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Immunohistochemical assessment of parafibromin in mouse and human tissues

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Abstract

Parafibromin is a protein encoded by the HRPT2 oncosuppressor gene, whose mutation causes the hyperparathyroidism–jaw tumour syndrome, characterized by the occurrence of parathyroid adenoma or carcinoma, fibro-osseous jaw tumours, and renal neoplastic and non-neoplastic abnormalities. Non-morphological techniques, such as Northern and Western blotting and reverse transcriptase-PCR, indicate that parafibromin is ubiquitously expressed, but extensive immunohistochemical studies have not been performed. To increase our knowledge of the distribution and patterns of expression of parafibromin, we examined its expression and location in many different mouse and human organs by immunohistochemistry. There were no substantial differences in parafibromin expression between mouse and human. We found widespread expression of parafibromin, except in connective tissue, smooth muscle, endothelium and some other types of epithelia (colonic, urinary, tubaric, uterine, thyroid). Heterogeneity of positivity intensity and subcellular location (nuclear, nucleocytoplasmic, cytoplasmic) was found between tissues and cell types, suggesting differential functional involvement of parafibromin. Moreover, higher parafibromin expression was found in cell types, such as hepatocytes, cells of the base of gastric glands, renal cortex tubules and the pars intermedia of the hypophysis, which are characterized by different proliferative capacity, thus indicating that the cellular function of parafibromin may not be reduced only to its anti-proliferative effect.

Key words HRPT2; human; hyperparathytoidism; immunohistochemistry; parafibromin.

Introduction

Hyperparathyroidism-jaw tumour (HPT–JT) syndrome is an autosomal dominant disorder characterized 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, hamartoma or polycystic disease (Szabo et al. 1995; Teh et al. 1996, 1998; Hobbs et al. 1999; Haven et al. 2000; Cavaco et al. 2001). Association with benign and malignant uterine tumours has also been described (Bradley et al. 2005) and some cases of Hurthle cell thyroid adenomas, pancreatic adenocarcinomas and testicular mixed germ cell tumours have been reported (Haven et al. 2000). HPT–JT syndrome is caused by mutation of the HRPT2 gene, which is located on chromosome 1q31.2, and encodes a 531 amino acid protein named parafibromin (Carpten et al. 2002).

The ~200 amino acid C-terminal segment of parafibromin displays homology with Cdc73, a *Saccharomyces cerevisiae* protein that is a component of the Paf1 complex. In the yeast, it has been reported that the Paf1 complex is associated with RNA polymerase II (Shi et al. 1997; Squazzo et al. 2002) and is involved in various steps of gene expression, such as transcript site selection (Stolinski et al. 1997), transcriptional elongation (Pokholok et al. 2002; Squazzo et al. 2002; Rondon et al. 2004), histone H2B ubiquitination and histone H3 methylation (Krogan et al. 2003; Ng et al. 2003a,b; Wood et al. 2003) and poly(A) length control and coupling of transcriptional and post-transcriptional events

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(Mueller et al. 2004). The Paf1 complex is involved in the expression of genes regulating metabolism and cell cycle control (Betz et al. 2002; Porter et al. 2002). An anti-proliferative effect due to down-regulation of cyclin D1 expression by wild-type parafibromin, but not by mutated HPRT2, has also been demonstrated (Woodard et al. 2005; Yart et al. 2005).

Parafibromin mRNA and protein expression was studied by Northern (Carpten et al. 2002) and Western blotting (Woodard et al. 2005; Juhlin et al. 2006) in human tissues and by reverse transcription PCR (RT-PCR) in a panel of human multiple-tissue cDNAs (Juhlin et al. 2006). However, detection of mRNA does not give direct information about the contents of the translated protein product, and Western blotting does not give information about the cell types expressing the protein. Immunohistochemical studies of the expression of parafibromin have been performed only in the human normal and neoplastic parathyroid (Tan et al. 2004; Juhlin et al. 2006), in the kidney (Tan & Teh, 2004), and in ossifying fibroma of the jaws (Pimenta et al. 2006). Identification of the tissue or cell types which express parafibromin would contribute towards clarifying its physiological function. The aim of the present study was to detect the expression of parafibromin in normal human and mouse tissues using immunohistochemistry, in order to establish its expression profiles.

