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Full Length Research Paper

Down regulation of fibulin-1 in squamous cell carcinoma of the oesophagus

Fred Wamunyokoli[1*], Denver Hendricks[2] and Iqbal Parker[2]

[1]Department of Biochemistry Jomo Kenyatta University of Agriculture and Technology P. O. Box 62000 City Square Post Office 00 200 Nairobi Kenya.
[2] Division of Medical Biochemistry Institute for infectious diseases and molecular medicine Faculty of Health Sciences University of Cape Town Observatory, 7925 Cape Town South Africa.

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The molecular events involved in the development of squamous cell oesophageal cancer remain poorly understood. To elucidate the probable genetic events involved, genes that are differentially expressed between normal and tumour oesophageal tissue were identified. Differential display reverse transcription polymerase chain reaction was carried out on both malignant and adjacent normal oesophageal tissue of the same patient to identify differentially expressed genes in oesophageal cancer patients. Differentially expressed genes were isolated, cloned, sequenced and identified in GenBank using BLAST. Fibulin-1 was identified as one of the differentially expressed genes. Northern blot analysis confirmed down regulation of fibulin-1 in oesophageal cancer tissues, while immunohistochemical studies using a larger patient sample localized fibulin-1 to both the cell membrane and cytoplasm of both cancer cells and corresponding normal epithelial tissue. Whereas the protein was abundant in the normal oesophageal epithelial cells, its expression was significantly reduced in dysplastic cells and in the different grades of oesophageal cancer cells. This observation suggests that fibulin-1 down regulation in oesophageal cancer is an early event and is probably influenced by the grade of the tumour. This is the first study to report on the relationship between fibulin-1 expression and oesophageal cancer tumour grade. It forms the basis for further evaluation of this gene as a candidate biomarker in cancer of the oesophagus and its probable role in the early events of tumourigenesis.

Key words: Oesophageal cancer, fibulin, differential display, tumour markers.

INTRODUCTION

Cancer of the oesophagus is the fifth most frequent cause of cancer deaths worldwide (World Health Organization WHO, 1997), and the fourth most common cancer in developing countries (Hennessy, 1996). In South Africa, squamous cell carcinoma of the oesophagus is a leading cause of death in the black population, where lifetime risk of the disease amongst black males is 1 in 59 (Sitas et al., 1998). Like other cancers, oesophageal cancer is a multi-step disease characterized by alterations in a variety of molecular pathways that ultimately result in aberrant morphological and functional characteristics of the cell (Kinzler and Vogelstein, 1996). Although the molecular mechanisms of these pathways are poorly understood in oesophageal cancer, the molecular, biochemical and cellular characteristics that manifest them during tumourigenesis are similar to those observed in other cancers (Hanahan and Weinberg, 2000).

Oesophageal cancer cells like other cancer cells display two important properties: they are able to invade the surrounding tissues through the basement membrane (Liotta et al., 1991) and secondly, can metastasise to distant tissues (Chambers et al., 1995). The success of extra-oesophageal spread is therefore in part, dependent

*Corresponding author. E-mail: fwamunyokoli@yahoo.com. Tel: +254 (67) 52711. Fax: +254 (67) 52164.

on the de-regulation of important cell adhesion and motility functions that facilitate tissue invasion and metastasis. Previous studies have shown that extracellular matrix proteins such as fibronectin, laminin and type IV collagen regulate the motility of both normal and malignant cells (Akiyama et al., 1995).

Fibulins are a class of extracellular matrix and blood glycoproteins found extensively in the basement membrane and connective tissues (Roark et al., 1995). They currently, constitute five members, namely fibulin-1 and 2 (Argraves et al., 1989; Balbona et al., 1992: Pan et al., 1993), fibulin-3 (S1-5/EFEMP1) (Lecka-Czernik et al., 1995), fibulin-4 (MBP/H411/EFEMP2) (Gallagher et al., 1999) and fibulin-5 (EVEC/DANCE) (Kowal et al., 1999). These proteins interact and are responsible for binding important extracellular matrix proteins such as fibronectin laminin, nidogen, endostatin, tropoelastin and fibrinogen (Balbona et al., 1992; Pan et al., 1993; Tran et al., 1995; Sasaki et al., 1999). These interactions suggest that fibulin may be involved in the organizational structure and functioning of the extracellular matrix.

