Human fibulin-4: analysis of its biosynthetic processing and mRNA expression in normal and tumour tissues

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Abstract Here, we report the identification of a human orthologue of fibulin-4, along with analysis of its biosynthetic processing and mRNA expression levels in normal and tumour tissues. Comparative sequence analysis of fibulin-4 cDNAs revealed apparent polymorphisms in the signal sequence that could account for previously reported inefficient secretion in fibulin-4 transfectants. In vitro translation of fibulin-4 mRNA revealed the presence of full-length and truncated polypeptides, the latter apparently generated from an alternative translation initiation site. Since this polypeptide failed to incorporate into endoplasmic reticulum membrane preparations, it was concluded that it lacked a signal sequence and thus could represent an intracellular form of fibulin-4. Using fluorescence in situ hybridisation analysis, the human *fibulin-4* gene was localised to chromosome 11q13, this region being syntenic to portions of mouse chromosomes 7 and 19. Considering the fact that translocations, amplifications and other rearrangements of the 11q13 region are associated with a variety of human cancers, the expression of human fibulin-4 was evaluated in a series of colon tumours. Reverse transcription-polymerase chain reaction analysis of RNA from paired human colon tumour and adjacent normal tissue biopsies showed that a significant proportion of tumours had ~2–7-fold increases in the level of fibulin-4 mRNA expression. Taken together, results reported here suggest that an intracellular form of fibulin-4 protein may exist and that dysregulated expression of the *fibulin-4* gene is associated with human colon tumourigenesis.

Key words: Fibulin; Colon cancer; Oncogene; 11q13; Cell growth regulation

1. Introduction

Members of the fibulin gene family are characterised by a unique C-terminal fibulin-type module and several repeated epidermal growth factor (EGF)-like domains, most of which possess a consensus motif for calcium binding [1]. Fibulin-1 and -2 proteins exhibit the capacity to self-associate [2,3] and display a similar, but not entirely overlapping, repertoire of extracellular matrix (ECM) protein binding partners [2,4–10]. These ECM protein partners include fibronectin, nidogen, laminin, collagen IV and endostatin. In some cases, the binding sites within fibulin-1 and -2 that mediate these various protein–protein interactions have been discovered [11,12]. Both of these fibulins exhibit alternative splicing of RNA transcripts. Other fibulins have been identified more recently: S1-5/EFEMP1 (fibulin-3), MBP1/H411/EFEMP2 (fibulin-4) and EVEC/DANCE (fibulin-5) [13–15]. This subset of the family (class B) is significantly smaller than either fibulin-1 or -2 (class A) and lacks certain structural/functional domains at the N-terminus when compared to the original progenitors of the family [12]. In addition, the terminal location(s) of fibulin-3 protein is still ill-defined, with the choice of whether to remain inside the cell or be secreted perhaps dependent on alternative RNA splicing and/or selection of different translation initiation sites [14].

Fibulin gene family members have been associated with a variety of biological processes including embryonic development and organogenesis, haemostasis and thrombosis, fibrogenesis, tissue homeostasis and remodelling [1]. Fibulins are thought to serve structural roles within the ECM. In addition, fibulins may interact, either directly or indirectly, with receptors at the cell surface, thus providing an avenue for regulation of cell behaviour. Indeed, fibulins have been shown to modulate cell morphology, growth, adhesion and motility [14,16–19]. Dysregulation of fibulin expression/activity may have pathobiological consequences. For example, up-regulation of fibulin-5 mRNA expression has been observed in diseased vasculature, notably in atherosclerotic and balloon-injured blood vessels, this being reflective of a putative role in tissue remodelling [15,19]. More specifically, a single non-conservative point mutation (Arg345Trp) within one of the EGF-like domains of fibulin-3 has been clearly associated with two autosomal dominant retinopathies, namely Malattia Leventinese and Doyne honeycomb retinal dystrophy [20].

Fibulins also appear to impact upon the process of tumourigenesis. There is increasing evidence pointing to a tumour suppressive role for fibulin-1 [17,18]. Ectopic overexpression of fibulin-1 in tumour cells has been shown to delay tumour formation in vivo and suppress anchorage-independent cell growth, motility and invasion in vitro [17,18]. One current hypothesis is that fibulin-1 acts as a negative regulator of cell motility, one of whose normal functions is to prevent invasion of tumour cells into surrounding tissues.

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However, a recent study showed elevated levels of fibulin-1 protein within the stroma of human ovarian carcinomas in comparison to normal ovaries [21]. Expression of fibulin-3 mRNA is induced during the processes of cell growth arrest and senescence [13]. This apparently negative growth regulatory role for fibulin-3 is contradicted by the observation of up-regulated fibulin-3 mRNA levels in transformed cell lines. In a similar vein, microinjection of in vitro transcribed fibulin-3 mRNA into normal human fibroblasts stimulated DNA synthesis of injected cells, as well as some surrounding cells. In summary, the role of fibulins in cancer is still controversial and further work is needed to fully resolve the above-mentioned seemingly ambiguous observations.

