RESEARCH ARTICLE

Effects of Parafibromin Expression on the Phenotypes and Relevant Mechanisms in the DLD-1 Colon Carcinoma Cell Line

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Abstract

Background: Parafibromin is a protein encoded by the HRPT2 (hyperparathyroidism 2) oncosuppressor gene and its down-regulated expression is involved in pathogenesis of parathyroid, breast, gastric and colorectal carcinomas. This study aimed to clarify the effects of parafibromin expression on the phenotypes and relevant mechanisms of DLD-1 colon carcinoma cells. Methods: DLD-1 cells transfected with a parafibromin-expressing plasmid were subjected to examination of phenotype, including proliferation, differentiation, apoptosis, migration and invasion. Phenotype-related proteins were measured by Western blot. Parafibromin and ki-67 expression was detected by immunohistochemistry on tissue microarrays. Results: The transfectants showed higher proliferation by CCK-8, better differentiation by electron microscopy and ALP activity and more apoptotic resistance to cisplatin by DNA fragmentation than controls. There was no difference in early apoptosis by annexin V, capase-3 activity, migration and invasion between DLD-1 cells and their transfectants. Ectopic parafibromin expression resulted in down-regulated expression of smad4, MEKK, GRP94, GRP78, GSK3β-ser9, and Caspase-9. However, no difference was detectable in caspase-12 and -8 expression. A positive relationship was noted between parafibromin and ki-67 expression in colorectal carcinoma. Conclusions: Parafibromin overexpression could promote cell proliferation, apoptotic resistance, and differentiation of DLD-1 cells.

Keywords: Colon carcinoma cells - parafibromin - cell phenotypes - molecular mechanisms

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Introduction

Parafibromin is a protein encoded by oncosuppressor gene HRPT2 (hyperparathyroidism 2), whose mutation causes the hyperparathyroidism- jaw tumor syndrome (Aldred et al., 2006). HRPT2 is located in human chromosome 1q31.2, consists of 17 exons, and spans 18.5kb in the genome. It encodes a 2.7kb transcript which is translated into a 60kD parafibromin protein. Parafibromin protein can be involved in the formation of polymerase-associated factor 1(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, coupling of transcriptional and post-transcriptional events (Newey et al., 2009). Parafibromin overexpression was documented to induce cell cycle arrest in G1 phase by repressing cyclin D1 via histone H3K9 methylation, indicating that parafibromin has a critical role in cell cycle (Zhang et al., 2006; Yang et al., 2010). Parafibromin or Paf1 knockdown was proved to stimulate cell proliferation and increase the c-myc level by stabilizing c-myc protein and activating the c-myc promoter (Lin et al., 2008). Reportedly, parafibromin interacts with the ring finger proteins RNF20 and RNF40 and is required for the maintenance of histone 2B monoubiquitination (Hahn et al., 2011).

Hyperparathyroidism-jaw tumor- 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 parathyroid carcinomas (Zhao et al., 2007). It was found that parafibromin expression was inversely linked to tumor size, pathologic stage, and lymphovascular invasion of breast carcinomas (Selvarajan et al., 2008). Another study showed that parafibromin was a novel immunohistochemical markers effectively discriminating chromophobe renal cell carcinoma and renal oncocytoma (Tan et al., 2010). In our previous work, it was found that down-regulated parafibromin expression might contribute to the pathogenesis, growth, invasion and metastasis and be regarded as a good marker to indicate the poor prognosis of gastric or colorectal carcinoma patients (Zheng et al., 2008; Zheng et al., 2011). We reported that parafibromin mRNA was downregulated in lung carcinoma and its protein expression was closely linked

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to favorable prognosis of the patients with pulmonary adenocarcinoma (Xia et al., 2011). It was documented that distinct parafibromin expression was strongly correlated with tumor types of major renal cell tumors, which may suggest that it plays a role in the tumorigenesis in renal cell tumors (Cui et al., 2012). In the present study, we observed the effect of ectopic parafibromin overexpression on proliferation, apoptosis, differentiation, invasion and migration of colorectal carcinoma cell, and analyzed the relevant mechanisms.

