higher	CCN3 is highly expressed in the bone metastases when compared with the other metastases (lung, brain, and liver)	[86]
		Higher	CCN3 is highly expressed in bone metastasis samples from breast cancer patients	[87]
	CCN6	Lower	CCN6 expression is inversely correlated with invasive breast carcinomas	[88]
Prostate cancer metastasize to bone	CCN1	N/A	CCN1 increases tumorigenesis and metastasis of prostate cancer cells	[89]
	CCN3	N/A	Knockdown of CCN3 expression decreases cell migration *in vitro* and tumor growth in bone and bone metastasis *in vivo*	[90]
		Higher	CCN3 expression levels are higher in bone metastasis patients and positively correlated with malignancy in human prostate cancer cells	[91]
	CCN4	Higher	Higher expression level of CCN4 has been found in the tissues and sera of prostate cancer patients in early stages	[92]

demonstrated to be involved in chondrosarcoma progression and malignancy except for CCN5. The CCN proteins have been shown to promote cell migration through upregulation of various genes such as MMP-2, MMP-13, and intercellular adhesion molecule 1 (ICAM-1) [68–72]. These results suggest that CCN proteins might regulate common biological functions in chondrosarcoma. Other cellular functions regulated by CCN proteins such as adhesion, proliferation, survival, apoptosis, and angiogenesis may also be involved in CCN protein-regulated tumorigenesis. Interestingly, CCN proteins promote expression of MMPs, important regulators of ECM, which might explain the prometastatic effects exerted by the CCN family. The tumor microenvironment could also significantly influence chondrosarcoma malignancies. A previous study indicated that the tumor microenvironment could affect CCN2 gene expression in Swarm rat chondrosarcoma tumors, suggesting that CCN2 may play a role in chondrosarcoma development and progression [78].

6. The Role of CCN Family Proteins in Metastatic Bone Cancers

Bone metastasis is a common complication of advanced cancer, occurring when cancer cells from the primary tumor spread to the bone. Prostate, breast, and lung cancers are most likely to result in bone metastasis. As CCN proteins have important roles in the differentiation and function of bone resident cells, they have been implicated in the progression of bone metastases from other cancers (Table 2).

6.1. Breast Cancer Metastasis to the Bone. Breast cancer shows a high predilection for metastasis to the bone, causing bone pain, pathological fractures, hypercalcemia, spinal cord compression, and immobility [93]. In a cohort of 122 human breast tumors and 32 normal breast specimens, significantly elevated levels of CCN1 were shown to be associated with poor prognosis, nodal involvement, and metastatic disease [79]. CCN1 is a potent proangiogenic molecule,

and a previously published study suggested the critical role of CCN1 in the Hedgehog-influenced proangiogenic tumor microenvironment [94]. CCN1 has also been recommended as a candidate target for breast cancer bone metastases. Espinoza et al. found that zoledronic acid, a bisphosphonate currently used to treat breast cancer bone metastases, downregulated CCN1, thus inhibiting tumor growth [80]. Moreover, the anti-human CCN1 antibody, denoted as 093G9, was shown to inhibit breast cancer cell migration and invasion through upregulation of the MMP inhibitors TIMP1 and TIMP2. *In vivo* mouse model results showed that 093G9 also inhibited primary tumor growth and spontaneous lymph node metastases [81].

CCN2 was found to be overexpressed in tumor cells from human bone metastases compared to a normal human epithelial cell line [82]. In addition, another report indicated that CCN2 was significantly overexpressed in metastatic tumor cells compared to disseminated tumor cells [83], further supporting the previously mentioned evidence. An *in vivo* mouse model study was performed to investigate the role of CCN2 in osteolytic metastasis by breast cancer cells. The results showed that CCN2 was crucial for osteolytic metastasis and was induced by protein kinase A- and protein kinase C-dependent activation of ERK1/2 signaling by parathyroid hormone-related protein (PTHrP). The authors also found that osteolytic metastasis accompanied by the PTHrP-CCN2 signaling pathway was efficiently abolished by a CCN2 neutralizing antibody [84]. Another previously published study investigated the mechanism of osteolytic bone metastasis by selecting human breast cancer cell line subpopulations with elevated metastatic activity and found that IL-11 and CCN2 expressions were further increased by the prometastatic cytokine TGFβ. These results elucidated a mechanism for the prometastatic activity of these cytokines in the bone [85].

However, studies on CCN3 have reported contradicting results. In a different cohort of 122 human breast tumors and 32 normal breast specimens, the expression of CCN3 was found to be lower in tumor tissues when compared to normal specimens [79]. Interestingly, in another study, a microarray profile derived from 58 breast cancer metastases showed CCN3 to be highly expressed in bone metastases when compared to other metastases (lung, brain, and liver) [86]. Moreover, Véronique et al. found that CCN3 was highly expressed in bone metastasis samples from breast cancer patients. They also demonstrated that CCN3 increased the bone metastatic potential of 66cl4 cells, which are breast cancer cells metastasizing to the lungs [87].

The balance between bone formation and resorption is a significant factor in the development of bone metastasis. In accordance with this opinion, CCN3 was shown to impair osteoblast differentiation and affect receptor activator of NF-κB ligand (RANKL)/osteoprotegerin ratios of osteoblasts, thereby enhancing osteoclastogenesis. CCN3 was also shown to promote osteoclast differentiation through a RANKL-dependent pathway, which involves calcium oscillations and nuclear factor of activated T-cell nuclear translocation [87].

In contrast, studies on CCN6 indicated that it inhibited breast cancer metastasis. In clinical specimens, CCN6 expression was shown to inversely correlate with invasive breast carcinomas. Moreover, CCN6 was shown to inhibit invasion and metastasis of breast cancer *in vivo*. The mechanism of CCN6-inhibited breast cancer progression was shown to be mediated by the BMP4/TAK1/p38 pathway, which could induce epithelial-mesenchymal transition, cell invasion, and metastasis [88]. Decline of CCN6 protein expression was demonstrated to sufficiently activate the phosphatidylinositol 3-kinase/Akt signaling pathway, thus promoting growth factor-independent survival that is triggered by resistance to detachment-induced cell death (anoikis) [95]. The role of CCN6 in breast cancer metastasis has been proved. However, the role of CCN6 in bone metastatic breast cancer remains poorly understood and further studies are necessary.

6.2. Prostate Cancer Metastasis to the Bone. Prostate cancer is the most commonly diagnosed malignancy in the United States and other Western countries, and bone metastasis is a common complication associated with advanced prostate cancer [96–98]. Prostate cancer bone metastases are most often characterized as osteoblastic lesions as opposed to osteolytic lesions with decreased bone mineral density. Increasing evidence suggests that prostate cancer cells synchronize the combined osteoclastic and osteoblastic activity occurring in the bone microenvironment [99–101]. However, the role of CCN proteins in prostate cancer metastasis to the bone is discussed relatively scarcely.

CCN1 has been implicated in tumorigenesis and metastasis of prostate cancer cells [89]. CCN1 activates Ras-related C3 botulinum toxin substrate 1 and its downstream targets, including phosphorylated c-Jun N-terminal kinase, E-cadherin, and p27 (kip1), key molecules involved in cell growth, migration, and invasion. *In vivo* mouse model results revealed that CCN1 increased the metastatic potential of prostate cancer cells. The correlation of CCN1 and prostate cancer bone metastases, however, needs to be confirmed.

CCN3 has been demonstrated to have prometastatic potential in prostate cancer in our previous study [90]. We found that CCN3 increased cell migration through the upregulation of ICAM-1 expression. Knockdown of CCN3 expression markedly inhibited cell migration *in vitro* and tumor growth in bone and bone metastasis *in vivo*. Moreover, our latest study revealed the critical role of CCN3 in prostate cancer bone metastases [91]. An immunohistochemistry study on normal prostate tissues, primary tumors, and bone metastasis samples obtained from patients revealed that CCN3 expression levels were higher in patients with bone metastasis and positively correlated with malignancy in human prostate cancer cells. In agreement with the study by Véronique et al. [87], our results showed that the prostate cancer-secreted CCN3 induced osteoclastogenesis through a RANKL-dependent pathway. Moreover, the focal adhesion kinase/Akt/p38/NF-κB signaling pathway was found to be involved in CCN3-mediated receptor activator of NF-κB expression and RANKL-dependent osteoclastogenesis. Experiments with intratibia injection of prostate cancer cells

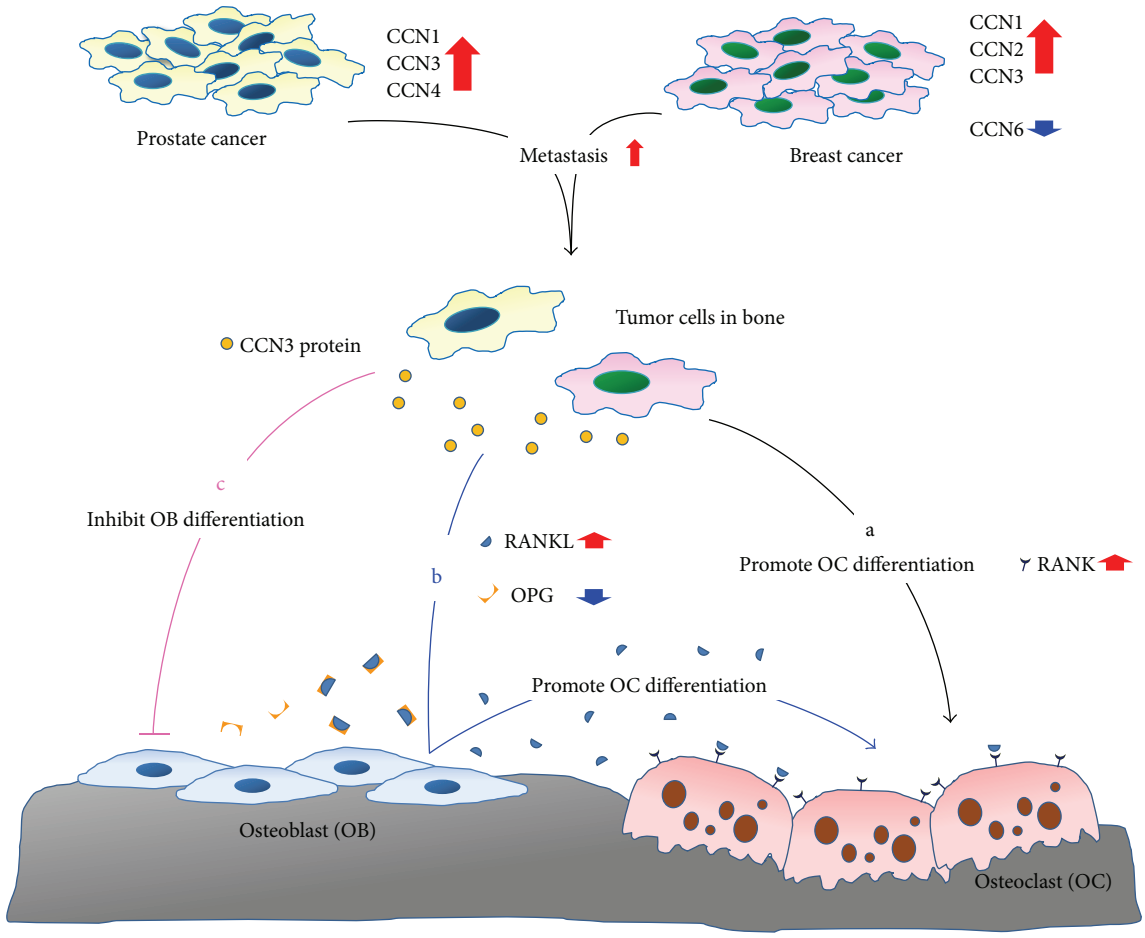

Figure 1: CCN family proteins involved in tumor metastasis and the mechanism of CCN3-modulated osteolytic bone metastasis. Tumors secrete different CCN proteins in prostate cancer (such as CCN1, CCN3, and CCN4) or breast cancer (such as CCN1, CCN2, CCN3, and CCN6) that regulate tumor metastasis. When tumor cells metastasize to the bone microenvironment, the secreted CCN protein (such as CCN3) promotes osteolytic bone metastasis in bone microenvironment through 3 different mechanisms. (a) CCN3 directly enhances osteoclasts formation through upregulating RANK expression, the crucial regulator of osteoclastogenesis. (b) CCN3 indirectly enhances osteoclast formation through affecting the RANKL/OPG secretion in osteoblasts, and the higher RANKL/OPG ratio increases the osteoclastogenesis. (c) CCN3 inhibits osteoblasts differentiation and thus reduces bone formation.

also proved that CCN3 enhanced osteoclast activity and bone metastasis *in vivo*.

CCN4 has been shown to play similar roles to CCN3 in prostate cancer [92]. An increased expression level of CCN4 has been found in prostate cancer tissue in the early stages, sera of patients, and carcinoma tissues of the mouse prostate cancer model TRAMP, which spontaneously develops to prostate carcinomas. Injections of CCN4 neutralizing antibodies were shown to reduce local tumor growth in a mouse xenograft model. These results suggest that CCN4 expression plays significant roles in both tumor growth and its metastasis to bone.

7. Perspectives

The CCN family proteins are multifunctional cytokines that regulate signals from the ECM. They are involved in many cellular processes and exert their functions through modulating various components including ECM proteins, transmembrane proteins, growth factors, and cytokines in the cell microenvironment. As they have crucial roles in osteo/chondrogenesis during development, abnormal expression of CCN proteins is implicated in tumors that grow in the bone microenvironment such as primary bone tumors and bone metastases. However, the roles of CCN proteins in osteo/chondrogenesis in development vary. The complicated interaction of CCN family proteins and other components outside the cells may contribute to unique activities and functions of CCN proteins. Emerging results suggest that the ultimate outcome of cellular responses modulated by CCN family proteins may also depend on the level of CCN expression. Although the available results suggest ambiguous roles of the CCN family proteins, they reveal the significance of CCN proteins in the regulation of bone homeostasis and turnover.

Interestingly, evidence on CCN family proteins in metastatic bone tumors indicates their pivotal role in bone microenvironment (Figure 1). However, there remains a lack

of sufficient studies on CCN proteins in prostate and lung cancer metastasis to the bone. Further studies are required to confirm the molecular basis of CCN proteins in metastatic bone tumors. Moreover, the correlation and commonality of CCN proteins in metastatic bone tumors will help elucidate the importance of CCN family proteins in the bone microenvironment.

CTGF, one member of the CCN family associated with tumorigenesis, is a novel therapeutic target for the treatment of pancreatic cancer and is currently being investigated in a Phase I clinical trial. The results showed that twice weekly i.p. administration of FG-3019, a fully human CTGF-specific monoclonal antibody, decreased tumor growth and metastasis and attenuated tumor angiogenesis and cancer cell proliferation [102, 103]. The other CCN proteins may use similar strategies to develop potential therapeutics which target CCN proteins and apply to bone tumor treatment.

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

Novel Strategies for the Treatment of Chondrosarcomas: Targeting Integrins

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Chondrosarcomas are a heterogeneous group of malignant bone tumors that are characterized by the production of cartilaginous extracellular matrix. They are the second most frequently occurring type of bone malignancy. Surgical resection remains the primary mode of treatment for chondrosarcomas, since conventional chemotherapy and radiotherapy are largely ineffective. Treatment of patients with high-grade chondrosarcomas is particularly challenging, owing to the lack of effective adjuvant therapies. Integrins are cell surface adhesion molecules that regulate a variety of cellular functions. They have been implicated in the initiation, progression, and metastasis of solid tumors. Deregulation of integrin expression and/or signaling has been identified in many chondrosarcomas. Therefore, the development of new drugs that can selectively target regulators of integrin gene expression and ligand-integrin signaling might hold great promise for the treatment of these cancers. In this review, we provide an overview of the current understanding of how growth factors, chemokines/cytokines, and other inflammation-related molecules can control the expression of specific integrins to promote cell migration. We also review the roles of specific subtypes of integrins and their signaling mechanisms, and discuss how these might be involved in tumor growth and metastasis. Finally, novel therapeutic strategies for targeting these molecules will be discussed.

1. Chondrosarcomas

Chondrosarcomas are a heterogeneous group of malignant bone tumors with diverse histopathology and clinical behavior, which are characterized by the production of cartilage matrix. They are the second most common type of skeletal malignancy after osteosarcomas [1]. Chondrosarcomas are usually found within flat bones; the pelvis and femur are two common sites of involvement, although any bone may be affected [2, 3]. These malignant cartilaginous tumors may either arise *de novo* or develop from pre-existing benign lesion (e.g., enchondromas and osteochondromas), termed primary (or conventional), and secondary chondrosarcomas, respectively. Tumors can arise in both skeletal (central) and extraskeletal (peripheral) locations [4]. The majority of cases are primary central chondrosarcomas; together, primary central and secondary peripheral chondrosarcomas constitute approximately 85% of all chondrosarcomas. Other specialized types of chondrosarcoma, such as dedifferentiated, clear cell, and mesenchymal chondrosarcomas, account for the remaining 10%–15% of cases [3, 5].

Chondrosarcomas are classified into three histological grades (grades 1–3), based on the extent of cellularity, nuclear atypia, nuclear staining (hyperchromasia), mucomyxoid matrix changes, and increased vascularization [6–9]. Approximately 90% of conventional chondrosarcomas are grade 1 or 2, which have an indolent clinical course, low metastatic potential, and good prognosis; the remaining 5–10% are grade 3 tumors, which have high metastatic potential and are associated with poor outcomes [3, 10, 11].

For chondrosarcomas, prognosis is strongly correlated with histological grade, as well as with the adequacy of the resection margins [12, 13]. Low-grade chondrosarcomas show little cellularity and an abundant matrix that resembles hyaline cartilage. These tumors rarely metastasize and are therefore often managed with intralesional curettage and resection. In contrast, high-grade conventional chondrosarcomas are highly cellular, with little or no cartilaginous matrix. High-grade tumors often metastasize, leading to lethality in most cases; for these, radical excision margins, or even amputation, may be recommended [3].

