Parafibromin expression in lung normal tissue and carcinoma: its comparison with clinicopathological parameters of carcinoma

Pu Xia1,2, Wei Wang1,2, Xiao-yan Xu2, Jian-ping Wang1,2, Yasuo Takano3 and Hua-chuan Zheng1,2

1Department of Biochemistry and Molecular Biology, College of Basic Medicine, China Medical University, Shenyang, China, 2Institute of Pathology and Pathophysiology, College of Basic Medicine, China Medical University, Shenyang, China and 3Clinical Research Institute, Kanagawa Cancer Center, Yokohama, Japan

Summary. Parafibromin is a protein encoded by the hyperparathyroidism 2 oncosuppressor gene and its down-regulated expression is involved in the pathogenesis of parathyroid, gastric and colorectal carcinomas. To clarify the roles of parafibromin expression in lung carcinomas, it was examined by immunohistochemistry and in situ hybridization on tissue microarray containing lung carcinomas (n=144) and normal lung tissue (n=20), with a comparison to clinicopathological parameters of carcinomas. Lung carcinoma cell lines and tissues were studied for parafibromin expression by Western blot and RT-PCR. Down-regulated expression of parafibromin mRNA was found in lung carcinoma in comparison with matched normal tissue (p<0.05). Parafibromin protein was found in the cilia and nuclei of pseudo-stratified columnar epithelium, and the nuclei of lung carcinoma. According to immunostaining and in situ hybridization, there was no difference in parafibromin expression between histological subtypes of lung carcinoma (p>0.05). The Kaplan-Meier method indicated that nuclear parafibromin expression was positively correlated with adenocarcinoma patients (p<0.05). Down-regulated parafibromin mRNA expression might play an important role in lung carcinogenesis, but not in its histogenesis. Strong parafibromin expression in cilia of the pseudo-stratified columnar epithelium indicated its possible involvement in cell mobility. Parafibromin expression could be employed to indicate the favorable prognosis of patients with adenocarcinoma.

Key words: Lung carcinoma, Parafibromin, Pathogenesis, Progression, Prognosis

Introduction

Parafibromin is a protein encoded by the HRPT2 (hyperparathyroidism 2) oncosuppressor gene, whose mutation causes hyperparathyroidism-jaw tumour syndrome (Shattuck et al., 2003; Bradley et al., 2005; Aldred et al., 2006; Pimenta et al., 2006). The 200 amino acid C-terminal segment of parafibromin shares 54% homology with Cdc73 protein forming the Paf1 complex, which is associated with 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 post-transcriptional events (Krogan et al., 2003; Wood et al., 2003; Rozenblatt-Rosen et al., 2005; Yart et al., 2005; Porzionato et al., 2006; Farber et al., 2010). Parafibromin overexpression was documented to inhibit colony formation and cellular proliferation and induce cell cycle arrest in the G1 phase by repressing cyclin D1 via histone H3 K9 methylation, indicating that parafibromin has a critical role in cell growth (Zhang et al., 2006; Yang et al., 2010). Parafibromin was proved to stimulate cell proliferation and increase levels of the c-myc by stabilizing c-myc protein and activating the c-myc promoter without alleviation of the c-myc transcriptional pause. Northern blot analysis showed HRPT2 expression in heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas (Lin et al., 2008). Western blot revealed parafibromin expression as a 60-kDa band in the adrenal gland, heart, pancreas and kidney, but 40-kDa bands in the heart and skeletal muscle of the human (Woodard et al., 2005).
Immunohistochemically, parafibromin expression was found to be widespread in glomerular mesangial cells, hepatocytes, cells of the base of gastric glands, renal cortex tubules and the pars intermedia of the hypophysis at nuclear and nucleocytoplasmic patterns (Porzionato et al., 2006).

