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ORIGINAL ARTICLE

Downregulated parafibromin expression is a promising marker for pathogenesis, invasion, metastasis and prognosis of gastric carcinomas

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Abstract Parafibromin is a protein encoded by the hyperparathyroidism 2 oncosuppressor gene and its downregulated expression is involved in pathogenesis of parathyroid carcinomas. To clarify the roles of parafibromin expression in tumourigenesis and progression of gastric carcinomas, it was examined by immunohistochemistry (IHC) on tissue microarray containing gastric carcinomas (n=508), adenomas (n=45) and gastritis (n=49) with a comparison of its expression with clinicopathological parametres of carcinomas. Gastric carcinoma cell lines (MKN28, AGS, MKN45, KATO-III and HGC-27) were studied for parafibromin expression by IHC and western blot. Parafibromin expression was localised in the nucleus of gastric epithelial cells, adenoma, carcinoma cells and cell lines. Its expression was gradually decreased from gastritis to gastric carcinoma, through gastric adenomas ($p<0.05$) and inversely correlated with tumour size, depth of invasion, lymphatic invasion, lymph node metastasis and Union Internationale Contre le Cancer (UICC) staging ($p<0.05$) but not with sex or venous invasion ($p>0.05$). Parafibromin was strongly expressed in older carcinoma patients compared with younger ones ($p<0.05$). There was stronger positivity of parafibromin in intestinal-type than diffuse-type carcinomas ($p<0.05$). Univariate analysis indicated cumulative survival rate of patients with positive parafibromin expression to be higher than without its expression ($p<0.05$). Multivariate analysis showed that age, tumour size, depth of invasion, lymphatic invasion, lymph node metastasis, UICC staging and Lauren's classification but not sex, venous invasion or parafibromin expression were independent prognostic factors for carcinomas($p<0.05$). Downregulated parafibromin expression possibly contributed to pathogenesis, growth, invasion and metastasis of gastric carcinomas. It was considered as a promising marker to indicate the aggressive behaviours and prognosis of gastric carcinomas.

Keywords Gastric carcinoma · Parafibromin · Pathogenesis · Progression · Prognosis

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Introduction

Parafibromin is a protein encoded by the hyperparathyroidism 2 (HRPT2) oncosuppressor gene, whose mutation causes the hyperparathyroidism–jaw tumour syndrome. The disease is an autosomal dominant disorder characterised by the occurrence of parathyroid adenoma or carcinoma, fibro-osseous jaw tumours of the mandible or maxilla and renal neoplastic and non-neoplastic abnormalities, such as Wilms' tumour, hamar-

toma or cystic renal disease [1, 16, 20]. HRPT2 gene is located in human chromosome 1q31.2, consists of 17 exons and spans 18.5 kb in the genome. It encodes a 2.7-kb transcript which is translated into a 531-amino-acid parafibromin protein with a molecular weight of 60 kd [3, 4, 23]. The 200-amino-acid C-terminal segment of parafibromin shares 32% identity and 54% homology with cell division cycle 73, a *Saccharomyces cerevisiae* protein forming the polymerase-associated factor 1 (Paf1) complex, which is associated with ribonucleic acid (RNA) polymerase II and involved in transcript site selection, transcriptional elongation, histone H2B ubiquitination, histone H3 methylation, poly (A) length control and coupling of transcriptional and posttranscriptional events [10, 17, 18, 24, 27]. Parafibromin overexpression was documented to inhibit colony formation and cellular proliferation and induce cell cycle arrest in the G1 phase, indicating that parafibromin has a critical role in cell growth [28]. Northern blot analysis showed HRPT2 expression in heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas [4]. Western blot study revealed parafibromin expression as a 60-kd band in the adrenal gland, heart, pancreas and kidney but 40-kd immunoreactive bands in the heart and skeletal muscle of human [25]. Immunohistochemically, higher expression of parafibromin was found widespread in glomerular mesangial cell, hepatocytes, cells of the base of gastric glands, renal cortex tubules and the pars intermedia of the hypophysis [17]. Subsequent investigations have revealed that mutations in HRPT2 are present in 66–100% of sporadic parathyroid carcinomas [7, 20]. Hyperparathyroidism–jaw-tumours-syndrome-related and sporadic parathyroid carcinomas are characterised by loss of parafibromin nuclear immunoreactivity [5, 22]. Selvarajan et al. [19] found that parafibromin expression was inversely linked to tumour size, pathologic stage and lymphovascular invasion of breast carcinomas using immunohistochemistry in a tissue microarray (TMA) study. These findings suggested the potential roles of parafibromin in pathogenesis and progression of malignancies.