Mouse fibulin-4, also known as MBP1, was originally identified by our research group [14]. Fibulin-4 was found as a specific protein partner for mutant p53, and displays both mutant p53-dependent and -independent oncogenic properties. In addition, fibulin-4 provides new insight into the mutant p53 'gain of function' phenotype, which may have significant implications for both cancer biology and therapeutic intervention [22]. One hypothesis whereby fibulin-4 interacts with mutant p53 is that this interaction occurs within the lumen of the endoplasmic reticulum as fibulin-4 follows the secretory pathway. Another possibility is that certain isoforms of fibulin-4 (lacking the leading signal sequence) stay within the cell, these being generated by differential post-transcriptional processing and/or translation initiation. Fibulin-4 and mutant p53 can synergise in functional terms to promote tumour cell growth and increase rates of neoplastic transformation. This provides a physiological basis for a relationship, whether interaction-dependent or not, between these disparate proteins. It should also be restated that fibulin-4 also exhibits oncogenic properties that are independent of mutant p53, with this concept being clearly worth further exploration. Indeed, a recent study from another research group has also provided evidence of a growth stimulatory role for fibulin-4 [23]; in this case the hamster version (H411) of fibulin-4 was studied.

The following report describes the characterisation, in both biochemical and genetic terms, of human fibulin-4.

2. Materials and methods

2.1. In vitro translation

Mouse and human fibulin-4 cDNA was transcribed and translated in vitro using the TNT coupled reticulocyte lysate system (Promega), with T7 RNA polymerase being used for transcription. 1 μg of fibulin-4 cDNA expression vector plasmid was placed in a final reaction volume of 50 μl containing 25 μl TNT rabbit reticulocyte lysate, 2 μl TNT reaction buffer, 1 μl TNT T7 RNA polymerase, 1 μl 1 mM amino acid mixture (minus Met), 1 μl 40 U/μl RNaseOUT (Gibco BRL) and 1 μCi ^{35}S-Met (NEN Life Science Products). Co-translational and initial post-translational processing events were mediated by inclusion of 3 μl nuclease-treated canine pancreatic microsomal membranes (CMM; Promega) to the above reaction mixture. The positive control mRNAs, the precursors for *Saccharomyces cerevisiae* α-*factor and Escherichia coli* β-lactamase, were used at 0.2 μg/50 μl reaction. Following a 1 h incubation at 30°C, 10 μl of each translation reaction was analysed on a 12% gel by SDS–PAGE. Gels were vacuum-dried using a gel dryer, with radioactive emissions detected by exposure to Kodak BMR film.

2.2. Proteinase K digestion and deglycosylation

15 μl of translated product was subjected to digestion with *Tritirachium album*-derived proteinase K (Boehringer Mannheim) in a final reaction volume of 20 μl, which contained 2 μl reaction buffer (250 mM Tris–HCl pH 7.5, 500 mM NaCl, 15 mM EDTA) plus one of the following: (a) 3 μl water, (b) 2 μl water and 1 μl 100 mg/ml proteinase K (having been pre-digested at 37°C for 30 min), or (c) 1 μl proteinase K (pre-digested) and 2 μl 10% Triton X-100. Digestion was carried out for 1 h on ice. Reactions were terminated by the addition of 4 μl 18 mM phenylmethylsulfonylfluoride, with the incubations being continued for a further 30 min. Digests were immediately boiled for 3 min following addition of conventional SDS sample buffer. 40 μl of each digest was analysed by SDS–PAGE as before.

Translated products were examined for evidence of glycosylation using the *N*-glycosidase F deglycosylation system (Boehringer Mannheim). In more detail, 5 μl of the translation mixture in 1% Triton X-100 was denatured at 95°C for 3 min in an equal volume of the reduced denaturation buffer contained within the above kit. The denatured samples were then mixed with equal volumes of reaction buffer (again kit-derived) and incubated overnight in the presence or absence of *N*-glycosidase F (42 U/reaction). It should be noted that Triton X-100 at this concentration allows access of the *N*-glycosidase enzyme to membrane-protected translated proteins without adversely affecting *N*-glycosidase activity. SDS sample buffer was added to each reaction followed immediately by boiling for 3 min and analysis by SDS–PAGE as above.

2.3. RNA hybridisation analysis

Human fibulin-4 and GAPDH-specific probes were generated from the above-mentioned testis cDNA library by polymerase chain reaction (PCR) amplification using the following primers:

Human fibulin-4
Forward, 5′-GCCCTGATGGTTACCGCAAGA-3′
Reverse, 5′-AGCCCCCATGGAAGTTGACAC-3′
Human GAPDH
Forward, 5′-CGGAGTCAACGGATTTGGTCGTAT-3′
Reverse, 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′

Hybridisation of PCR-derived probes to Northern blots proceeded as indicated previously [14]. Quantitation of hybridisation signals was achieved by electronic autoradiography using an Instant Imager plus associated analysis software (Packard).

2.4. Fluorescence in situ hybridisation (FISH)

FISH analysis was performed by Genaxis Biotechnology (Montigny-le-Bretonneux, France) using a full-length human fibulin-4 cDNA probe on cytogenetic preparations from a normal human male. Metaphase chromosomes were prepared according to conventional procedures and normal karyotypes verified. The probe was labelled by nick translation with 11-dUTP-biotin (Genaxis Biotechnology), with hybridisation occurring overnight in a denaturing moist chamber. Washes, signal detection and amplification were performed according to the standard chromosome in situ hybridisation protocol. The chromosomes were counter-stained with 4,6-diamidino-2-phenylindole antifade and visualised with an Olympus AX 70 fluorescent microscope equipped with a PSI Power Gene FISH System analyser.