Chemotherapy and radiotherapy are largely ineffective for treating chondrosarcomas, due to the slow growth, abundant extracellular matrix (ECM), low percentage of dividing cells, and poor vascularity of these tumors [14–17]. Moreover, some studies indicate that chondrosarcoma cells can express multidrug-resistance gene products, such as P-glycoprotein, thereby reducing the absorption of drugs, and giving rise to chemotherapy resistance [18, 19]. Surgical resection remains the primary mode of treatment for chondrosarcomas. In a minority of patients, local recurrence or metastasis occurs, and can result in death; this is more prevalent in those with high-grade tumors [3, 16]. The above features make the clinical management of chondrosarcomas particularly challenging, and new therapeutic strategies are urgently needed. One type of approach focuses on inhibiting the processes of metastasis and invasion, and may facilitate the development of effective adjuvant therapy. Integrins have been considered potential therapeutic targets because they are exposed on the cell surface and are sensitive to pharmacological blockade.

2. Characteristics of Integrins

Integrins are a family of heterodimeric transmembrane glycoproteins that are found on nearly all cells, where they function as adhesion receptors, mediating dynamic cell-cell and cell-extracellular matrix interactions. Through these interactions, integrins play critical roles in cancer cell migration, invasion, and metastasis—processes that contribute to tumor progression [20]. To date, at least 24 unique integrin heterodimers have been identified. These heterodimers are formed from various combinations of 18 α-subunits and 8 β-subunits, which interact noncovalently. Each integrin subunit consists of a large extracellular domain, a single type I transmembrane domain, and a short intracellular cytoplasmic tail domain [21]. The ligand-binding site of an integrin heterodimer lies at the interface between the two subunits. Their cytoplasmic domains form connections with the cytoskeleton, enabling integrins to serve as a link between the ECM and the cytoskeleton.

Ligand specificity is determined by the extracellular domain of the integrins. Five main groups have been identified: arginine-glycine-aspartate (RGD)-binding, the α4 family, laminin-binding, I-domain collagen-binding, and leukocyte adhesion integrins. Approximately one third of integrins have binding sites for the RGD sequence, which is found on many ECM proteins. Although the RGD sequences within collagen and laminin are not normally exposed, denaturation or cleavage of these proteins may result in exposure of the RGD sequence and subsequent integrin binding. Generally, α4 integrins recognize the leucine-aspartic acid-valine (LDV) tripeptide, collagen-binding integrins recognize a triple helical collagen peptide containing the glycine-phenylalanine-hydroxyproline-glycine-glutamate-arginine (GFOGER) motif [22, 23]. Integrins do not simply act as adhesion molecules; they can also function as bidirectional signaling molecules, controlling a variety of cell functions such as proliferation, differentiation, survival/apoptosis, cell shape, polarity, or motility, as well as gene expression [21].

3. Integrin-Dependent Signaling

Although integrins lack intrinsic kinase activity, signal transduction can be induced by the assembly of signaling complexes on the cytoplasmic domains of integrin subunits. Through interactions of their cytoplasmic domains with a wide variety of adaptor proteins, integrins are able to deliver signals into the cell in response to extracellular cues ("outside-in" signaling). In addition, some cytoplasmic interactions can induce conformational changes in integrin molecules. This can affect their activation state by modulating their binding affinity for extracellular ligands ("inside-out" signaling) [24–26]. In the inactive or low-affinity state, integrins are in a "bent" conformation, with the transmembrane and cytoplasmic domains close together; this impedes ligand engagement and maintains the low-affinity state. The binding of talins and kindlins to their respective binding regions of the β integrin cytoplasmic tails induces conversion from the bent to the extended conformation. This separates the cytoplasmic and transmembrane subunits and results in a shift to the activated or high-affinity state. When activated integrins bind to ligands, they cluster at the plasma membrane. This clustering promotes intracellular signaling, resulting in the formation of tight focal adhesions, actin cytoskeletal assembly, and activation of multiple downstream signaling pathways that influence a variety of cellular functions [27–29]. Precise regulation of ligand binding affinity is therefore critical for proper integrin function.

The canonical view has been that ECM ligands bind to their cognate integrins and initiate signaling via specific pathways, to give rise to distinct cell responses. However, accumulating evidence reveals that several integrins are able to crosstalk with oncogenic signal transducers, such as ErbB, Ras, and Src, to promote tumorigenesis [30–34]. Cooperative signaling between integrins, growth factor receptors, and cytokine receptors has also been implicated in tumor progression [35–41]. Upon ligand binding, integrins may trigger cells to secrete growth factors and/or cytokines, which in turn can bind to their receptors in an autocrine or paracrine manner to induce further signaling. For example, the activation of integrin $\alpha v\beta 3$ can trigger phosphorylation of p66 Shc; this has been shown to upregulate the expression of vascular endothelial growth factor (VEGF), leading to tumor growth and angiogenesis in human prostate and breast cancer cells

[38]. In pancreatic cancer cells, the $\alpha 6\beta 1$ and $\alpha 3\beta 1$ integrins interact with laminin-1 to mediate cell migration; this process involves the upregulation of CXC chemokine receptor 4 (CXCR4) and IL-8 expression in response to the chemokine ligand CXCL12, also known as stromal cell-derived factor-1 (SDF-1) [42]. Integrin activation of growth factor receptors, through collaborative mechanisms, has also been reported to induce downstream signaling [43]. Alternatively, both growth factor and chemokine signaling may regulate integrin function by directly controlling integrin expression levels.

4. Regulation of Integrin Gene Expression

A number of growth factors and chemokines/cytokines have been found to regulate the expression of integrins in many malignancies, indicating a critical role in cancer progression. For example, heparin-binding EGF-like growth factor has been observed to increase integrin expression in human breast and esophageal cancer cells [67, 68]. Binding of the CXCL12 chemokine to its receptor (CXCR4) may regulate tumor dissemination in prostate tumor cells by enhancing expression of $\alpha v\beta 3$ integrins [40]. More recently, a study demonstrated that CXCL12 strongly induced $\alpha v\beta 6$ integrin expression in ovarian cancer, leading to enhanced urokinase plasminogen activator (uPA)-mediated ECM degradation and cell invasion [69]. In human osteosarcoma cells, the CCL5/CCR5 axis can induce increased expression of $\alpha v\beta 3$ integrin via the MEK, ERK, and NF-κB pathways, thereby contributing to cell migration [70]. The pro-inflammatory cytokine interleukin-1α (IL-1α) can induce selective upregulation of $\alpha 6\beta 1$ integrin in pancreatic cancer cells and has been suggested to modulate tumor aggressiveness [71, 72]. Transforming growth factor-β1 (TGF-β1), a multifunctional cytokine, can promote human hepatocellular carcinoma (HCC) cell invasion by stimulating $\alpha 3$ integrin expression [44]. Another study found that TGF-β1 treatment promotes gastric carcinoma cell adherence by increasing $\alpha 3$ integrin levels [67].

In human chondrosarcoma cells, numerous studies have shown that growth factors, chemokines/cytokines, and other inflammation-related molecules can control the expression of specific integrins to promote cell migration. Among the growth factors, insulin-like growth factor-I (IGF-I) is able to enhance the migration of chondrosarcoma cells by increasing $\alpha v\beta 1$ integrin expression, through the IGF-I receptor/PI3K/Akt/NF-κB pathway [45]. Brain-derived neurotrophic factor (BDNF) is a small protein from the neurotrophin family of growth factors whose expression has been associated with disease status and outcomes in various cancers. Recent research has shown that BDNF enhances the migration of chondrosarcoma by increasing $\beta 5$ integrin expression, through the TrkB receptor, PI3K, Akt, and NF-κB pathways [46] (Table 1).

Interleukin-8 (IL-8), a chemokine also known as CXCL8, interacts with the CXCR1 and CXCR2 receptors to activate PI3K and Akt pathways, and induce AP-1 activation. In human chondrosarcoma cells, IL-8 induced upregulation of $\alpha v\beta 3$ integrin expression and increased cell migration [47]. Metastasis, particularly to the lungs, is often observed with high-grade chondrosarcomas. Interestingly, the CXCL12/SDF-1 chemokine, which is constitutively secreted by human lung epithelium cells, has been shown to enhance the invasiveness of chondrosarcoma cells by increasing $\alpha v\beta 3$ integrin expression, through the CXCR4/ERK/NF-κB pathway. It has also been observed that the expression of CXCR4 in human chondrosarcoma tissues and chondrosarcoma cell lines is higher than in normal cartilage and in human chondrocytes. This could potentially account for the homing of chondrosarcoma cells to the lung [48] (Table 1).

Accumulating evidence suggests that fat tissue can function as an endocrine organ, producing and secreting a variety of bioactive substances that are referred to as adipocytokines or adipokines. Most adipocytokines are pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α). Various adipocytokines, including TNF-α, leptin, and adiponectin, have been reported to enhance chondrosarcoma cell migration by increasing the expression of specific integrins. A range of signaling pathways are involved. For example, TNF-α and leptin were found to increase $\alpha v\beta 3$ integrin expression, through their effects on the MEK/ERK/IKKα/β/NF-κB and the OBR1/IRS-1/PI3K/Akt/NF-κB pathways, respectively [49, 50]. Adiponectin is a member of the C1q and tumor necrosis factor superfamily, and structurally resembles TNF-α. Adiponectin can promote migration of human chondrosarcoma cells by upregulating $\alpha 2\beta 1$ integrin, via AdipoR-, AMPK-, p38-, IKKα/β-, and NF-κB-dependent pathways [51]. Macrophage migration-inhibitory factor (MIF), a pro-inflammatory cytokine involved in macrophage migration and activation, is able to enhance the migration of chondrosarcoma cells by increasing $\alpha v\beta 3$ integrin expression, mediated via PI3K/Akt/NF-κB signaling [52] (Table 1).

The transforming growth factor-β (TGF-β) superfamily includes the prototypical member TGF-β, and numerous others, such as bone morphogenetic proteins (BMPs) and glial cell derived neurotrophic factor (GDNF). Many of these proteins are known to play pivotal roles in tumor progression, invasion, and metastasis. TGF-β has been previously shown to increase cell motility and $\alpha v\beta 3$ integrin expression in human chondrosarcoma cells, via pathways involving PI3K, Akt, and NF-κB [53]. BMPs are proteins originally isolated from bone tissue, and are capable of ectopically inducing new cartilage and bone formation. BMP-2 has been found to act through PI3K/Akt, IKKα/β, and NF-κB, resulting in increased $\beta 1$ integrin expression and migration of human chondrosarcoma cells [54]. GDNF is a factor required for survival, proliferation, and activation of glioma cells. GDNF has been shown to promote the migration of human chondrosarcoma cells by upregulating $\alpha v\beta 3$ integrin expression, through activation of the MEK/ERK, IKKα/β, and NF-κB pathways [55]. A novel cytokine system, consisting of receptor activator of NF-κB ligand (RANKL), its receptor, RANK, and the protein osteoprotegerin (OPG), has been identified and extensively characterized for its role in bone remodeling. The RANKL/RANK signaling axis has also been found to regulate cell migration in human chondrosarcoma cells, through MEK, ERK, IKKα/β, and NF-κB signaling and upregulation of $\beta 1$ integrin [56] (Table 1).

Table 1: Regulation of integrin expression in human chondrosarcoma cells.

Groups	Activators	Integrins	Pathway	References
Growth factors	Insulin-like growth factor-I (IGF-I)	$\alpha5\beta1$	IGF-I receptor/PI3K/Akt/NF-κB	[44]
	Brain derived neurotrophic factor (BDNF)	$\beta5$	TrkB receptor/PI3K/Akt/NF-κB	[45]
Chemokines	IL-8/CXCL8	$\alpha v\beta3$	CXCR1 and CXCR2/PI3K/Akt/AP-1	[46]
	CXCL12/SDF-1	$\alpha v\beta3$	CXCR4/ERK/NF-κB	[47]
Pro-inflammatory cytokines	TNF-α	$\alpha v\beta3$	MEK/ERK/IKKα/β/NF-κB	[48]
	Leptin	$\alpha v\beta3$	OBR1/IRS-1/PI3K/Akt/NF-κB	[49]
	Adiponectin	$\alpha2\beta1$	AdipoR/AMPK/p38/IKKα/β/NF-κB	[50]
	Macrophage migration-inhibitory factor (MIF)	$\alpha v\beta3$	PI3K/Akt/NF-κB	[51]
Cytokines	TGF-β	$\alpha v\beta3$	PI3K/Akt/NF-κB	[52]
	Bone morphogenetic proteins (BMPs)	$\beta1$	PI3K/Akt/IKKα/β/NF-κB	[53]
	Glial cell derived neurotrophic factor (GDNF)	$\alpha v\beta3$	MEK/ERK/IKKα/β/NF-κB	[54]
	Receptor activator of nuclear factor kappa-B ligand (RANKL)	$\beta1$	RANK/MEK/ERK/IKKα/β/NF-κB	[55]
Inflammatory-related molecules	Cyclooxygenase-2 (COX-2)	$\alpha2\beta1$	EP1/PLC/PKCα/c-Src	[56]
	Bradykinin (BK)	$\alpha2\beta1$	BK receptors/PLC/PKCδ/NF-κB	[57]
	High mobility group box chromosomal protein 1 (HMGB1)	$\alpha5\beta1$	RAGE (receptor for advanced glycation end products)/PI3K/Akt/c-Jun/AP-1	[58]

Certain inflammation-related molecules may also play important roles in regulating migration in human chondrosarcoma cells. Cyclooxygenase-2 (COX-2), an inducible enzyme that catalyzes the formation of prostaglandin E2 (PGE_2) during inflammation, is one such molecule. PGE_2 appears to upregulate the expression of the $\alpha2\beta1$ integrin via the EP1/PLC/PKCa/c-Src signaling pathways, leading to increased cell migration [57]. Bradykinin (BK) is a vasoactive peptide that mediates inflammatory responses and can also stimulate cell proliferation. BK was found to enhance chondrosarcoma cell migration by increasing $\alpha2\beta1$ integrin expression, through the BK receptor and PLC/PKCδ/NF-κB signal transduction pathways [58]. High mobility group box chromosomal protein 1 (HMGB-1) was originally identified as a nuclear protein that plays important roles in chromatin organization and transcriptional regulation. HMGB-1 has multiple functions, including the release of pro-inflammatory cytokines, cell proliferation, and cell migration. In human chondrosarcoma cells, HMGB-1 appears to promote cell migration by increasing $\alpha v\beta1$ integrin expression, through the RAGE (receptor for advanced glycation end products)/PI3 K/Akt/c-Jun/AP-1 signal transduction pathway [59] (Table 1).

5. Integrins as Signaling Receptors Regulating Chondrosarcoma Progression

High levels of integrin expression have been found in chondrosarcomas. Often, this is correlated with metastasis and poor prognosis. In light of this, it is noteworthy that integrins can regulate a wide range of signaling pathways critical for tumor growth and metastasis.

Increasing evidence suggests that ECM and its degradation products could play important roles in cancer progression and metastasis. Many of the underlying mechanisms are likely to involve integrin signaling. Proteomic comparison of human chondrogenic tumors revealed that the protein C-propeptides of procollagens I$\alpha1$ (PC1CP) were highly expressed in human chondrosarcomas, but not in benign enchondromas. Soluble PC1CP can induce the expression of VEGF and CXCR4 in a $\beta1$ integrin-dependent manner, and this has been linked to chondrogenic tumor vascularization and progression [60]. On the other hand, a different extracellular matrix protein, the NH_2-propeptide of type IIB procollagen (PIIBNP), was found to be capable of inducing cell death in chondrosarcoma, cervical and breast cancer cell lines, via its interaction with the integrins $\alpha v\beta3$ and $\alpha v\beta5$ [61]. Osteopontin (OPN) is an important component of the extracellular matrix in bone. The OPN protein has also been found to play a crucial role in determining the metastatic potential of various cancers. For example, OPN enhances the migration of chondrosarcoma cells by upregulating MMP-9 expression, through the $\alpha v\beta3$ integrin receptor, FAK (Focal Adhesion Kinase), MEK, ERK, and NF-κB-dependent signaling pathways [62, 63] (Table 2).

The CCN family of small secreted cysteine-rich proteins has six members (CCN1 to CCN6). The name CCN is derived from the first three members of the family to be discovered, namely, CYR61 (cysteine-rich angiogenic protein 61 or CCN1), connective tissue growth factor (CTGF/CCN2), and nephroblastoma overexpressed (NOV/CCN3). CCNs

Table 2: Integrin as a receptor regulates signalings in human chondrosarcoma cells.