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 [9]. Tumourigenesis and progression of gastric carcinoma is a multistage process with the involvement of a multifactorial aetiology, which mainly results from gene–environment interactions [32, 33]. Gastric carcinomas are classified into early and advanced ones on the basis of whether the carcinomas invade into the muscularis propria of the stomach [12]. In 1965, Lauren [13] classified gastric carcinomas into intestinal- and diffuse-type ones based on the morphological appearances. Intestinal-type carcinomas are characterised by cohesive carcinoma cells forming gland-like tubular structures with expanding or infiltrative growth pattern. However, the cell cohesion is less apparent or absent in diffuse-type carcinoma and cancer cells diffusely spread in the gastric wall [31]. Generally, there is a favorable prognosis for the patients with early or intestinal carcinoma compared with the other type. In our study, parafibromin expression was examined in gastric carcinoma, adenoma, gastritis and gastric carcinoma cell lines and compared with the clinicopathological parametres of carcinomas, as well as prognosis to explore the clinicopathological significance and molecular roles of parafibromin expression in stepwise development of gastric carcinoma.

Materials and methods

Subjects

Gastric carcinomas (n=508) were collected from the surgical resection, adenoma (n=45) from endoscopic biopsy or polypectomy and gastritis (n=49) from the endoscopic biopsy in our affiliated hospital, Himi Citizen Hospital and Kouseiren Takanoka Hospital between 1993 and 2006. All carcinomas were adenocarcinomas and the adenoma group was free from non-neoplastic polyp types, leiomyomas and benign gastrointestinal stromal tumours. The patients with gastric carcinoma were 354 men and 154 women (29~91 years, mean=65.4 years). Among them, 191 cases have carcinomas accompanied with lymph node metastasis. None of the patients underwent chemotherapy or radiotherapy 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.

Pathology

All 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. The staging for each gastric carcinoma was evaluated according to the Union Internationale Contre le Cancer (UICC) system for the extent of tumour spread [21]. Histological architecture of gastric carcinoma was expressed in terms of Lauren's [13, 31] classification. Furthermore, tumour size, depth of invasion, lymphatic and venous invasion were determined.

Tissue microarray

Representative areas of solid tumours were identified in HE-stained sections of the selected tumour cases and a 2-mm-in-

diametre tissue core per donor block was punched out and transferred to a recipient block with a maximum of 48 cores using a Tissue Microarrayer (AZUMAYA KIN-1, Japan). Four-micrometre-thick sections were consecutively incised from the recipient block and transferred to polylysine-coated glass slides. HE staining was performed on TMA for confirmation of tumour tissue.

Cell lines and culture

Gastric carcinoma cell lines come from the Japanese Physical and Chemical Institute, including MKN28 (well-differentiated adenocarcinoma), AGS (moderately differentiated adenocarcinoma), MNK45 (poorly differentiated adenocarcinoma), KATO-III (poorly differentiated adenocarcinoma) and HGC-27 (undifferentiated adenocarcinoma). They were maintained in Roswell Park Memorial Institute 1640 (MKN28, MKN45 and KATO-III), minimum essential (HGC-27) or Ham's F12 (AGS) medium supplemented with 10% foetal bovine serum, 100-units/ml penicillin, and 100-μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Total protein was prepared from all cells by cell disruption buffer according to Protein And RNA Isolation System manual (Arctiris Bioscience, USA). All cells were collected by centrifugation, rinsed with phosphate-buffered saline, fixed by 10% formalin and then embedded in paraffin as routinely processed.