2.5. RNA isolation and semi-quantitative reverse transcription (RT)-PCR

Frozen tissue biopsies, i.e. colon tumour and paired normal samples, were homogenised in RNA NOW solution (Ozyme) using a Polytron probe. At least 1.0 ml of RNA NOW solution was used per 100 mg of tissue and total RNA isolation proceeded according to the manufacturer's recommendations. RNA yields were quantified by spectrophotometry, with RNA integrity being assessed by conventional agarose gel electrophoresis. Single-stranded cDNA was produced using 1.5 μg total RNA in combination with the First-Strand cDNA Synthesis kit (Amersham Pharmacia). The first-strand reaction was primed by the *Not*I-d(T)$_{18}$ oligonucleotide contained within this kit. For semi-quantitative RT-PCR analysis of human fibulin-4 (primers shown above) and β-actin (primers indicated below) mRNA, serial dilutions were made of the above cDNA preparations and PCR amplification performed on these using the primers listed below. PCR products were analysed by agarose gel electrophoresis and visualised by ethidium bromide staining.

Human β-actin
Forward, 5′-GTGGGGCGCCCCAGGCACCA-3′
Reverse, 5′-CGGTTGGCCTTGGGGTTCAGGGGGG-3′

3. Results

3.1. Identification and characterisation of human fibulin-4 cDNA

Human fibulin-4 was identified by a PCR-based approach from a commercially available testis-derived cDNA library (GenBank accession AX023967; see Section 5). The open reading frame (ORF) within the human fibulin-4 cDNA was easily determined by comparison with both mouse and hamster fibulin-4 forms (Fig. 1). All three fibulin-4 cDNAs contain a major ORF of 443 amino acids in length, which includes a putative signal sequence at the N-terminus. This leaves 5′ and 3′ untranslated terminal regions (UTRs) of 57 and 90 bp, respectively, for human fibulin-4. Considerable conservation, in terms of primary protein structure, was noted between the species homologues of fibulin-4 (amino acid sequence identities, mouse versus hamster: 97.1%; human versus hamster: 96.8%; human versus mouse: 95.3%), with the only difference being single amino acid substitutions. Indeed, all forms exhibit the same number of EGF-like repeats (six in all), along with a short proline-rich region inserted within the first EGF-like domain. In addition, a similar profile was seen with respect to consensus sites for potential post-translational modifications, such as *N*-glycosylation and β-hydroxylation. One notable exception is the presence of a putative *O*-glycosylation site (with Ser30 being the affected residue) within the N-terminal end of both human and hamster fibulin-4 proteins, which is not found in the mouse equivalent (Pro30).

In the past year, several groups have either published [24,25], or solely deposited into GenBank (AK000980; AB030655), nucleotide sequences (cDNA only) encoding for human fibulin-4 protein. Using the CLUSTALW (1.8) multiple sequence alignment program [26], the respective nucleotide and predicted amino acid sequences, as defined by the assumed major ORF, were compared (data not shown). There were found to be notable sequence differences between the different versions of human fibulin-4, with the cDNA that we have obtained appearing to represent the definitive consensus. Apparent single nucleotide polymorphisms (SNPs) within the ORF portion of the human fibulin-4 were as follows: codon 5 (**G**CC to **A**CC), missense mutation (Ala to Thr); codon 96 (C**C**C to C**T**C), missense mutation (Pro to Leu); codon 191 (GG**C** to GG**T**), silent mutation; codon 355 (AG**C** to AG**A**), missense mutation (Ser to Arg); codon 356 (GT**G** to GT**A**), silent mutation.

It is unclear, as yet, whether the above alterations are authentic or due to sequencing errors within the individual laboratories concerned. The apparent SNPs at codons 96 (AK000980), 355 [26] and 356 [26] have been seen only once, while those at codons 5 and 191 were observed at the same time by two independent laboratories ([25] and AB030655). The described tissue sources of origin for human fibulin-4 cDNA isolation, apart from the testis-derived cDNA library used in this study, are melanoma [25], foetal brain [26] and 10 week old embryo (AK000980), consisting mainly of tissue around the head region. Finally, there appears to be