Materials and methods

Human normal tissues were sampled during autopsy of six subjects (mean age 42 years; age range 33–69 years; three males, three females). Death–autopsy intervals were less than 48 h. Samples of oesophagus, stomach, small intestine, colon, rectum, liver, testis, prostate, ovary, uterus, oviduct, thyroid and parathyroid were also taken from surgical specimens. Mouse tissues were obtained from four 8-week-old healthy mice, two males and two females. The series of mouse and human tissues sampled is listed in Table 1. All tissues were fixed in 10% buffered-formalin for 48 h and paraffin-embedded.

Sections were hydrated gradually through decreasing concentrations of ethanol and then washed in deionized H_2O. Antigen unmasking was performed with 10 mM sodium citrate buffer, pH 6.0, in a microwave oven at 96 °C for 30 min. Sections were incubated in 0.3% hydrogen peroxide for 10 min at room temperature, to remove endogenous peroxidase activity, and then in blocking serum for 30 min. Sections were incubated with primary mouse monoclonal antibody recognizing human and mouse parafibromin [mouse antibody raised against a peptide corresponding to amino acids 87–100 of parafibromin of mouse origin, Parafibromin (2H1) sc-33638, Santa Cruz Biotechnology Inc., USA] diluted 1 : 20 in phosphate-buffered saline (PBS) for 3 h at 37 °C. Sections were then washed three times for 5 min in PBS, revealed with anti-mouse serum for 30 min (Universal Immuno-peroxidase Polymer, Histofine, Japan), and developed in 3,3′-diaminobenzidine (DAB, Sigma-Aldrich, Milan, Italy). Lastly, sections were counterstained with haematoxylin. Negative controls were performed by omission of primary antibody. Parafibromin immunoreaction detected in normal parathyroid was used as positive control. In order to verify the immunohistochemical specificity of the reaction, absorption tests with parafibromin were also performed.

Immunohistochemical staining for parafibromin was evaluated by scanning entire tissue specimens under low-power magnification (×5), later confirmed under higher power magnification (×10, ×20, ×40). Immunohistochemical evaluation included number of positive cells, pattern of subcellular immunostaining (nuclear, cytoplasmic, nucleocytoplasmic) and staining intensity. Overall staining intensity was stratified as – (absent), ± (barely detectable), + (weak), ++ (moderate) or +++ (strong).

Results

Parafibromin was found in almost all normal human and mouse tissues examined, although expression levels differed among tissues and cell populations. The various cell types were characterized by different subcellular location of parafibromin, i.e. nuclear, cytoplasmic or nucleocytoplasmic. The distribution and the quantitative expression of parafibromin is described in detail below and further summarized in Table 1. There were no significant differences in immunostaining between autoptic and surgical human specimens.

Muscles

Both in mouse and in human, heart (Fig. 1A) and skeletal (Fig. 2A) muscles showed heterogeneous staining. Most fibres showed only moderate cytoplasmic staining and some strong nuclear staining and moderate cytoplasmic staining; others did not stain at all. In human samples, nuclear positivity was less frequent.

Table 1 Parafibromin expression in normal mouse and human tissues

		Mouse		Human	
Tissues	Histological location	Nuclear immunostaining intensity	Cytoplasmic immunostaining intensity	Nuclear immunostaining intensity	Cytoplasmic immunostaining intensity
Heart	Myocardial cells	+	++	+/–	++
Skeletal muscle	Muscle cells	+	++	+/–	++
Smooth muscle	Muscle cells	–	–	–	–
Bone	Osteoblasts	NA	NA	+	–
	Osteoclasts			+	–
	Marrow			++	++
Skin	Epidermis	NA	NA	++	++
Digestive system					
Oesophagus	Epithelia	–	+/–	–	+/–
Stomach	Superficial epithelia	–	–	–	–
	Base of glands	–	+++	–	+++
Small intestine	Epithelia	–	+	–	+
Colon	Epithelia	–	–	–	–
Rectum	Epithelia	–	–	–	–
Liver	Hepatocytes	–	+++	+++	+++
Pancreas	Acinar epithelia	–	–	–	–
	Duct epithelia	–	–	–	–
	Langerhans cells	–	+/–	–	+/–
Respiratory system	Alveolar epithelia	++	+	+	+/–
	Bronchial epithelia	–	+/–	–	+/–
Urinary system					
Kidney	Renal corpuscles	+/–	–	+/–	–
	Cortex tubules	+	+++	+++	+
	Medullary tubules	–	–	–	–
Bladder	Epithelia	–	–	–	–
Male germinal system					
Testicle	Spermatogonias	NA	NA	+	–
	Spermatocytes			–	+
	Sertoli cells			–	+
	Leydig's cells			–	+
Prostate	Glandular epithelia	NA	NA	+	+/–
Female germinal system					
Ovary	Oocytes	NA	NA	–	+
	Follicular epithelia			–	+
	Thecal cells			–	–
	Luteal cells			–	–
Oviduct	Epithelia	–	–	–	–
Uterus	Endometrial cells	–	–	–	–
Vagina	Epithelia	NA	NA	–	+
Endocrine glands					
Hypophysis	Anterior lobe	NA	NA	–	+
	Pars intermedia			++	+++
	Posterior lobe			–	–
Thyroid	Follicular epithelia	–	–	–	–
	Parafollicular epithelia	–	–	–	–
Parathyroid	Principal cells	++	+	++	+
Adrenal gland	Cortex	++	++	++	++
	Medulla	++	+	++	+
Langerhans isle	Langerhans cells	–	+/–	–	+/–
Lymphatic tissues					
Lymphnode	Lymphocytes	–	–	–	–
Spleen	Red pulp	–	–	–	–
	Lymphocytes	–	+	++	+