Table 1. Some clinico-pathologic characteristics of the patients used in immunohistochemical studies.

Charactersitics	Number of Patients
Histological stage	
0	1
1	4
2A	19
2B	6
3	20
4	0
Tumour grade	
Carcinoma *in situ*	2
Well differentiated	11
Moderately differentiated	28
Poorly differentiated	9
Survival	
Alive	9
Lots to follow up	6
Deceased	35
Race	
Black	22
Mixed ancestry	22
Caucasian	6
Gender	
Males	36
Females	14

Multiple forms of fibulin-1, namely, A, B, C and D exist. They are produced through alternative RNA splicing (Argraves, 1990). These variants differ in their c-terminal regions and are expressed in a wide range of cancer cell lines (Qing et al., 1997; Tran et al., 1997) and human tissues (Roark et al., 1995; Tran et al., 1997). Fibulin-1 is identical in its first 566 residues to other isoforms, however it has a unique 137 amino terminal segment encoded by alternatively spliced portion of its transcript (Tran et al., 1997).

Currently, there is no data that associates the expression of specific fibulin variants with particular cancer phenoltypes. In addition, although the expression of fibulin-1 has been reported in cancer cell lines, there are no reports on the expression levels and patterns in the human oesophagus and cancer of the oesophagus. This is the first study to report on the expression of fibulin-1 in oesophageal cancer tumours compared to their corresponding normal tissues.

MATERIALS AND METHODS

Samples for RT-PCR

Oesophageal cancer tumour and their corresponding normal specimens were obtained from the Oesophageal clinic of the Cardiothoracic Surgery Department at Groote Schuur Hospital, Cape Town, South Africa. These biopsies were excised from patients and immediately snap-frozen in liquid nitrogen. All sections were histopathologically classified according to the World Health Organization (WHO) classification system (WHO, 1999). Pathologic staging was classified using International Union against cancer (UICC) criteria (Sobin and Wittekind, 1997). All specimens were pathologically and histologically certified to be predominantly either tumour or normal prior to their use in these studies.

Samples for immunohistochemistry

Formalin-fixed paraffin embedded sections from 50 patients who underwent oesophageal resection at the Department of Cardiothoracic Surgery were used in these studies. These patients were resected between 1983 and 2000 and constituted 36 men and 14 women with ages ranging from 23 to 80 years (mean 53.3 ± 10.1 years). Ethics approval (Reference number: 085/2002) for the study was obtained from the Ethics Committee at the University of Cape Town. Some of the patient clinico-pathological information is summarized in Table 1.

RNA isolation

Total RNA from human biopsies and cell lines was isolated using the guanidine thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Residual chromosomal DNA was removed by incubating 5 µg of RNA with 5 U of RNA free DNAse enzymes in the presence of RNA inhibitor (Promega, USA). After incubation, the reaction volume was made up to 50 µl using diethylpyrocarbonate (DEPC) (0.05%) treated water. RNA was extracted using phenol: chloroform (3:1 v/v) and precipitated by the addition

of one-tenth volume of 3 M sodium acetate (pH 5.2) and four volumes of ethanol. The precipitated RNA was collected by centriugation (13,000 *g*, 10 min, and 4°C). The RNA was washed with 70% ethanol and re-suspended in DEPC-treated water. The purity of the RNA was ascertained by spectrophotometry ($A_{260/280}$) and formaldehyde agarose gel electrophoresis prior to its storage at –70°C in aliquots of 1 µg.

