RESEARCH COMMUNICATION

The Clinicopathological Significance of Bmi-1 Expression in Pathogenesis and Progression of Gastric Carcinomas

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Abstract

Background: Oncogenic Bmi-1 (B-lymphoma Moloney murine leukemia virus insertion region-1) belongs to the Polycomb- group (PcG) family of proteins and plays an important role in the regulation of proliferation, senescence, cell cycle and apoptosis, chromosome stability, activation of gene transcription. Methods: To clarify the roles of Bmi-1 in tumourigenesis and progression of gastric carcinomas, it was examined by immunohistochemistry (IHC) and real-time RT-PCR in gastric carcinomas, dysplasia, intestinal metaplasia (IM), and gastritis with a comparison of its expression with clinicopathological parameters of carcinomas. Results: There was gradually increased Bmi-1 protein expression from gastritis, IM, dyplasia to carcinoma (p<0.001). Bmi-1 expression was positively linked to tumor size, depth of invasion, lymph node metastasis and worse prognosis of carcinomas (p<0.001), but not to age or sex of carcinoma patients (p>0.05). There was higher Bmi-1 protein expression in intestinal- type carcinomas than diffuse-type ones (p<0.001). At mRNA level, Bmi-1 protein expression was increased from gastritis, IM, dysplasia and carcinoma (p<0.001). Bmi-1 overexpression was observed in gastric carcinoma with larger diameter, deeper invasion, lymph node metastasis, and intestinal-type carcinoma (p<0.05). Conclusion: These findings indicate that up-regulated Bmi-1 expression is positively linked to pathogenesis, growth, invasion, metastasis and differentiation of gastric carcinomas. It was considered as a promising marker to indicate the aggressive behaviors and prognosis of gastric carcinomas.

Keywords: Bmi-1 - gastric carcinoma - pathogenesis - progression - prognosis

Introduction

Gastric carcinoma ranks as the world's second leading cause of cancer mortality behind lung cancer despite a sharp worldwide decline in both its incidence and mortality since the second half of the 20th century. It continues to be a major health problem because of the slow decrease in incidence in Asia and high mortality of diagnosed gastric carcinoma in West (Yu et al., 2010). Therefore, it is of much significance for the prevention, treatment and prognosis evaluation of gastric cancer to clarify its molecular mechanisms and find out a good biomarker to indicate its carcinogenesis and subsequent progression.

Bmi-1(B-lymphoma Moloney murine leukemia virus insertion region-1) gene was first isolated as an proto-oncogene that cooperated with c-myc in generating lymphomas in a transgenic murine model. It is a transcriptional repressor belonging to the Polycomb- group (PcG) family of proteins which play an important role in axial patterning, hematopoiesis, regulation of proliferation, senescence, cell cycle and apoptosis, chromosome stability, activation of gene transcription, the self-renewal and propagation of normal and cancer stem cells. Bmi-1 is localized in 10p11.23 with its 3434b

mRNA and 36.9 kDa protein of 326 amino acids. Bmi1 has been shown to interact with zinc finger and BTB domain-containing protein 16, PHC2, PHC1 and RING1. The N-terminal RING finger domain of Bmi-1 cooperates with the tumor suppressor c-myc and the central conserved DNA binding helix-turn-helix-turn motif is involved in transcriptional repression. The both above-mentioned domains is required for inducing telomerase activity and immortalization of human epithelial cells. Bmi-1 has been reported as an oncogene by regulating p16 and p19, which are cell cycle inhibitor genes (Grinstein and Mahotka, 2009; Jiang et al., 2009).

The overexpression of Bmi-1 enhances the motility and invasiveness of immortalized human mammary epithelial cells, facilitates concurrent epithelial- mesenchymal-transition-like molecular changes, and promotes the stabilization of Snail and the activation of the Akt/GSK3β pathway (Song et al., 2009). The critical negative target of Bmi-1 is the Ink4a/Arf locus, which encodes the p16Ink4a and p14Arf tumor suppressor proteins and Bmi-1-deficient mouse embryonic fibroblasts overexpressed p16Ink4a and p19Arf (mouse homologue of human p14Arf). Bmi-1 overexpression leads to activation of human telomerase reverse transcriptase transcription and induction of telomerase activity in immortalized mammary epithelial

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cells (Milyavsky et al., 2003). Bmi-1 can inhibit the apoptosis and senescence induced by p53 and prB growth regulatory pathways (Itahana et al., 2003).