Table 1 *Continued*

Tissues	Histological location	Mouse		Human	
		Nuclear immunostaining intensity	Cytoplasmic immunostaining intensity	Nuclear immunostaining intensity	Cytoplasmic immunostaining intensity
Brain					
Brain cortex	Neurons	+/–	++	+	++
	Glia	–	–	–	–
Cerebellum	Purkinje cells	+/–	++	+	++
	Other neurons	+/–	+	+/–	+
	Glia	–	–	–	–
Basal ganglia	Neurons	+/–	++	+	++
	Glia	–	–	–	–
Brainstem	Neurons	+/–	++	+	++
	Glia	–	–	–	–

Immunostaining intensity graded as –, negative; –/+, barely detectable; +, weak; ++, moderate; +++, strong. NA, tissue not available.

Smooth muscle cells of the gastrointestinal tract, urinary system and vessels did not show parafibromin immunostaining.

Bone

In human marrow (Fig. 2B), all cells showed moderate cytoplasmic parafibromin immunoreactivity, and many showed strong nuclear staining as well. Some osteoblasts and osteoclasts with moderate nuclear positivity were also found.

Skin

In the human epidermis, most cells showed moderate cytoplasmic and nuclear positivity. Positive cells were mainly located in the spinous, granular and cornified layers. Dermal structures were negative.

Digestive system

Oesophageal epithelium showed no or weak cytoplasmic staining throughout its thickness. Nuclei were negative. There were no differences between the two species.

In the stomach of both mouse (Fig. 1B,C) and human (Fig. 2C,D), the surface epithelial cells and cells of the isthmus and neck of gastric glands were negative or weakly stained in the cytoplasm. Most epithelial cells at the base of the gastric glands showed moderate or strong cytoplasmic parafibromin staining, although with no nuclear staining (Figs 1C and 2D). Only a few cells did not show immunostaining.

In the small intestine of both mouse (Fig. 1D) and human (Fig. 2E), the epithelial cells of the villi showed weak cytoplasmic parafibromin immunostaining, but no or weak staining was observed in crypt cells.

In colon and rectum of both mouse and human (Fig. 2F), epithelial cells did not show parafibromin immunostaining.

In mouse liver (Fig. 1E), most hepatocytes showed strong cytoplasmic staining, with a weak nuclear immunoreaction. In human liver (Fig. 2G), most hepatocytes showed strong immunostaining in both cytoplasm and nucleus. Only a few cells, with no specific distribution on the basis of hepatic lobule, were not stained. In both species, sinusoidal lining cells, portal tracts, terminal hepatic venules and hepatic arterioles were negative. Bile ducts and connective tissue also showed no immunostaining.

In the pancreas of both species, no immunoreaction was found in ductal epithelia or acinar secretory cells (Fig. 1F).

Respiratory system

In both mouse and human samples, the tracheal, bronchial and bronchiolar epithelial cells and cells of the bronchial submucosal glands exhibited no or weak cytoplasmic staining. No staining was seen in tracheal or bronchial cartilage. In distal lung parenchyma, the great majority of pneumocytes were negative, but rare alveolar lining cells with strong nuclear immunoreaction were found (Figs 1G and 2H), more frequently in mouse specimens.