Materials and Methods

Cell culture and transfection

Colorectal carcinoma cell line (DLD-1) were kindly presented by Prof. Sugiyama, Department of Gastroenterology, Graduate School of Medical and Pharmaceutical Sciences, University of Toyama. It was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. The cells were transfected with pEGFP-N1-parafibromin plasmid at 70% confluence 20 to 24 h after seeding on dishes using (QIAGEN, USA) method according to the manufacturer's instructions.

All cells were harvested by centrifugation, rinsed with PBS, and subjected to total protein extraction by sonication in RIPA lysis buffer. Cells were fractionated into cytosolic and nuclear fraction using NE-PER Nuclear and Cytoplasmic Extraction Reagents (78833; Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions.

Proliferation assay

Cell Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan) was employed to determine the number of viable cells. In brief, 2.5×10^3 cells/ well were seeded on 96-well plate and allowed to adhere and grow in 10% FBS-containing RPMI 1640. At different time points, 10 μL of CCK-8 solution was added into each well of the plate and the plate were incubated for 3 h in the incubator and measured at 450 nm. Furthermore, the cell viability curve was made to confirm the data of CCK8 by cell counting.

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was used as an additional marker of the degree of colonic differentiation. The cells were harvested, washed, broken and subjected to the determination of ALP activity using the Sigma Diagnostics ALP reagent (Sigma, USA) according to the manufacture's protocol. The protein content of the samples was determined using the Coomassie Protein Assay Reagent Kit (Biorad, USA). ALP activity was calculated as units of activity per mg of protein.

Transmission electron microscopy

Specimens were immersed in 2% (wt/vol) cacodylate-buffered glutaraldehyde (pH 7.4) for 6 h. They were then rinsed in cacodylate buffer supplemented with 15% (wt/vol) sucrose, postfixed with 1% (wt/vol) phosphate-buffered OsO_4 (pH 7.4) for 2 h, dehydrated with graded alcohol, clarified in propylene oxide, and embedded in Epon using flat molds. Ultrathin sections were made with ultramicrotome, stained with uranyl acetate, followed by a saturated solution of bismuth subnitrate and finally examined under a JEOL 1010 electron microscope.

Immunofluorescence

Cells were grown on glass coverslips, washed twice with PBS, fixed with 4% formaldehyde for 10 min at room temperature, and permeabilized with 0.25% Triton X-100 for 10 min at room temperature. After washing with PBS, cells were incubated overnight at 4 °C with the mouse antibody against E-cadherin (Takara) or β-catenin (BD Bioscience). We incubated Alexa Fluor 568 (red) anti-mouse IgG (Invitrogen, USA; 1:500) for 1h as the secondary antibody. Alternatively, the sections were mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, USA). Finally, the microphotography was performed under the Bio-zero fluorescence microscopy (BZ-8000, KEYENCE, Osaka, Japan)

Apoptosis assay by flow cytometry

Flow cytometry was performed with 7-amino-actinomycin (7-AAD) and phycoerythrin (PE)-labeled annexin V (BD Pharmingen, San Diego, CA92121,USA) to detect phosphatidylserine externalization (on the surface of cell membrane) as an endpoint indicator of early apoptosis as the recommended protocol. The CaspGLOW Red Caspase-3 Staining Kit was used to monitor the intracellular caspase-3 activity according to the manufacturer's recommendations (BioVision, Linda Vista Avenue, Mountain View, CA 94043 USA).

DNA fragmentation

Quantitative DNA fragmentation assay was carried out according to the method of Sellins and Cohen (1987). Briefly, the cells were lysed and supernatant was precipitated into pellet. was added to 12.5% trichloroacetic acid (TCA) at 4°C and quantified using a diphenylamine reagent after hydrolysis in 5% TCA at 90°C for 20 min. Percentage of DNA fragmentation refers to the ratio of DNA in the supernatant ("fragmented") to the total DNA recovered in both supernatant and pellet ("fragmented plus intact").

Wound healing assay

Cells were seeded at a density of 1.0×10^6 cells/well in 6-well culture plates. After they had grown to confluence, the cell monolayer was scraped with a pipette tip to create a scratch, washed by PBS for three times and cultured in the FBS-free medium. Cells were photographed at 24 h and the scratch area was measured using Image software.