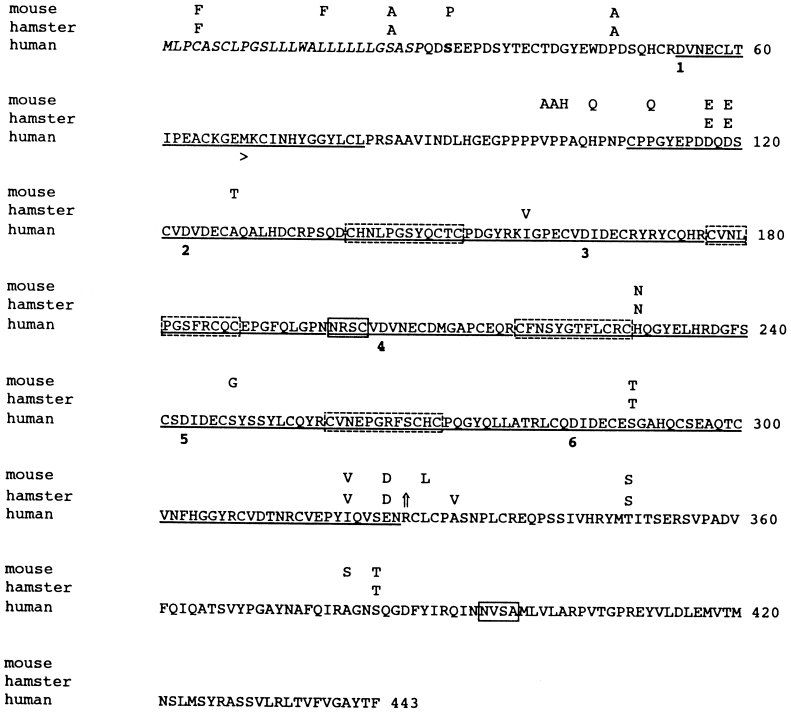

Fig. 1. Amino acid sequence comparison between human, mouse and hamster fibulin-4 proteins. Human fibulin-4 is the main sequence shown, numbers starting from the primary initiation codon, with amino acid variations from the mouse and hamster forms indicated above the respective residues. The six calcium-binding EGF-like domains are numbered sequentially, with the location of the numerals demarcating the start of these repeats. The fibulin-type module begins at amino acid position 326 (Arg), as highlighted by the vertical arrow. The putative N-terminal signal sequence of human fibulin-4 is italicised, with the signal peptidase cleavage site between amino acid positions 27 (Pro) and 28 (Gln) being marked by a solid triangle. Candidate motifs for post-translational modifications include two *N*-glycosylation sites (solid boxes) and four asparagine hydroxylation sites (hatched boxes). Potential *O*-glycosylation sites in human and hamster fibulin-4 at amino acid position 30 (Ser) are indicated by bold typeface. The putative alternative translation initiation site at amino acid position 69 is shown by the following symbol, >.

sequence differences at both the 5′ and 3′ UTRs of the various fibulin-4 cDNAs, suggestive of alternative splicing events in these regions (data not shown).

From here on, we will refer to our sequence as human fibulin-4, with the other human variants defined when appropriate.

3.2. In vitro translation of fibulin-4

The calculated molecular weight of both mouse and human precursor fibulin-4 proteins, without taking into consideration the putative post-translational modifications mentioned above, is 49.4 kDa. In vitro translation of recombinant mouse and human fibulin-4 produced major polypeptides of 54 kDa (Fig. 2A, lanes 1 and 2). Mouse and human fibulin-4 proteins are relatively rich in negatively charged amino acids, with aspartate and glutamic acid residues comprising 12.4% of the total number of amino acids in both cases. The estimated isoelectric points are 4.76 and 4.79, respectively. Takano et al. [27] have suggested that such acidic characteristics may contribute to overestimation of molecular mass by SDS–PAGE.

Translation of fibulin-4 also yielded a minor protein product of 46 kDa (Fig. 2A, lane 2), suggesting usage of a downstream translation initiation site. This phenomenon was much more prevalent in the case of human, rather than mouse, fibulin-4, i.e. with the former reaching nearly 10% of the total translated protein. While several candidate initiation codons occur just downstream from the major translation start site, only one of these had a corresponding termination codon that would allow production of a polypeptide of this size. Translation from this AUG leads to a theoretical in-frame product of 42.0 kDa, encompassing amino acids 69–443 of the major translated protein. The reduction in relative translation efficiency of the minor product observed in the case of mouse fibulin-4 may be due to further divergence of the concerned translation initiation site, by comparison to human fibulin-4, from an optimal consensus sequence for translation initiation [28]. It will be interesting to see if the alternative fibulin-4 polypeptide is produced under physiological conditions, and whether it has specific functions of its own.

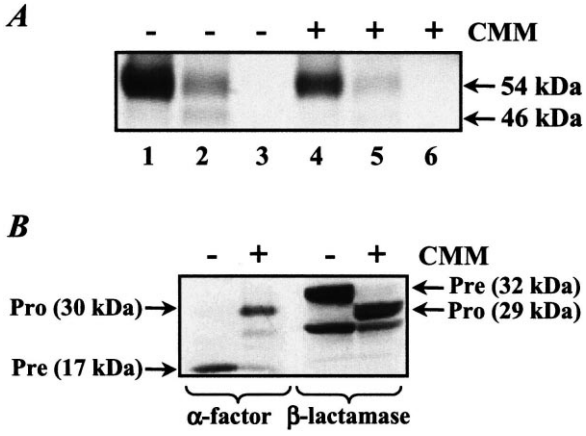

Fig. 2. A: In vitro translation of mouse and human fibulin-4. Translation reactions were performed either in the absence (−) or presence (+) of CMM. Apparent molecular weights of the translated products were determined by comparison with ^{14}C-labelled protein size markers. Lanes 1 and 4, mouse fibulin-4 cDNA expression vector; lanes 2 and 5, human fibulin-4 cDNA expression vector; lanes 3 and 6, vector alone control. B: Co-translational processing and post-translational modification of control mRNAs. Upon exposure to CMM, the precursor form (17 kDa) of the α-factor is converted to a 30 kDa protein due to extensive glycosylation. Similarly, pre-β-lactamase (32 kDa) is reduced in size upon cleavage of a 3 kDa signal peptide, forming mature β-lactamase. Precursor and processed species are indicated by Pre and Pro, respectively.