Differential display RT-PCR

Differential display RT-PCR was performed as previously described (Liang et al., 1993). Reverse transcription was performed using 1 µg of total RNA. RNA was mixed with 11 µl of 0.5 µg/µl degenerate anchor primer (5' TTT TTT TTT TTT (G/A/C) G3'), heated (70°C, 10 min) and quickly chilled on ice for 5 min. The contents were collected by brief centrifugation at maximum speed. Thereafter, first strand buffer (Life Technologies, USA), 10 mM DTT and 20 µM 4dinucleotide triphosphates (dNTPs) mix was added. After gentle mixing by pipetting, the mixture was incubated (42°C, 10 min) followed by addition of 200 U of Superscript II reverse transcriptase enzyme (Life technologies, USA) and incubation at 42°C for 1 h. The enzyme was inactivated by heat (95°C, 5 min) and the contents collected by centrifugation (13,000 *g*, 4°C, 2 min).

PCR amplification was carried out using 2 µl of the reverse transcription reaction mixture in a total reaction volume of 20 µl containing 1 x PCR amplification buffer (Takara, Japan), 2 µM 4dNTP mix, 1 µM degenerate anchor primer, 1 µl (10 µCi/µl)[α-^{32}P]-dCTP, 15 mM $MgCL_2$, 1U of Taq polymerase (Takara, Japan) and 2 µM arbitrary decamer (5' AGC CAG CGA A 3'). Amplification was carried out in a Hybaid Omnigene thermocycler (Hybaid, UK) with initial denaturation at 94°C for 2 min followed by 40 cycles of 94°C for 30 s, 40°C for 2 min, 72°C for 30 s, followed by 10 min at 72°C. PCR products were resolved on a 45 cm denaturing polyacrylamide gel 6% containing 6 M urea. 3.5 µl of the PCR products were mixed with formamide loading buffer heated (80°C, 2 min) and immediately loaded onto a pre-electrophoresed gel (50°C). Electrophoresis was performed for ~3h at 55 W. Gels were dried under vacuum on Whatman 3 MM paper and subjected to autoradiography overnight.

Recovery of differentially expressed gene sequences

A fragment was considered differentially expressed if differential expression between tumour and normal sample was observed in at least two patients. This was done by visually assessing the intensity of each band across the patient profiles by at least 2 independent observers. The cDNA was recovered by excision from the PAGE gel and ethanol precipitation. The recovered cDNA was PCR amplified using the conditions previously described by Liang et al., (1993). The PCR products were analyzed on a 2.0% agarose gel and the products recovered using Nucleospin elution columns (Macherey-Nagel, Germany) according to the manufacturer instructions.

Cloning and sequence analysis

Recovered PCR products were cloned into pGemT-easy vector (Promega, USA) according to the manufacturer instructions. Cloned PCR products were sequenced using the dideoxy chain termination method (Sanger et al., 1977) using the T*7* sequenase quick-denature plasmid sequencing kit (USB Corporation, USA). Sequence reactions were separated on a 6% polyacrylamide gel containing 7 M urea as described above. Sequences were read manually and homologous sequences searched in Genbank and EMBL databases using the program BLAST (Altschul et al., 1990).

Northern analysis

1 µg of total RNA per sample was electrophoresed on a 1% agarose gel containing 8% formaldehyde. The RNA was transferred onto Hybond-N nylon membranes (Amersham, UK) overnight using the sandwich method (Sambrook et al., 1989). Nucleic acids were fixed to the membrane by ultraviolet cross-linking using a spectrolinker, XL-1000UV crosslinker (Spectronics Corporation, USA). Probes were labelled with [α^{32}P]-dCTP using the megaprime DNA labelling system (Amersham, UK) according to the instructions of the manufacturer. Radiolabelled probe was hybridized to the membrane using a concentration of 1-2 x 10^6 cpm/ml of hybridization buffer. Hybridization was performed at 42°C for 16 h using 10 ml of UltraHyb hybridization buffer (Ambion, USA). The membranes were washed using 2 changes of 2x SSC, 0.1% SDS at 42°C for 15 min each followed by 2 high stringency washes of 0.1 x SSC, 0.1% SDS at 65°C for 15 min. The total radioactivity of each band was determined using the Instant Imager 2024 electronic autoradiograph (Parkard, USA). Beta actin was used as an internal control.