Groups	Ligand	Integrin signaling	Regulation	Function	References
Extracellular matrix and its degradation fragments and by-products	PC1CP	$\beta 1$	VEGF expression ↑ CXCR4 expression ↑	Inducing chondrogenic tumor vascularization and progression	[59]
	PIIBNP	$\alpha v\beta 3$ and $\alpha v\beta 5$		Inducing cell death	[60]
	OPN	$\alpha v\beta 3$/FAK/MEK/ERK/NF-κB	MMP-9 expression ↑	Increasing cell migration	[61]
CCN family	CCN1	$\alpha v\beta 3$/FAK	MMP-13 expression ↑	Increasing cell migration	[62]
	CCN2	$\alpha v\beta 3$/FAK	MMP-13 expression ↑	Increasing cell migration	[63]
	CCN3	$\alpha v\beta 3$/FAK	MMP-13 expression ↑	Increasing cell migration	[64]
	CCN4	$\alpha 5\beta 1$/FAK/MEK/ERK/IKKα/β/NF-κB	MMP-2 activity ↑	Increasing cell migration	[65]
	CCN6	$\alpha v\beta 3$ and $\alpha v\beta 5$/FAK/MEK/ERK/c-Jun/AP-1	ICAM-1 expression ↑	Increasing cell migration	[66]

appear to regulate numerous biological processes, such as differentiation, migration, proliferation, and cell adhesion. Notably, aberrant expression of CCNs has been identified in a broad range of tumor types. In human chondrosarcoma cells, CCN1, CCN2, and CCN3 have been found to enhance cell migration by increasing MMP-13 expression; this is mediated via the $\alpha v\beta 3$ integrin receptor and FAK-dependent signaling mechanisms [64–66]. Other members of the CCN family have also been studied, including CCN4 (WISP-1) and CCN6 (WISP-3). These integrin-binding proteins appear to regulate cell migration in human chondrosarcoma cells by inducing integrin-dependent signaling. CCN4 (WISP-1) increases the activity of MMP-2, via the $\alpha v\beta 1$ integrin receptor and the FAK, MEK, ERK, IKKα/β, and NF-κB pathways, leading to enhanced migration of human chondrosarcoma cells [73]. Likewise, CCN6 (WISP-3) appears to function by increasing ICAM-1 expression through the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin receptor, FAK, MEK, ERK, c-Jun, and AP-1 pathways [74] (Table 2).

6. Integrins as Therapeutic Targets in Chondrosarcomas

Given the important roles of integrin-mediated signaling in metastasis and cancer progression, there has been increasing interest in therapeutic strategies to target these proteins. In human chondrosarcomas, increased expression of integrins, including $\alpha 2\beta 1$, $\alpha v\beta 1$, $\alpha v\beta 3$, $\beta 1$, and $\beta 5$, is closely associated with tumor progression and metastasis. Signaling through integrin receptors, such as $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, and $\beta 1$, may also promote cancer progression by regulating cell migration. This review discusses a selection of emerging therapeutic approaches for chondrosarcoma, together with their underlying molecular mechanisms. These include (i) integrin antagonists, (ii) inhibition of the RANK/RANKL/OPG axis, (iii) inhibition of FAK, (iv) inhibition of the IGF-I/IGF-IR axis, and (v) herbal medicines.

6.1. Integrin Antagonists. Since the discovery of the integrin-binding RGD sequence motif and its importance in mediating cell attachment, efforts have been made to develop RGD-related small molecules as integrin antagonists. Cilengitide, a cyclic RGD pentapeptide, is the first antiangiogenic small molecule developed to target the integrins $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha v\beta 1$ [75]. This drug is currently being tested in phase III clinical trials for treatment of glioblastomas, and in phase II trials for several other tumor types [76]. Chemical modifications to the cilengitide molecule, including N-methylation at distinct positions, can modulate its biological, structural, and pharmacokinetic properties; this could enhance selectivity, particularly for the $\alpha v\beta 3$ subtype [77]. In addition, since integrin $\alpha v\beta 3$ is expressed on the blood vessels that supply tumors, as well as on the tumor cells themselves, antagonists to this integrin might be particularly useful for treatment of chondrosarcoma. Another drug, ATN-161, is a non-RGD-based peptide inhibitor of $\alpha v\beta 1$ that is currently in clinical trials for cancer. In patients with advanced solid tumors who were given ATN-161, prolonged stable disease was observed in up to a third of the patients [78]. In a murine model of metastatic colorectal cancer, combination therapy with ATN-161 and 5-fluorouracil was found to reduce metastasis and improve survival [79]. ATN-161 has also been shown to reduce growth and metastasis of breast cancer cells implanted in mice [80]. The above findings suggest that this $\alpha v\beta 1$-inhibiting drug holds promise for the treatment of human chondrosarcomas.

Etaracizumab (also known as vitaxin, Abegrin, or MEDI-522), a humanized anti-$\alpha v\beta 3$ antibody, was the first anti-integrin monoclonal antibody to be tested in clinical trials for cancer. In a phase I study on etaracizumab, prolonged disease stabilization was observed in a number of cancer patients with metastatic lesions, who received the drug beyond the first cycle of therapy [81]. Etaracizumab was also shown to decrease osteoclastic bone resorption by impairing osteoclast attachment, without affecting osteoclast formation and multinucleation; this could be useful for reducing metastatic bone loss in cancer patients [82]. Volociximab (M200) is a chimeric mouse-human anti-$\alpha v\beta 1$ monoclonal antibody, which has shown anti-angiogenic activity *in vitro* and *in vivo* [83, 84]. In clinical trials, volociximab was well tolerated, and there is support for its efficacy in metastatic melanoma and renal cell carcinoma [85]. Consequently, these integrin antagonists may also have therapeutic potential for chondrosarcomas, to reduce metastasis and control tumor progression.

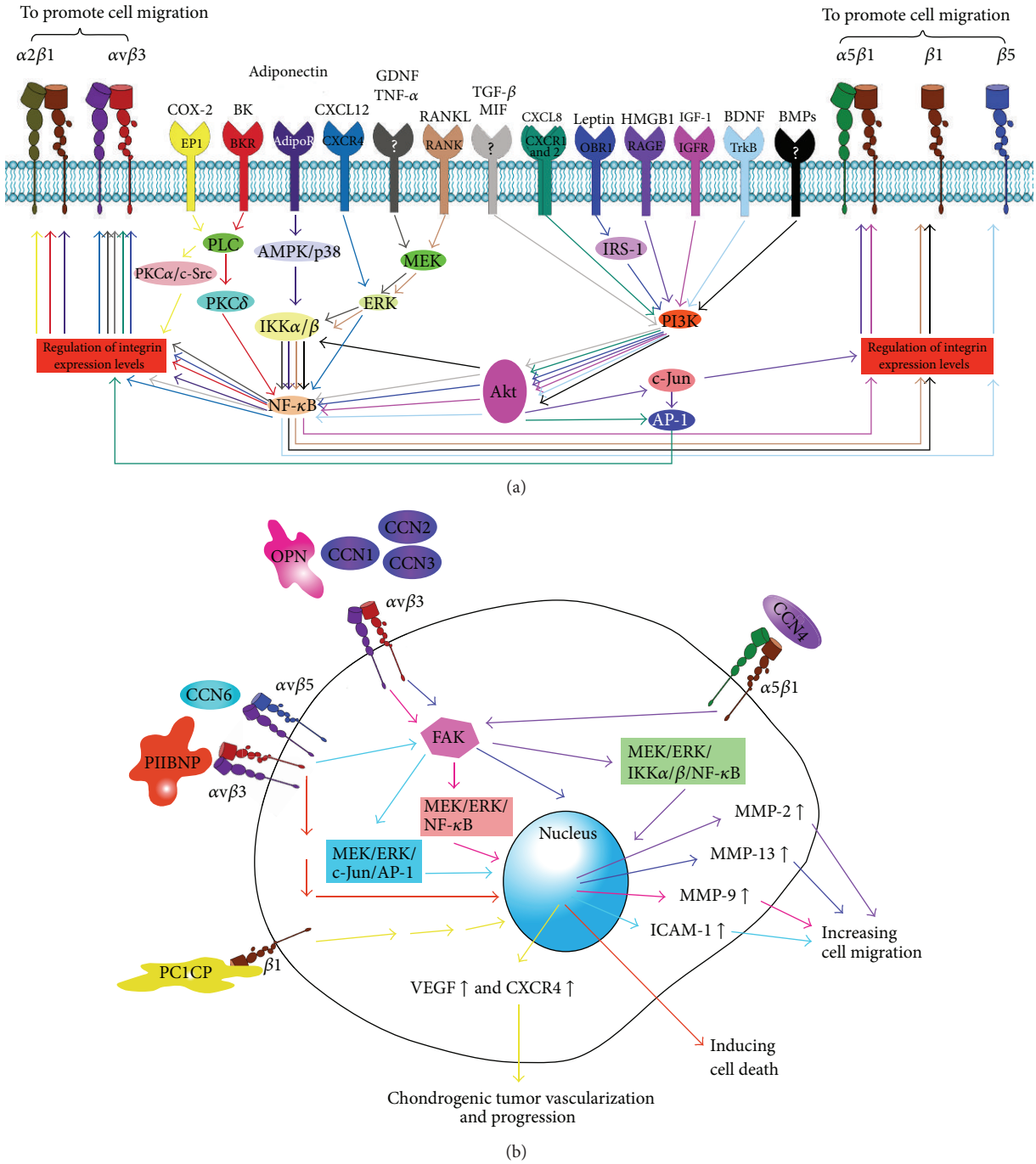

Figure 1: Schematic representation of the mediators that increase surface expression of integrin (a) and integrin-mediated signalings (b) which are shown to be novel therapeutic targets for chondrosarcomas.

The small molecule compound L-000845704 is an orally bioavailable nonpeptide $\alpha v\beta 3$ antagonist, which has been tested in preclinical and clinical trials for the treatment of osteoporosis [86, 87]. Another orally active nonpeptide $\alpha v\beta 3$ antagonist, SB 273005, has been shown to prevent and reduce edema and inflammation in a rat model of adjuvant-induced arthritis [88]. The potential applications of these integrin antagonists in the treatment of chondrosarcomas could be explored.

6.2. Inhibition of the RANK/RANKL/OPG Axis. In human chondrosarcoma tissues, RANKL and RANK expressions are higher than those in normal cartilage. Activation of the RANK/RANKL axis leads to the upregulation of $\beta 1$ integrin, and contributes to enhanced migration in human chondrosarcoma cells [56]. These observations have prompted efforts to develop therapies targeting RANKL. One promising approach involves the targeting of RANKL signaling with a decoy receptor, OPG, or with a soluble receptor form

(RANK-Fc); this has been shown to inhibit bone metastasis in a number of murine models [89–91]. A number of clinical trials involving denosumab, a fully human monoclonal antibody against RANKL, support its use as an alternative treatment option for bone metastases [92–94].

6.3. Inhibition of FAK (Focal Adhesion Kinase). Numerous studies indicate that integrin signaling through FAK plays a role in promoting migration of chondrosarcoma cells [62, 64–66, 73, 74, 95]. Inhibition of endogenous FAK activity by adenoviral overexpression of the C-terminal domain of FAK effectively interrupts FAK signaling and its downstream events; this was found to decrease cell invasiveness in chondrosarcoma cell lines [96]. A recent phase I trial of an inhibitor of FAK showed antitumor efficacy and minimal toxicity in patients with advanced solid tumors. Such results indicate that FAK might be another promising therapeutic target [97].

6.4. Inhibition of the IGF-I/IGF-IR Axis. Insulin-like growth factor 1 (IGF-1) can enhance the migration of chondrosarcoma cells by upregulating integrin expression. In addition, integrin binding can also regulate IGF-1 receptor (IGF-1R) signaling [98]. Consistent with this, blocking ligand occupancy of integrins reduced IGF-1-stimulated receptor phosphorylation, and inhibited cellular migration and DNA synthesis in response to IGF-1 [99]. This suggests that the IGF-1 signaling pathway may be another potential therapeutic target in chondrosarcoma [100]. Various IGF-1R monoclonal antibodies, including R1507, figitumumab, and ganitumab (AMG 479), have emerged as promising drugs for the treatment of Ewing's sarcoma, a small round-cell tumor that typically arises in the bones and soft tissues. A number of clinical trials to test these novel therapies are ongoing [101–105]. These drugs could potentially be explored for the treatment of other sarcomas, including chondrosarcoma.

6.5. Herbal Medicine. Berberine, an isoquinoline alkaloid, is a bioactive molecule found in the Ranunculaceae and Papaveraceae plant families. Berberine, which has been shown to inhibit cancer cell migration, was shown to downregulate $\alpha v\beta 3$ integrin expression through the PKCδ, c-Src, and AP-1 pathways [106].

7. Conclusion

Chondrosarcomas are the second most common form of bone malignancy. These tumors are relatively resistant to chemotherapy and radiotherapy; currently, surgical resection is the only effective therapeutic option. However, 5–10% of conventional chondrosarcomas are high-grade tumors, which show high metastatic potential and poor outcomes after resection alone. It is therefore crucial to identify and develop effective adjuvant treatments. Integrins, which are cell surface proteins involved in diverse biological processes, have been implicated in cancer cell migration, invasion, and metastasis, during tumor progression. Consequently, targeting of integrin expression and signaling has been considered a promising approach in cancer therapy. Nevertheless, integrins play a crucial role in many physiological processes; for example, tissue morphogenesis, inflammation, wound healing, and regulation of cell growth and differentiation. Any inhibition of these may cause serious adverse effects that must be taken to into account. Clinical and preclinical studies aimed at inhibiting integrin expression and signaling are ongoing. To date, however, integrin-targeted therapeutics in chondrosarcomas have not yet been successfully translated into clinical practice. This review summarizes recent progress in elucidating the molecular basis for integrin function in cancer. We have discussed various mechanisms and mediators that regulate the expression of integrins and integrin-mediated signaling (Figure 1). This understanding of molecular mechanisms could be translated into effective therapies for chondrosarcoma.

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Review Article

Poly-*S*-Nitrosated Albumin as a Safe and Effective Multifunctional Antitumor Agent: Characterization, Biochemistry and Possible Future Therapeutic Applications

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Nitric oxide (NO) is a ubiquitous molecule involved in multiple cellular functions. Inappropriate production of NO may lead to disease states. To date, pharmacologically active compounds that release NO within the body, such as organic nitrates, have been used as therapeutic agents, but their efficacy is significantly limited by unwanted side effects. Therefore, novel NO donors with better pharmacological and pharmacokinetic properties are highly desirable. The *S*-nitrosothiol fraction in plasma is largely composed of endogenous *S*-nitrosated human serum albumin (Mono-SNO-HSA), and that is why we are testing whether this albumin form can be therapeutically useful. Recently, we developed SNO-HSA analogs such as SNO-HSA with many conjugated SNO groups (Poly-SNO-HSA) which were prepared using chemical modification. Unexpectedly, we found striking inverse effects between Poly-SNO-HSA and Mono-SNO-HSA. Despite the fact that Mono-SNO-HSA inhibits apoptosis, Poly-SNO-HSA possesses very strong proapoptotic effects against tumor cells. Furthermore, Poly-SNO-HSA can reduce or perhaps completely eliminate the multidrug resistance often developed by cancer cells. In this review, we forward the possibility that Poly-SNO-HSA can be used as a safe and effective multifunctional antitumor agent.

1. Nitric Oxide Delivery Systems

Nitric oxide (NO) is a unique, diffusible molecular messenger that plays a central role in mammalian physiology and pathophysiology [1–7]. The effects of NO are pleiotropic, including vascular smooth muscle relaxation [8, 9], inhibition of platelet aggregation [10], and regulation of immune and neuronal functions [11]. However, under certain circumstances NO can be cytotoxic. For example, high concentrations of NO can inhibit tumor cell growth and induce apoptosis. Recent studies have revealed that NO is associated with not only apoptosis of cancer cells but also with inhibition of cancer progression and metastasis, as well as cancer angiogenesis. It also functions as a modulator for chemo/radio/immunotherapy. Unfortunately, despite such highly useful properties, the use of NO has been impeded by the fact that its *in vivo* half-life is so short (~0.1 s) that NO itself cannot be used as a therapeutic agent. Therefore, pharmacologically active compounds that can release NO or lead to its formation in the body have been synthesized. For example, organic nitrates and nitrite esters have been used for many years to treat patients with ischemic heart disease. However, there are well-known side effects and limitations to these NO donors, including potentially adverse hemodynamic effects, drug tolerance, lack of selectivity and limited bioavailability [12, 13]. Thus, it is essential to

develop reliable NO donors with better pharmacological and pharmacokinetic parameters.

It is also important to be able to control reaction selectivity and dose of the NO donor against reactive oxygen species such as in NO therapy in inflammatory diseases. A high concentration of NO produced by inducible nitric oxide synthase (iNOS) is protective against bacterial infection in inflammatory processes, but too much NO will induce apoptosis and cellular damage [1, 15–17]. The latter effect is due to the formation of peroxynitrite ($ONOO^-$), the reaction product of the interaction between superoxide (O_2^-) and NO, a potent proinflammatory nitroxide implicated in acute and chronic inflammatory conditions of many etiologies [18–21]. In addition, tissue injury and inflammation often accompany rapid development of hypersensitivity to noxious and nonnoxious stimuli (hyperalgesia and allodynia, resp.). In fact, sodium nitroprusside, which has been parenterally administered for the treatment of hypertension and heart failure, also induces an increase in the vascular production of superoxide leading to the formation of $ONOO^-$, which is associated with cytotoxic effects of sodium nitroprusside [22, 23].

Local application of NO may be a very effective and safe form of NO therapy. To develop a method for the targeted delivery of NO, several groups of researchers have synthesized NO donors that hopefully can release NO selectively at a target site. For example, O(2)-vinyl-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (V-PYRRO/NO) and 2-(acetyloxy)benzoic acid-3-(nitrooxymethyl)phenyl ester (NCX-1000) can selectively release NO in the liver. NO release from V-PYRRO/NO is mediated by cytochrome P450 which removes the vinyl group of the drug to generate free PYRRO/NO ion [24–26]. NCX-1000 is a prototype of a family of NO-releasing derivatives of ursodeoxycholic acid. The two compounds are selectively metabolized in the liver and biologically active NO enters the liver microcirculation without a detectable effect on systemic circulation [27]. However, these NO donors have not yet been applied to clinical situations, because their reaction mechanisms are not yet fully clarified. Thus, although NO release from V-PYRRO/NO is mediated by cytochrome P450, the isoform of cytochrome P450 catalyzing the process has not been identified. The enzyme that mediates NO release from NCX-1000 is still unknown. The enzymes that mediate NO release from V-PYRRO/NO and NCX-1000 must be identified in order to optimize their therapeutic efficacy [28].