Subsequent investigations have revealed that mutations in HRPT2 are present in 66-100% of sporadic parathyroid carcinomas (Howell et al., 2003; Shattuck et al., 2003). Hyperparathyroidism–jaw tumors (HPT-JT) syndrome-related and sporadic parathyroid carcinomas are characterized by loss of nuclear parafibromin immunoreactivity (Gill et al., 2006). In contrast, methylation of the HRPT2 CpG islands and mutations of HRPT2 in the 5’-untranslated region of HRPT2 were not identified in any specimens of parathyroid carcinomas (Hahn et al., 2010). Selvarajan et al. (2008) found parafibromin expression was inversely linked to tumor size, pathologic stage, and lymphovascular invasion of breast carcinomas. In our previous work, it was found that down-regulated parafibromin expression might contribute to pathogenesis, growth, invasion and metastasis of gastric carcinomas and be regarded as a promising marker to indicate the aggressive behaviors and prognosis of gastric and colorectal carcinomas (Zheng et al., 2008; 2011). These findings suggested its potential roles in pathogenesis and progression of malignancies.

Lung cancer is one of the most common malignancies and greatest causes of cancer-related death in Japan and worldwide, despite the increased survival of cancer patients who receive advanced chemotherapy (Travis et al., 2004; Alberg et al., 2005). The development of lung carcinoma is due to multistep processes with involvement of genetic or epigenetic alteration of oncogenes and tumor suppressor genes, interacting with numerous environmental factors. In this paper, parafibromin expression was for the first time examined in lung normal tissues, carcinoma cell lines and tissues, and compared with the clinicopathological parameters of carcinomas, as well as prognosis to explore molecular roles of parafibromin expression in stepwise development of lung carcinoma.

Materials and methods

Cell lines and culture

Lung carcinoma cell lines, AoI and SQ-5 (squamous cell carcinoma), PC-14 (adenocarcinoma), H-460 (large cell carcinoma), and MS-1 and H-446 (small cell carcinoma) came from the Japanese Physical and Chemical Institute. They were maintained in RPMI 1640 (PC-14, SQ-5, MS-1, H-446 and H-460) and MEM (AoI) medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/mL streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. All cells were collected by centrifugation, rinsed with phosphate buffered saline (PBS, pH 7.2), and fractionated into cytosolic and nuclear fraction according to our own protocol (Xu et al., 2010).

Subjects

Lung carcinoma (n=144) and adjacent non-cancerous lung tissues (n=20) were collected from Kouseiren Takaoka Hospital (Takaoka, Japan) and the Affiliated Hospital, University of Toyama (Toyama, Japan) from 1993 to 2006 including 100 men and 44 women (40-85 years, mean=69.2 years). Among them, 49 were complicated by lymph node metastasis. Eighteen cases of lung carcinoma and paired normal tissue were collected from the First Affiliated Hospital of China Medical University and frozen in -80°C until protein or RNA extraction. None of the patients underwent chemotherapy, radiotherapy or adjuvant treatment before surgery. All patients or their relatives provided consent for use of tumor tissue for clinical research and our University Ethical Committee approved the research protocol. We followed up 135 patients by consulting their case documents and by telephone.

Pathology and tissue microarray (TMA)

144 cases of lung cancer and 20 normal control tissues were fixed in 10% neutral formalin, embedded in paraffin and sectioned at 4 µm. Sections were stained with hematoxylin-and-eosin (H&E) to confirm their histological diagnosis and histologically classified according to World Health Organization (WHO) criteria (Travis et al., 2004). The staging for each lung carcinoma was evaluated according to the Union Internationale Contre le Cancer (UICC) system to indicate the extent of tumor spread (Sobin and Wittekind, 2002). Differentiation, lymphatic or venous invasion was also determined.

H&E stained sections were examined and representative areas of solid tumor were identified for sampling. Two 2 mm-in-diameter or one 4 mm-in-diameter tissue cores per donor block were punched off and transferred to a recipient block carrying a maximum of 48 or 24 cores using a Tissue Microarrayer (AZUMAYA KIN-1, Tokyo, Japan) respectively. Four µm thick sections were consecutively incised from each recipient block and transferred to polylysine-coated glass slides. H&E staining was performed on the TMA to confirm carcinoma and normal tissues.