Immunohistochemistry

Samples analyzed included, tumour sections (2 µM thickness), corresponding adjacent normal oesophageal epithelium and 5 patients with Dysplasia. Human skin tissue sections were used as a positive control (Roark et al., 1995). For the negative control, the incubation of tissue sections without the primary antibody was carried out. Tissue sections were dewaxed in 4 changes (5 min each) of xylol and rehydrated in 4 changes (5 min each) of 96% ethanol. After a brief rinse in distilled water, the fibulin-1 antigen was retrieved by digesting the sections in 1% trypsin in 50 mM Tris-HCl (pH 7.6) for 5 min at 37°C. The trypsin was removed by rinsing twice (2 min each) in phosphate buffered saline (PBS). The sections were blocked for endogenous peroxidase using 1% hydrogen peroxide (in methanol) for 20 min, followed by a 2 min rinse in PBS. Sections were subsequently blocked for non-specific binding by incubation in 5% normal rabbit serum (in PBS) for 10 min. Incubation with the primary antibody, goat anti-fibulin-1 (Santa Cruz Biotechnology, USA), detection using the secondary antibody and colour development was carried out in an automated DAKO universal stainer using the IHC software (DAKO, Denmark). Briefly, the sections were incubated with the primary antibody (1:30 dilution) for 30 min at room temperature. After 2 washes with PBS, they were incubated with rabbit anti-goat serum (1:400 dilution) (DAKO, Denmark) for 30 min. The antibody was removed with 2 rinses in PBS and overlayed with Avidin horseradish peroxidase (1:500 dilution) (DAKO, Denmark) for 30 min. The chromogenic substrate, diaminobenzene was added and the colour developed for an additional 30 min. The stained slides were removed from the stainer and briefly counterstained in hematoxylin, mounted in Entellen (Merck, Germany) and examined under an Axiophot microscope (Zeiss, Germany) using Leica Image Manager Version 2.0 software.

Criteria were developed for quantitating the immunoreactivity of the fibulin stain in both the normal and tumour section using a score range of 0 to 3. A value of 0 indicated 0 - 25% of area stained; +1, 25-50%; +2, 50-75% and +3 >75% stained. Similarly, the same score range was used for none stained or none expressing cells. Each location (membrane or cytoplasm) was scored individually in 5

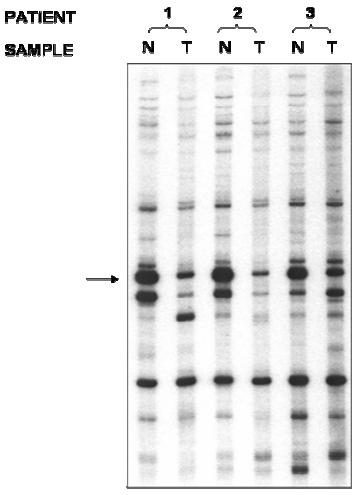

Figure 1. Differential display RT-PCR using oligo TG and arbitrary primer. Total RNA was reverse transcribed using a degenerate oligo TG primer followed by PCR with the same primer and a 10 mer arbitrary decamer. The products were resolved on a denaturing 6% polyacrylamide gel. The differentially expressed gene corresponding to Fibulin-1D is indicated by the arrow. Abbreviations: N, normal tissue; T, tumour biopsy.

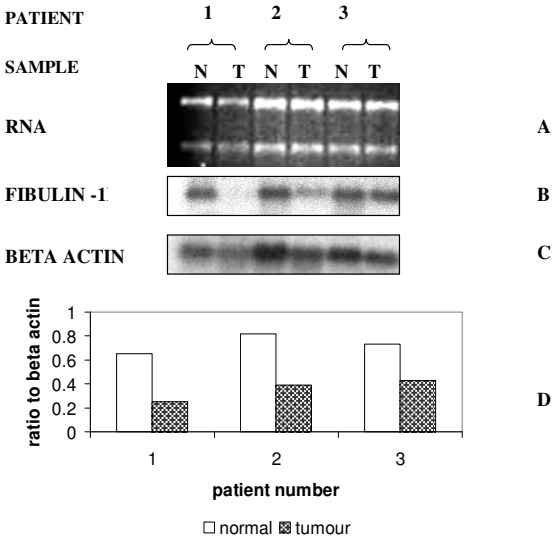

Figure 2. Expression of fibulin-1 in normal and tumour biopsies. RNA from normal (N) and tumour (T) specimens of the different patients were separated on a formaldehyde 1% gel (A) and transferred to nylon membranes as described in the materials and methods section. Radiolabelled fibulin-1 probe was hybridized to the membrane overnight, washed and exposed to x-ray film (B). Beta actin was used as an internal control (C). The final radioactive counts of each sample were determined using a phosphoimager (D) as described in the materials and methods section.