Immunohistochemistry

Consecutive sections were deparaffinised with xylene, dehydrated with alcohol and subjected to antigen retrieval by irradiating in target retrieval solution citrate pH 6.0 (TRS, DAKO, Carpinteria, CA 93013, USA) for 15 min with microwave oven (Oriental Rotor Lmt. Co., Tokyo, Japan). Five percent bovine serum albumin was then applied for 1 min to prevent non-specific binding. The sections were incubated with mouse anti-parafibromin antibody (Clone 2H1, SC-33638, Santa Cruz, CA, USA; 1:40) for 15 min, then treated with the anti-mouse Envison-PO (DAKO, CA, USA) antibody for 15 min. Binding sites were visualised with 3, 3′-diaminobenzidine with the 5-min reaction. All the incubations were performed in a microwave oven to allow intermittent irradiation as described previously [11]. After each treatment, the slides were washed with Tris-buffered saline with Tween 20 (TBST; 10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) three times for 1 min. After being counterstained with Mayer's haematoxylin, the sections were dehydrated, cleared and mounted. Omission of the primary antibody was used as a negative control.

One hundred cells were randomly selected and counted from five representative fields of each section blindly by three independent observers (Takano Y, Li XH and Zheng HC). 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%.

Western blot

Fifty-microgramme denatured protein was separated on an SDS-polyacrylamide gel (10% acrylamide) and transferred to Hybond membrane (Amersham, Germany), which was then blocked overnight in 5% milk in TBST. For immunoblotting, the membrane was incubated for 1 h with mouse anti-parafibromin antibody as described above. Then, it was rinsed by TBST and incubated with anti-mouse immunoglobulin G conjugated to horseradish peroxidase (DAKO, CA, USA, 1:1,000) for 1 h. Bands were visualised with X-ray film (Fujifilm, Japan) by ECL-Plus detection reagents (Amersham, Germany). After that, membrane was washed with WB Stripping Solution (pH 2–3, Nacalai, Tokyo, Japan) for 30 min and treated as described above except mouse anti-β-actin antibody (Sigma, MO, USA, 1:5,000) as an internal control.

Statistical analysis

Statistical evaluation was performed using *Spearman* correlation test to analyse the rank data. *Kaplan–Meier* survival plots were generated and comparisons between survival curves were made with the log–rank statistic. The Cox's proportional hazards model was employed for multivariate analysis. $P<0.05$ was considered as statistically significant. SPSS 10.0 software was employed to analyse all data.

Results

Parafibromin expression in gastric tumours and carcinoma cell lines

As shown in Fig. 1, parafibromin was positively immunostained in the nucleus of MKN28, AGS, MKN45, KATO-III and HGC-27, and its expression level was consistent with the data of Western blot. Parafibromin was strongly expressed in the nucleus of gastric epithelial cells, adenomas and early carcinomas but not in given advanced carcinomas. Occasionally, it also appeared in stromal fibroblasts and lymphocytes but much weaker than epithelial cells or adenomas (Fig. 2).Generally, the stromal lymphocytes and fibroblasts were negative in cases where the tumour was negative. Overall, parafibromin expression was detected respectively in all gastritis (100.0%), 36 out of