Western blot

The denatured protein from the frozen samples and carcinoma cell lines 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 tris buffered saline with Tween 20 (TBST, 10 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% Tween 20). For immunoblotting, the membrane was incubated for 1 hour with mouse anti-
parafibromin antibody (Santa Cruz; 1:500). Then, it was rinsed by TBST and incubated with anti-mouse IgG conjugated to horseradish peroxidase (DAKO, USA, 1:1000) for 1 hour. Bands were visualized with X-ray film (Fujifilm, Japan) by ECL-Plus detection reagents (Amersham, Germany). After that, membrane was washed with WB Stripping Solution (pH2-3, Nacalai, Tokyo, Japan) for 30 minutes and treated as described above, except for mouse anti-β-actin (Sigma, USA, 1:5000) antibody to label the cytosolic fraction or goat anti-Lamin B (Santa Cruz; 1:500) to label the nuclear fraction.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from lung carcinoma, paired normal tissue and carcinoma cell lines using RNeasy mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Two micrograms of total RNA were subjected to cDNA synthesis using Avian Myeloblastosis Virus (AMV) transcriptase and random primer (Takara, Otsu, Japan). According to the Genbank, oligonucleotide primers were forward: 5'-GCGACAGTACAA CATCCAGAA -3' and reverse: 5'-CCAATGTTTCCCCAA AACGG TAAG -3' for parafibromin (NM_024529, 194-1725; 1532bp). The primers for an internal control, GAPDH, were forward: 5'-CAGACAGTACAA CATCCAGAA -3' and reverse: 5'-TGGAAGATGGTGATGGGATT-3' (201-335, 135bp; NM_002046.3). PCR amplification of cDNA was performed in 25 µL mixtures containing 0.125 µL Pfu polymerase (Stratagene, West Cedar Creek, USA) and primers (forward: 5'-CGGACAGTACAA CATCCAGAA -3 and reverse: 5'-GGTACAGTTTCCCCAA AACGG TAAG -3' for parafibromin (NM_024529, 194-1725; 1532bp). The conditions for PCR were: 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min.

Real-time RT-PCR

To further confirm the results of general RT-PCR, we carried out real-time PCR using SYBR Premix Ex Taq™ II kit using the RNA samples from frozen samples and carcinoma cell lines. According to the Genbank, oligonucleotide primers for parafibromin were designed as follows: forward: 5'-CACGGAATTG AGGATGAAGAG TG -3' and reverse: 5'-CTGTTCA GTCTGTACAATC CCT-3'. The primers for the internal control, GAPDH, were forward: 5'-CCAATGTTTCCCCAA AACGG TAAG -3' and reverse: 5'-TGGAAGATGGTGATGGGATT-3' (201-335, 135bp; NM_002046.3). Primer amplification of cDNA was performed in 20 µL mixtures containing 10 µL SYBR Premix Ex Taq (x2) with 0.08 µL of each primer, 0.4 µL of ROX Reference Dye, and 1µL of template cDNA (50 µg/µL). The protocol included the following parameters: an initial 30 seconds of incubation at 95°C followed by 40 cycles of denaturation at 95°C for 5 seconds and annealing at 90°C for 20 seconds.