randomly selected fields. The scoring was carried out under x10 magnification eye objective. Two investigators of whom one was a pathologist performed the scoring. This evaluation was carried out blind with respect to tumour stage and grade.

Statistical analysis

The relationship between the amount of the protein expressed and the tissue type; normal or tumour was analysed using the Kruskl Wallase test. In case of tumour, 3 grades namely well, moderately and poorly differentiated cancer cells were analyzed. In addition, a precancerous condition - dysplasia was included in the analysis. Differences were considered significant at $p<0.05$.

RESULTS

Down regulation of fibulin-1 mRNA expression in oesophageal cancer tissues

Differential display RT-PCR allowed us to identify a cDNA fragment that was differentially expressed in oesophageal cancer tissue and adjacent normal tissue obtained from oesophageal cancer patients (Figure 1). The differentially expressed fragment was isolated, PCR re-amplified and sequenced. BLAST analysis indicated that the cDNA fragment corresponded to the 3' end of the fibulin-1D gene (Accession number G6AD126110). Down-regulation of the fibulin-1D gene was confirmed by Northern blot analysis using RNA isolated from matched (tumour and normal) biopsies from 3 different oesophageal cancer patients (Figure 2). Fibulin-1 was down regulated by factors of 2.6, 2.1, and 1.7 for patients 1, 2 and 3, respectively. These results clearly demonstrated for the first time that fibulin-1D is expressed in the normal human oesophagus, however, this expression is significantly down regulated in oesophageal cancer tissue.

Immunohistochemistry (IHC)

Since the RNA analysis could be done on a limited number of patients only, immunohistochemical analysis on paraffin sections was performed on a larger number of tissues. In addition to tumour and normal sections, dysplastic sections were included in the study for comparison. The positive control, sebaceous glands of the skin positively stained for fibulin-1 (data not shown).

This observation has been previously reported (Roark et al., 1995). The negative controls (tissue sections that were incubated without the primary antibody) failed to stain (data not shown).

Fibulin-1 was limmunolocalized in both the membrane and cytoplasm of maturing or differentiating cells of normal oesophageal epithelial cells (Figure 3). This observation is in agreement with previously reported studies using organs such as human lungs, skin, brain, cervix,

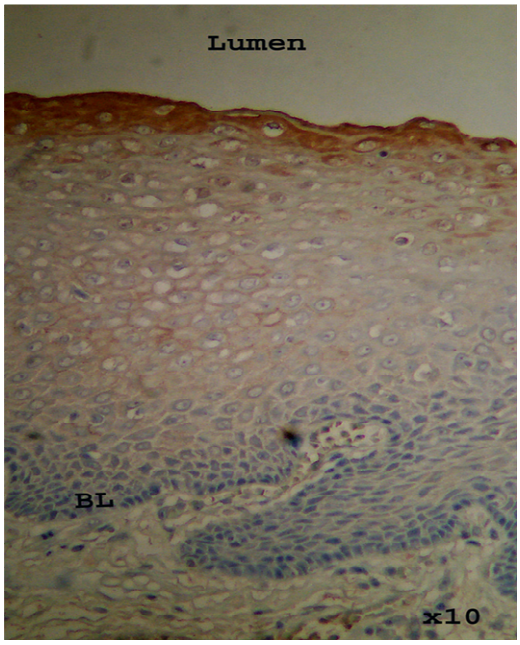

Figure 3. Normal squamous epithelium of the oesophagus. The cytoplasm and membrane of the maturing epithetlial cells stained positively for fibulin-1 protein while the basal layer did not stain. The magnification used is x10 of the original magnification. Abbreviations: BL, basal layer cells; and Lumen, lumen of the oesophagus.