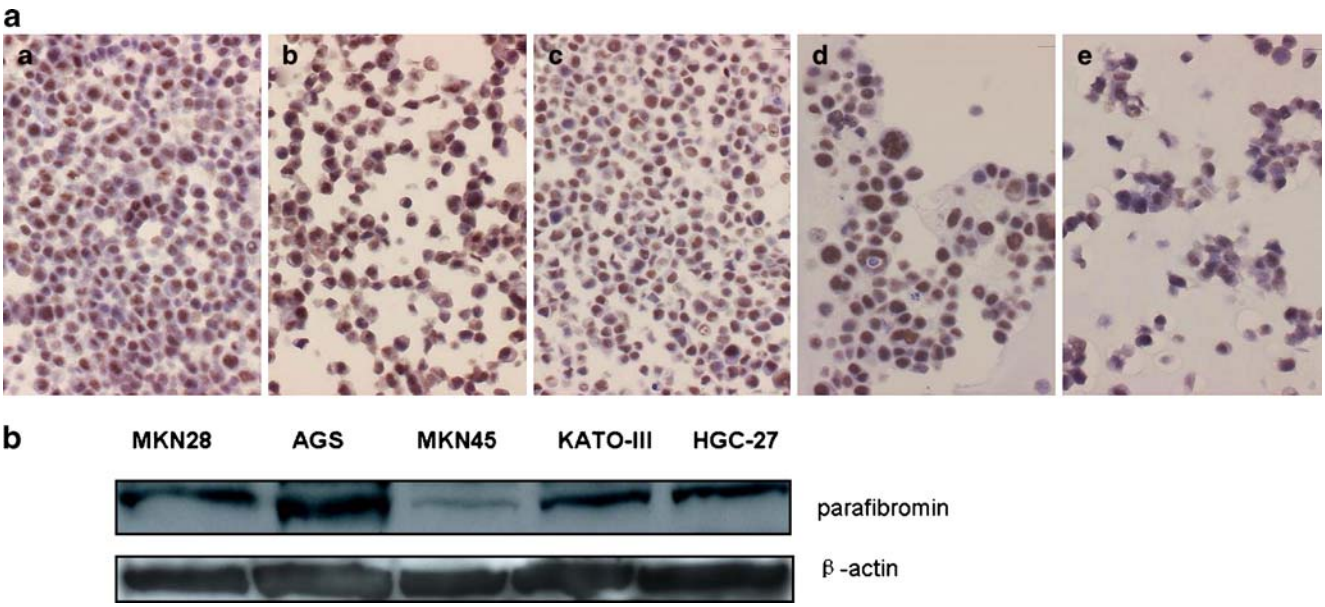

Fig. 1 Parafibromin expression in gastric carcinoma cell lines. **a** Parafibromin was positively immunostained in the nucleus of MKN28 (*a*), AGS (*b*), MKN45 (*c*), KATO-III (*d*) and HGC-27(*e*). **b** Cell lysate (50 μg) was loaded and probed with anti-human parafibromin antibody (60 kd) with β-actin (42 kd) as an internal control. *Lane #1*: MKN28; *#2* AGS; *#3* MKN45; *#4* KATO-III; *#5* HGC-27

45 adenoma patients (80.0%) and 233 out of total 508 gastric carcinoma patients (45.9%). Statistically, gradually reduced expression of parafibromin was seen from gastritis to gastric carcinoma through gastric adenoma ($p<0.05$, Table 1). As summarised in Table 2, parafibromin expression was inversely correlated with tumour size, depth of invasion, lymphatic invasion, lymph node metastasis and UICC staging ($p<0.05$) but not with sex or venous invasion ($p>0.05$). Parafibromin was strongly expressed in older carcinoma patients compared with younger ones ($p<0.05$). Intestinal-type carcinomas exhibited more frequent expression of parafibromin than diffuse-type ones ($p<0.05$).