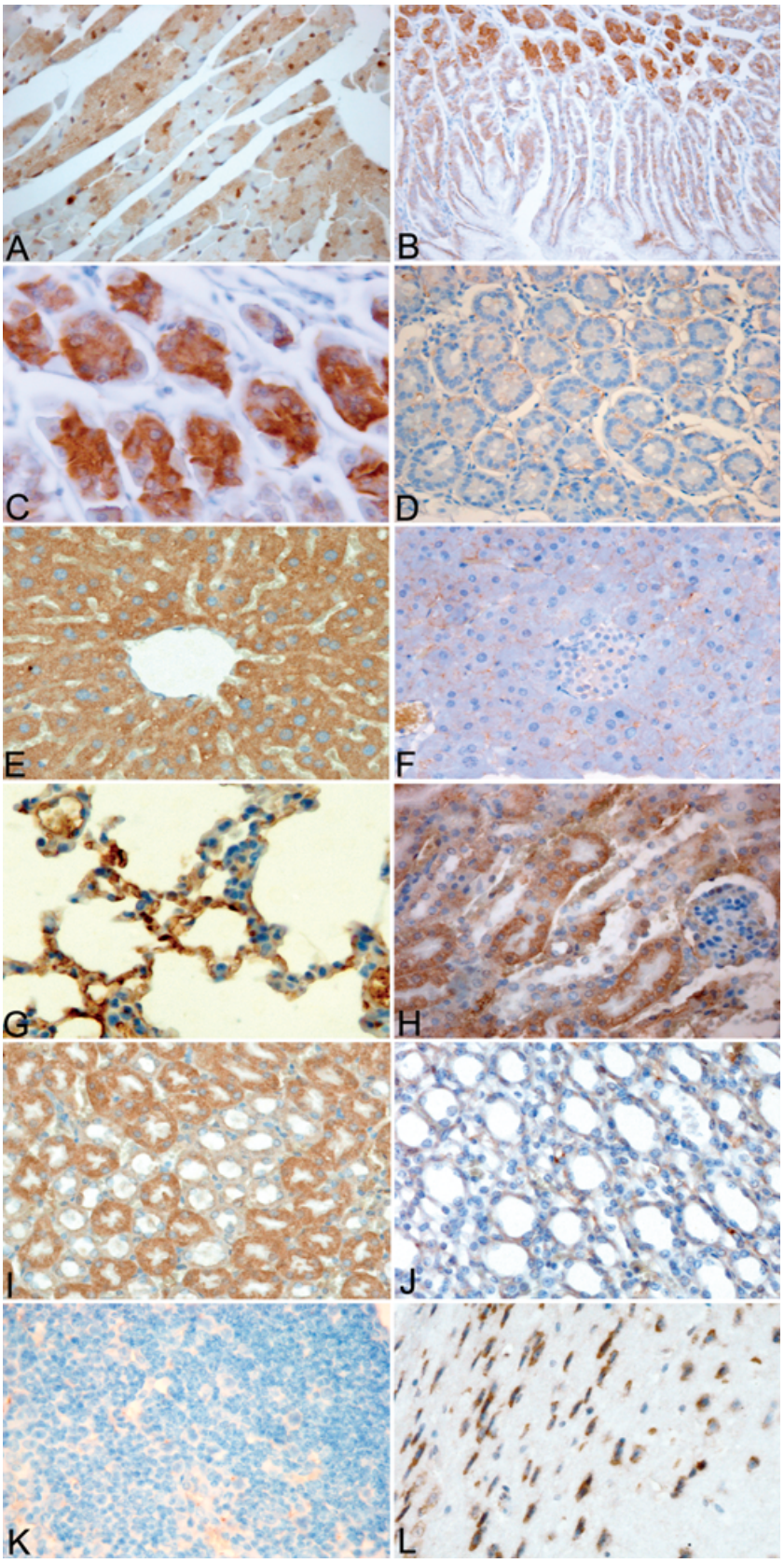

Fig. 1 Immunohistochemical staining of normal mouse tissues with antiparafibromin. (A) Myocardial muscle, ×40; (B,C) gastric mucosa, ×20, ×63; (D) small intestine, ×40; (E) liver, ×40; (F) pancreas, ×20; (G) lung, ×63; (H,I) renal cortex, ×40; (J) renal medulla, ×40; (K) lymph node, ×63; (L) brain, ×40.