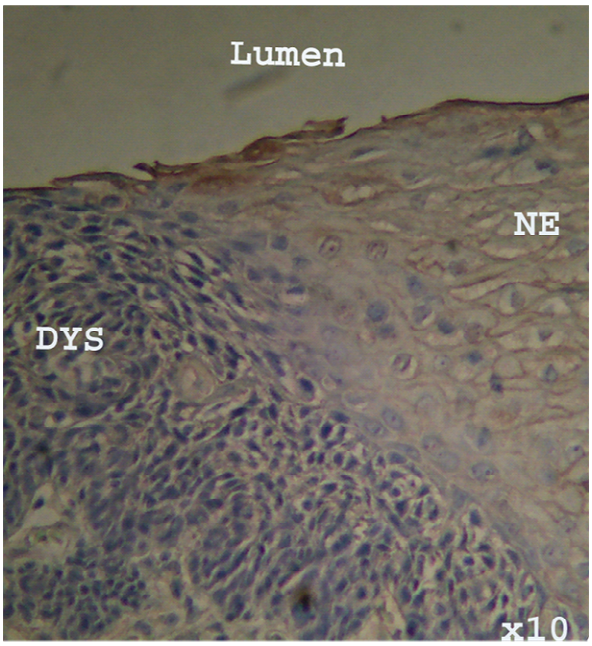

Figure 4. Dysplasia of the oesophagus. Dyspalstic cells adjacent to normal squamous epithelial cells. The dysplastic cells failed to stain for fibulin-1D. X10 of original magnification. Abbreviations: DYS (dysplastic cells), NE (normal epithelial cells) and Lumen (lumen of the oesophagus).

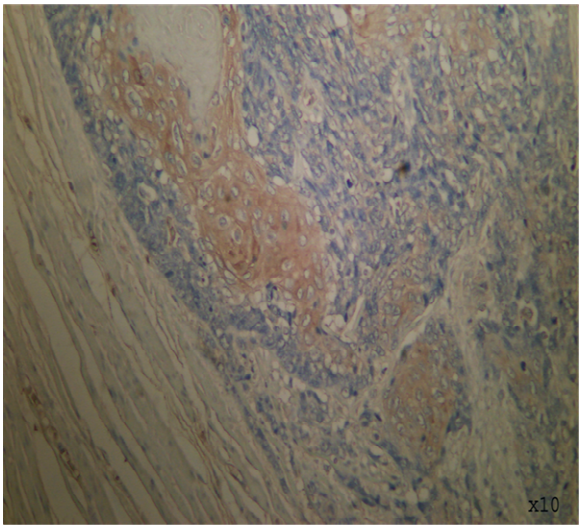

Figure 5. Well differentiated squamous cell carcinoma. Fibulin-1D was localized in the membrane and cytoplasm of well differentiated cancer cells. X10 of the original magnification.

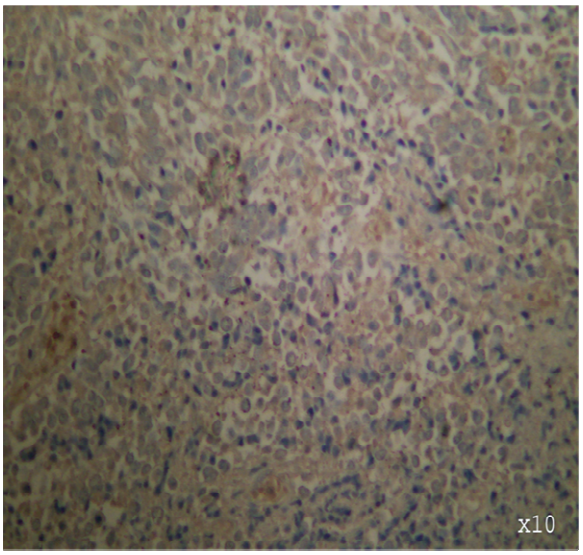

Figure 6. Poorly differentiated squamous cell carcinoma. Poorly differentiated cells show reduced staining for fibulin-1D protein compared to the normal epithelial cells. X10 of the original magnification.