Transwell chamber assay

For the invasive assay, 2.5×10^5 cells were resuspended in serum-free RPMI 1640, and seeded in the matrigel-coated insert on the top portion of the chamber (BD Bioscience, 354481). The lower compartment of the chamber contained 10% v/v FBS as a chemoattractant. After incubated at 37 °C, 5% CO_2 for 24 h, cells on the

Table 1. The Primary Antibodies Used in the Present Study

Names	Source	Company
Smad4	Mouse	Santa Cruz Biotech. Inc.
MEK kinase-1 (F-11):	Mouse	Santa Cruz Biotech. Inc.
GRP94	Goat	Santa Cruz Biotech. Inc.
GRP78	Goat	Santa Cruz Biotech. Inc.
GSK3β-ser9	Rabbit	Applied Biological Materials Inco
Caspase-12	Mouse	Life Span Biosciences. Inc.
Caspase-9	Mouse	Santa Cruz Biotech. Inc.
Caspase-8	Rabbit	Santa Cruz Biotech. Inc.
α-tubulin	Mouse	Labvision
β-actin(C-4)	Mouse	Santa Cruz Biotech. Inc.

membrane were scrubbed, washed with PBS and fixed in 100% methanol and stained with Giemsa dye for the measurement. For the migration assay, the procedures were the same as above excluding the control-membrane insert (BD Bioscience, 354578). To confirm the migration assay, wound healing assay was performed as well.

Real-time PCR

Total RNA was extracted from colorectal carcinoma cell lines and tissues using RNeasy mini kit (QIAGEN, Germany). Two micrograms of total RNA was subjected to cDNA synthesis using avian myeloblastosis virus (AMV) transcriptase and random primer (Takara, Japan). The primers for parafibromin were forward: 5'- GCGACAGTACAACATCCAGAA -3' and reverse: 5'-CAT TCTTGGGCCAGGAGAAC-3'(83bp,194-276, NM_024529). The primers for an internal control, GAPDH, were forward: 5'- CAATGACCCCTTCATTGACC -3' and reverse: 5'- TGGA AGAT GGTGATGGGATT-3' (135 bp, 201-335, NM_002046.3). Real-time PCR was carried out according to the protocol of SYBR Premix Ex TaqTM II kit (Takara) in 20 μL mixture. These primers were synthesized by Takara Biotech company (Dalian, China). Real- time PCR was performed according to the protocol of SYBR Premix Ex TaqTM II kit (Takara). The expression level of REIC was expressed as $2^{-\Delta Ct}$, where ΔCt =Ct (parafibromin)–Ct (GAPDH). Additionally, the expression level of the control cells was considered as "1".

Western blotting

Protein assay were performed using Biorad protein assay kit. The denatured protein was separated on 10% SDS-polyacrylamide gel and transferred to Hybond membrane (Amersham, Germany), which was then blocked overnight in 5% milk in TBST. For immunobloting, the membrane was incubated for 1 h with primary antibodies (Table 1). Then, it was rinsed by TBST and incubated with anti-mouse, anti-rabbit or anti-goat IgG conjugated to horseradish peroxidase (DAKO, USA) for 1 h. Bands were visualized by ECL-Plus detection reagents (Santa cruz, USA). Densitometry quantification was performed with a β-actin control using Scion Image software.