In our search for a reliable and safe NO donor, we have followed a different approach, namely, to examine the possibility of using a NO-traffic protein. By a NO-traffic protein is meant a protein with (i) high efficiency of *S*-nitrosation, (ii) high stability of the *S*-nitroso form in the circulation, and (iii) high efficiency of *S*-transnitrosation into cells which need NO. As a candidate in this respect we focus on human serum albumin (HSA), because HSA is the most abundant plasma protein (35–50 g/L) and because endogenous *S*-nitrosothiol in human plasma is largely associated with HSA [31, 32].

2. Native, *S*-Nitrosated HSA as a NO Carrier

S-nitrosated HSA (SNO-HSA) is significantly more stable than low molecular weight *S*-nitrosothiols [31, 32]. In addition to us, also other researchers have attempted to produce NO delivery systems using a NO-albumin conjugate. Thus, Marks et al. [34] and Ewing et al. [35] have synthesized a macromolecular *S*-nitrosothiol, Poly-SNO-BSA, in which several *S*-nitrosothiols are formed in bovine serum albumin (BSA) after reduction of the protein's disulfide bonds. Independently, Beak et al. have developed a macromolecular NONOate, diazeniumdiolated BSA, in which several NONOate moieties have been conjugated to native BSA [36]. In a porcine coronary angioplasty model, the two BSA-forms, Poly-SNO-BSA and diazeniumdiolated BSA, were applied locally to a site of vascular injury and showed high retention at the administration site and reduced platelet attachment and activation. These effects were due to high binding of the modified albumins to the injured vessel. In the development of targeted NO delivery systems for intravenous use, tissue distribution characteristics of the NO-carrier conjugate should be evaluated *in vivo* in order to identify the various obstacles to targeted delivery, such as extensive uptake by mononuclear phagocyte systems and rapid loss by glomerular filtration. Katsumi et al. have examined the pharmacokinetic properties of SNO-BSA. The results showed that serum albumin is a promising carrier to control pharmacokinetic properties of NO after intravenous injection, because *S*-nitrosated albumin shows a relatively high retention in the blood circulation after intravenous injection into mice. However, targeted NO delivery after intravenous injection using a macromolecular carrier has not been successfully achieved so far [37].

To achieve targeted NO delivery from SNO-HSA after intravenous injection, we need to understand the method of *S*-nitrosation, the structure of HSA, and its biological fate in detail. Therefore, we have recently examined the structure and the biological effects of Mono-SNO-HSA, HSA with one (or less) *S*-nitrosothiol, and Poly-SNO-HSA.

Endogenous *S*-nitrosated human serum albumin (Mono-SNO-HSA) is a large molecular weight NO carrier in human plasma, which has shown many beneficial effects in different animal models. In an attempt to construct more efficient SNO-HSA preparations, we have prepared SNO-HSA with many conjugated SNO groups (Poly-SNO-HSA) using chemical modification with 2-iminothiolane. We have compared the properties of such a preparation to those of Mono-SNO-HSA using C26 and HepG2 cells [14]. We found that cellular uptake of NO from Mono-SNO-HSA partly takes place via low molecular weight thiol, and it results in cytoprotective effects by induction of heme oxygenase-1. By contrast, transfer of NO from Poly-SNO-HSA into the cells was faster and more pronounced. The influx mainly takes place by cell-surface protein disulfide isomerase. Instead of cytoprotection, the considerable NO inflow resulted in apoptotic cell death caused by reactive oxygen species (ROS) induction, caspase-3 activation, and other means. Thus, increasing the number of SNO groups on HSA does not simply intensify the cellular responses to NO but can also

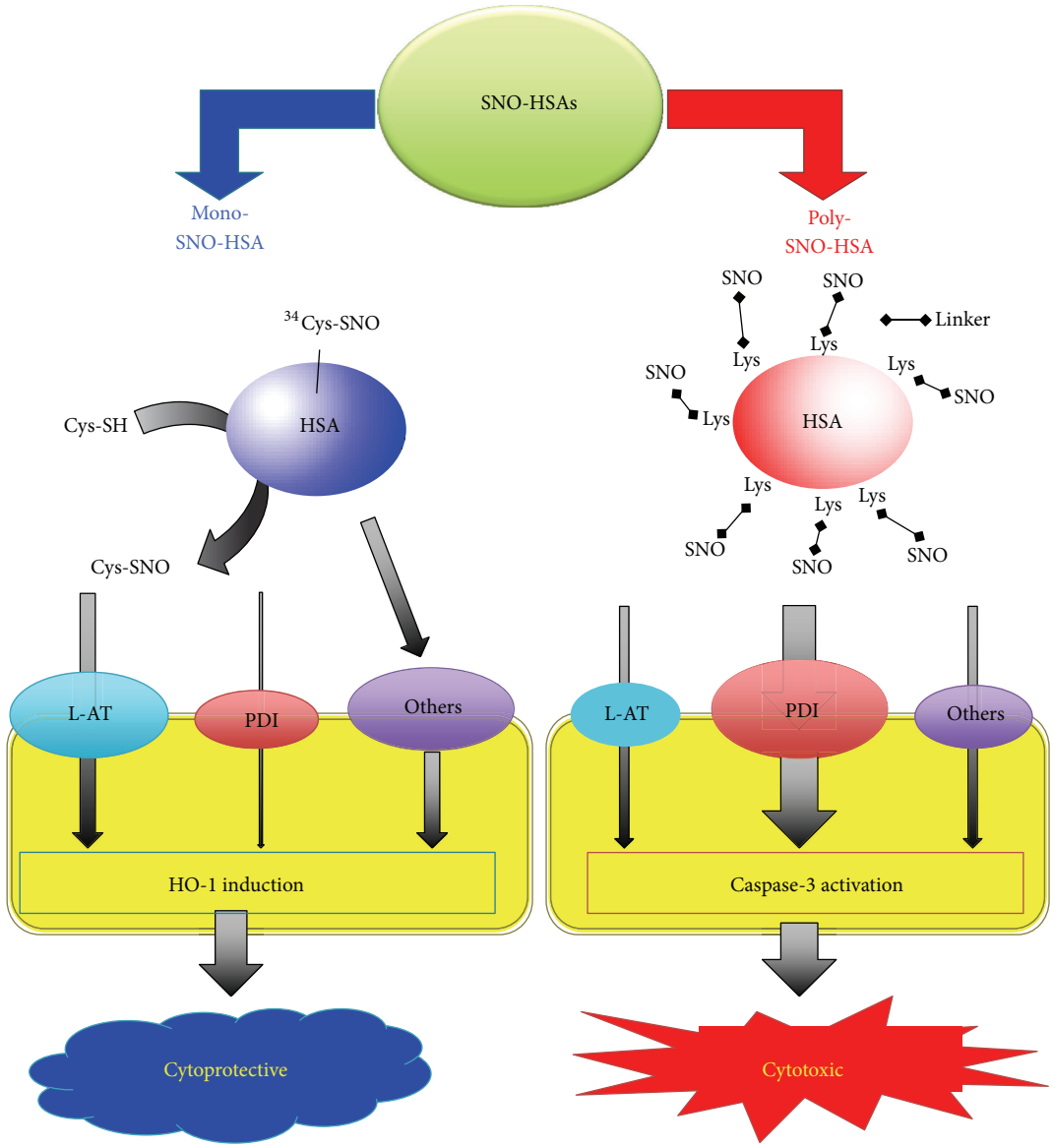

Figure 1: Differences in the mechanisms and consequences of NO traffic from Mono-SNO-HSA and Poly-SNO-HSA to cells. NO transfer from the SNO group of Cys-34 on Mono-SNO-HSA to the cell is partly mediated by the L-amino acid transporter (L-AT) via *S*-transnitrosation to free low molecular weight thiol. By contrast, NO transfer from Poly-SNO-HSA is mainly mediated by cell-surface protein disulfide isomerase (PDI) without *S*-transnitrosation to free low molecular weight thiol. The relatively slow transfer of NO from Mono-SNO-HSA avoids the presence of high intracellular NO concentrations and leads to cytoprotective activity through heme oxygenase-1 (HO-1) induction. On the other hand, the NO influx from Poly-SNO-HSA is very fast and pronounced and leads to cell death caused by apoptosis [14].

result in very different effects (Figure 1). The number of moles of NO per mole of HSA in the Poly-SNO-HSA preparation used in our studies was estimated to be 6.6 ± 0.5 mol NO/mol HSA. The half-life ($T_{1/2}$) of Poly-SNO-HSA in phosphate buffered saline, pH 7.4, is 21 ± 3 days at 25°C. On the other hand, $T_{1/2}$ of Poly-SNO-HSA in mice is only 2.1 ± 0.3 h. A metabolite of Poly-SNO-HSA, iminothiolane-modified HSA which had been exposed to UV-light, had no modified effect on LDH release as compared with normal HSA [14]. Thus, Mono-SNO-HSA could be a cytoprotective agent, whereas Poly-SNO-HSA could have potential as an antitumor agent. In this review, we discuss the possibility of using Poly-SNO-HSA as a safe and effective multifunctional antitumor agent in biological systems.

3. Nitric Oxide Donor as Cancer Therapeutics Agent

NO is a cell signaling molecule that can be a potent inducer of cell death in cancers at elevated concentrations [38]. For example, increased intracellular NO levels lead to

growth inhibition of both androgen-dependent and castration-resistant prostate tumors through a mechanism that involves androgen receptor function inactivation by *S*-nitrosylation of a single C601 residue present in the DNA-binding domain [39]. Furthermore, NO donors such as spermine/NO and diethylenetriamine/NO present cytotoxic activity on ovarian cancer cell lines, mainly through induction of apoptosis through inhibition of phosphorylation of STAT3 and AKT3 signaling proteins [40]. NO-donating nonsteroidal anti-inflammatory drugs (NSAIDs), especially NO-aspirin (NO-ASA), have also been shown to be able to reduce the growth of cultured HT-29 colon adenocarcinoma cells [41]. NO-ASA consists of traditional ASA to which an NO-releasing moiety is bound via a spacer. This agent induces oxidative stress by increasing intracellular peroxide and superoxide, thereby inducing apoptosis via activation of the intrinsic apoptosis pathway [42]. More recently, glutathione-*S*-transferase activated NO generators have shown some promise for NO therapy of cancer [43]. Intravenous injection of one such agent, JS-K, into mice bearing subcutaneously implanted multiple myeloma tumors on the flank, resulted in an impressive inhibition of tumor growth and induction of extensive apoptosis throughout the tumor. Finally, Duan et al. have designed a polymeric carrier system to deliver nitric oxide locoregionally to tumorigenic tissues at micromolar concentrations, and treatment of tumor-bearing nude mice with this polymeric carrier resulted in 50% tumor inhibition and in a 7-week extension of the average survival time, compared to intravenous therapy with the above mentioned prodrug JS-K [44]. Thus, cancer therapy by NO donors has been actively investigated. However, free NO can also be toxic to normal tissues, and chronic exposure at low levels can induce tumor growth. In addition, systemic use of NO donor drugs at high doses can result in hypotension [40]. In comparison, HSA has several advantages as a tumor targeting NO carrier as mentioned in Native, *S*-Nitrosated HSA as a NO Carrier.

4. Direct Antitumor Effect of Poly-SNO-HSA via Apoptosis

Poly-SNO-HSA as a NO donor has been investigated for its potential therapeutic applications, but there had been no reports describing the effects of Poly-SNO-HSA on cancer. Therefore, our research group produced Poly-SNO-HSA using the chemical linker 2-iminothiolane and observed that this HSA form can induce apoptosis in cancer cells [14]. The apoptosis occurs via activation of the intrinsic apoptosis pathway in, for example, murine colon 26 carcinoma cells and in the rat tumor cell line LY-80 (a variant of Yoshida sarcoma) both *in vivo* and *in vitro*.

The process was studied in some detail. For example, mitochondria seem to play a pivotal role in the regulation of this process in mammals, because it is believed that loss of mitochondrial membrane potential is an essential element of apoptosis. To evaluate the effects of Poly-SNO-HSA on mitochondrial function and membrane potential, LY-80 cells were loaded with a mitochondrion-selective fluorescent cation (rhodamine 123), and we found that Poly-SNO-HSA treatment decreased rhodamine fluorescent intensity in a dose-dependent manner [29, 30]. HSA also attenuated fluorescence, but to a lesser extent than Poly-SNO-HSA (Figure 2(a)). These observations indicate that Poly-SNO-HSA induces depolarization of the mitochondrial membrane.

Caspase-3 is a cell-death protease that is involved in the downstream execution phase of apoptosis, during which cells undergo morphological changes, such as DNA fragmentation, chromatin condensation, and formation of apoptotic bodies. As compared to controls, LY-80 cells treated with 25, 50, or 100 μM Poly-SNO-HSA showed relative increases in caspase-3 activity of 21-, 34-, and 42-fold, respectively (Figure 2(b)). The caspase-3 activity of HSA-treated cells was equivalent to that of cells treated with buffer alone. As additional controls, in order to elucidate the effect of 2-iminothiolane on the activation of caspase-3, HSA-2-iminothiolane modified (HSA-I) and UV-reduced Poly-SNO-HSA (Poly-SNO-HSA-R) were also incubated with LY-80 cells. Predictably, Poly-SNO-HSA-R as well as HSA-I did not activate caspase-3, which suggested that 2-iminothiolane did not participate in the activation of caspase-3 and that NO bound via functional thiols introduced by reaction of 2-iminothiolane with amino groups of lysine residues on HSA might be more efficient for its release than NO bound to free thiols obtained by reduction (Figure 2(b)). As an interesting observation supporting this result, also uptake of NO from Poly-SNO-HSA to HepG2 cells was much higher than that from Mono-SNO-HSA [14]. To further confirm that Poly-SNO-HSA induced apoptosis in LY-80 cells, DNA fragmentation, which is a morphological change characteristic of the execution phase of apoptosis, was examined [30]. DNA fragmentation was observed in LY-80 cells after 4 h of incubation with 100 μM Poly-SNO-HSA and stabilized after 8 h. By contrast, DNA fragmentation was not detected until 24 h of incubation with 100 μM HSA. Moreover, the DNA ladder observed after 12 h of incubation in Poly-SNO-HSA increased in a dose-dependent manner. To determine the mechanism by which Poly-SNO-HSA causes DNA fragmentation, LY-80 cells were simultaneously incubated with Poly-SNO-HSAs and Z-VAD-FMK (a caspase inhibitor). DNA fragmentation induced by 100 μM Poly-SNO-HSA was completely abolished by treatment with Z-VAD-FMK, indicating that caspases are positive, upstream regulators of the DNA fragmentation that is elicited by Poly-SNO-HSA.

To determine the effect of Poly-SNO-HSA on cell growth, the viability of LY-80 cells was examined after treatment with either HSA or various concentrations of Poly-SNO-HSA. We found that Poly-SNO-HSA inhibited growth of LY-80 cells in a concentration-dependent manner. HSA also tended to abrogate cell proliferation, but to a lesser extent than Poly-SNO-HSA. To further characterize Poly-SNO-HSA-induced LY-80 cell death, cytotoxicity was examined using an assay of lactate dehydrogenase (LDH) activity (Figure 2(c)). LDH is a stable enzyme that is rapidly released from cells into the cell culture medium upon damage to the plasma membrane. Cell death increased with incubation time for the cultures incubated with 100 μM Poly-SNO-HSA. After 48 h of incubation, LDH was released from nearly all cells. In contrast, HSA was not cytotoxic. We also observed that