heart and kidney (Roark et al., 1995). The cells of the basal layer of the normal epithelium of the oesophagus failed to stain for fibulin-1 (Figure 3). The same was observed in dysplastic cells (Figure 4). Well-differentiated tumour cells stained positively for fibulin-1 (Figure 5) while poorly differentiated tumour cells displayed little or no fibulin-1 staining (Figure 6) compared to both the normal oesophageal epithelium cells and well-differentiated

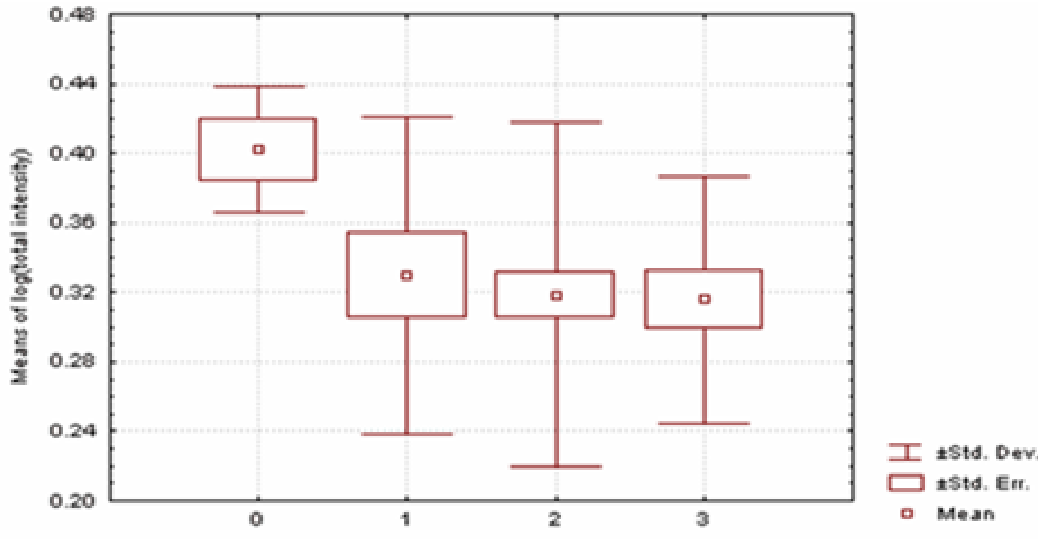

Figure 7. The relationship between the amount of fibulin-1D protein and the various cell types. The mean log of the total protein scored was plotted verses the different cell types studied using the box plot. There was a progressive reduction in the amount protein from normal oesophageal epithelial cells to poorly differentiated squamous cell carcinoma. Abbreviations: 1 (normal oesophageal epithelial cells), 2 (well differentiated squamous cell carcinoma), 3 (moderately differentiated squamous cell carcinoma) and 4 (poorly differentiated squamous cell carcinoma). Abbreviations: Std. Dev., standard deviation; and Std. Err., standard error.

tumour cells. Moderately differentiated tumour cells stained positively for fibulin-1 (data not shown).

Statistical analysis of the amount of fibulin-1 protein expressed in the normal oesophageal epithelial cells, well differentiated and poorly differentiated tumour cells revealed a significant and progressive reduction (in descending order) in fibulin-1 expression from the normal tissue, well differentiated, moderately differentiated to poorly differentiated tumour cells (Figure 7). These results suggest that fibulin-1 is progressively down regulated in oesophageal cancer and this down regulation correlates with the grade of the tumour.