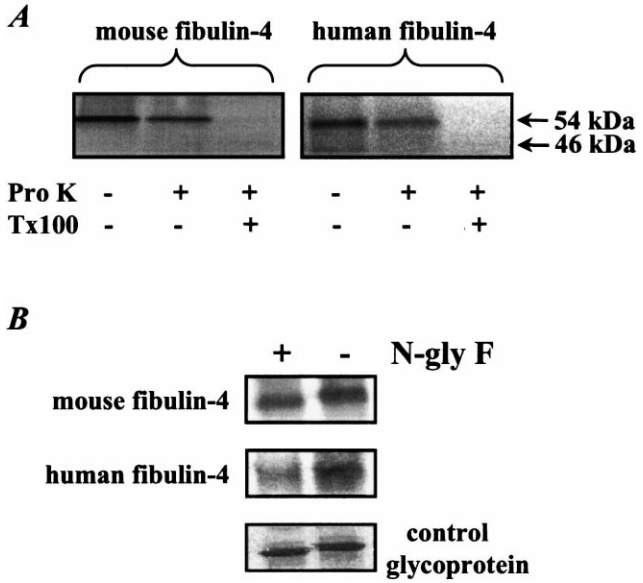

Fig. 3. Signal peptide cleavage and *N*-glycosylation of mouse and human fibulin-4. A: Proteinase K digestion of in vitro translated fibulin-4. Mouse and human fibulin-4 proteins, translated in the presence of CMM, were incubated in either the absence (−) or presence (+) of proteinase K (Pro K) and Triton X-100 (Tx100). B: Enzymatic deglycosylation of in vitro translated fibulin-4. Translated fibulin-4 proteins, following incubation with CMM, were digested with *N*-glycosidase F (*N*-gly F). The control glycoprotein was transferrin, and was visualised by conventional staining with Coomassie Blue. +, *N*-gly F; −, absence of *N*-gly F.

The respective molecular weight of processed mouse and human fibulin-4 proteins, assuming signal peptide cleavage at predicted sites and lack of post-translational modification, are 46.6 and 46.7 kDa. Microsomal membranes, which are portions of the endoplasmic reticulum, can be used with standard in vitro translation reactions in order to simulate certain key co-translational processing and post-translational modification events, such as signal sequence cleavage and core *N*-glycosylation [29]. Interestingly, no change was seen with respect to the size of in vitro translated mouse and human fibulin-4 protein when the translation reactions were performed in either the presence or absence of CMM (Fig. 2A, lanes 4 and 5). Under the same conditions, controls for signal sequence cleavage (β-lactamase) and core glycosylation (α-factor) showed clear evidence of co-translational and post-translational processing, respectively (Fig. 2B). Giltay et al. [24] reported the apparent molecular weight of secreted human fibulin-4 protein to be 54 kDa, as determined by SDS–PAGE under reducing conditions. In keeping with the in vitro translation results presented above, this would suggest that mature human fibulin-4 is post-translationally modified to some extent, most likely by *N*-glycosylation (see Fig. 1), such that direct evidence of signal peptide cleavage is masked.

In addition, Giltay et al. mentioned an inherent difficulty in obtaining secreted recombinant human fibulin-4 protein fol-

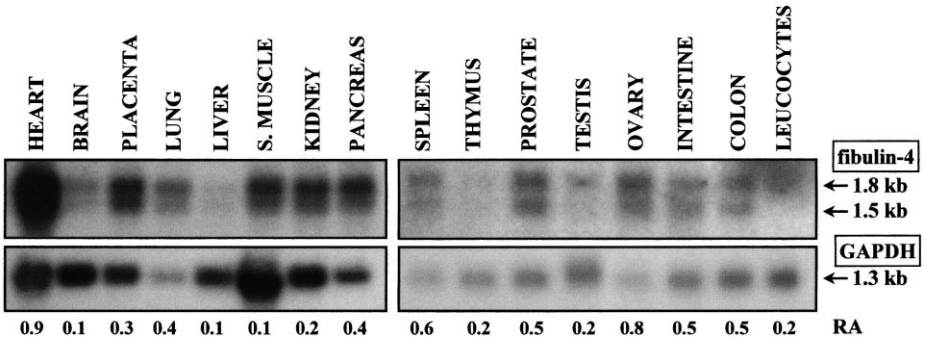

Fig. 4. Expression of fibulin-4 mRNA in human adult tissues. RNA hybridisation analysis of the levels of human fibulin-4 mRNA found within two commercially available multiple tissue Northern blots (Clontech). RNA loading was evaluated by hybridisation with a GAPDH-specific probe. The relative abundance (RA) of fibulin-4 mRNA is expressed as a function of the GAPDH mRNA signal.

lowing transfection of an episomal fibulin-4 cDNA expression vector into human kidney cells [24]. The discrepancies noted above with respect to the predicted amino acid sequence of human fibulin-4 protein may provide an explanation. The amino acid residue envisaged at codon 5 is either alanine or threonine depending upon the research group involved. The human fibulin-4 cDNA cloned in this study encodes for an alanine residue at the amino acid position concerned. It should also be noted that both the mouse and hamster forms of fibulin-4 code for an alanine residue at the equivalent amino acid position [14,23]. Signal sequence function is determined, amongst other factors, by the balance between the central hydrophobic core of the signal peptide and the N-terminal charge [30]. The presence of a polar residue such as threonine within the hydrophobic segment may adversely affect the function of the fibulin-4 signal sequence, thus leading to inefficient co-translational processing. Indeed, use of the SignalP (V2.0) signal sequence prediction server [31,32] indicated that the threonine-containing signal peptide should be slightly less effective, than the alanine-based version, with respect to being cleaved after insertion into the endoplasmic reticulum (data not shown). This may explain the low levels of secreted recombinant fibulin-4 protein, when directed from native signal sequence, mentioned by Giltay et al. [24].