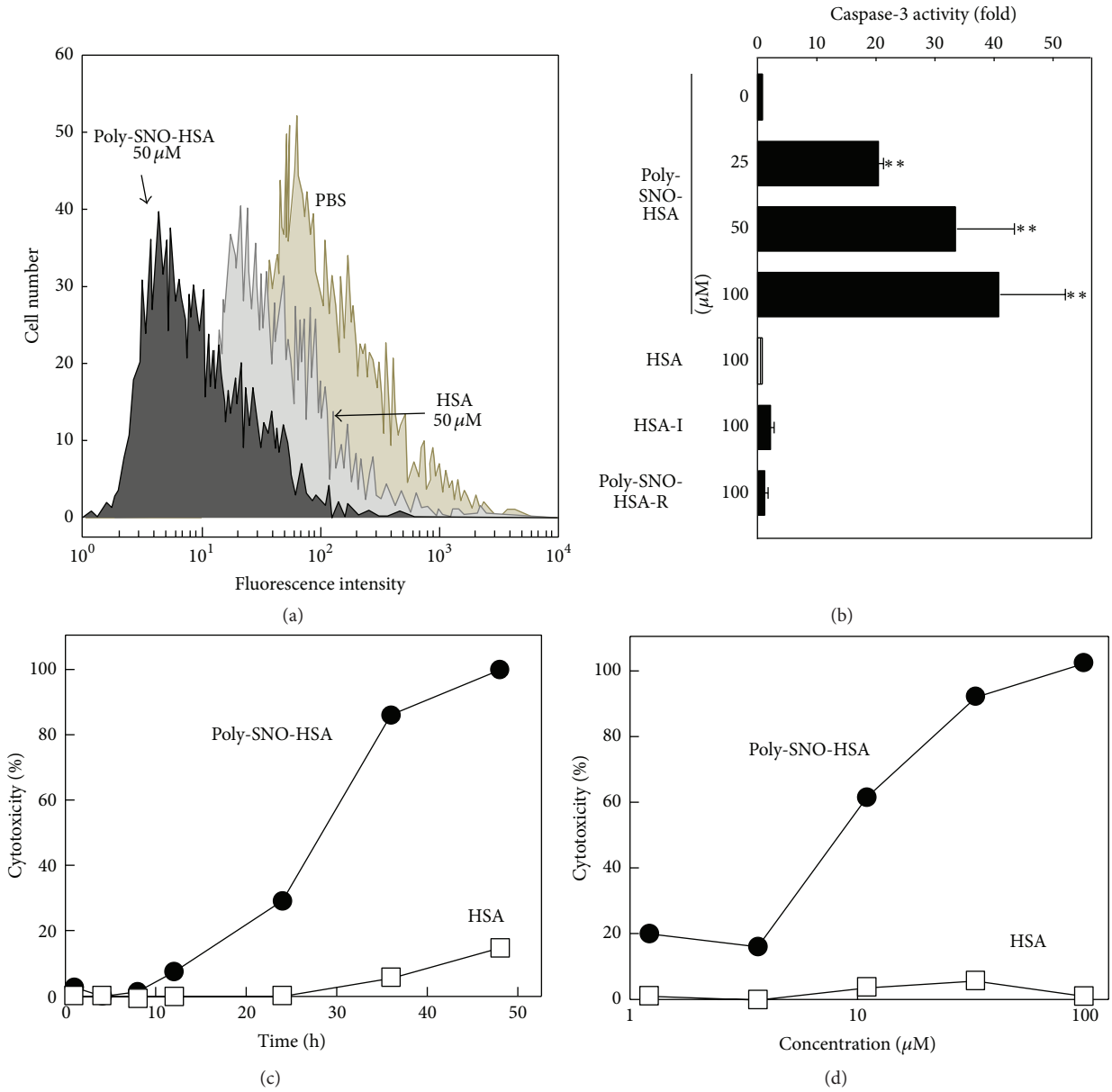

FIGURE 2: (a) Mitochondrial transmembrane potential is altered by Poly-SNO-HSA treatment. LY-80 cells were cultured with either phosphate buffered saline (PBS), 50 μM HSA, or 50 μM Poly-SNO-HSA for 2 h, followed by addition of rhodamine 123. Results are for one representative experiment. (b) Activation of caspase-3 after Poly-SNO-HSA treatment. LY-80 cells were incubated with either PBS (0), 100 μM of HSA, HSA-I or Poly-SNO-HSA-R or with different concentrations of Poly-SNO-HSA for 24 h. HSA-I and Poly-SNO-HSA-R represent 2-iminothiolane modified HSA and UV-reduced Poly-SNO-HSA, respectively. Results are means ± SD of three separate experiments. (c) Effect of Poly-SNO-HSA on LDH release (cytotoxicity). LY-80 cells were incubated for the indicated times with 100 μM HSA (open squares) or 100 μM Poly-SNO-HSA (closed circles). (d) Effect of Poly-SNO-HSA on LDH release (cytotoxicity). LY-80 cells were treated for 48 h with various concentrations of HSA (open squares) or Poly-SNO-HSA (closed circles). Results from three separate experiments are presented as means. $^{**}P < 0.01$, compared with control.

the cytotoxicity of Poly-SNO-HSA towards LY-80 cells is dose-dependent (Figure 2(d)). These, and the above, results suggest that Poly-SNO-HSA induces cell death via activation of an intrinsic apoptosis-signaling pathway.

To investigate the antitumor effects of Poly-SNO-HSA *in vivo*, LY-80 tumor-bearing rats received either intravenous or direct intratumor injections of either saline, HSA, or Poly-SNO-HSA. Mean tumor volume increased with time in the saline-treated group. A similar trend was observed in the HSA-treated group. However, tumor growth in animals that received direct intratumor injections of Poly-SNO-HSA was only one-third that observed in the saline- and HSA-treated animals (Figure 3). This observation suggests that Poly-SNO-HSA has antitumor effects *in vivo*, presumably due

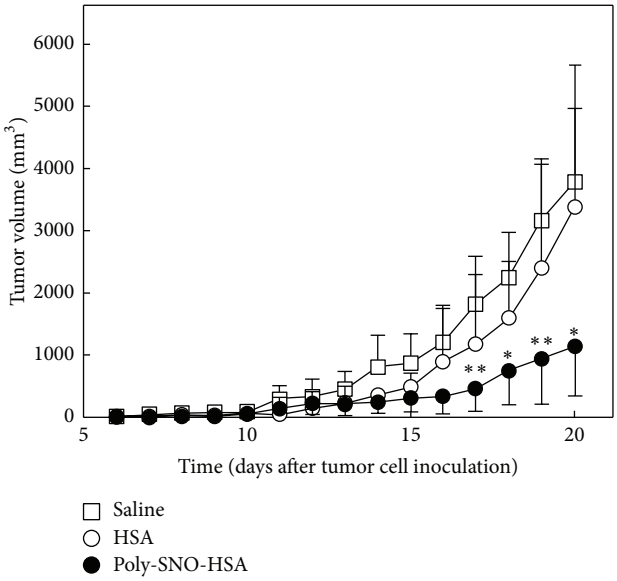

Figure 3: Effect of Poly-SNO-HSA on tumor growth in LY-80 tumor-bearing rats. LY-80 tumor-bearing rats were given daily intratumor injections of saline (5 mL/kg; open squares), HSA (10 μmol/5 mL/kg; open circles), or Poly-SNO-HSA (10 μmol/5 mL/kg; closed circles) for 7 days (day 5–11 after inoculation with tumor cells). Results are means ± SD; $n = 4$ animals per experimental group. *Statistically significant reduction in tumor growth as compared with treatment with saline ($P < 0.05$) or HSA ($P < 0.05$) at the corresponding time. **Statistically significant reduction as compared with treatment with saline ($P < 0.01$) or HSA ($P < 0.05$) at the corresponding time [29, 30].

to induction of apoptosis. We observed similar results, when we used mice bearing other cell types such as murine colon 26 carcinoma cells or SW480 cells [29].

The latter study [29] also revealed that treatment with Poly-SNO-HSA caused no significant changes in total serum protein, serum creatinine, blood urea nitrogen, aspartate aminotransferase, or alanine aminotransferase. These findings propose that Poly-SNO-HSA does not cause kidney or liver damage and suggest that Poly-SNO-HSA may not interfere with cell cycle in nonmalignant cells.

5. Poly-SNO-HSA Can Overcome MDR

Cancer treatment remains one of the most important clinical challenges. One difficulty in treating various cancers is the development of multidrug resistance (MDR) by cancer cells. Various approaches have been tested to overcome MDR such as using agents that inhibit P-glycoprotein (P-gp) directly or indirectly through altering the cell membrane and using targeted drug delivery. Most of the approaches have shown some success in small animal models, but its clinical application has been limited. However, NO is one of the compounds that have shown promises in treating cancer.

In addition to its apoptosis effects, the antitumor effect of Poly-SNO-HSA could be due to its ability to overcome MDR. Therefore, we have performed *in vitro* and *in vivo* experiments to shed some light on this effect. For example, preliminary experiments showed, as expected, that doxorubicin- (dx-)resistant K562 (K562/dx) cells had significantly better survival as compared with K562 parental cells while treated with dx alone for 24 h (Figure 4(a)). To determine the effect of Poly-SNO-HSA on resistant tumor cell growth, the viability of K562 and K562/dx cells was examined after incubation with various concentrations of Poly-SNO-HSA. We found that Poly-SNO-HSA inhibited growth of both the K562 parental cells and the doxorubicin resistant K562/dx cells, in a similar way and in a dose-dependent manner (Figure 4(b)). Some of our earlier studies had shown that Poly-SNO-HSA also induces cell death in C26 cells and in LY-80 cells [29, 30]. In a following study, we first evaluated the transfer of NO from Poly-SNO-HSA into K562 cells and K562/dx cells by the DAF-FM DA fluorescence assay. It was seen that treatment of the cells with Poly-SNO-HSA resulted in a dose- and time-dependent uptake of NO the amount of which reached saturation in approximately 2 h. Therefore, in the following experiments different concentrations of Poly-SNO-HSA (0.5–10 μM) were added to the cells 2 h before treatment with 5 μM dx. The results showed a significant attenuation in the resistance to dx in the K562/dx cells as the concentration of Poly-SNO-HSA increased (Figure 4(c)). The inhibitory effects of Poly-SNO-HSA and dx on the growth of K562/dx cells were evaluated for synergistic action by using isobologram analysis. These results propose that Poly-SNO-HSA has the following two antitumor effects: it inhibits cell growth and attenuates the resistance to dx.

To investigate the effect of Poly-SNO-HSA on chemoresistance *in vivo*, we prepared K562 and K562/dx tumor-bearing mice by injection of 2×10^7 K562 or K562/dx cells into the left hind flank of female BALB/cAJcl-nu/nu mice. After the tumors had grown to 150–200 mm^3, the mice received either intraperitoneal or intravenous injections of either saline, dx, and/or Poly-SNO-HSA biweekly. Treatment with dx decreased significantly the time-dependent tumor growth in K562 tumor-bearing mice (Figure 5(a)). By contrast, in K562/dx mice dx did not affect tumor growth significantly (Figure 5(b)). A slightly more pronounced, but significant, effect was observed, when the K562/dx mice were treated with Poly-SNO-HSA. However, combining dx and Poly-SNO-HSA decreased tumor volume to one-third as compared with treatment with them alone (Figure 5(c)). This observation proposes that Poly-SNO-HSA overcomes dx resistance *in vivo*. In the two types of mice, we also measured the tumor amounts of dx. The results showed that Poly-SNO-HSA increased several-fold the dx concentration in the tumor tissue of the K562/dx mice, suggesting that the antitumor effect of Poly-SNO-HSA was enhanced, at least in part, by increasing the local concentration of dx.

We have also examined the mechanism of Poly-SNO-HSA-induced chemosensitivity. NO shows multiple physiological actions via the cGMP-dependent way which involves activation of a soluble guanylate cyclase resulting in production of cGMP and activation of a protein kinase (PKG) [45, 46]. To determine whether the effect of Poly-SNO-HSA

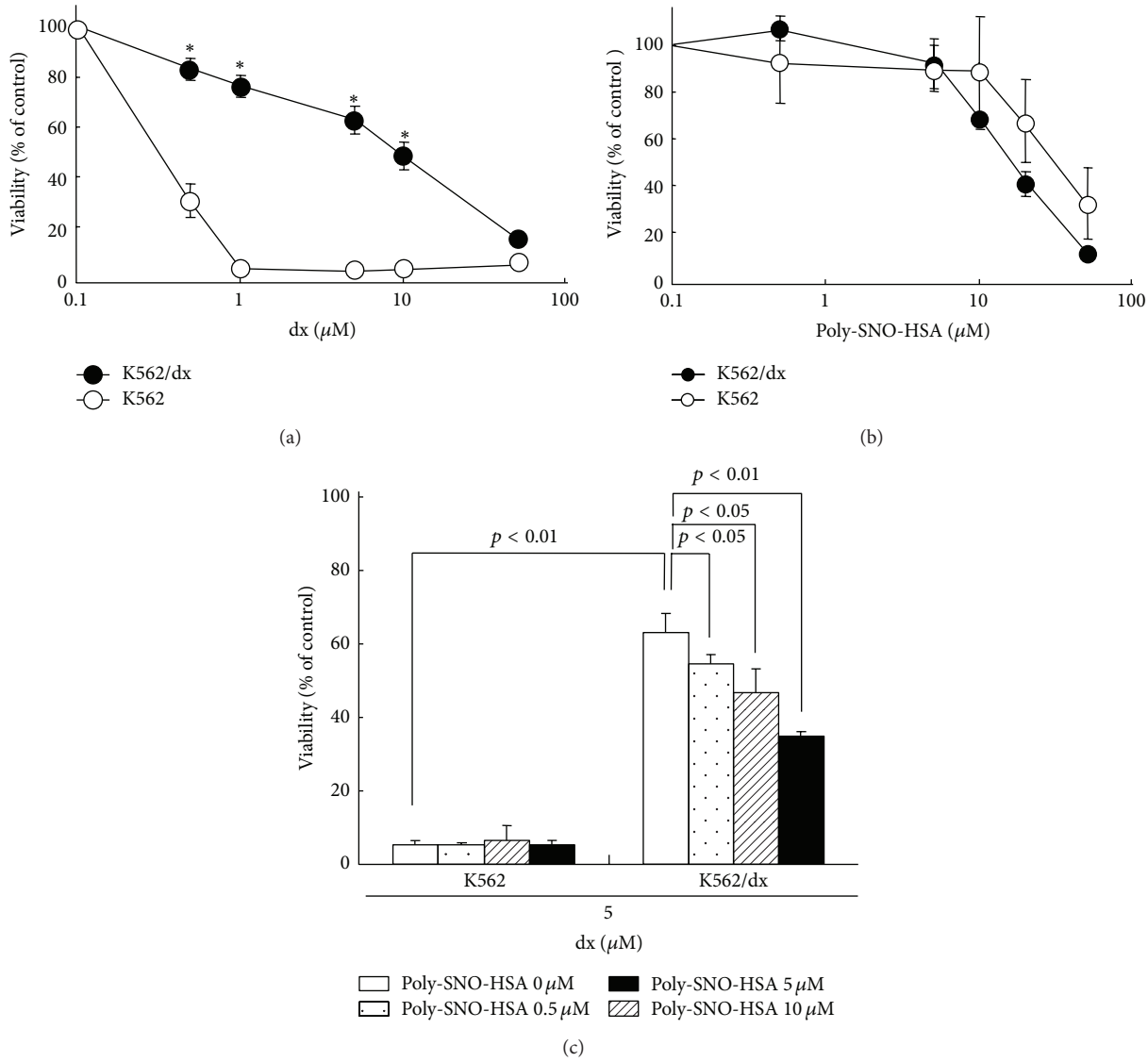

Figure 4: Synergistic effects of doxorubicin (dx) and Poly-SNO-HSA on the viability of K562 parental cells and doxorubicin-resistant K562 (K562/dx) cells. K562 cells (open circles) and K562/dx cells (closed circles) were treated for 24 h with various concentrations of dx (a) or Poly-SNO-HSA (b). In panel (c), K562 cells and K562/dx cells were treated with dx (5 μM) for 24 h following incubation with various concentrations of Poly-SNO-HSA for 2 h. Data are expressed as means ± SD ($n = 3$). $^*P < 0.01$, compared with K562 cells [29, 30].

on resistant tumor cells was mediated by cGMP signaling, we used the soluble guanylate cyclase inhibitor 1-H-(1,2,4)oxadiazolo-(4,3-a)quinoxalin-1-one (ODQ) and the nonhydrolyzable cGMP analogue 8-Br-cGMP. ODQ in K562/dx cells suppressed the effect of Poly-SNO-HSA on reverting the resistance to dx. In contrast, the resistance to dx in K562/dx was significantly diminished following administration of 8-Br-cGMP. These data strongly indicate that Poly-SNO-HSA reverts dx resistance partly via the cGMP signaling pathway. Thus, activation of the NO-cGMP-dependent signaling pathway by NO donors or cGMP analogs could represent a novel approach to cancer therapy including chemoresistance.

Another mechanism of Poly-SNO-HSA-induced chemosensitivity could be intracellular accumulation of dx. Therefore, that potential effect was examined by flow cytometry [33]. We found that, after 1 h incubation with dx, the fluorescence intensity of dx in K562/dx cells was about 60% of that in K562 cells. Furthermore, in K562/dx cells, pretreatment for 2 h with 0.5–10 μM Poly-SNO-HSA before dx addition significantly enhanced the dx accumulation.

In order to explain the modulating effects of Poly-SNO-HSA on dx in K562/dx cells, we also analyzed the expression of P-gp by Western blotting [33]. The P-gp protein was strongly expressed in K562/dx cells, while it was not clearly detected in K562 cells. The overexpression of P-gp in K562/dx was decreased by Poly-SNO-HSA pretreatment. These results indicate that Poly-SNO-HSA enhances intracellular accumulation of dx by downregulating P-gp.