Specimen and immunohistochemistry

Colorectal carcinomas (CRCs, n=292) were collected

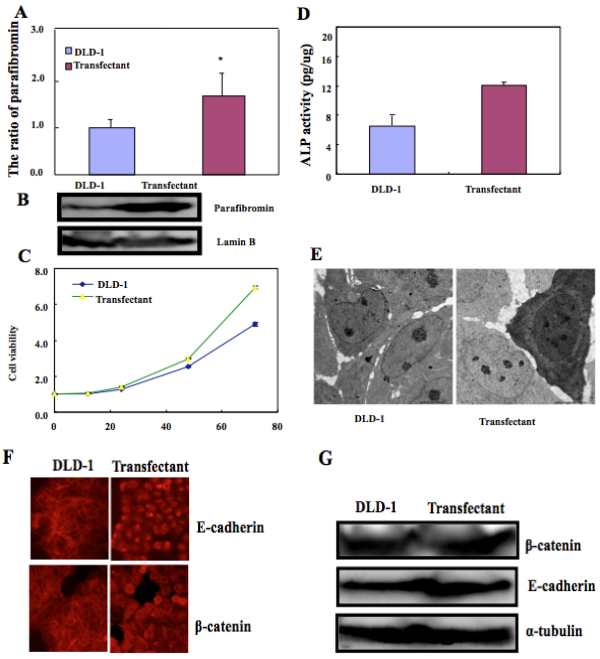

Figure 1. The Effects of Parafibromin on the Proliferation Aand Differentiation Of DLD-1 Cells. Ectopic parafibromin expression was confirmed in DLD-1 transfectant by real-time PCR(A) and Western blot (B). The transfectants showed higher proliferation (C) and good differentiation, evidenced by ALP activity (D). However, transmission electric microscopy showed that DLD-1 transfectants became regular and wide between cell-cell junction (E) There was no difference in β-catenin and E-cadherin expression by immunofluorescence (F) and Western blot (G)

from the surgical resection in the Affiliated Hospital, University of Toyama, and Kouseiren Takaoka Hospital between 1993 and 2002. The patients with colorectal carcinoma were 166 men and 126 women (18~90years, mean=68.9 years). None of the patients had undergone chemotherapy, radiotherapy or adjuvant treatment prior to surgery. Patients were followed up by consulting their case documents and by telephone. Informed written consent was obtained from all participants and the study was approved by China Medical University Ethics Committee. All the paraffin-embedded specimens were subjected to the establishment of tissue microarray using a Tissue Microarrayer (Japan).

The immunohistochemistry was performed according to the procedures recommended as previously described (Kumada et al., 2004). The mouse anti-parafibromin or rabbit anti-ki-67 antibody were purchased from Santa cruz and DAKO respectively. Immunoreactivity for parafibromin and ki-67 was localized in the nucleus (Figure 5). One hundred cells were randomly selected and counted from 5 representative fields of each section blindly by three independent observers (Zhao S 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%.

Statistical analysis

Statistical evaluation was performed using Spearman

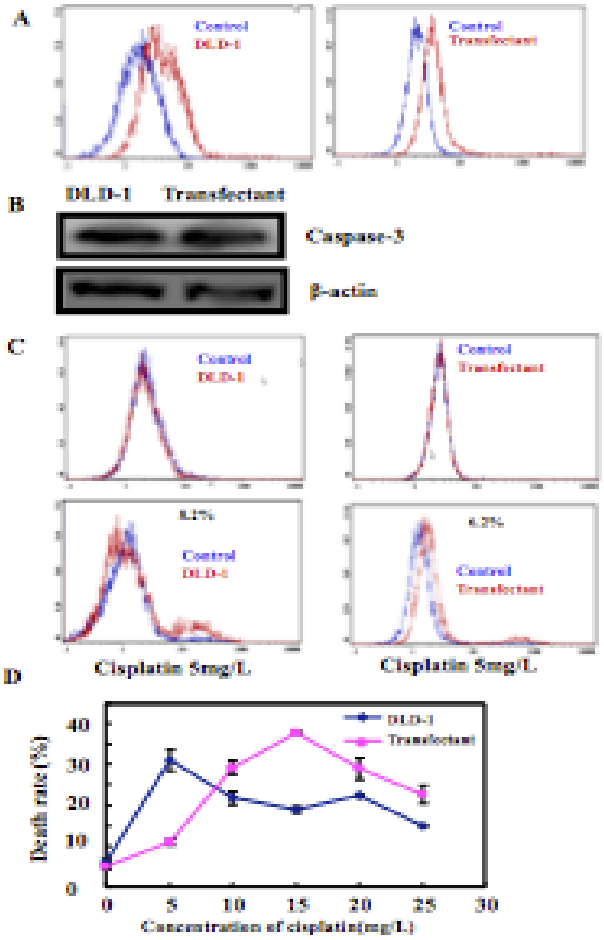

Figure 2. The Effects of Parafibromin on the Apoptosis of DLD-1 Cells. There was no difference in early apoptosis between DLD-1 and its transfectants by Annexin V staining (A). Western blot (B) and flow cytometry(C) showed similar expression level and activity of Caspase-3 between both kinds of cells. DLD-1 transfectants displayed cisplatin- resistance, evidenced by Annexin staining (D) and DNA fragmentation (E)

correlation test to analyze the rank data and student t test to compare the means of different groups. $p<0.05$ was considered as statistically significant. SPSS 10.0 software was employed to analyze all data.