With the rapid development of molecular biology, Bmi-1 has been shown to be overexpressed across a broad spectrum of human cancers including breast cancer, lung squamous cell cancer, esophageal squamous cell cancer, ovarian cancer, endometric carcinoma, colon cancer, bladder cancer, and pancreatic cancer (Qin et al., 2009; Honig et al., 2010; Li et al., 2010; Liu et al., 2010; Song et al., 2010; Huang et al., 2011) and Bmi-1 is tightly associated with the development of tumors, including uterine cervical cancer, breast cancer, pancreatic cancer, ovarian cancer, colon cancer, bladder cancer,endometric cancer, and renal clear cell carcinoma (Engelsen et al., 2008; Kozakowski et al., 2008; Qin et al., 2009; Li et al., 2010; Song et al., 2010; Zhang et al., 2010; Yang et al., 2010; Guo et al., 2011; Min et al., 2011). In the present study, we aimed to observe Bmi-1 expression at both protein and mRNA levels and compared its expression with clinicopathological features of gastric cancer.

Materials and Methods

Subjects and pathology

Gastritis, intestinal metaplasia (IM), dysplaisa, and carcinomas were collected from endoscopic biopsy, polypectomy, or surgical resection in the first affiliated hospital of Liaoning Medical College between 1993 and 2006. None of the patients underwent chemotherapy, radiotherapy or adjuvant treatment before surgery. They all provided consent for use of tumour tissue for clinical research and our University Ethical Committee approved the research protocol. We followed up all patients by consulting their case documents or through telephone.

Some tissues were fixed in 4% neutralised formaldehyde, embedded in paraffin and incised into 4 μm sections. These sections were stained by haematoxylin-and-eosin (HE) to confirm their histological diagnosis and other microscopic characteristics. Histological architecture of gastric carcinoma was expressed in terms of Lauren's classification (Zheng et al., 2007; Zheng et al., 2008). Furthermore, tumour size, depth of invasion, and lymph node metastasis were determined. The fresh samples were also collected, frozen in liquid nitrogen and stored in -80 °C until RNA extraction.

Tissue microarray and immunohistochemistry

All tissue slides were histopathologically re-evaluated by one pathologist. Two 2.0-mm tissue cores were taken for tissue microarray (TMA) from representative areas of gastric samples using a manual arraying device (MTA-1; Beecher Inc., Sun Prairie, WI, USA) and inserted in a new recipient block. Four-µm-thick sections were consecutively incised from the recipient block, transferred to poly-lysine- coated glass slides, deparaffinized with xylene and rehydrated through an alcohol gradient. The sections were quenched with 3% hydrogen peroxide in absolute methanol for 20 min to block endogenous peroxidase activity, and heated in a microwave for 15 min in citrate buffer (0.01 mol/L, pH 6.0) to retrieve

the antigen. The sections were incubated with rabbit Bmi-1 antibody (Abcam, USA; 1:80), followed by exposure to the anti-rabbit Envison-PO (DAKO, USA) antibody for 60 min. Binding sites were visualized with 3, 3'-diaminobenzidine (DAB) with the 5-min reaction. After each treatment, the slides were washed with TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) three times for 1min. After counterstained with Mayer's haematoxylin, the sections were dehydrated, cleared and mounted. Omission of the primary antibody was used as a negative control.

As indicated in Figure 1, Bmi-1 was positively localized in the cytoplasm. One hundred cells were randomly selected and counted from 5 representative fields of each section blindly by three independent observers. The positive percentage of counted cells was graded semi-quantitatively according to a four-tier scoring system: negative (-), 0~5%; weakly positive (+), 6~25%; moderately positive (++), 26~50%; and strongly positive (+++), 51~100%.