3.3. Signal peptide cleavage and N-glycosylation

Mouse and human fibulin-4 proteins, having been translated in vitro in the presence of CMM, were subjected to proteolytic digestion by proteinase K (Fig. 3A). In the absence of a membrane solubilising detergent, such as Triton X-100, there was no change in the amount of either mouse or human fibulin-4 protein after proteinase K digestion. This indicates that both fibulin-4 proteins are translocated in entirety across the lipid bilayer. While the major fibulin-4 proteins (54 kDa) were unaffected in this scenario, the minor polypeptide (46 kDa) seen clearly in the case of human fibulin-4 was completely digested by proteinase K even in the absence of detergent. This is in agreement with the concept that this minor polypeptide does not contain a signal peptide and, thus, would not undergo co-translational processing. In the presence of Triton X-100, both the mouse and human fibulin-4 major protein products were found to undergo complete digestion. This further indicates that the lipid bilayer of the microsomal membrane protects against proteinase K-mediated proteolytic digestion of fibulin-4 [33].

As indicated in Fig. 1, mouse and human fibulin-4 have two consensus sites for *N*-glycosylation, with Asn198 and Asn394 being the potentially modified residues. Taking the molecular weight of both signal peptides to be approximately 3 kDa, this being equivalent to that estimated for two standard asparagine (*N*)-linked carbohydrate side chains, simultaneous signal sequence cleavage and *N*-glycosylation might explain the lack of size difference between fibulin-4 proteins translated with/without microsomal membranes mentioned above. This scenario was tested by subjecting fibulin-4, translated in the presence of microsomal membranes, to digestion with *N*-glycosidase F (Fig. 3B). This enzyme acts by cleaving *N*-linked oligosaccharides from the protein backbone. The electrophoretic mobility of both mouse and human fibulin-4 protein increased following digestion with *N*-glycosidase F, with the apparent molecular weight of the deglycosylated protein corresponding to ~51 kDa (as opposed to 54 kDa for undigested protein). A control glycoprotein, transferrin, which has a molecular weight of 65 kDa and also contains two *N*-linked glycan chains, showed a similar reduction in size by 3 kDa upon digestion with *N*-glycosidase F.

In conclusion, the signal sequences of both mouse and human fibulin-4 are expected to be fully functional with respect to insertion of translated protein into the lumen of the endoplasmic reticulum. In addition, we have confirmed here that fibulin-4 is *N*-glycosylated.

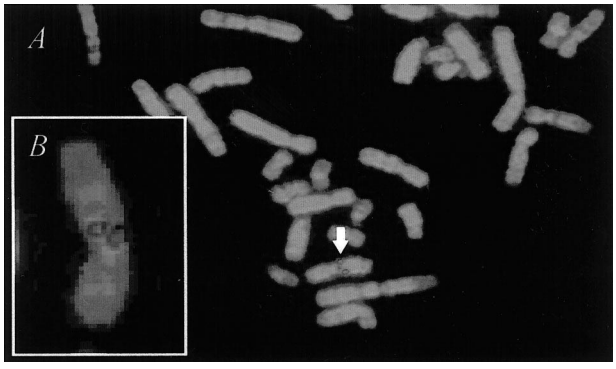

Fig. 5. Mapping of the human *fibulin-4* gene to chromosome 11q13 by FISH. A: Metaphase spread of chromosomes with twin fluorescent hybridisation spots indicated by the white arrow, these being obtained with human fibulin-4 cDNA as the probe. B: Close-up view of metaphase chromosome 11, with fibulin-4 hybridisation signals appearing adjacent to the centromere on the distal arm of the chromosome.

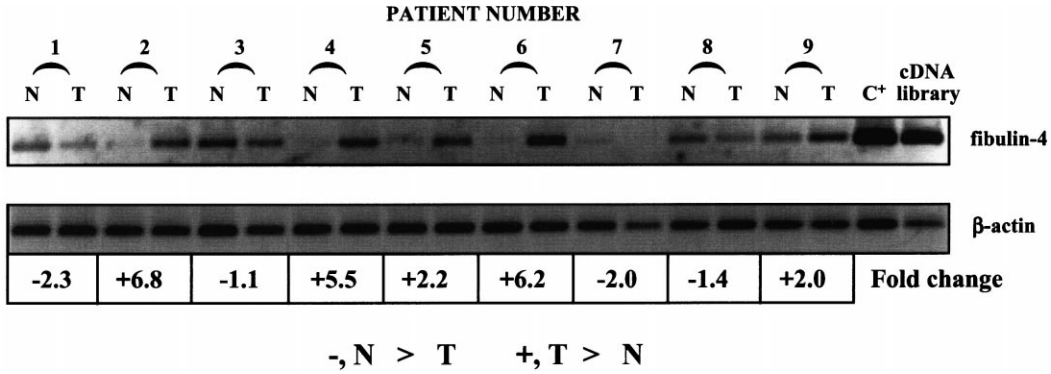

Fig. 6. RT-PCR analysis of fibulin-4 mRNA expression in human colon tumours. The main details of the approach used for semi-quantitative RT-PCR analysis are detailed in Section 2. Paired normal (N) and tumour (T) tissue biopsies from different patients are indicated by numbers 1–9. Positive DNA controls for human fibulin-4 and β-actin are indicated by C^+. The human testis cDNA library was also used as a secondary control for PCR amplification from complex cDNA populations. The level of fibulin-4 mRNA was expressed as a function of β-actin gene expression, with this data being used to calculate the fold differences between normal and tissue samples from the same patient. A fold difference (− or +) of greater than or equal to 2.0 was deemed to be within the limit of sensitivity in terms of quantitation.