Results

To clarify the role of parafibromin overexpression, we transfected pEGFP-N1-parafibromin plasmid into DLD-1 cells because it showed lower parafibromin expression (Zheng et al., 2012). According to the results of real-time PCR (Figure 1A) and Western blot (Figure 1B), ectopic parafibromin expression was confirmed in DLD-1 transfectants. The transfectants showed higher proliferation than the control (Figure 1C), which was verified by cell counting. There was high ALP activity (Figure 1D) and regular appearance (Figure 1E) in transfectants, in comparison with the control. We found no difference in the expression level of both cell adherent proteins (membranous E-cadherin and β-catenin) by Western blot (Figure 1F) and immunofluorescence (Figure 1G).

As for apoptosis, we employed Annexin V staining (Figure 2A) to measure the early apoptosis, Western blot (Figure 2B) and flow cytometry (Figure 2C) to detect Caspase-3 expression level and activity. No effects of parafibromin expression on the apoptosis were observed in DLD-1 cells. To observe the effects of parafibromin overexpression on drug-induced apoptosis, DLD-1 cells and their transfectants were exposed to cisplatin and subjected to DNA fragmentation for the measurement of late apoptosis (Figure 2D). After treated with cisplatin, the apoptotic level of transfectants was lower than the control because more cell death might decrease the ratio of apoptotic to living cells. Wound healing indicated no difference in migration between DLD-1 and its

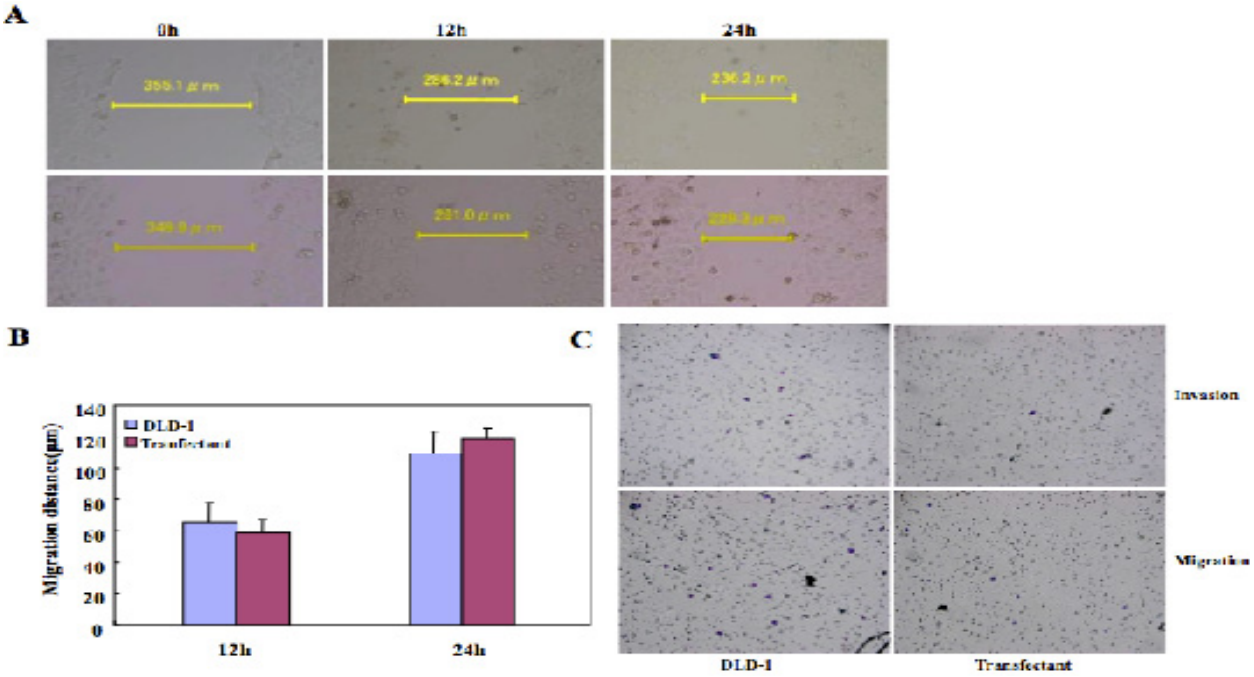

Figure 3. The Effects of Parafibromin on the Migration and Invasion Of DLD-1 Cells. Wound healing indicated no difference in migration between DLD-1 and its transfectants (A and B). There was no DLD-1 cells and its transfectants to migrate and invade by tranwell chamber assay (C)