Real-time Reverse transcriptase-polymerase chain reaction (Real-time RT-PCR)

Total RNA was extracted from gastric tissue samples using Trizol (Takara, Japan) according to the manufacturer's protocol. Two micrograms of total RNA was subjected to cDNA synthesis using the AMV transcriptase and random primer (Takara, Japan). Oligonucleotide primers for PCR were 5'-TGACAAATGCTGGAGAACTG-3' and 5'-ATGTGAGGAAACTG TGGATG-3' for Bmi-1 (139bp, 1234-1372, NM_005180.8), and sense, 5'-CAATGACCCCTTCATTGACC-3' and anti-sense: 5'-TGGAAGATGGTGATG GGATT-3' for GAPDH (135bp, 201-335, NM_ 002046.3). Real-time PCR was performed according to the protocol of SYBR Premix Ex TaqTM II kit (Takara).

Statistical Analysis

Statistical evaluation was performed using Spearman correlation test to analyze the rank data, and and Wilcoxon test to differentiate the means of different groups. Kaplan-Meier survival plots were generated and comparisons between survival curves were made with the log-rank statistic. $p < 0.05$ was considered as statistically significant. SPSS 10.0 software was employed to analyze all data.

Results

As showed in Figure 1, Bmi-1 was positively immunostained in the cytoplasm of gastric epithelial cells, intestinal metaplasia (IM), adenomatous dysplasia and carcinomas. Overall, Bmi-1 expression was immunohistochemically detected respectively in 33.5% of gastritis (n=45), 23 out of 60 IM patients (38.3%), 46.2% of dysplasia (n=60) and 208 out of total 309 gastric carcinoma patients (65.0%). According to its expression frequency and density, there was gradually increased Bmi-1 protein expression from gastritis, IM, dyplasia to carcinoma (p<0.001, Table 1). As summarized in Table 2, Bmi-1 expression was positively linked to tumor size, depth of invasion, and lymph node metastasis (p<0.001),
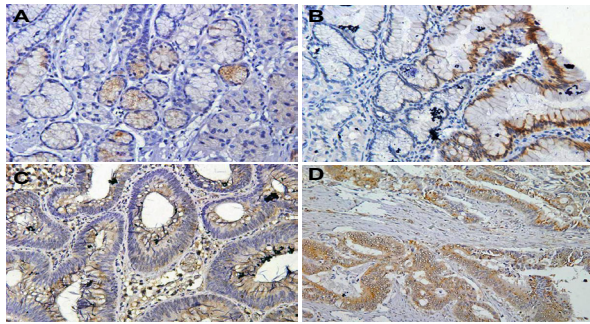
Figure 1. Immunohistochemical Staining of Bmi-1 Protein in Gastric Lesions. Bmi-1 protein was positively expressed in the cytoplasm of gastritis (A), intestinal metaplasia(B), adenomatous dysplasia(C), and carcinoma(D)
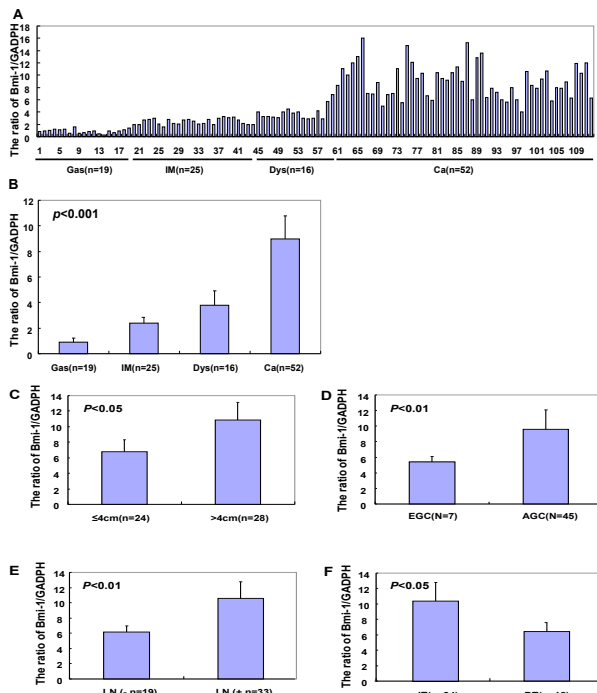