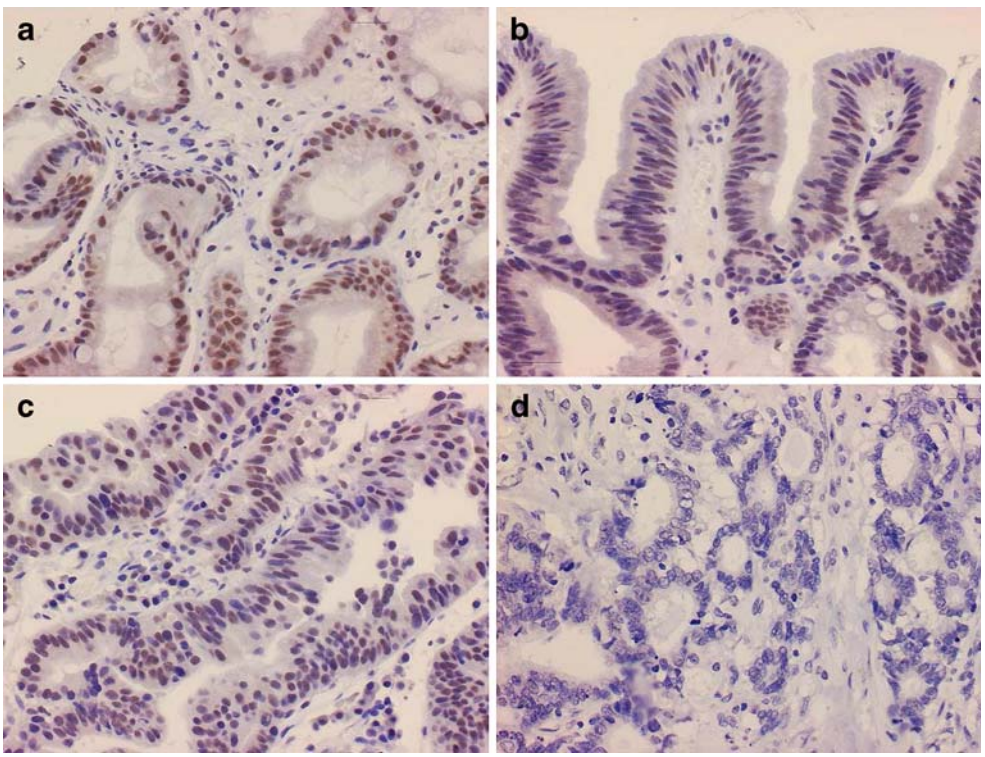

Fig. 2 Immunohistochemical staining of parafibromin in gastritis, adenoma and carcinoma. Note parafibromin positivity was strongly observed in the nucleus of gastric superficial epithelium (**a**), and adenoma (**c**) and early gastric carcinoma (**b**), occasionally weaker in the stromal fibroblasts and lymphocytes (**a**, **c**), but not in given advanced gastric carcinomas (**d**), indicating that the internal positive control (stromal cells) was negative adjacent to the negative staining carcinoma cells but positive adjacent to the positive epithelial cells

Table 1 Parafibromin expression in gastric tissue samples

Groups	Number	Parafibromin expression				
		–	+	++	+++	PR (%)
Gastritis	49	0	1	7	41	100.0[a]
Gastric adenoma	45	9	5	8	23	80.0[b]
Gastric carcinoma	508	275	55	60	118	45.9

PR Positive rate
[a] Compared with gastric adenoma or carcinoma, $p<0.001$
[b] Compared with gastric carcinoma, $p<0.001$

Univariate and multivariate survival analysis

Follow-up information was available on 508 gastric carcinoma patients for periods ranging from 0.2 months to 12.2 years (median=67.2 months). Figure 3 showed survival curves stratified according to parafibromin expression for gastric carcinomas. Univariate analysis using the *Kaplan–Meier* method indicated cumulative survival rate of patients with weak, moderate or strong parafibromin expression to be obviously higher than without its expression ($p<0.05$; Fig. 3a). The significant difference disappeared if stratified according to the depth of invasion (Fig. 3b, c). Multivariate analysis using Cox' s proportional hazard model indicated that age, tumour size, depth of invasion, lymphatic invasion, lymph node metastasis, UICC staging and Lauren's classification ($p<0.05$) but not sex,

Table 2 Relationship between parafibromin expression and clinicopathological features of gastric carcinomas