3.4. Tissue-specific expression of the human fibulin-4 gene

Expression of human fibulin-4 mRNA was, as determined by Northern blot analysis, shown to be differentially regulated in a tissue-specific manner in the human adult (Fig. 4). For example, a high level of *fibulin-4* gene expression was observed in heart and ovarian tissue, with more moderate expression levels seen in the spleen, prostate, intestine and colon. Significantly lower expression levels for fibulin-4 mRNA were noted in all other tissues examined, with notably weak signals in the brain, liver and skeletal muscle. The expression pattern for the *fibulin-4* gene observed in certain adult tissues, as detailed in the left hand panel of Fig. 4, agrees with fibulin-4 mRNA expression results previously shown by Giltay et al. [24]. Furthermore, we also observed the same two mRNA isoforms of 1.5 and 1.8 kb in size, these being most likely obtained by alternative RNA splicing. Interestingly, the ratio in terms of expression between the major (1.8 kb) and minor (1.5 kb) mRNA forms appeared to be the same independent of tissue type. While the expression profile of fibulin-4 mRNA between mouse and human adult tissues are quite similar, there are some notable differences, e.g. low level of *fibulin-4* gene expression in the mouse heart compared to a markedly high level in humans, and vice versa with respect to the expression of fibulin-4 mRNA in lung tissue [14].

3.5. Physical mapping of the human fibulin-4 gene

The chromosomal localisation of the human *fibulin-4* gene was determined by FISH using full-length human fibulin-4 cDNA as a probe. Of 30 normal human metaphase cells examined, 25 displayed a specific hybridisation signal with twin spots at 11q13 (Fig. 5). Secondary hybridisation signals at other chromosomal regions were not observed. Utilising PCR-based amplification of a portion of the 3′ UTR of the human fibulin-4 gene from monochromosomal and radiation cell hybrids as well as previously mapped genomic clones, a recent study mapped the human *fibulin-4* gene to the same region [25]. 11q13 is notably gene-rich and contains an extensive number of disease-related loci, including those involving bone disorders, retinopathies and cancer [34]. This human chromosomal band displays regions of homology with portions of mouse chromosomes 7 and 19 [34], with the latter chromosome being the most likely location for the mouse *fibulin-4* gene when taking into consideration the genetic mapping data presented elsewhere [25].

3.6. Aberrant expression of fibulin-4 mRNA in human colon tumours

Levels of fibulin-4 mRNA were assessed by semi-quantitative RT-PCR in a small panel of paired normal/tumour human colon tissue biopsies ($n = 9$). Expression of the fibulin-4 gene in normal colon tissue varied considerably in different patients (Fig. 6), preventing direct comparison of gene expression levels in colon tumour tissue between individuals. Analysis of colon tumour tissue from each patient showed evidence of aberrant fibulin-4 mRNA expression in seven out of the nine patients. Five of these patients displayed up-regulation of fibulin-4 mRNA in the tumour relative to normal tissue, with fold differences ranging from 2.0 to 6.8. Down-regulation of fibulin-4 mRNA was observed in two patients, with fold differences being 2.0 and 2.3 in these cases.

4. Discussion

An accumulating body of evidence suggests a growth stimulatory role for fibulin-4 [14,23]. As indicated in a previous study from our group, mouse fibulin-4 promoted cell growth when overexpressed in a human lung tumour cell line [14]. In addition, hamster fibulin-4 stimulated the growth rate of stably transfected macrophages [23]. This potential role for fibulin-4 in mediating proliferative responses may be shared by other fibulin family members, with fibulin-3 being a notable example [13]. These growth regulatory actions are thought to primarily occur through an autocrine mechanism of action, with secreted fibulin-4 protein acting either directly or indirectly on, as yet unidentified, cell surface receptors. The observation of a downstream translation initiation codon in fibulin-4, which as seen above produces a mature protein minus signal sequence, may implicate a cytoplasmic role for this protein. While the physiological relevance of such an intracellular form of fibulin-4 is not readily apparent, we have shown before that N-terminally deleted mouse fibulin-4 retains some oncogenic activity, albeit not as markedly as that observed with full-length protein [14]. This study supports the hypothesis that an intracellular form of fibulin-4 exists, and

that mutant p53/fibulin-4 protein–protein interactions may occur in either of two ways: (a) mutant p53 enters the lumen of the endoplasmic reticulum and physically interacts with fibulin-4 as it passes along the secretory pathway [14], and/or (b) an intracellular form of fibulin-4 physically interacts with mutant p53 within the cytoplasm.