The hypoxia-inducible factor-1α (HIF-1α) is activated in hypoxia solid tumor, and it is involved in regulating the transcription of the ATP-binding cassette transporters

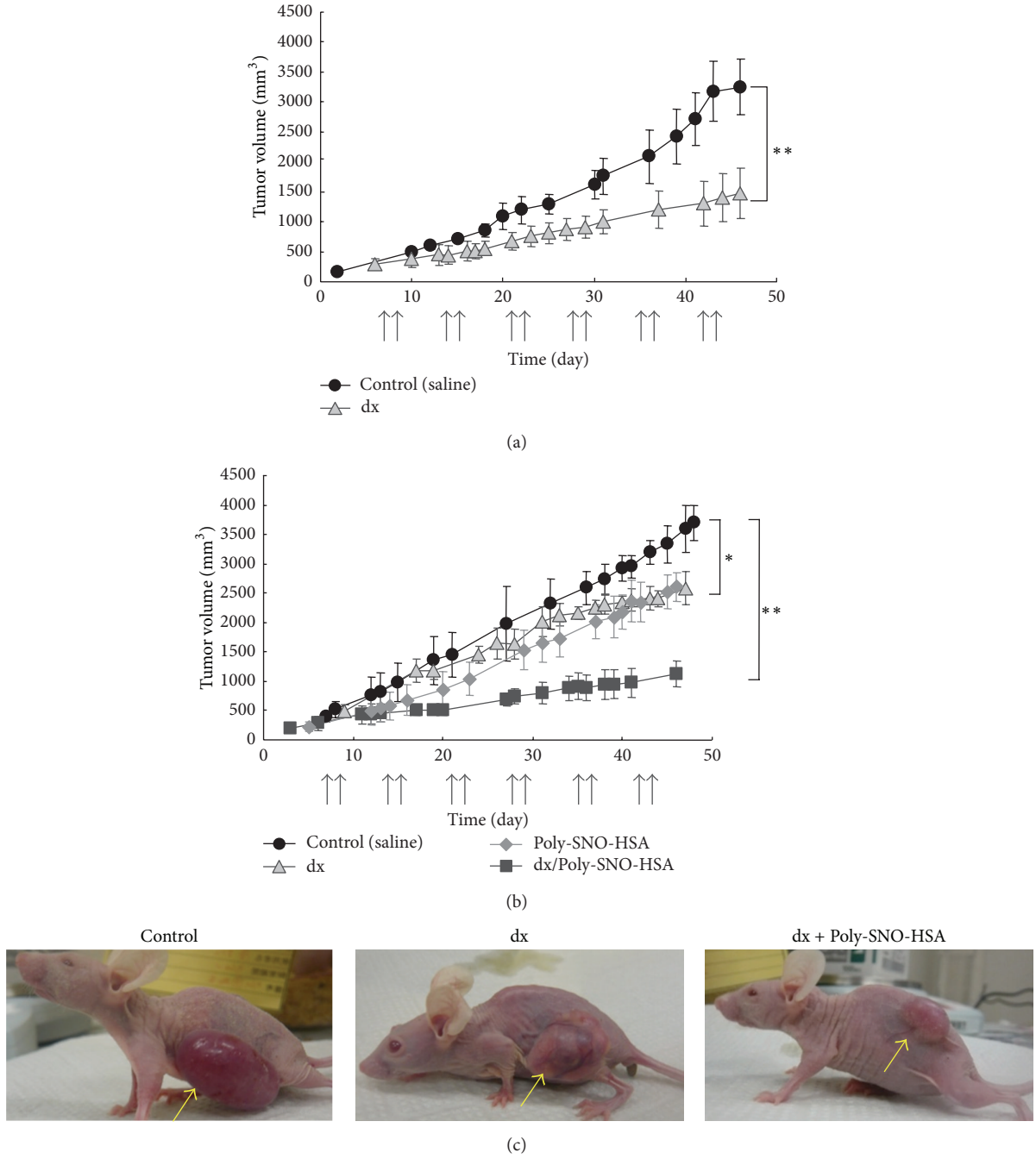

Figure 5: Effects of dx and Poly-SNO-HSA on tumor growth *in vivo*. K562 tumor-bearing mice (a) and K562/dx tumor-bearing mice (b) were given injections of saline (5 mL/kg), dx (4 mg/kg), Poly-SNO-HSA (10 μmol/5 mL/kg), or dx combined with Poly-SNO-HSA biweekly as noted (↑). Results are means ± SD; n = 3-4 animals per experimental group. (c) Results are shown for one representative experiment of K562/dx tumor-bearing mice. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control (saline) [33].

family including P-gp genes [47, 48]. To check for the involvement of HIF-1α in the above described effects of Poly-SNO-HSA, K562 cells were cultured for 24 h under hypoxic conditions [33]. Western blotting analysis showed clear HIF-1α expression in K562 cell lysates incubated to hypoxia. The high expression of HIF-1α was significantly suppressed in the presence of Poly-SNO-HSA. This finding is in full accordance with that observed for P-gp expression.

Similar results were obtained by immunostaining of P-gp and HIF-1α in K562 and K562/dx tumor-bearing mice [29].

Finally, we checked the effects of normoxia and hypoxia conditions on the *in vitro* resistance of K562 cells to dx [33]. The data showed that hypoxia condition significantly induced resistance to dx (0.5–10 μM). Intriguingly, Poly-SNO-HSA could inhibit growth of K562 cells under hypoxia condition in a dose-dependent manner, indicating that Poly-SNO-HSA

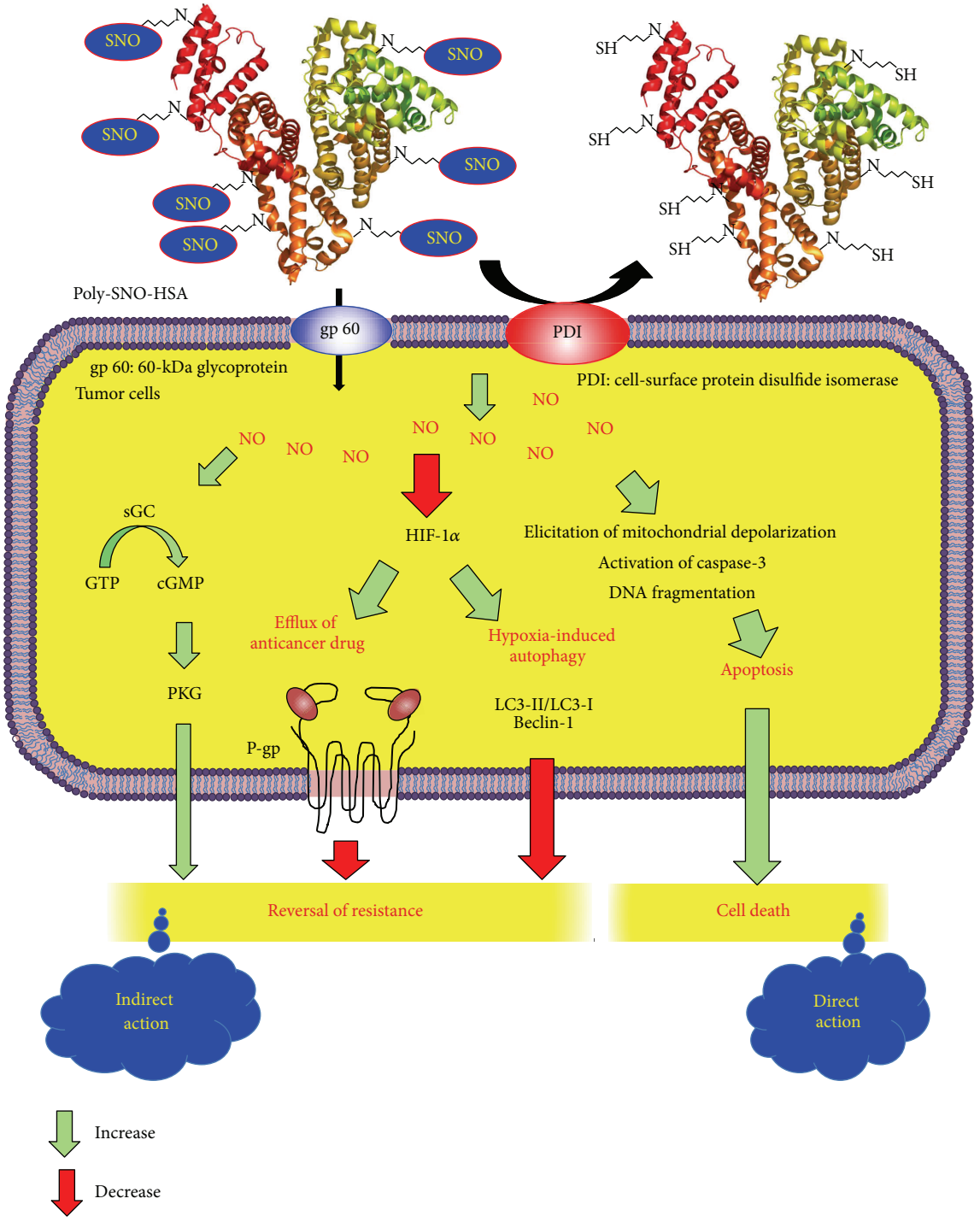

Figure 6: Mechanisms of Poly-SNO-HSA as a safe and strong multiple antitumor agent. Fast and pronounced transfer of NO from Poly-SNO-HSA into the cell mainly takes place via cell-surface protein disulfide isomerase (PDI) and to a minor extent via 60 kDa glycoprotein (gp60). Within the cell, a high concentration of NO induces apoptosis by the mechanisms mentioned, and perhaps by other means not yet identified. NO also reverts dx resistance partly by activating a cGMP dependent pathway. Finally, NO reverts drug resistance by decreasing the efflux of the drug. The latter effect is brought about by decreasing the expression of hypoxia-inducible factor-1α (HIF-1α) and P-glycoprotein (P-gp). In addition, Poly-SNO-HSA inhibits hypoxia-induced autophagy via downregulation of the cell signaling factors LC3-II/LC3-I and Beclin-1. The green and red arrows represent increasing and decreasing effects, respectively.

inhibits the expression of HIF-1α, which is a major factor for the resistance to dx. This is the case for both hypoxia condition and for the K562/dx cells (Figure 6).

Our findings suggest that Poly-SNO-HSA can be developed as a safe and strong, multifunctional antitumor agent. This is especially true if the Poly-SNO-HSA can be formulated into suitable carriers targeted to tumors. One of the limitations of the current nanovehicles for targeted drug delivery is accumulation of the vehicles in nontarget organs, causing serious side effects. Since NO is rather benign unless the concentration is too high, it presents a unique opportunity to achieve treating tumors without significant side effects. The current approach of nanotechnology-based drug delivery to tumors can benefit significantly through understanding of the underlying mechanisms of drug actions, for example, therapeutic mechanisms of NO [33, 49].

6. Future Direction of Poly-SNO-HSA

The hypothesis that tumor progression can be curbed by antiangiogenic agents targeting abnormal tumor vessels is supported by preclinical evidence and clinical trials [50]. However, these initial successes were tempered by the failure of angiogenesis inhibitors to produce enduring clinical responses. For example, in clinical trials of the vascular endothelial growth factor- (VEGF-) neutralizing antibody bevacizumab in glioblastoma, 40% to 60% of the tumors progressed after initially successful treatment [51], consistent with the development of resistance to antiangiogenic therapy, a state exhibiting a poor prognosis and poor response to available treatments [52]. The molecular basis of acquired resistance to antiangiogenic treatments causing this lack of sustained responses remains unclear. However, Hu et al. [53] have demonstrated that the devascularization caused by antiangiogenic therapy increases tumor hypoxia and that this hypoxia mediates resistance to antiangiogenic therapy. Furthermore, recent reports suggest that hypoxia activates autophagy, a lysosomal degradation pathway which may promote tumor cell survival [54, 55]. In fact, the devascularization caused by antiangiogenic therapy increases tumor hypoxia, and this hypoxia mediates resistance to antiangiogenic therapy [53]. The mechanisms by which hypoxia induces autophagy need clarification, but the finding that BNIP3, a HIF-1α downstream target gene, is essential to hypoxia-induced autophagy suggests one possible mechanism [56, 57]. Therefore, autophagy inhibitors may help prevent resistance to antiangiogenic therapy. In our studies, we found that Poly-SNO-HSA possesses very strong HIF-1α inhibition in tumor cells *in vivo*. We hope that future studies will clarify whether Poly-SNO-HSA also can be used as a specific and potent autophagy inhibitor.

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

Gemifloxacin, a Fluoroquinolone Antimicrobial Drug, Inhibits Migration and Invasion of Human Colon Cancer Cells

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Gemifloxacin (GMF) is an orally administered broad-spectrum fluoroquinolone antimicrobial agent used to treat acute bacterial exacerbation of pneumonia and bronchitis. Although fluoroquinolone antibiotics have also been found to have anti-inflammatory and anticancer effects, studies on the effect of GMF on treating colon cancer have been relatively rare. To the best of our knowledge, this is the first report to describe the antimetastasis activities of GMF in colon cancer and the possible mechanisms involved. Results have shown that GMF inhibits the migration and invasion of colon cancer SW620 and LoVo cells and causes epithelial mesenchymal transition (EMT). In addition, GMF suppresses the activation of NF-κB and cell migration and invasion induced by TNF-α and inhibits the TAK1/TAB2 interaction, resulting in decreased IκB phosphorylation and NF-κB nuclear translocation in SW620 cells. Furthermore, Snail, a critical transcriptional factor of EMT, was downregulated after GMF treatment. Overexpression of Snail by cDNA transfection significantly decreases the inhibitory effect of GMF on EMT and cell migration and invasion. In conclusion, GMF may be a novel anticancer agent for the treatment of metastasis in colon cancer.

1. Introduction

Colon cancer is the leading cause of cancer morbidity and mortality and the third most lethal malignancy worldwide [1, 2]. The incidence rate of colon cancer has increased worldwide over the last 20 years, due to changes in the environment, life styles, and nutritional habits [3, 4]. About 50% of patients with colon cancer will develop metastasis, which is defined as colon cancer cells migrating to and invading organs such as the lungs and liver [5]. Metastasized cancer is particularly lethal and challenging because it is highly resistant to radiation and conventional chemotherapeutic agents, and only one-fourth of patients with metastasis can be treated by surgery [6]. Consequently, novel therapeutic agents are needed to deal with the increasing incidence of the disease, to advance the efficacy of chemotherapy in human colon cancer and increase the number of alternative regimens.

It has been widely reported that persistently activated nuclear factor κB (NF-κB) in tumor cells plays a critical oncogenic role in modulating malignancy transformation and cancer progression [7, 8]. NF-κB signaling, induced by various growth factors, inflammatory factors, and genetic transfection, promotes cancer invasion and metastasis. Inhibition of NF-κB by genetic knockdown or chemical inhibitors

has been shown to decrease cancer cell proliferation, migration, invasion, and metastasis and to reduce the chemoresistance of cancer cells to anticancer drugs [9, 10]. Epithelial mesenchymal transition (EMT) is an important process in cancer development, enabling cancer cells to metastasize [11, 12]. Snail, an important transcription factor, has been reported to be involved in the regulation of EMT by repressing the expression of the E-cadherin gene [13]. Recent study has demonstrated that the NF-κB-related cascade modulates Snail expression, leading to EMT in various cell types [14]. Therefore, we hypothesized that inhibition of NF-κB would suppress tumor cell metastasis through inhibition of the downstream target Snail.

Gemifloxacin (GMF) is a fluoroquinolone antimicrobial agent which acts through inhibiting bacterial DNA gyrase and topoisomerase IV [15]. The treatment indications for GMF are community-acquired pneumonia and acute exacerbation of chronic bronchitis [16]. Fluoroquinolones have been reported to decrease tumor growth and DNA synthesis resulting in S- and G2/M-phase cell cycle arrest [17, 18]. This type of antibiotics has also been reported to induce cell apoptosis by causing mitochondrial dysfunction in lung, bone, bladder, and prostate cancer cells [19]. The synergistic effect of GMF on the enhancement of the cytotoxicity of other chemotherapeutic agents, such as doxorubicin and etoposide, has also been reported. In addition, fluoroquinolones have also been reported to reduce the production of inflammatory cytokines/chemokine induced by lipopolysaccharide [20]. In this study, we investigated the anticancer effects of GMF on human colon cancer, with an emphasis on inhibition of cell migration, invasion, and inflammatory stimulation.

2. Materials and Methods

2.1. Cell Culture and Cell Viability Assay. The human colorectal adenocarcinoma cell line SW620, together with the LoVo cell line, was purchased from the Bioresource Collection and Research Center in Taiwan. SW620 cells were cultured in Leibovitz's L-15 medium (Life Technologies, Inc., Grand Island, NY), supplemented with 10% FBS, 0.1 mg/mL streptomycin, and 100 units/mL penicillin (Life Technologies, Inc., Grand Island, NY), and incubated at 37°C. LoVo cells were cultured at 37°C in a humidified 95% air-5% CO_2 atmosphere in F12 medium supplemented with 10% FBS. For the cell viability assay, cells (5×10^4/well) were plated in 96-well culture plates. After 24 hours of incubation, the cells were treated with vehicle control (0.1% DMSO) or various concentrations of GMF or TNF-α for 48 hours. Viability of the SW620 and LoVo cells was determined by Premixed WST-1 Cell Proliferation Reagent (Clontech Laboratories Inc., Mountain View, CA, USA) according to the manufacturer's instructions.

2.2. Scratch Wound-Healing Assay, Cell Migration, and Invasion Assay. The SW620 and LoVo cells were allowed to grow to full confluence in 24-well plates. The following day, a uniform scratch was made down the center of the well using a micropipette tip, followed by washing once with PBS. Various concentrations of GMF were added to the respective wells for the indicated times. Photographic imaging was performed using a Nikon inverted microscope. Quantitative migration and invasion assays were conducted using a QCM 24-well Cell Migration Assay and Invasion System (Millipore Corp., Billerica, MA, USA). Briefly, 1×10^5 SW620 and LoVo cells were seeded into the top chamber and treated with different concentrations of GMF. Ten percent of FBS or TNF-α was added to the bottom wells for 48 hours as the chemoattractant. At the end of the treatment, the cells were poststained with CyQuant GR dye in cell lysis buffer for 15 minutes at room temperature. Fluorescence of the migratory and invading cells was then read using a fluorescence plate reader at excitation/emission wavelengths of 485/540 nm.

2.3. Immunoblot/Immunoprecipitation. Cells (8×10^6/dish) were seeded in a 10 cm dish. After 24 hours of incubation, the cells were treated with various concentrations of GMF for the indicated times. Total cell extracts were prepared in RIPA lysis buffer (Millipore Corp). Equivalent amounts of protein were resolved by SDS-PAGE and transferred to PVDF membranes. After the membranes had been blocked in Tris-buffer saline containing 0.05% Tween 20 (TBST) and 5% nonfat powdered milk, they were incubated with primary antibodies at 4°C for 1–16 hours. Following three 5-minute washes with TBST, the membranes were incubated with horseradish peroxidase-labeled secondary antibody for 1 hour and then washed again. Detection was performed using an enhanced chemiluminescence blotting detection system (Millipore Corp).

For TAK1 immunoprecipitation, cell lysates (200 μg of total protein) were incubated with 2 μg of anti-TAK1 overnight and then 20 μL of protein A-agarose beads (Millipore Corp., Billerica, MA, USA) for 2 h at 4°C. Association of TAK1 with TAB2 was detected by incubating the blots with anti-TAB2 antibodies (Cell Signaling).

2.4. NF-κB DNA Binding Assay. NF-κB activity was measured using an ELISA-based kit according to the manufacturer's specifications (Active Motif, Carlsbad, CA). Briefly, nuclear extracts containing NF-κB were prepared using a Nuclear Extract kit (Active Motif, Carlsbad, CA), and the capture was accomplished by binding to a consensus oligonucleotide (5′-GGGACTTTCC-3′) immobilized on a 96-well plate (Millipore Corp). The p65 subunit of NF-κB was determined in a colorimetric reaction using a specific primary antibody and a secondary horseradish peroxidase-conjugated antibody. Spectrophotometric data were expressed as a ratio of the absorbance of each experimental condition compared with control cells exposed to vehicle alone.