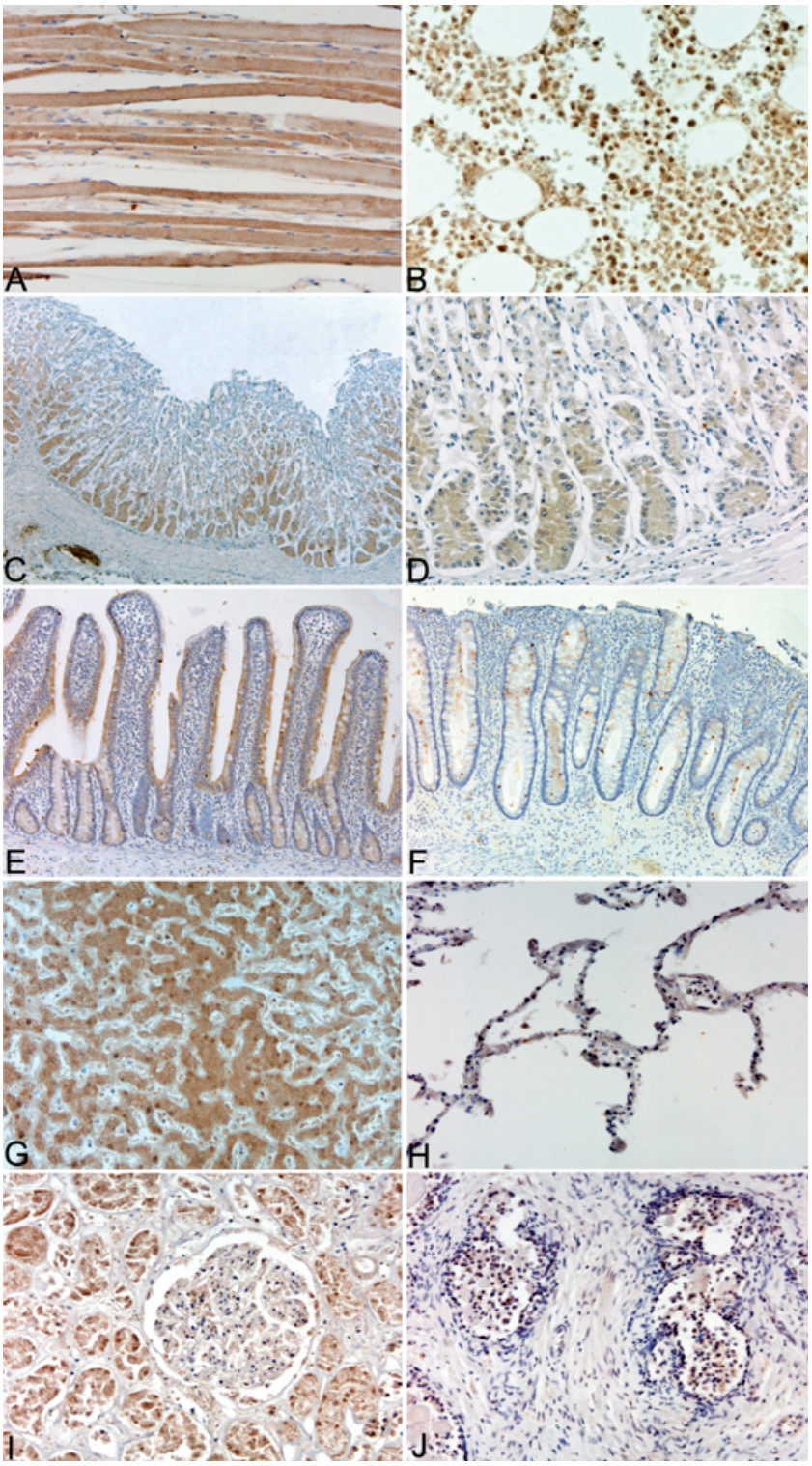

Fig. 2 Immunohistochemical staining of normal human tissues with antiparafibromin. (A) Skeletal muscle, ×20; (B) marrow, ×40; (C,D) gastric mucosa, ×5, ×20; (E) small intestine, ×10; (F) rectum, ×10; (G) liver, ×20; (H) lung, ×20; (I) kidney, ×20; (J) prostate, ×20.

Urinary system

There were no substantial differences between mouse and human tissues. The renal corpuscles were usually negative, although mesangial epithelial cells with positive nuclear immunoreaction were found in some (Figs 1H and 2I). Positive nucleocytoplasmic immunoreaction was observed in the epithelium of the majority of the cortex tubules, although some tubules without immunoreaction could be found adjacent to others

showing strong positivity (Figs 1I and 2I). In mouse, positivity of tubules was mainly cytoplasmic; in human samples, it was mainly nuclear. The epithelium of the collecting tubules (Fig. 1J) and renal pelvis was negative or showed weak staining. The surrounding fibrous stroma was negative.

Urether and bladder epithelium was negative.