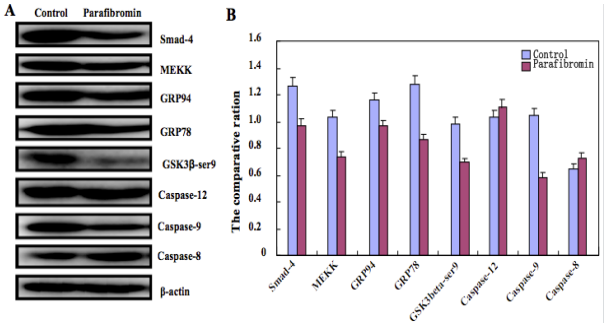

Figure 4. The Effects of Parafibromin Expression on the Expression of Phenotype- Related Proteins. The phenotype-related proteins were determined in DLD-1, mock and parafibromin transfectants by Western blot (A). The denometry analysis was performed to measure the protein level with an internal control of β-actin (B)

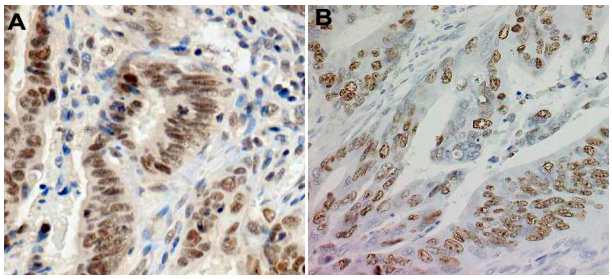

Figure 5. The Parafibromin And Ki-67 Expression In Colorectal Carcinoma. Parafibromin (A) and ki-67(B) were positively expressed in the nuclei of colorectal carcinoma cells

Table 2. The Relationship Between Parafibromin and Ki-67 Expression in Colorectal Carcinomas

Ki-67 expression	n	Parafibromin expression				PR(%)	Rs	*p* value
		-	+	++	+++			
-	21	17	3	1	0	19.0	0.425	<0.001
+	79	59	10	6	4	25.3		
++	119	44	34	33	8	63.0		
+++	73	17	18	28	10	76.7		

transfectants (Figure 3A and 3B). There was no DLD-1 cells and its transfectants to migrate and invade by tranwell chamber assay (Figure 3C).

Finally, Western blot and densimometry was employed to determine the expression of phenotype-related proteins (Figure 4). It was found that forced parafibromin overexpression resulted in down-regulated expression of Smad4, MEKK, GRP94, GRP78, GSK3β-ser9, and Caspase-9. However, there was no difference in Capase-12 and -8 expression between DLD-1 cells and their transfectants. Immunohistochemically, there was positive correlation between parafibromin and ki-67 expression in colorectal carcinoma (Figure 5; Table 2).

Discussion

Parafibromin might inhibit cyclin D1 and c-myc by recruiting SUV39H1 histone methyltransferase (Lin et al., 2008; Hahn et al., 2011; Takahashi et al., 2011). In sporadic parathyroid carcinomas and hyperparathyroidism-jaw tumors, LOH or mutation of HRPT2 might cause the loss and inactivation of parafibromin protein (Howell et al., 2003; Shattuck et al., 2003; Gill et al., 2006; Zhao et al., 2007). Previously, parafibromin expression was down- regulated in lung, gastric and colorectal carcinoma (Zheng et al., 2008; 2012; Xia et al., 2012). These findings supported its role of parafibromin in cancers as a tumor suppressor gene. In contrast, we found higher proliferation and cisplatin-induced apoptotic resistance in parafibromin transfectants of colorectal carcinoma cell (DLD-1). The positive relationship between parafibromin and ki-67 expression also supported this result because ki-67 antigen is present in the nuclei of cells in the G1-, S- and G2- phases of the cell cycle as well as in mitosis (Xiao et al. 2013). In our experiment, it is more difficult for typsin to digest DLD-1 into monocells than DLD-1 transfectant, suggesting that parafibromin overexpression weakened adherent ability. The lower proliferation of DLD-1 might be due to the difficulty to spread for more proliferating space. Reportedly, parafibromin can play an oncogenic role by binding to β-catenin, thereby activating promitogenic/ Wnt signaling upon tyrosine dephosphorylation by SHP2 (Takahashi et al., 2011). As a result, the parafibromin/β-catenin interaction overrides parafibromin/SUV39H1- mediated transrepression and induces expression of Wnt target genes, including cyclin D1 and c-myc (Takahashi et al., 2011). Therefore, parafibromin might function as a double -edged sword.