Clinicopathological features	Number	Parafibromin expression						
		–	+	++	+++	PR (%)	Rs	*p* value
Age (years)								
<65	209	125	24	24	36	40.2	0.095	<0.05
≥65	299	150	31	36	82	49.8		
Sex								
Male	354	188	37	38	91	46.9	0.054	>0.05
Female	154	87	18	22	27	43.5		
Tumour size (cm)								
<4	263	116	26	34	85	55.9	−0.237	<0.001
≥4	245	159	27	26	33	35.1		
Depth of invasion								
T_{is-1}	263	102	30	40	91	61.2	−0.344	<0.001
T_{2-4}	245	173	25	20	27	29.4		
Lymphatic invasion								
–	331	157	42	45	87	52.6	−0.168	<0.001
+	177	118	13	15	31	33.3		
Venous invasion								
–	443	236	49	51	107	46.7	−0.051	>0.05
+	65	39	6	9	11	40.0		
Lymph node metastasis								
–	317	138	38	44	97	56.5	−0.285	<0.001
+	191	137	17	16	21	28.3		
UICC staging								
0–I	292	123	36	41	92	57.9	−0.292	<0.001
II-IV	216	152	19	19	26	29.6		
Lauren's classification								
Intestinal-type	273	108	34	38	93	60.4	−0.322	<0.001
Diffuse-type	225	157	21	22	25	30.2		

PR Positive rate, T_{is} carcinoma in situ, T_1 lamina propria and submucosa, T_2 muscularis propria and subserosa, T_3 exposure to serosa, T_4 invasion into serosa, *UICC* Union Internationale Contre le Cancer

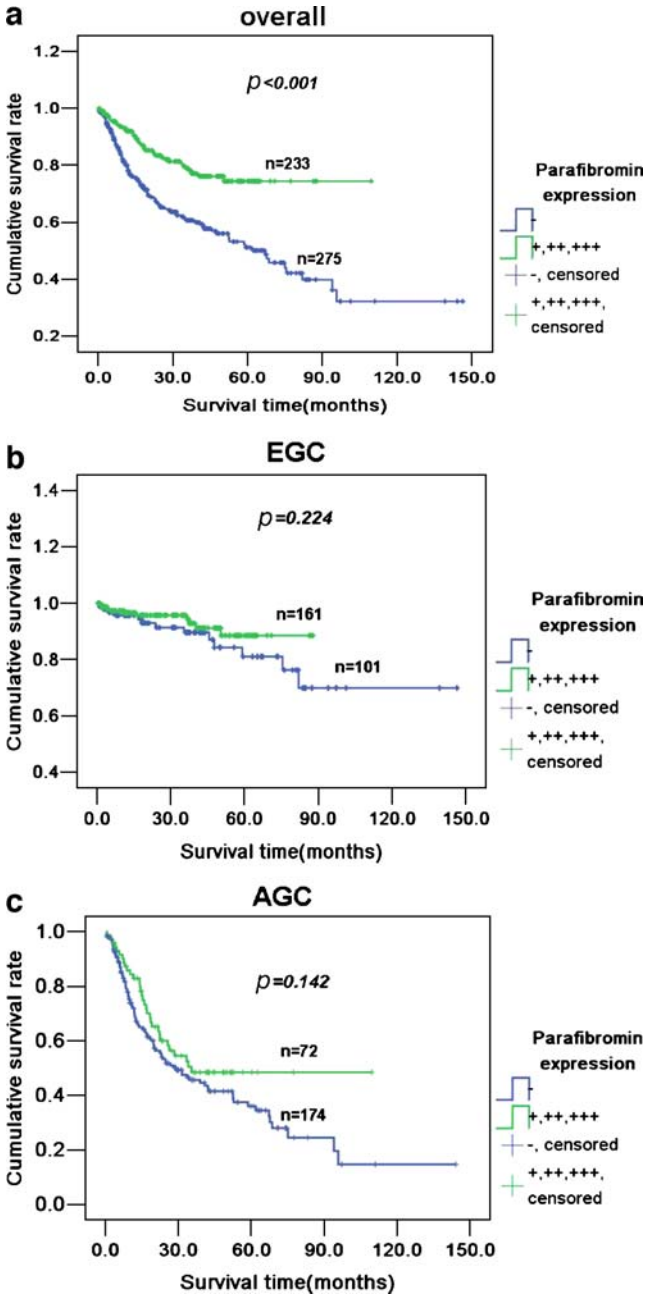

Fig. 3 Correlation between parafibromin status and prognosis of the gastric carcinoma patients. *Kaplan–Meier* curves for cumulative survival rate of patients with gastric carcinomas according to the parafibromin expression in overall (**a**), early (**b**, *EGC*) and advanced (**c**, *AGC*) gastric carcinomas

venous invasion or parafibromin expression were independent prognostic factors for overall gastric carcinomas ($p>0.05$; Table 3).