Immunohistochemistry

Consecutive sections of TMA were deparaffinized with xylene, rehydrated with alcohol, and subjected to antigen retrieval by irradiating in target retrieval solution (DAKO, Carpinteria, USA) for 15 minutes with a microwave oven (Oriental rotors, Co., Tokyo Japan). Five percent bovine serum albumin was then applied for 1 minute to prevent non-specific binding. The sections were incubated with mouse anti-parafibromin (Santa Cruz; 1:50) antibody for 15 minutes and then treated with the anti-mouse Envison-PO (DAKO) antibodies for 15 minutes. All the incubations were performed in a microwave oven to allow intermittent irradiation as described previously (Kumada et al., 2004). After each treatment, the slides were washed with TBST three times for 1 minute. Omission of the primary antibody was considered as a negative control and appropriate positive controls were utilized as recommended by the manufacturers. For evaluation, one hundred cells were randomly selected and counted from 5 representative fields. The positive percentage of counted cells was graded semi-quantitatively with a four-scale scheme: negative (-), less than 5%; weakly positive (+), 6-25%; moderately positive (++), 26-50%; and strongly positive (+++), more than 51%. Inconsistent data points were reanalyzed by both observers (Xia and Zheng) until agreement was reached.

In situ hybridization (ISH)

To perform RNA-DNA ISH for parafibromin, a digoxigenin-labeled Parafibromin probe was made in 30-cycle PCR of 100 µL mixture using 30 ng template pEGF-N1- parafibromin plasmid DNA, Pfu polymerase (Stratagene, USA) and primers (forward: 5'-CGGAGCATCAATCCAGAA -3 and reverse: 5'-CTGTTACTGTTGACATCCACCTCCC T-3', 194-677, 484bp, Tm=55°C, CCDS1382.1). Four-µm-thick sections of TMA were dewaxed and digested with 20µg/mL proteinase K in 50 mmol/L Tris-HCl at 37°C for 10 minutes. Then 20 µL of a 1:20 probe dilution in hybridization buffer (22 mmol/L Tris-HCl, pH7.4, 2.75 mmol/L EDTA, 660 mmol/L NaCl, 1x Denhardt solution, 5.5% dextran sulfate, 0.33% dimethyl sulfoxide, 0.55% ethoquad18/25 and 44% deionized formamide) was added to each slide. After coverslipping, heating at 95°C for 5 minutes, and incubation overnight in a humidified chamber at 37°C, sections were rinsed for 10 minutes in TBST and incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche Diagnostics, Germany) for 20 minutes at 37°C. The slides were then washed for 5 minutes and immersed in solution II (100 mmol/L Tris-HCl pH9.5, 100 mmol/L NaCl and 50mmol/L MgCl2) for 15 minutes and followed by exposure to NBT (nitro-blue tetrazolium chloride) / BCIP (5-Bromo-4-Chloro-3'-Indolyolphosphatase p-Toluidine salt) as chromogens. Finally, counterstaining was performed using methyl green for 2 minutes. Omission of the probe or RNase digestion was used as a negative control. For evaluation, one hundred cells were randomly selected and counted from 5 representative
fields by both researchers (Xia P and Zheng HC). The percentages of counted cells were scored as follows: 0-5%-negative (-), 6-100%-positive (+).

Statistical analysis

Statistical evaluation was performed using Spearman correlation test to analyze the rank data and using Fisher’s Exact Probability Test to compare the positive rates. 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 analyze all data.

Results

Parafibromin expression in lung carcinoma cell lines

Parafibromin protein was positively distributed to the nuclear fraction of H-446, H-460, SQ-5 and MS-1, whereas weakly in PC14 and AoI carcinoma cells (Fig. 1a). There was strong expression of parafibromin mRNA in H460, SQ-5 and MS-1, but weak in H-446, PC-14 and AoI (Fig. 1b).

Table 1. Relationship between parafibromin expression and clinicopathological parameters of lung carcinomas.

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
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<th>PR (%)</th>
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PR: positive rate; Rs: Spearman correlation co-efficient; NS: not significant; UICC: Union Internationale Contre le Cancer.

Parafibromin expression in frozen lung carcinoma and matched normal tissues

In 18 cases of fresh samples, only 1 case of lung
carcinoma showed a lack of parafibromin expression (Fig. 2a). Statistically, no difference in total parafibromin protein expression was found between carcinoma and paired normal tissue (p>0.05, Fig. 2d). Using general RT-PCR and primers spanning the full-length parafibromin cDNA, we only detected its mRNA expression in 8 carcinomas and 9 normal tissues (Fig. 2b). Compared with matched normal tissue, lung carcinoma showed a low tissue account of Parafibromin mRNA (p<0.05, Fig. 2c,e) by real-time PCR.