Although there was negative association between parafibromin expression and aggressiveness of malignancies (Selvarajan et al., 2008; Zheng et al., 2008; 2012), several cells of DLD-1 and parafibromin transfectants were observed by the transwell membrane, suggesting that both cells had weak ability to invade and migrate, and parafibromin possibly could not influence both biological events of DLD-1 cells. Additionally, our in vitro experiment showed that forced parafibromin overexpression caused the differentiation of carcinoma cells evidenced by enhanced ALP activity and regular appearance. Although both differentiation markers (E-cahderin and β-catenin) were examined by western blot and immunofluorescence (Kambhampati et al., 2010), we found no difference in their expression between both cells, indicating the parafibromin-induced differentiation might be independent of both cells adherent molecules in DLD-1 cells.

Although Lin et al. (2008) reported that overexpression of wild-type parafibromin induced apoptosis in transfected HeLa cells, but it was not true in DLD-1 cells, evidenced by Annexin V, Caspase-3 expression and activity. Caspase-3 is activated in the apoptotic cell both by extrinsic and intrinsic pathways directly or indirectly by Caspase- 8, 9, 10, and 12, finally to initiate apoptosis (Xiao e t al., 2013; Yamaguchi, 2013). No alteration in Caspase-8 and Caspase-12 expression also supported this datum. Interestingly, decreased expression of GRP78 and 94 was seen in parafibromin transfectants cell, suggesting parafibromin overexpression showed cisplatin-resistance by attenuating ER stress because GRP94 and 78 are ubiquitously expressed in ER and able to assist in protein folding and assembly, and induced by tumor growth or toxic damage (Zheng et al., 2008). Glycogen synthase kinase 3β is reported to repress Wnt/β-catenin pathway (Zheng et al., 2010). MEK kinase 1 occupies

a pivotal role in a network of phosphorylating enzymes integrating cellular responses to mitogenic and metabolic stimuli and mediates the anti-apoptotic effect of the Bcr-Abl oncogene (O'Neill and Kolch, 2004). In response to TGF-β, Smad4 promotes the binding of the Smad2/Smad4/FAST-1 complex to DNA and induces apoptosis by modulating Bcl-2/Bax balance (Owens, 2008). So, ectopic parafibromin overexpression resulted in down- regulated expression of Smad4, MEKK and GSK3β-ser9, which in part underlay the molecular mechanisms of parafibromin functions in DLD-1 cells.

In summary, parafibromin overexpression could promote cell proliferation, apoptotic resistance, and differentiation of DLD-1 cells possibly via regulate the expression of Smad-4, MEKK, GRP94, GRP78 and Caspase-9. The biological functions about parafibromin need further study in other cell lines of colorectal carcinoma.

Acknowledgements

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References

Aldred MJ, Talacko AA, Savarirayan R, et al (2006). Dental findings in a family with hyperparathyroidism- jaw tumour syndrome and a novel HRPT2 gene mutation. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, **101**, 212-8.

Cui C, Lal P, Master S, et al (2012). Expression of parafibromin in major renal cell tumors. *Eur J Histochem*, **56**, e39.

Gill AJ, Clarkson A, Gimm O, et al (2006). Loss of nuclear expression of parafibromin distinguishes parathyroid carcinomas and hyperparathyroidism- jaw tumour (HPT-JT) syndrome-related adenomas from sporadic parathyroid adenomas and hyperplasias. *Am J Surg Pathol*, **30**, 1140-9.

Hahn MA, Dickson KA, Jackson S, et al (2011). The tumor suppressor CDC73 interacts with the ring finger proteins RNF20 and RNF40 and is required for the maintenance of histone 2B monoubiquitination. *Hum Mol Genet*, **21**, 559-68.

Howell VM, Haven CJ, Kahnoski K, et al (2003). HRPT2 mutations are associated with malignancy in sporadic parathyroid tumours. *J Med Genet*, **40**, 657-63.