Discussion

HRPT2 has been isolated from complementary DNA libraries of parathyroid, kidney and bone tissue and encodes tumour suppressor protein parafibromin [4]. In the present study, the nuclear expression pattern was observed in the gastric epithelial cells, adenomas, adenocarcinomas and carcinoma cell lines consistent with previous reports in the gastric superficial mucosa, hepatocytes, kidney cortex tubules, adrenal gland, spleen lymphocytes, parathyroid tissue, adenoma and carcinomas, breast carcinoma [5, 8, 17, 19]. Although the result was in contrast with the paper of Porzionato et al. [17] possibly due to different incubation times of primary antibody, the great majority of immunohistochemical and cell transfection studies supported our observation of the nuclear staining [2, 5, 6, 8, 14, 19]. This study again demonstrates that parafibromin is nuclear and not cytoplasmic or nucleocytoplasmic in location as initially thought. The weaker expression of parafibromin in stromal cells than epithelial cells and adenoma might be due to the specificity of its cellular distribution as described previously [17]. It was found that the translocation of parafibromin to the nuclear compartment involved a function monopartite nuclear localisation signal at residues 136–139 [2, 6]. Parafibromin is a component of Paf1 complex in the nucleus, where it plays a role in cell cycle regulation, histone methylation, lipid and nucleic acid metabolism [10, 17]. The distribution pattern of parafibromin protein in gastric epithelial cells or tumour cells demonstrated its biological function in the nucleus.

Statistically, parafibromin expression was gradually reduced from gastritis to carcinoma through adenoma in line with parathyroid carcinogenesis, suggesting that downregulated parafibromin expression might contribute to the malignant transformation of gastric epithelial cells as an early event. The positive rate of parafibromin expression was reduced to 80% in gastric adenoma and reached about 46% of gastric adenocarcinoma, supporting the involvement of parafibromin in the gastric adenoma–adenocarcinoma sequence. Actually, the adenoma can progress into and be incorporated with gastric well-differentiated carcinoma when it grows bigger and de novo carcinogenesis is well understood, especially in diffuse-type gastric carcinomas [34]. Higher parafibromin expression in adenoma and intestinal-type carcinoma indicated that decreased parafibromin expression might play an important role in de novo diffuse-type carcinogenesis but less in intestinal carcinogenic pathway.

A body of evidences indicated that downregulation of tumour suppressor protein expression was due to genetic or epigenetic changes, like allelic loss, mutation, loss of heterozygosity (LOH), hypermethylation and microsatellite instability in malignancies [29, 30]. In the sporadic parathyroid carcinomas and hyperparathyroidism–jaw tumours, LOH or mutation of HRPT2 might cause the loss and inactivation of parafibromin protein [20, 25, 26]. Furthermore, the reduced expression of parafibromin was

Table 3 Multivariate analysis of clinicopathological variables for survival with gastric carcinomas

Number	Clinicopathological parametres	Relative risk (95%CI)	*p* value
A	Age (≥65 years)	1.929 (1.357–2.743)	<0.001
B	Sex (female)	1.463 (0.983–2.179)	>0.05
C	Tumour size (≥4 cm)	1.606 (1.013–2.547)	<0.05
D	Depth of invasion ($T_{2–4}$)	6.530 (3.110–13.710)	<0.001
E	Lymphatic invasion (+)	2.626 (1.516–3.374)	<0.001
F	Venous invasion (+)	0.959 (0.633–1.452)	>0.05
G	Lymph node metastasis (+)	2.773 (1.525–5.043)	<0.01
H	UICC staging (II-IV)	0.294 (0.139–0.622)	<0.01
I	Lauren's classification (diffuse-type)	1.796 (1.212–2.661)	<0.01
J	Parafibromin expression (+~+++)	0.792 (0.529–1.187)	>0.05