Male germinal system

In the human testis, some spermatogonias showed moderate nuclear parafibromin immunostaining, but the majority was negative. Spermatocytes, Sertoli cells and Leydig cells showed only weak cytoplasmic positivity.

Some glandular epithelial cells of the human prostate showed moderate nuclear and weak cytoplasmic staining (Fig. 2J).

Female germinal system

Oocytes and follicular epithelial cells showed only weak cytoplasmic immunostaining. Thecal and luteal cells were negative.

The epithelial cells of oviduct (Fig. 3A) and endometrium (Fig. 3B) were negative in both mouse and human, as with their connective and muscular structures.

Vaginal human epithelium showed weak or moderate cytoplasmic parafibromin staining, mainly in superficial layers.

Endocrine glands

The human adenohypophysis showed some cells with moderate cytoplasmic parafibromin immunostaining. The cellular elements of neurohypophysis were all negative. In the pars intermedia, about half the cells showed very strong cytoplasmic or nucleocytoplasmic immunostaining, whereas the others were negative (Fig. 3C,D).

In the thyroid of both mouse and human, follicular and parafollicular epithelial cells did not immunostain. The principal cells of mouse and human parathyroid presented moderate nuclear immunostaining and weak cytoplasmic positivity.

In both species, adrenal cortex cells showed moderate cytoplasmic parafibromin immunostaining; about half of the cells also showed strong nuclear staining. Adrenal medullary cells showed weak or moderate cytoplasmic positivity, and many of them also revealed strong nuclear immunostaining.

The cells of the islets of Langerhans (Fig. 1F) showed no or weak cytoplasmic parafibromin immunostaining in both species.

Lymphatic tissues

Lymph nodes (Fig. 1K) and lymphatic aggregates in the mucosa and submucosa of the gastrointestinal tract were negative.

In the human spleen, about half the lymphatic cells showed moderate nuclear and weak cytoplasmic parafibromin immunostaining (Fig. 2H). In mouse, parafibromin immunostaining of spleen lymphatic cells was mainly cytoplasmic. Sinusoid lining cells were negative in both species. Red pulp showed aspecific parafibromin immunostaining in both species.

Brain

In mouse and human, many neurons with positive immunostaining were located in all the districts examined, i.e. brain cortex, basal ganglia, cerebellum and brainstem. In mouse, the immunostaining pattern was mainly cytoplasmic (Fig. 1L) whereas nuclear immunostaining was also frequently found in human (Fig. 3I,J).

Glial cells were negative in both species and in all districts studied.

Discussion

Northern blot analysis and RT-PCR showed expression of HRPT2 in heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas (Carpten et al. 2002; Juhlin et al. 2006). Western blot study revealed expression of parafibromin as a ~60-kDa band in the adrenal gland, heart and kidney of both human and mouse, and in the liver and ovary of mouse but not of human. In mouse kidney, a 53-kDa immunoreactive band was also found. In both human and mouse, the heart and skeletal muscle expressed 40-kDa immunoreactive bands. Parafibromin immunoreactivity was not found in the brain of either species (Woodard et al. 2005). Juhlin et al. (2006) revealed a 60-kDa band in human pancreas and kidney. In normal human parathyroid, immunohistochemistry shows strong nuclear or nucleocytoplasmic reactivity (Tan et al. 2004; Woodard et al. 2005; Juhlin et al. 2006). In the kidney, parafibromin has been detected by immunohistochemistry in the nucleus of glomerular mesangial cells and in the nucleus and