In situ parafibromin expression in lung normal tissue and carcinoma

Parafibromin protein was found in the cilia and nuclei of pseudo-stratified columnar epithelium, but not in the alveolar epithelial cells (Fig. 3a,b). In contrast, parafibromin was distributed in the nuclei of squamous cell carcinoma (SQ, Fig. 3c), adenocarcinoma (AD, Fig. 3d), large cell carcinoma (LCC, Fig. 3e), small cell carcinoma (SCC, Fig. 3f) and infiltrating inflammatory cells. Immunohistochemically, parafibromin expression was not correlated with sex, age, histological classification, differentiation, lymphatic and venous invasion, lymph node metastasis or UICC staging of total carcinomas (p>0.05, Table 1) or even adenocarcinomas (p>0.05, data not shown).

According to the observation of ISH, the signal of parafibromin mRNA was detected in the alveolar and pseudo-stratified columnar epithelium, SQ, AD, LCC

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**Fig. 2.** Parafibromin expression in lung carcinomas and matched normal tissues. a. Tissue lysate was loaded and probed by anti-parafibromin antibody (Panel 1, 60kDa) and with β-actin (Panel 2, 42kDa) as an internal control. b. Parafibromin amplicon (1532bp) was detected by RT-PCR with an internal control of GAPDH (135bp). c. Real-time quantitative PCR assay of Parafibromin mRNA expression in carcinoma and paired normal tissues. d. There was no difference in parafibromin protein expression between carcinoma and paired normal tissues (p>0.05). e. Parafibromin mRNA expression level was higher in normal tissues than matched carcinomas (p<0.05). N: normal tissue; C: carcinoma.
Fig. 3. Immunohistochemical staining of parafibromin protein in lung tissues. Parafibromin positivity was not detectable in alveolar epithelial cells (a), but was detectable in the cilia and nuclei of pseudo-stratified columnar epithelium (b). Additionally, it was strongly expressed in the nuclei of squamous carcinoma (c), adenocarcinoma (d), large cell carcinoma (e) and small cell carcinoma (f), occasionally in the stromal fibroblasts and lymphocytes (b-f).

Fig. 4. Parafibromin mRNA expression in lung tissues by in situ hybridization. Parafibromin mRNA signal was observed in alveolar epithelial cells (a), pseudo-stratified columnar epithelium (b), squamous carcinoma (c), adenocarcinoma (d), large cell carcinoma (e) and small cell carcinoma (f).
and SCC. The positive rates of parafibromin mRNA expression were 47.4% (9/19), 45.6% (21/46), 38.5% (5/13), and 38.5% (5/13) in SQ, AD, LCC, and SCC respectively with no statistical significance (p>0.05, Fig. 4).

Univariate and multivariate survival analysis

Follow-up information was available on 135 patients with lung carcinomas for periods ranging from 1 month to 12 years (mean=20.5 months). Univariate analysis using Kaplan-Meier method indicated that nuclear parafibromin expression was not related with the survival rate of the patients with lung carcinoma (p>0.05), but positively with those with adenocarcinoma (p<0.05, Fig. 5). Multivariate analysis using Cox’s risk proportional model showed parafibromin was not an independent factor for lung adenocarcinoma (p>0.05, data not shown).