The human *fibulin-4* gene has now been mapped by two independent research groups, each using different approaches, to chromosome 11q13. Based on this chromosomal localisation and analogy to the macular dystrophy-causing mutation in fibulin-3 [20], this marks fibulin-4 a prime candidate for the following inherited retinopathies which have been linked to 11q13: neovascular inflammatory vitreoretinopathy, familial exudative vitreoretinopathy, Bardet-Biedl syndrome 1 and congenital fibrosis of the extraocular muscles. Several hereditary diseases involving dysregulated bone homeostasis have been mapped to this region also, such as osteoporosis-pseudoglioma syndrome, high bone mass and osteopetrosis. Individuals with some of these bone disorders often exhibit progressive blindness and deafness, along with other complications. Taking into consideration the potential role of other fibulins in the development of skeletal structures [35], it may prove to be informative to look more closely at the involvement of fibulin-4 in the above, as well as other, bone disorders.

Other inherited disorders mapped to this region, which have not as yet been connected to a disease-causing gene or genes, include insulin-dependent diabetes mellitus 4, spinocerebellar ataxia 5, non-syndromic hearing loss, Meckel syndrome type 2 and acromegaly/gigantism. In specific relation to carcinogenesis, the distal portion of 11q13 undergoes amplification in a number of common human tumours, including oesophageal, head and neck, bladder, lung and breast cancer [36,37]. Gene amplification at 11q13 is complex, with multiple amplicons having been reported which occur primarily at the distal portion of 11q13. Oncogenes resident at 11q13 include CCND1, EMS1, FGF3, FGF4 and FOSL1, with amplification events generally involving different combinations of these genes. In addition, 11q13 is a common target for chromosomal translocations and other rearrangements during tumourigenesis. Previous evidence suggests that the human *fibulin-4* gene is located at the proximal portion of 11q13 close to the centromere [25], which may obviate against this candidate oncogene being part of the major 11q13 cancer-related amplicon.

The up-regulation of fibulin-4 mRNA expression noted in human colon tumours relative to normal tissue is consistent with the putative oncogenic role for this gene. While it is possible that more discrete amplifications comprising the *fibulin-4* gene may occur, the increased expression of the *fibulin-4* gene is probably due to either enhanced translation or increased mRNA stability. Interestingly, expression of the *fibulin-1* gene has recently been shown to be regulated through selective translation [38]. The down-regulation of fibulin-4 mRNA levels seen in two of the colon tumours is perhaps reflective of non-fibulin-4-specific chromosomal rearrangements. The lack of consistent trend with respect to fibulin-4 mRNA expression in the colon tumour samples versus normal colon specimens may be reflective of the multi-factorial basis of this disease. Although this pilot study was relatively limited in terms of sample size, this work provides the basis for much larger clinical studies assessing the role of fibulin-4 in colon cancer, along with other tumour types.

5. Supplementary material

5.1. Cloning of human fibulin-4

The nucleotide sequence of the predicted ORF from mouse fibulin-4 cDNA [14] was used to search for homologous human expressed sequence tags (ESTs) present within the GenBank database. The ESTs used for this inquiry were taken from the human dbEST library. Over 60 human ESTs displayed a high degree of sequence similarity ($>85\%$ sequence identity) with mouse fibulin-4 cDNA [14]. These ESTs spanned various sections of mouse fibulin-4, with the majority being localised to the 3′ end of the ORF. Using one of the most 5′ situated human ESTs from this 3′ subset (GenBank accession: AA055998), primers were designed so as to allow overlapping PCR-based amplification of the 5′ and 3′ ends of human fibulin-4 DNA. A human testis cDNA library was chosen as the starting material for PCR amplification. This choice was influenced by the previous observation in the mouse of a high expression level of mouse fibulin-4 mRNA within this particular tissue type [14]. Thus, the commercially available SuperScript human testis cDNA library (Gibco BRL) was used as the substrate for PCR-based amplification of fibulin-4. Internal fibulin-4 and library vector-specific primer pairs were used as follows:

5′ fibulin-4 cDNA fragment
Forward, 5′-AGCTATTTAGGTGACACTATAG-3′ (vector-specific: SP6).
Reverse, 5′-CTCCGCTCCGAGGTGATGGTC-3′ (internal fibulin-4).
3′ fibulin-4 cDNA fragment
Forward, 5′-TGTAGCTACTCCAGCTACCTC-3′ (internal fibulin-4).
Reverse, 5′-TAATACGACTCACTATAGGGAGA-3′ (vector-specific: T7).

Use of the above primer sets allowed PCR-mediated amplification of overlapping 5′ and 3′ fragments of human fibulin-4 cDNA, with the largest of these being approximately 1.20 and 0.75 kb in size, respectively. Multiple independent PCR products were placed into the pCR II cloning vector (Invitrogen), with insert-containing plasmid clones being sequenced at a number of times. Subsequently, a consensus sequence for full-length human fibulin-4 cDNA was derived taking into account the 326 bp overlapping sequence. The longest 5′ and 3′ cDNA fragments were joined together using the restriction enzymes *Eco*RI, *Nco*I and *Not*I and the intermediary recipient plasmid, pBC-SK+/− (Stratagene). The entire human fibulin-4 cDNA was then cloned into the pcDNA3 mammalian gene expression vector (Invitrogen) using the restriction enzymes *Hin*dIII and *Not*I. Finally, the reconstituted human fibulin-4 cDNA was confirmed several times by DNA sequencing. The total nucleotide sequence for human fibulin-4 cDNA was 1479 bp.

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