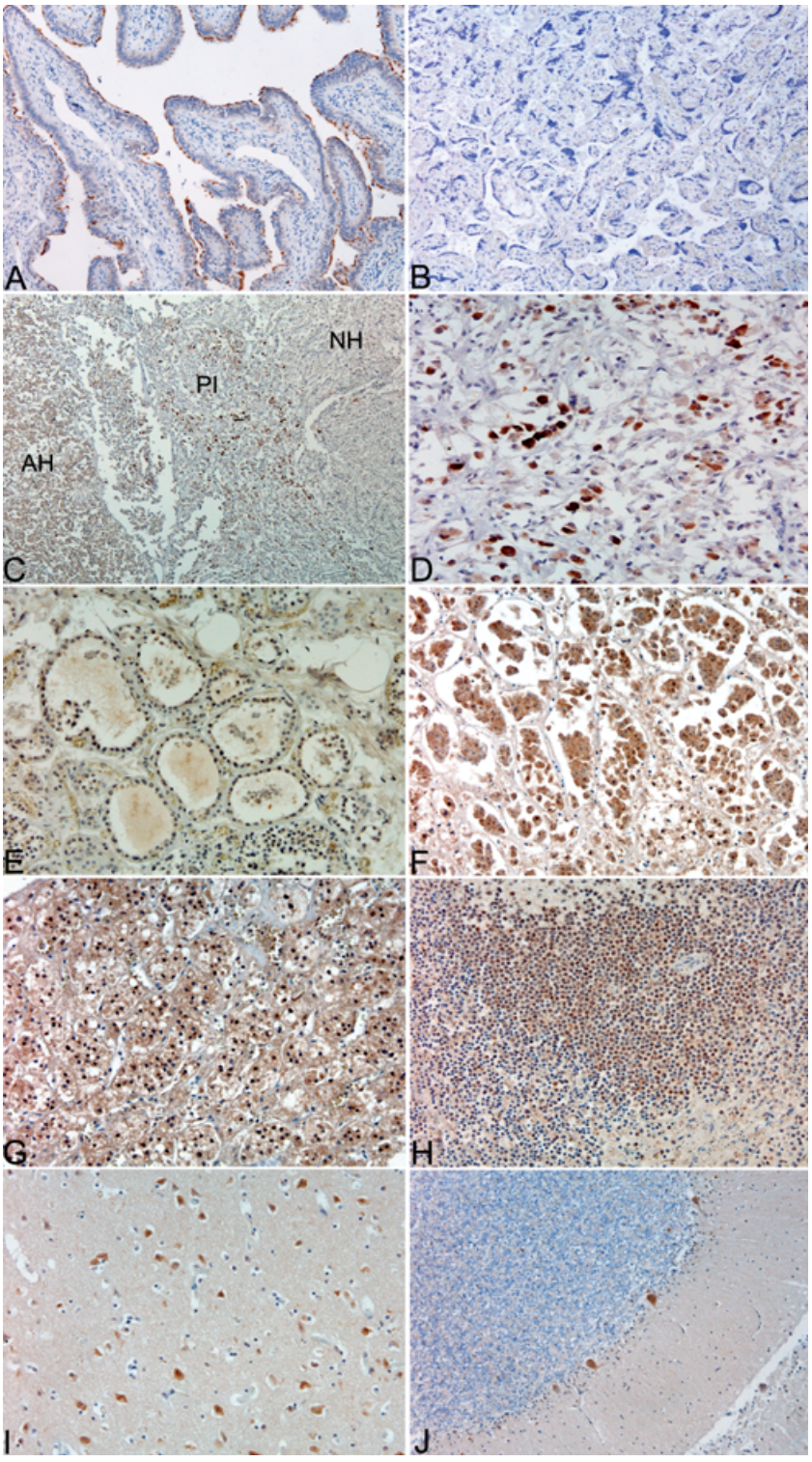

Fig. 3 Immunohistochemical staining of normal human tissues with anti-parafibromin. (A) Oviduct, ×10; (B) endometrium, ×10; (C) hypophysis (AH, adenohypophysis; PI, pars intermedia; NH, neurohypophysis), ×5; (D) hypophysis, pars intermedia, ×20; (E) thyroid, ×20; (F) adrenal cortex, ×20; (G) adrenal medulla, ×20; (H) spleen, white pulp, ×20; (I) cerebral cortex, ×20; (J) cerebellum, ×10.

cytoplasm of tubular cells by Tan & The (2004), who also examined a range of human tissues and observed that parafibromin immunoreactivity was present in all organs but was specific to cell type. However, they did not specify which tissues they examined and did not describe the different patterns of immunoreactivity in the samples examined.

The present study is the first in the literature which evaluates the expression of parafibromin protein in a series of normal mouse and human tissues. There were

no substantial differences in patterns of parafibromin expression between mouse and human samples, with the exception of subcellular location in a few cell types. In hepatocytes and cells of the renal cortex tubules, human samples showed strong nuclear positivity compared with no or weak immunostaining of mouse specimens. Parafibromin was detected in many different tissues and cell types, but not in all of them. In particular, parafibromin immunostaining was not found in connective tissue, smooth muscle, endothelium or some types of epithelia (colonic, urinary, tubaric, uterine, thyroid). With respect to Western blot studies, we also found moderate to strong parafibromin immunostaining in liver and brain and weak positivity in ovary. In the various tissues, heterogeneity of positivity was found between and within cell types. In the kidney, for instance, cortex tubules showed strong positivity, whereas medullary tubules and most cells of the renal corpuscles did not stain (heterogeneity between cell types). Moreover, in the renal cortex, some tubules without immunoreaction were also found (heterogeneity within a cell type). These findings suggest the functional involvement of parafibromin in specific cell types and in cells in a specific functional state. In the literature, the anti-proliferative function of parafibromin has been stressed (e.g. Woodard et al. 2005; Yart et al. 2005). However, in our study, cell types which showed higher parafibromin expression were hepatocytes, cells of the base of gastric glands, renal cortex tubules and the pars intermedia of the hypophysis. These cell types are characterized by different proliferative capacity, thus indicating that the cellular function of parafibromin may not be reduced only to its anti-proliferative effect. In this regard, parafibromin has been recently shown to bind to β-catenin and to mediate nuclear transduction of the Wnt signalling pathway. Thus, parafibromin might represent a bridge between β-catenin-mediated signalling and the Paf1 complex, thereby activating transcriptional initiation and elongation by RNA polymerase II (Mosimann et al. 2006).