Figure 2. Bmi-1 mRNA Expression Level During Gastric Carcinogenesis and Its Correlation with Clinicopathological Features of Carcinoma. Bmi-1 mRNA was detectable in gastritis (Gas), intestinal metaplasia(IM), dysplasia(Dys) and carcinoma(Ca) by real-time RT-PCR (A). Its expression level was gradually increased in subsequently-mentioned lesion (p<0.001, B). Bmi-1 mRNA expression was higher in gastric carcinoma with larger diameter (C), deeper invasion (D), frequent lymph node metastasis (E), and intestinal-type (F) carcinoma than that in their counterparts. Note: EGC, early gastric cancer; AGC, advanced gastric cancer; LN, lymph node metastasis; IT, intestinal type; DT, diffuse type

but not to age or sex of carcinoma patients (p>0.05). There was higher Bmi-1 expression in intestinal- type carcinomas than diffuse-type ones (p<0.001).

Additionally, we designed Bmi-1 primers and performed real-time RT-PCR to quantify its mRNA expression level with housekeeping GAPDH as an internal control. The increased Bmi-1 mRNA expression was observed from gastritis, IM, dysplasia and carcinoma (p<0.001, Figure 2). Bmi-1 overexpression was observed in gastric carcinoma with larger diameter, deeper invasion, lymph node metastasis, and intestinal-type carcinoma (p<0.05).

Follow-up information was available on 309 gastric

Table 1. Bmi-1 Expression in Gastric Carcinogenesis

Groups	n	Bmi-1 expression					
		-	+	++	+++	PR(%)	p value
Gastritis	45	30	9	4	2	33.3	<0.001
Intestinal metaplasia	60	37	10	8	5	38.3	
Dysplasia	52	28	12	6	6	46.2	
Gastric carcinoma	309	108	110	42	49	65	

PR, positive rate

Table 2. Relationship Between Bmi-1 Expression and Clinicopathological Features of Gastric Carcinomas

| Clinicopathological features	n	Bmi-1 expression					
		-	+	++	+++	PR(%)	p value
Age(years)							>0.05
<55	147	52	49	20	26	64.6	
≥55	162	56	61	22	23	65.4	
Sex							>0.05
Male	98	33	32	15	18	66.3	
Female	211	75	78	27	31	64.5	
Tumor size (cm)							<0.001
≤4	135	68	32	15	20	49.6	
>4	174	40	78	27	29	77	
Depth of invasion							<0.001
T_{is}-T_1	41	20	8	7	6	51.2	
T_2-T_4	268	88	102	35	43	67.2	
Lymph node metastasis							<0.001
-	104	50	25	16	13	51.9	
+	205	58	85	26	36	71.7	
Lauren's classification							<0.001
Intestinal-type	172	52	50	37	33	69.8	
Diffuse-type	137	56	60	5	16	59.1	

PR, positive rate
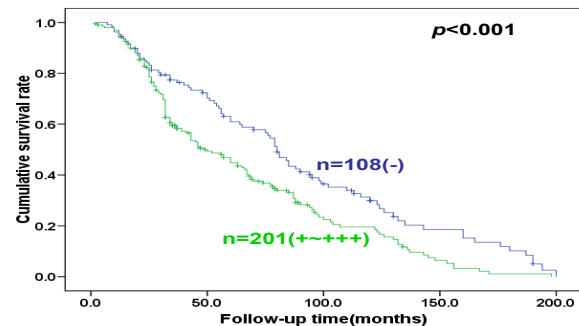
Figure 3. Correlation Between Bmi-1 Protein Expression Status and Prognosis of the Gastric Carcinoma Patients. Kaplan-Meier curves for cumulative survival rate of patients with gastric carcinomas according to Bmi-1 protein expression in gastric carcinomas

carcinoma patients for periods ranging from 2 months to 16.7 years (median=67.8months). Figure 3 showed survival curves stratified according to Bmi-1 protein expression for gastric carcinomas. Univariate analysis using Kaplan-Meier method indicated cumulative survival rate of patients with weak, moderate or Bmi-1 expression to be obviously lower than without its expression (p<0.05, Figure 3).