DISCUSSION

Using differential display RT-PCR, this study identified fibulin-1D as one of the genes that are significantly down regulated in oesophageal cancer tissues compared to normal oesophageal epithelial cells. Previous studies have shown fibulin-1 proteins to be multi-modular proteins with a consensus motif for calcium binding (Argraves et al., 1990; Pan et al., 1993). Their ability to interact with themselves, bind either directly or indirectly with cell surface receptors and important extracellular matrix components underscores their importance in the regulation of cell behaviour. Fibronectin, one of the important components to which fibulin binds, has been shown to be involved in the cell motility. For example, studies using fibroblasts have shown that cells add fibulin-1, which is either endogenously synthesized or exogenously added, into extracellular matrix fibrils that also contain fibronectin. Addition of fibronectin matrix antagonist blocks this incorporation (Godyna et al., 1995). Further studies by Twal et al. (2001) demonstrated that the rate of cell migration on fibronectin-fibulin-1 matrix was slower than on fibronectin matrix alone suggesting that fibulin is an inhibitor of *in vitro* cell adhesion and motility. Similarly, fibulin-1 has been shown to inhibit the migration of fibronectin-induced BG-1 cells *in vitro* (Hayashido et al., 1998). Furthermore constitutive over expression of fibulin-1 in tumour cells delays tumour formation *in vivo* and suppress anchorage-independent cell growth, motility and invasion *in vitro* (Qing et al., 1997; Hayashido et al., 1998). The above experiments clearly demonstrate that fibulin-1 is a negative regulator of cell motility. It is therefore probable that fibulin-1 plays an important role in tumour metastasis by preventing cell migration thus suppressing invasion of tumour cells into surrounding tissues. The down regulation of fibulin-1 in oesophageal cancer tissues supports the above observations and underscores the probable importance of fibulin-1 in tumourigenesis.

Immunohistochemical studies demonstrated the absence of fibulin-1 in the basal layer of the normal epithelium of the oesophagus. These results suggest that differentiating and maturing cell types rather than undifferentiated cells in the normal oesophageal epithelium secrete this

protein. It is probable that the fibulin-1 gene is only actively transcribed in actively differentiating cells of the normal epithelium. Dysplasia is characterized by cells proliferating from the basal layer without matching maturation during the process. If indeed dysplastic cells represent an enlarged population of sub-basal cells, the absence of fibulin-1 staining is not unexpected. This study found a consistent absence of fibulin-1 protein in all the different grades of dysplasia studied. Although the regulation of the fibulin-1 gene is not well documented, these results suggest that the factor(s) regulating the expression of fibulin-1 may essentially be the same in undifferentiated cell types. Since dysplasia is an early symptom in probable cancer development, it is plausible that fibulin-1 gene down regulation is an early event. A pertinent question is whether the mechanism of fibulin-1 gene deregulation is intrinsically linked to some of the important pathways that are known to be dysfunctional in the early stages of transformation of normal cells into tumour cells.

There was a statistically significant decrease in the amount of fibulin-1 protein expressed in the different grades of tumour cells compared to the normal oesophageal epithelial cells. This data confirms the results of differential display RT-PCR. Of interest however, is that the progressive under-expression of fibulin-1 protein from normal to well differentiated, moderately differentiated and finally to poorly differentiated tumour cells does not occur in a linear fashion. This suggests that the down regulation of the fibulin-1 gene does not occur in a linear fashion. Our data clearly demonstrates a correlation between the levels of fibulin-1 expressed and the grade of the tumour cells. What is of interest however, is whether the down regulation of fibulin-1 correlates with the expression levels and patterns of several fibulin-1 target proteins such as laminin and fibronectin. It is probable that the progressive down regulation of fibulin-1 gene and therefore the decrease in fibulin-1 amounts will affect the integrity of the extracellular matrix, influence cell adhesion and migration thus contributing positively to the progression of oesophageal cancer tumourigenesis.

In summary, by using differential display RT-PCR we identified fibulin-1D as a gene that is down regulated in human oesophageal cancer. We found that fibulin-1D protein is expressed at significantly lower levels in oesophageal cancer sections compared to normal ($p < 0.00001$). We demonstrated that fibulin-1 expression in both normal and cancerous oesophageal cells shows distinct patterns with total absence in dysplastic cells. These distinct expression patterns can be used to distinguish the dysplastic cells and the different grades of cancer cells from normal oesophageal epithelial cells. This study demonstrated a gradual reduction in fibulin-1 protein amounts in the different tumour grades heralding the probable importance of fibulin-1 in tumourigenesis and strengthening the important contribution of extra-cellular matrix components in cancer development.

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