In the literature, the subcellular location of parafibromin has been studied with reference to its function. The Paf1 complex is located in the nucleus (Porter et al. 2005), where it plays a role in cell cycle regulation, histone methylation, and lipid and nucleic acid metabolism (Betz et al. 2002; Hampsey & Reinberg, 2003). Cultured cells transfected with parafibromin showed the nuclear location of parafibromin, and a functional bipartite nuclear location signal was also found (Hahn & Marsh, 2005; Rozenblatt-Rosen et al. 2005). However, immunohistochemical study of ossifying fibroma of the jaws showed strong nuclear and cytoplasmic parafibromin reactivity (Pimenta et al. 2006). Immunohistochemical and immunofluorescence studies on human parathyroid tissues have also reported a nucleocytoplasmic location of this protein (Woodard et al. 2005; Juhlin et al. 2006). Woodard et al. (2005) found both a nuclear and a cytoplasmic location in COS cells transfected with AU5-tagged human wild-type parafibromin, by immunoblotting of nuclear and cytoplasmic fractions.

Our data regarding both the nuclear and the cytoplasmic location of parafibromin in some tissue and cell types are consistent with the above-mentioned studies showing its nucleocytoplasmic location. In some cell types of both mouse and human, we also found parafibromin immunostaining restricted only to cytoplasm. For instance, in the base of gastric glands of both mouse and human, cells showed strong cytoplasmic positivity without nuclear staining. These findings suggest the involvement of parafibromin not only in nuclear functions but also in some cellular processes of the cytoplasm. Hahn & Marsh (2005) suggested that this protein, in certain conditions, is transported to the cytoplasm, as with other proteins which regulate transcription, such as Iκβ-α and p53. These proteins have sequences which permit export from the nucleus, the so-called nuclear export sequences (NESs) (Stommel et al. 1999; Henderson & Eleftheriou, 2000; Huang & Miyamoto, 2001), and putative NESs have been identified in parafibromin (Hahn & Marsh, 2005). Our study revealed that different cell types are characterized by different subcellular locations of parafibromin (nuclear, nucleocytoplasmic, cytoplasmic), suggesting its differential functional involvement in different cell types.

In the literature, some authors have studied the expression of parafibromin in benign and malignant neoplasms in order to verify its possible role in tumorigenesis. Parathyroid adenomas, hyperplasias and MEN1 tumour groups displayed heterogeneity of staining, whereas parathyroid carcinomas, both HPT–JT syndrome-related and sporadic, are characterized by loss of parafibromin nuclear immunoreactivity (Tan et al. 2004; Juhlin et al. 2006). These studies strongly suggest that the involvement of the tumorigenesis pathway is mediated by the HPRT2 gene in parathyroid neoplasms. In four cases of ossifying fibroma of the jaws and in one

case of juvenile ossifying fibroma, immunohistochemistry displayed a similar pattern of immunolocation, with strong nuclear and cytoplasmic staining (Pimenta et al. 2006). Owing to the wide expression of parafibromin, it may be hypothesized that HPRT2 also plays a role in the tumorigenesis of other neoplasms, both HPT–JT syndrome-related and sporadic. Knowledge of the patterns of expression of parafibromin in normal tissues may suggest organs and tissues where the oncosuppressor role of this protein is more important and where loss of expression is more probably involved in tumorigenesis.

In summary, besides confirming the wide expression of parafibromin in mouse and human tissues, our study demonstrates the differential expression and/or subcellular location of parafibromin among tissues, cell types and single cells, suggesting differential functional involvement. On the basis of these findings, future studies will have to consider the possible role played by parafibromin not only in parathyroid carcinoma but also in other types of tumours.

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