2.5. Gene Knockdown and Overexpression. SW620 cells were transfected with either 0.5 μg of the Snail-expressing plasmid or control plasmid by Lipofectamine 2000. Snail cDNA overexpressing SW620 stable colonies were established by G418 selection.

2.6. Statistical Analysis. Data were expressed as the mean ± SD of three determinations. Statistical comparisons of

the results were made using analysis of variance (ANOVA). Significant differences ($P < 0.05$) between the means of the two test groups were analyzed by Student's t test.

3. Results

3.1. GMF Inhibited Cell Migration and Invasion in SW620 and LoVo Cells. We first assessed the effects of GMF on the viability of SW620 and LoVo cells. As shown in Figure 1(a), GMF did not affect the viability of SW620 and LoVo cells at concentrations ranging from 1 to 20 μg/mL. However, GMF exhibited an inhibitory effect on the migration of SW620 and LoVo cells, as determined by wound-healing assay (Figure 1(b)). In addition, quantitative transwell analysis also revealed that GMF decreased the migration of SW620 and LoVo cells in a dose-dependent manner (Figure 1(c)). Next, we assessed the effect of GMF on the invasive ability of colon cancer cells. Compared to vehicle-treated cells, GMF treatment attenuated SW620 and LoVo cell invasion in a dose-dependent manner after treatment for 48 hours (Figure 1(d)).

3.2. GMF Decreased Snail Expression and Caused EMT in SW620 Cells. EMT is a critical process in the development of invasive cancer cells, and restoration of the epithelial-like characteristics can reduce tumor invasive capacity [21]. We assessed the effect of GMF on EMT markers in SW620 cells and found that GMF treatment enhanced epithelial markers, including E-cadherin and claudin-3 levels. In contrast, GMF reduced mesenchymal markers such as vimentin and N-cadherin expression (Figure 2(a)).

Next, we investigated whether Snail, an important transcription factor in regulating cell migration and EMT, was involved in the GMF-mediated switch of EMT phenotype. Immunoblot analysis revealed that protein levels of Snail in the nuclei were reduced by GMF treatment of the SW620 cells (Figure 2(b)).

3.3. The Role of Snail on GMF-Mediated Inhibition of Cell Migration and EMT. To investigate the role of Snail on GMF-mediated cancer migration inhibition and EMT, we generated overexpressing Snail human colon cancer SW620 cells steadily expressing Snail cDNA and then confirmed the exogenous protein expression by immunoblot (Figure 3(a)). Snail overexpression was not found to affect the viability of SW620 cells after 48 h analysis (Figure 3(b)). We then examined the effect of GMF on the Snail-overexpressing SW620 cells. As shown in Figures 3(c) and 3(d), Snail overexpression blocked the effects of GMF on cell migration and invasion. In addition, the effects of GMF on the upregulation of E-cadherin were also blocked by Snail overexpression (Figure 3(a)). These data suggest that GMF decreases cancer cell progression by inhibiting Snail.

3.4. GMF Inhibits Constitutive and Inducible NF-κB Nuclear Translocation and Activity in SW620 Cells. Increased NF-κB signaling has been reported to act as a critical regulator and transcriptional activator in colon cancer [22]. We consequently tested whether GMF has a direct impact on NF-κB activation. As shown in Figure 4(a), the protein level of NF-κB in the nuclei was decreased by GMF treatment (Figure 4(a)). In addition, NF-κB activity (nuclear fraction) in SW620 cells decreased after GMF treatment (Figure 4(b)). Because phosphorylation and degradation of IκB by IκB kinase (IKK) is an important step in the process of NF-κB activation, we investigated the effect of GMF on the status of IκB. GMF decreases the phosphorylation of IκB and increases the amount of IκB in the SW620 cells, suggesting that the effect of GMF on NF-κB operates by decreasing IκB phosphorylation (Figure 4(c)).

Inflammatory factors such as TNF-α are thought to be major causes of elevated NF-κB in cancer cells [23]. We therefore investigated whether GMF also inhibited TNF-α-mediated NF-κB activation. As shown in Figure 5(a), TNF-α (20 ng/mL) increases the nuclear translocation of NF-κB in SW620 cells. In addition, GMF also reduces the DNA binding activity of NF-κB in a dose-dependent manner after 3 hours of treatment (Figure 5(b)). These data suggest that GMF is a potential inhibitor of oncogenic transcription factors.

Because IKK-α/β are upstream activator of IκB in the NF-κB signal pathway, we assessed the effect of GMF on TNF-α treated colon cancer cells. It was found that GMF markedly decreases TNF-α-induced IKK-α/β phosphorylation, without affecting the total amounts of IKK-α/β (Figure 5(c)). Furthermore, because TAK1 has been implicated in the regulation of IKK-α/β phosphorylation by inflammatory cytokines, we further examined the effect of GMF on the TNF-α induced phosphorylation of TAK1. Our results show that TNF-α treatment increases TAK1 phosphorylation, which was significantly inhibited by GMF. To determine whether GMF decreases TAK1 phosphorylation by decreasing the interaction of the TAK1 with TAB2, we assessed the association of TAK1/TAB2 by immunoprecipitation. Binding of TAK1 to TAB2 was observed after TNF-α treatment, but the interaction was significantly decreased after treatment with GMF (Figure 5(d)). These findings suggest that GMF suppresses TNF-α-induced NF-κB activation by downregulation of the TAB2/TAK1-mediated NF-κB pathway.

3.5. GMF Decreases Inflammatory TNF-α-Mediated Cell Migration, Invasion, and EMT in SW620 Cells. The inflammatory factor TNF-α is known to promote metastasis by enhancing EMT and invasiveness in colon cancer [23]. We therefore investigated whether GMF decreases the enhancement of TNF-α on the biologic events of cancer progression. Since TNF-α did not affect the viability of SW620 cells (Figure 6(a)), we further assessed the effect of GMF on cell migration and invasion. As shown in Figures 6(b) and 6(c), treatment of the SW620 cells with TNF-α (20 ng/mL) increases cell migration, but this effect is abrogated by GMF. In addition, GMF also abolishes TNF-α-induced cell invasion (Figure 6(d)). Furthermore, treatment with GMF also inhibits Snail upregulation and E-cadherin downregulation induced by TNF-α in SW620 cells (Figure 6(e)).

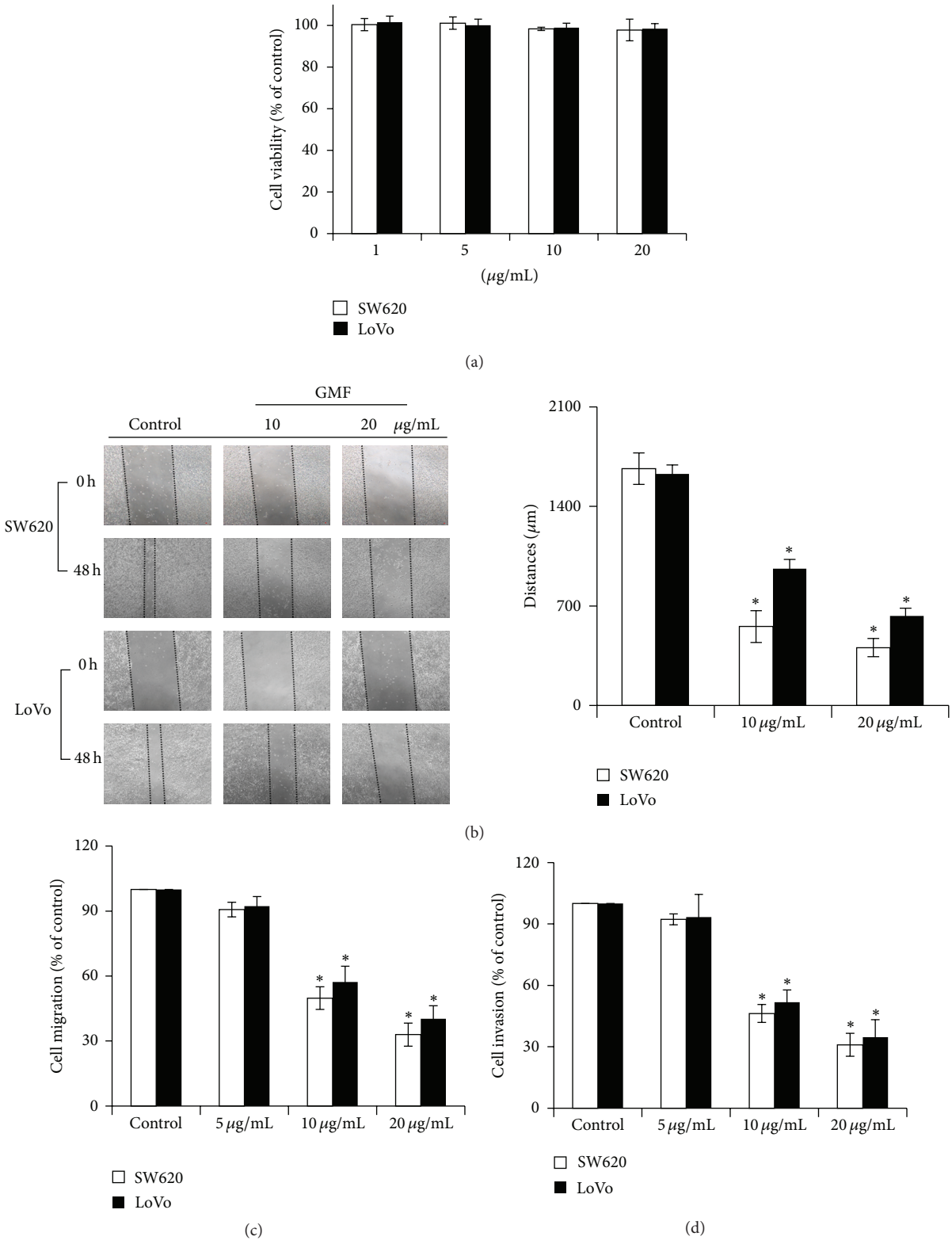

Figure 1: GMF decreased cell migration and invasion in SW620 and LoVo colon cancer cells. (a) The effect of GMF on viability of the SW620 and LoVo cells. GMF reduced cell migration as determined by wound healing analysis (b) and the transwell system (c). (d) GMF decreased cell invasion. The migration and invasion abilities of the SW620 and LoVo cells were quantified by QCM 24-well Cell Migration and Invasion assay kits, as described in Section 2. Ten percent of FBS acted as the chemoattractant for cancer migration and invasion. All results are representative of at least three independent experiments. Each value is the mean ± SD of three determinations. The asterisk (∗) indicates a significant difference between the two test groups, as analyzed by Student's t test ($P < 0.05$).

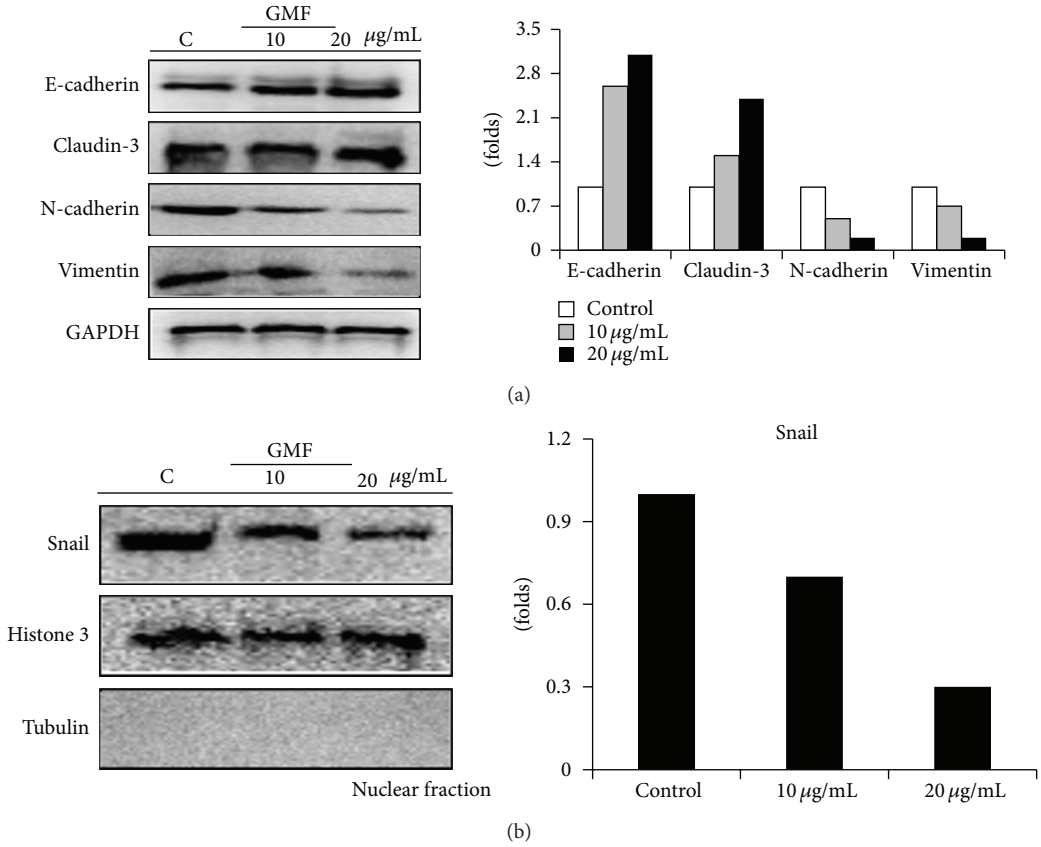

Figure 2: GMF changed the expressions of mesenchymal markers in SW620 cells. (a) GMF increased the levels of epithelial protein and reduced mesenchymal factors in the SW620 cells. (b) GMF reduced Snail levels. Cells were treated with various concentrations of GMF for 24 hours, and the protein expression was assessed by immunoblot assay. All results are representative of at least three independent experiments.

4. Discussion

Discovering new uses for preexisting drugs with well-defined profiles, such as side effects, pharmacokinetics, and pharmacodynamics, may prove to be beneficial for patients. These drugs have the potential and offer the advantage of extensive clinical experience in other therapeutic areas [24]. Therefore, their potential value in the prevention of metastasis is high. The current study shows that GMF, a clinical used antibiotic, decreases the metastasis of colon cancer SW620 and LoVo cells by decreasing cell migration and invasion. This study also investigates the effect of GMF in reversing inflammatory cytokine-mediated cancer progression. Our findings suggest that GMF is capable of preventing the progression of colon cancer.

The NF-κB signaling pathway is involved in the pathogenesis of various cancers, including colon cancer [25, 26]. Elevated activation of NF-κB and upstream triggers such as TNF-α supports the hypothesis that this pathway plays a crucial role in cancer development [27, 28]. When inactive, NF-κB dimers are associated with inhibitory IκB proteins and sequestered in the cytoplasm. Stimulus-induced phosphorylation and ubiquitination of IκB by the IκB kinase complex have been reported to result in proteasome-mediated degradation, which in turn causes nuclear translocation and DNA binding of NF-κB [29, 30]. The phosphorylation of IκB is catalyzed by IκBα kinase (IKK), which is necessary for NF-κB activation [30]. TAK1 has also been indicated to be an upstream activator of IKK in the canonical NF-κB signaling pathway, activated by inflammatory cytokines through its interaction with TAB1 and TAB2 [31, 32]. The current study reveals that GMF not only decreases the constitutive NF-κB nuclear translocation and activity but also blocks inducible NF-κB activation mediated by TNF-α. The inhibitory effect of GMF on inflammatory factor NF-κB-mediated cancer progression was also revealed by decreasing the oncogenic potential of TNF-α-mediated cell migration, invasion, and EMT after GMF treatment of the SW620 cells. This inhibitory effect of GMF on NF-κB is associated with a decrease in the interaction of TAK1 and TAB2, which in turn reduces IKK activation, thereby resulting in IκB phosphorylation and degradation. These data suggest that GMF is a potential inhibitor targeting both constitutive and inducible activation of NF-κB.