Discussion

In the present study, Bmi-1 protein is observed to exist in the cytoplasm of gastric lesions, opposite to its nuclear expression pattern in breast (Yin et al., 2011;

Wang et al., 2012), pancreatic (Yin et al., 2011), cervical (Zhang et al., 2010), esophageal squamous (Liu et al., 2010), tongue squamous (Häyry et al., 2010), colon (Li et al., 2010), bladder (Qin et al., 2009) and endometric (Engelsen et al., 2008) cancer cells. Another group also found cytoplasmic Bmi-1 expression in oropharyngeal squamous cell carcinoma (Huber et al., 2011). The discrepancy might be attributed to different antibodies and immunostaining approaches employed. Another explanation is tissue specificity of Bmi-1 expression with special functions because Bmi-1 is a transcriptional factor and can translocate from nucleus to cytosol under some physiological or pathological conditions.

Here, we found gradually reduced expression of Bmi-1 from gastritis, IM, dysplasia and carcinoma at both mRNA and protein. IM is believed to be an adaptive condition for gastric epithelium with injury and inflammation and could develop into globoid dysplasia, which is closely linked to signet ring cell carcinomas, evidenced by morphological appearance and biological characters (Zheng et al., 2010). Pathological and genetic observations demonstrate that gastric dysplasia precedes the majority of carcinoma and could undergo malignant transformation and is classified as cryptal, globoid, regenerative, and adenomatous subtypes (Zhang, 1994). Our data suggested that higher Bmi-1 expression might contribute to the carcinogenesis in agreement with previous literatures (Qin et al., 2009; Liu et al., 2010; Honig et al., 2010; Li et al., 2010; Song et al., 2010; Huang et al., 2011; Zhang et al., 2012).

To the role of Bmi-1 protein in the progression of gastric carcinoma, its expression was compared with the aggressive behaviors of carcinoma and found that Bmi-1 protein or mRNA expression was positively linked to tumor size, depth of invasion and lymph node metastasis. These findings suggested that Bmi-1 overexpression was involved in the growth, invasion and metastasis of gastric carcinoma and might be employed to indicate the biological behaviors of gastric carcinoma in clinicopathological practice. It was reported that Bmi-1 knockdown can enhance the chemosensitivity, cell cycle arrest and apoptosis, and inhibit invasive ability possibly via the inhibition of the PI3K-Akt pathway (Xiao and Deng, 2009; Li et al., 2010; Song et al., 2010; Wang et al., 2010; Guo et al., 2011; Xu et al., 2011; Yin et al., 2011). Overexpression of Bmi-1 increases the motility and invasive properties of immortalized human mammary epithelial cells. In addition, the repression of Bmi-1 reverses the expression of epithelial mesenchymal transition markers and inhibits the Akt/GSK3β/Snail pathway (Guo et al., 2011). To clarify the prognostic significance of Bmi-1 expression, we here analyzed their relation with the survival of 309 patients with gastric carcinoma and found a negative relationship link between the positivity of Bmi-1 expression and favorable survival, in line with other reports (Zheng et al., 2008; Xiao et al., 2009; Li et al., 2010; Liu et al., 2010; Yang et al., 2010; Zhang et al., 2010; Huber et al., 2011; Min et al., 2011; Zhang et al., 2012). It might be attributed to Bmi-1 overexpression in gastric cancer with more aggressive behaviors.

Although gastric cancer is malignant tumor originating from the same gastric epithelium, its morphological features vary substantially with the individual patients. According to Lauren's classification, intestinal-type carcinomas are characterized by cohesive carcinoma cells forming gland-like tubular structures with expanding or infiltrative growth pattern. The cell cohesion is less apparent or absent in diffuse-type carcinoma and cancer cells diffusely spread in the gastric wall lesions. Tumors that contain approximately equal quantities of intestinal and diffuse components are called mixed carcinoma (Zheng et al., 2007; Zheng et al., 2008). Here, it was noted that Bmi-1 expression was higher in intestinal- than diffuse-type carcinomas, indicating that it might play an important role in intestinal-type carcinogenesis, but less in de novo carcinogenic pathway and underlie the molecular basis for differentiation of both carcinomas.

In summary, up-regulated expression of Bmi-1 might play an important role of malignant transformation of gastric epithelial cells, was closely related to growth, invasion, metastasis and prognosis of gastric carcinomas and was considered as a promising marker to indicate the pathobiological behaviors. The distinct expression of Bmi-1 could be employed to differentiate the intestinal- and diffuse-type carcinomas and underlay the molecular mechanism about the differentiation of both carcinomas.

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