Kambhampati S, Banerjee S, Dhar K, et al (2010). 2-methoxyestradiol inhibits Barrett's esophageal adenocarcinoma growth and differentiation through differential regulation of the beta-catenin-E-cadherin axis. *Mol Cancer Ther*, **9**, 523-34.

Kumada T, Tsuneyama K, Hatta H, Ishizawa S, Takano Y (2004). Improved 1-h rapid immunostaining method using intermittent microwave irradiation: practicability based on 5 years application in Toyama Medical and Pharmaceutical University Hospital. *Mod Pathol*, **17**, 1141-9.

Lin L, Zhang JH, Panicker LM, Simonds WF (2008). The parafibromin tumor suppressor protein inhibits cell proliferation by repression of the c-myc proto-oncogene. *Proc Natl Acad Sci U S A*, **105**, 17420-5.

Newey PJ, Bowl MR, Thakker RV (2009). Parafibromin-functional insights. *J Intern Med*, B, 84-98.

O'Neill E, Kolch W (2004). Conferring specificity on the ubiquitous Raf/MEK signalling pathway. *Br J Cancer*, **90**, 283-8.

Owens P, Han G, Li AG, Wang XJ (2008). The role of Smads in skin development. *J Invest Dermatol*, **128**, 783-90.

Sellins KS, Cohen JJ (1987). Gene induction by gamma-irradiation leads to DNA fragmentation in lymphocytes. *J Immunol*, **139**, 3199-206.

Selvarajan S, Sii LH, Lee A, et al (2008). Parafibromin expression in breast cancer: a novel marker for prognostication? *J Clin Pathol*, **61**, 64-7.

Shattuck TM, Välimäki S, Obara T, et al (2003). Somatic and germ-line mutations of the HRPT2 gene in sporadic parathyroid carcinoma. *N Engl J Med*, **349**, 1722-9.

Takahashi A, Tsutsumi R, Kikuchi I, et al (2011).SHP2 tyrosine phosphatase converts parafibromin/Cdc73 from a tumor suppressor to an oncogenic driver. *Mol Cell*, **43**, 45-56.

Tan MH, Wong CF, Tan HL, et al (2010).Genomic expression and single-nucleotide polymorphism profiling discriminates chromophobe renal cell carcinoma and oncocytoma. *BMC Cancer*, **10**, 196.

Xia P, Wang W, Xu XY, et al (2011). Parafibromin expression in lung normal tissue and carcinoma: its comparison with clinicopathological parameters of carcinoma. *Histol Histopathol*, **26**, 1039-47.

Xiao LJ, Zhao S, Zhao GH, et al (2013). The clinicopathological and prognostic significance of ki-67, Caspase-3 and p53 expression in gastric carcinomas. Oncol Lett in press.

Yamaguchi M (2013). The anti-apoptotic effect of regucalcin is mediated through multisignaling pathways. Apoptosis in press.

Yang YJ, Han JW, Youn HD, Cho EJ (2010). The tumor suppressor, parafibromin, mediates histone H3 K9 methylation for cyclin D1 repression. *Nucleic Acids Res*, **38**, 382-90.

Zhang C, Kong D, Tan MH, et al (2006). Parafibromin inhibits cancer cell growth and causes G1 phase arrest. *Biochem Biophys Res Commun*, **350**, 17-24.

Zhao J, Yart A, Frigerio S, et al (2007). Sporadic human renal tumors display frequent allelic imbalances and novel mutations of the HRPT2 gene. *Oncogene*, **26**, 3440-9.

Zheng HC, Takahashi H, Li XH, et al (2008). Downregulated parafibromin expression is a promising marker for pathogenesis, invasion, metastasis and prognosis of gastric carcinomas. *Virchows Arch*, **452**, 147-55.

Zheng HC, Takahashi H, Li XH, et al (2008). Overexpression of GRP78 and GRP94 are markers for aggressive behavior and poor prognosis in gastric carcinomas. *Hum Pathol*, **39**, 1042-9.

Zheng HC, Wei ZL, Xu XY, et al (2011). Parafibromin expression is an independent prognostic factor for colorectal carcinomas. *Hum Pathol*, **42**,1089-102.

Zheng HC, Xu XY, Xia P, et al (2010). Involvement of inactive GSK3beta overexpression in tumorigenesis and progression of gastric carcinomas. *Hum Pathol*, **41**, 1255-64.