Discussion

HRPT2 has been isolated from cDNA libraries of parathyroid, kidney and bone tissue and encodes the tumor suppressor protein parafibromin (Bradley et al., 2005). Here, the nuclear expression pattern of parafibromin was observed in lung carcinoma and pseudo-stratified columnar epithelium, whereas the cytoplasmic parafibromin was strongly localized in the cilia of the pseudo-stratified columnar epithelium. However, the result was in contrast to the paper of Porzionato et al. (2006), possibly due to different secondary antibodies, different incubation times and approaches. The cytoplasmic localization of parafibromin might result from its nuclear export sequences, as with other proteins which regulate transcription, such as IκB-α and p53 (Hahn and Marsh, 2005; Porzionato et al., 2006). It was found that the translocation of parafibromin to the nuclear compartment involved a function monopartite nuclear localization signal at residues 136-139 (Hahn and Marsh, 2005; Bradley et al., 2007), where parafibromin is a component of Polymerase-associated factor 1 (Paf1) complex and plays a role in cell cycle regulation, histone methylation, lipid and nucleic acid metabolism (Krogan et al., 2003; Wood et al., 2003; Rozenblatt-Rosen et al., 2005; Yart et al., 2005; Porzionato et al., 2006; Farber et al., 2010). On the other hand, we for the first time reported the cilia and nucleic immunoreactivity of parafibromin protein in the pseudo-stratified columnar epithelium, suggesting that subcellular localization of parafibromin was closely linked to its biological function. For example, cytoplasmic parafibromin interacted with muscle alpha-actinins (actinin-2 and actinin-3), but not with non-muscle alpha-actinins (actinin-1 and actinin-4) (Agarwal et al., 2008). The specific cilia distribution of parafibromin indicated its role in the cytoplasmic compartment and cell mobility.

A body of evidence indicates that down-regulation of tumor suppressor protein expression in carcinogenesis (Tan et al., 2004; Gill et al., 2006; Zheng et al., 2008). Our RT-PCR data also showed a reduced expression of parafibromin mRNA in lung carcinoma, compared with its matched normal tissue. In normal lung tissue, parafibromin mRNA was distributed to the alveolar and pseudo-stratified columnar epithelium. Reportedly, reduced expression of parafibromin protein was found to be closely linked to the tumor size, depth of invasion, lymphatic or venous invasion and UICC staging in breast (Selvarajan et al., 2008), gastric (Zheng et al., 2008) and colorectal (Zheng et al., 2011) carcinomas. It was suggested that down-regulated parafibromin expression might be involved in the pathogenesis of lung carcinoma, although various histological types of lung...
carcinomas have different carcinogenic pathways and biological behaviors. However, the correlation of parafibromin expression was not found to be linked to aggressive behaviors of lung carcinoma. It is worth noting that there were no differences in parafibromin protein and mRNA expression between SQ, AD, LCC and SCC. It was suggested that parafibromin expression was not related to the histological differentiation of lung carcinoma. Here, we for the first time analyzed the relation of parafibromin expression with the survival rate of 135 patients with lung carcinoma. The results revealed no significant association between nuclear parafibromin expression and outcome of the patients with lung carcinomas overall, but parafibromin staining was a favorable prognostic factor for pulmonary adenocarcinomas if stratified by histological classification, which might be linked to a large number of adenocarcinoma cases, and higher parafibromin expression was found in the LCC and SCC with poor prognosis. Until now, only our group reported that parafibromin expression was significantly linked to the favorable prognosis of patients with gastric and colorectal carcinoma (Zheng et al., 2008, 2011). Further Cox's analysis indicated parafibromin immunoreactivity was not independent factor for patients with lung adenocarcinoma. No correlation between parafibromin expression and investigated clinicopathological features of lung adenocarcinoma makes us speculate that other parameters might influence parafibromin expression and then determine its prognostic significance. These findings suggested that parafibromin expression could be employed to indicate the favorable prognosis of adenocarcinoma patients, albeit not independently.

In summary, down-regulated parafibromin mRNA expression might play an important role in lung carcinogenesis, but not in histogenesis. Strong parafibromin expression in cilia of the pseudo-stratified columnar epithelium indicated its involvement in cell mobility. Parafibromin expression is a good indicator for the favorable prognosis of adenocarcinoma patients, albeit not independently.

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Parafibromin expression in lung carcinomas


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