CI Confidence interval, *UICC*= Union Internationale Contre le Cancer

found to closely link to the tumour size, depth of invasion, lymphatic or venous invasion and UICC staging in line with the observation in breast carcinomas [19], indicating the inhibitory effects of parafibromin on tumour growth, invasion, metastasis and progression of gastric carcinomas. Drosophila Hyrax and its human orthologue, parafibromin, are required for nuclear transduction of the Wnt/Wg signal and bind directly to the C-terminal region of beta-catenin–Armadillo, thereby controlling transcriptional initiation and elongation by RNA polymerase II [15]. Parafibromin overexpression can inhibit colony formation, anchorage-dependent cell growth and cellular proliferation and induce cell cycle arrest in the G1 phase [28]. These findings demonstrated that loss of parafibromin expression had impact on the pathogenesis and progression of malignancies by promoting cellular proliferation. Additionally, parafibromin was expressed with a higher incidence in intestinal-type gastric cancer, which is presumed to arise from preceding dysplastic lesions, than diffuse-type one, which evolves without any precedent dysplastic changes. It is also demonstrated that distinct parafibromin expression underlies the molecular mechanisms for the differentiation of intestinal- and diffuse-type carcinomas.

Until now, there is yet no paper describing the prognostic significance of parafibromin expression in malignancies. Here, for the first time, we analysed the relation of parafibromin expression with the survival rate of 508 patients with gastric carcinoma. The results revealed a close link between its loss and worse survival. If stratified according to the depth of invasion, the significant link disappeared, indicating that the relationship between parafibromin expression and prognosis depends on the depth of invasion. The multivariate analysis demonstrated that age, depth of invasion, lymphatic invasion, lymph node metastasis, UICC staging and Lauren's classification but not parafibromin expression, venous invasion or sex were independent prognostic factors for carcinomas. These findings suggested that parafibromin expression is a promising indicator for the favorable prognosis of gastric carcinoma patients, albeit not independent.

In the present study, a large number of gastric carcinoma cases were screened by TMA, which takes the advantages of high throughput, identical immunohistochemical conditions, and economy of samples, antibodies and time [33]. Although we used 2-mm-in-diametre needles, which are large enough to evaluate the morphological appearance and carefully selected representative regions with the reference of HE slides, it was difficult to avoid selection bias. Gill et al. [5] found that stronger parafibromin staining and more positive cells sometimes appeared at the edges of the parathyroid tumour than in the centre. This could be due to fixation methods, other processing issues or a biological phenomenon, for example tissue hypoxia in the centre of large tumours. Additionally, the collection of our samples (e.g. gastritis, adenoma and adenocarcinoma) respectively from the endoscopic biopsy, polypectomy, or surgical resection put forward their another possibility of selection bias because of different fixation and processing methods. Selvarajan et al. [19] found that parafibromin underexpression was correlated particularly with large tumour size which is in line with our finding. It was possible that weaker staining could be attributable to poor fixation properties in the centre of large tumours. Because tumour size is a key prognostic indicator, this artifact could explain the prognostic significance of parafibromin in gastric carcinomas. Therefore, it is a limitation of the present study not to separate the edge and centre of gastric carcinomas when establishing TMA. In the current study, the negatively staining carcinomas are associated with a negative internal control (stromal cells and lymphocytes) whereas the positive staining epithelium is adjacent to positive staining internal controls. Therefore, the negative staining of the carcinoma might be artificial, which should be considered as another limitation of the study. Our study might be mentioned as a preliminary experiment and the staining with original-size sections is an extensive work in

the future using the gastric carcinoma samples, fixed and processed by the same approach.

In summary, downregulated parafibromin expression might play an important role in malignant transformation of gastric epithelial cells. Its reduced expression was closely related to growth, invasion, metastasis and worse prognosis of gastric carcinomas. Its expression could be employed to differentiate the intestinal- and diffuse-type carcinomas and underlay the molecular mechanism about the differentiation of both carcinomas. It was considered as a promising marker to indicate the pathobiological behaviours and prognosis of gastric carcinomas.

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Conflict of interest statement No conflict of interest

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