Snail, a transcription factor, is a critical modulatory factor of cell motility which operates by changing the cell phenotype from an epithelial characteristic to mesenchymal. Its expression has been reported to be elevated in several cancer types, including colon cancer [33, 34]. NF-κB signaling is known to be a regulator of Snail in various types of cells

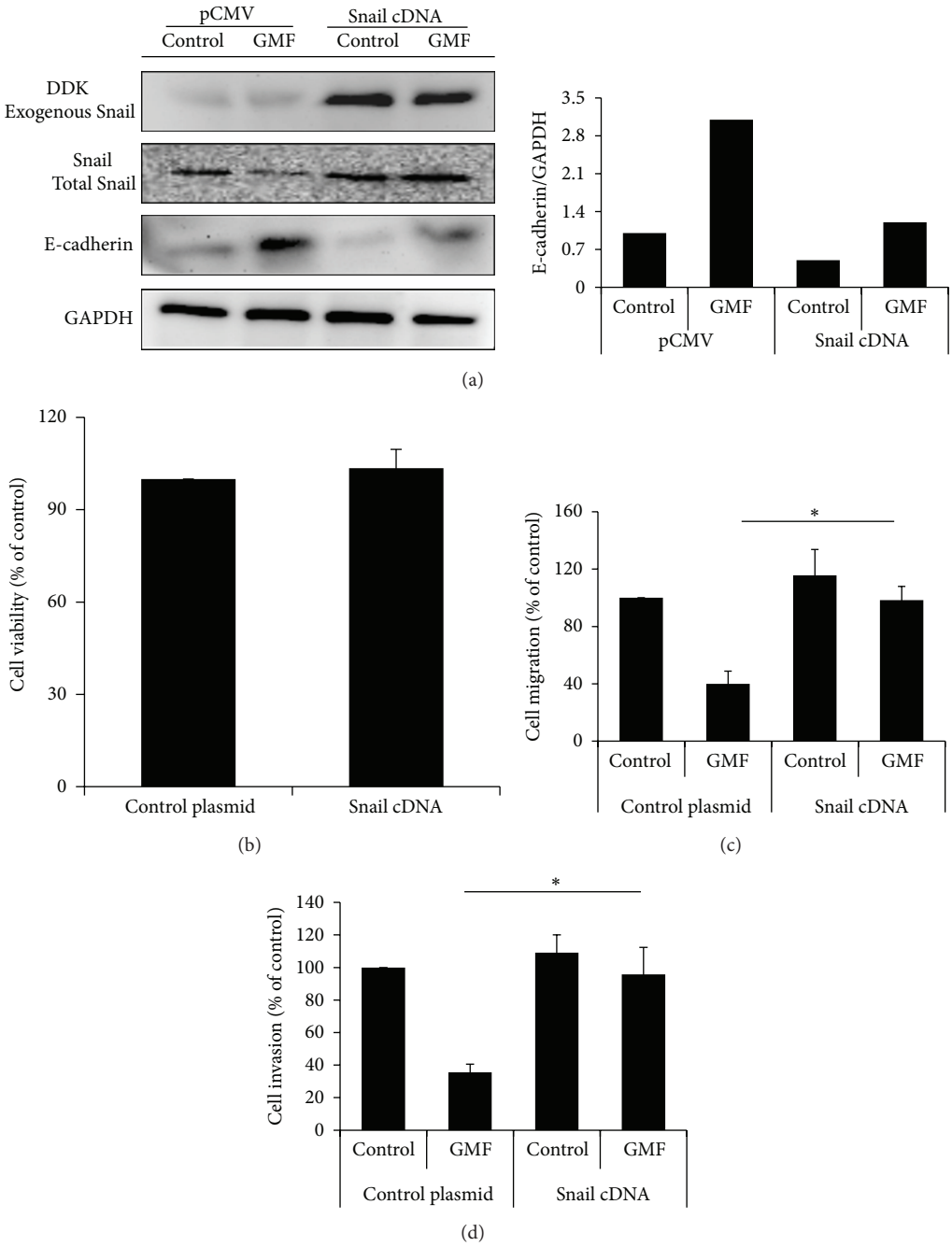

Figure 3: The role of Snail in GMF-mediated cell migration and invasion inhibition. (a) The effect of overexpression of Snail in GMF-treated SW620 cells. (b) Snail overexpression did not affect viability of SW620 cells. Snail overexpression decreased the inhibitory effect of GMF on cell migration (c) and invasion (d). Cells were transfected with pCMV or pSnail plasmids, and stable colonies were established by G418 selection. Snail-overexpressing cells were treated with GMF (20 μg/mL) (24 hours for E-cadherin), and various protein levels were assessed by immunoblot assays. Each value is the mean ± SD of three determinations, and all results are representative of at least three independent experiments. The asterisk (∗) indicates a significant difference between the two test groups, as analyzed by Student's t test ($P < 0.05$).

[14]. Triggering of the NF-κB pathway regulates EMT in human cancers by increasing Snail [14, 35]. In addition, Snail inhibits the expression of tight junction E-cadherin protein by binding to E2-box type elements within its promoter, resulting in EMT [13, 36]. E-cadherin downregulation and EMT have been implicated in the increase of metastatic ability and are strongly associated with a poor prognosis [37]. In contrast, mesenchymal-like tumors can revert to an epithelial-like characteristic via the MET process, resulting in a reduction in metastatic capacity [21, 38]. We found that GMF had a significant inhibitory effect on Snail expression, which was consistent with the blockade of NF-κB by GMF.

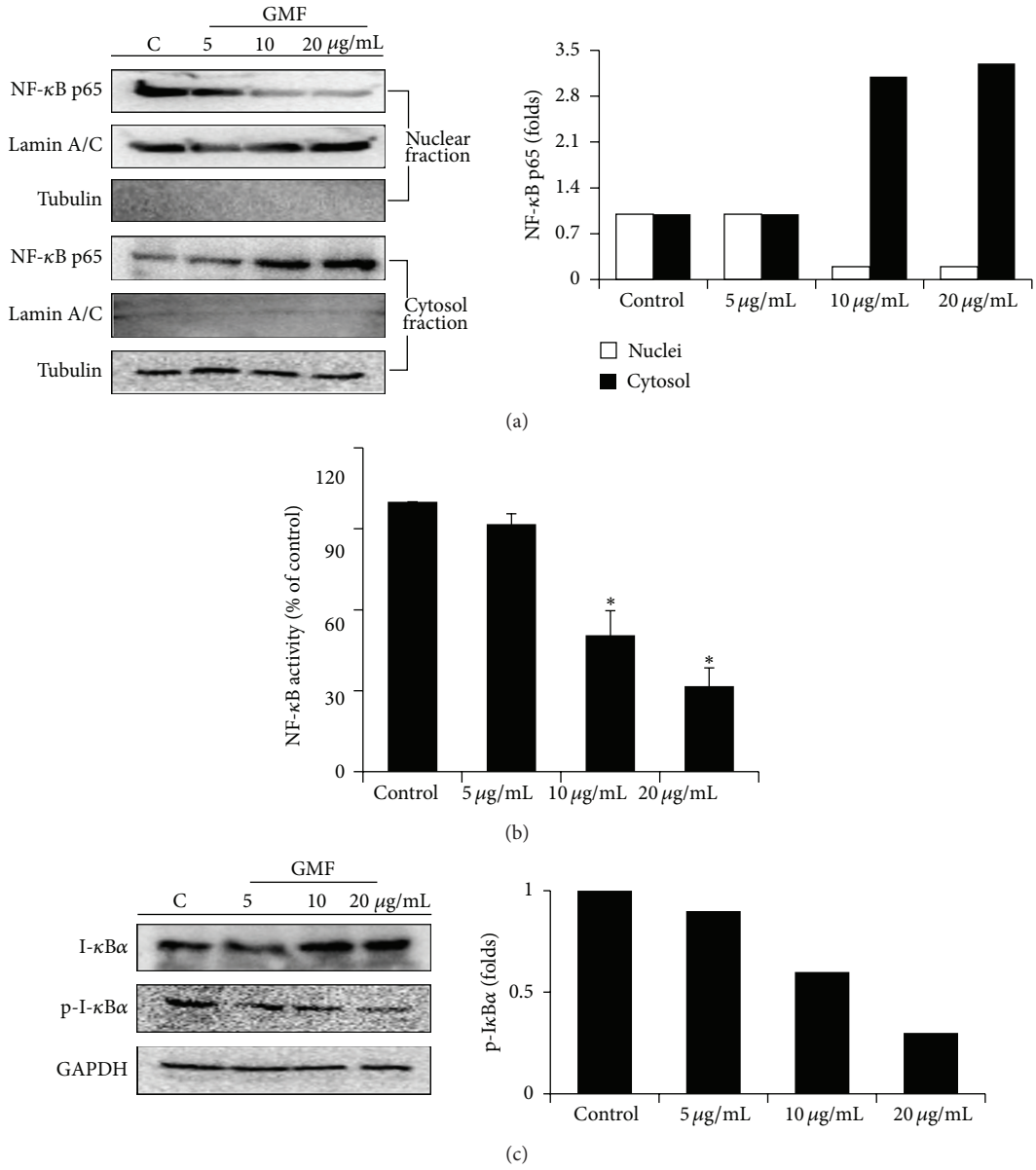

Figure 4: GMF inhibited NF-κB activity in SW620 cells. GMF decreased NF-κB nuclear translocation (a) and activity (b) in the SW620 cells. (c) GMF increased I-κBα levels in the cytosol. Cells were treated with various concentrations of GMF. Nuclear and cytoplasm fractions were separated by a nuclear extract kit, and protein expressions were assessed by immunoblot assay. The DNA binding activity of NF-κB in the nuclear fraction was assessed by a Trans-AM ELISA kit. All results are representative of at least three independent experiments. Each value is the mean ± SD of three determinations. The asterisk (∗) indicates a significant difference between the two test groups, as analyzed by Student's t test ($P < 0.05$).

The inhibition of Snail directly contributes to the restoration of E-cadherin and inhibition of mesenchymal gene markers (N-cadherin and vimentin). The ectopic expression of Snail blocks antimigration, and E-cadherin upregulates the effect of GMF in SW620 cells. These results suggest that inhibition of Snail may be pivotal for GMF-mediated E-cadherin induction and promotion of EMT.

Taken together, our findings provide strong evidence that GMF decreases colon cancer metastasis. The molecular mechanism of GMF in reducing the capacity of cell migration and invasion by decreasing NF-κB contributes to the change of metastatic phenotypes in colon cancer. Therefore, GMF may serve as a potential agent for the development of therapies against early metastatic events in colon cancer.

Authors' Contribution

Jaw-Yuan Wang and Po-Lin Kuo contributed equally to this work.

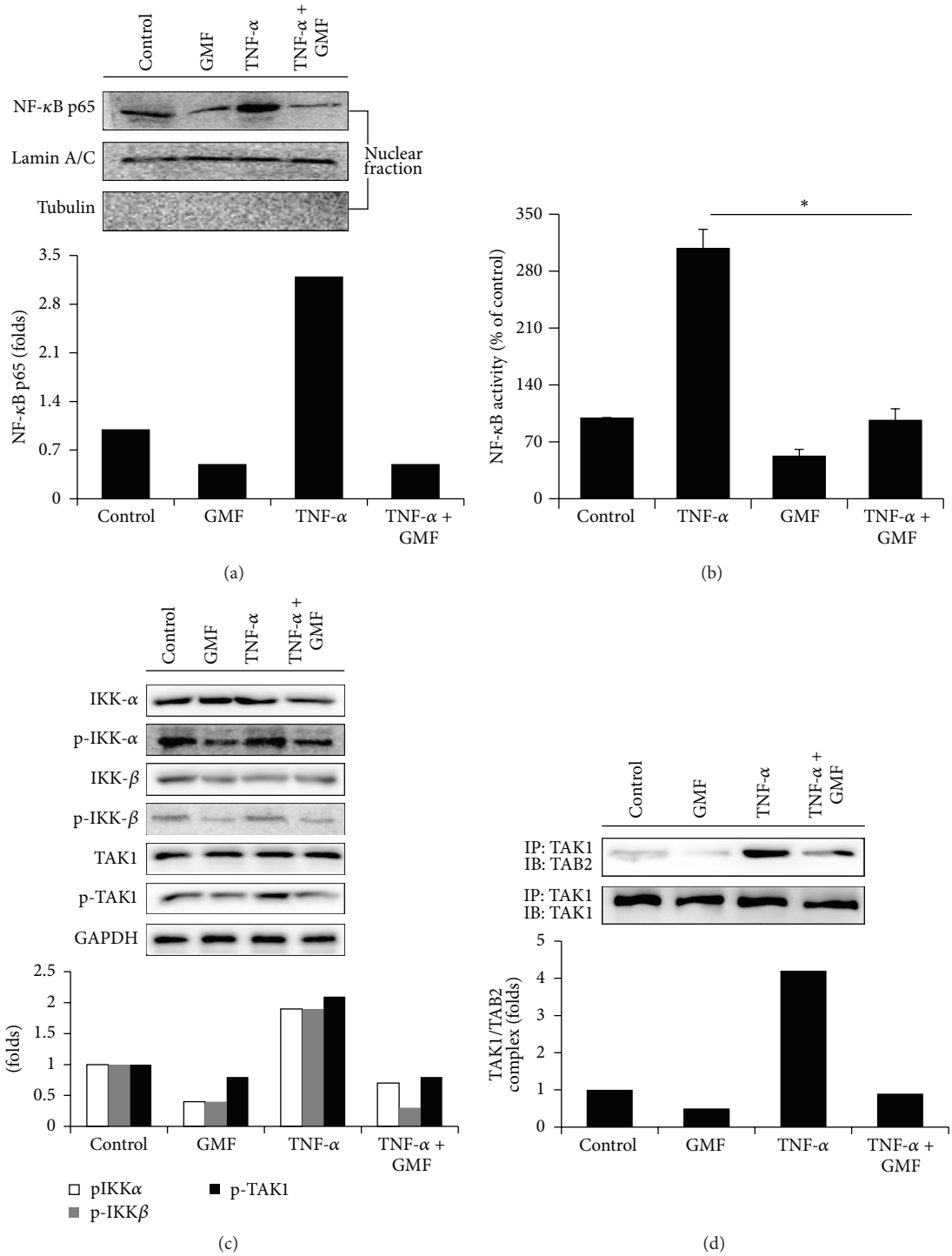

Figure 5: GMF inhibited inducible NF-κB activity in SW620 cells. GMF reduced TNF-α-mediated NF-κB nuclear translocation (a), activation (b), IKK phosphorylation (c), and TAK1/TAB2 interaction (d). Cells were pretreated with GMF (20 μg/mL) for 1 hour; then TNF-α (20 ng/mL) was added for another 3 hours. Protein expression was assessed by immunoblot, and protein-protein interaction was examined by immunoprecipitation. The DNA binding activity of NF-κB in the nuclear fraction was assessed by a Trans-AM ELISA kit. All results are representative of at least three independent experiments. Each value is the mean ± SD of three determinations. The asterisk (∗) indicates a significant difference between the two test groups, as analyzed by Student's t test ($P < 0.05$).

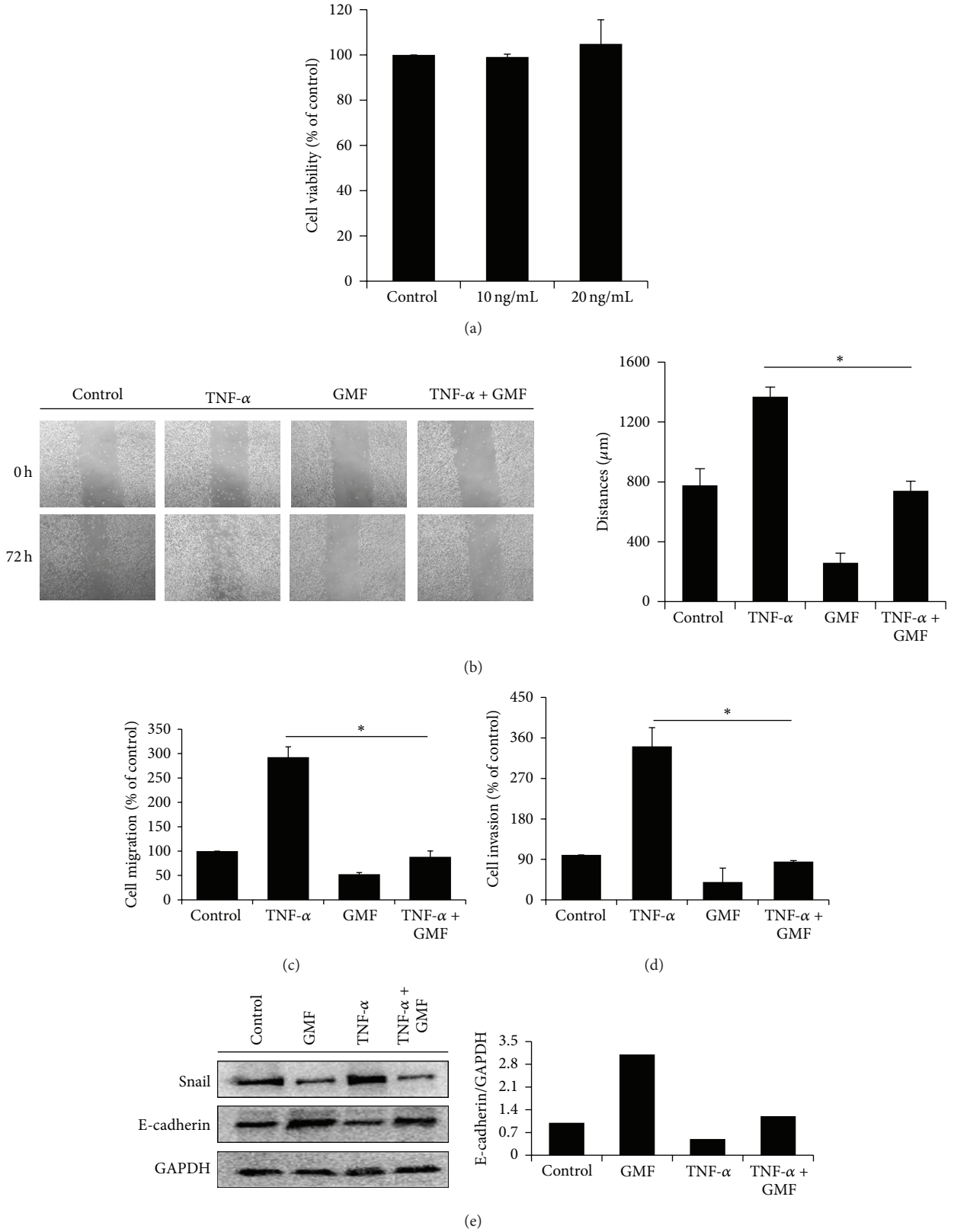

Figure 6: GMF reduced inflammatory factor TNF-α-mediated cell migration, invasion, and EMT in SW620 cells. (a) The effect of TNF-α on cell viability. GMF inhibited cell migration, as determined by wound-healing (b) and transwell system (c) and invasion (d) and EMT (e) were induced by TNF-α. The migration and invasive abilities of the SW620 cells were quantified by QCM 24-well Cell Migration and Invasion assay kits. TNF-α (20 ng/mL) acted as a chemoattractant of cancer migration and invasion. Each value is the mean ± SD of three determinations. The asterisk ($*$) indicates a significant difference between the two test groups, as analyzed by Student's t test ($P < 0.05$).

Acknowledgments

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