Cancer Biomarkers 12 (2012/2013) 1–9
DOI 10.3233/CBM-120289
IOS Press

Evaluation of protein expression pattern of stanniocalcin 2, insulin-like growth factor-binding protein 7, inhibin beta A and four and a half LIM domains 1 in esophageal squamous cell carcinoma

Manoj Kumar Kashyap[a,b,g,h,1], Harsh A. Pawar[a,c,e], Shivakumar Keerthikumar[a,2], Jyoti Sharma[a,f], Renu Goel[a], Riaz Mahmood[b], M. Vijaya Kumar[d], K.V. Veerendra Kumar[d], Akhilesh Pandey[g,h,i,j,k,*], Rekha V. Kumar[c,e,*], T.S. Keshava Prasad[a,f,l,*] and H.C. Harsha[a,*]
[a]*Institute of Bioinformatics, International Technology Park, Bangalore, India*
[b]*Department of Biotechnology, Kuvempu University, Shimoga, India*
[c]*Rajiv Gandhi University of Health Sciences, Bangalore, India*
[d]*Department of Surgical Oncology, Kidwai Memorial Institute of Oncology, Bangalore, India*
[e]*Department of Pathology, Kidwai Memorial Institute of Oncology, Bangalore, India*
[f]*Manipal University, Madhav Nagar, Manipal, Karnataka, India*
[g]*McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA*
[h]*Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD, USA*
[i]*Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA*
[j]*Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD, USA*
[k]*Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD, USA*
[l]*Centre of Excellence in Bioinformatics, School of Life Sciences, Pondicherry University, Pondicherry, India*

Abstract. The pathogenesis of esophageal squamous cell carcinoma (ESCC) involves both genetic and environmental factors. Previously, we have carried out gene and protein expression profiling of ESCC using DNA microarrays and mass spectrometry-based quantitative proteomics, respectively. These studies resulted in identification of several potential biomarkers of ESCC, some with known reports of differential expression in the scientific literature and others that were novel observations from our studies. We report systematic validation of selected markers from our studies on a larger cohort of cancer tissue sections by immunohistochemical labeling of tissue microarrays. We have validated expression of insulin-like growth factor-binding protein 7 (*IGFBP7*), stanniocalcin 2 (*STC2*), inhibin beta A (*INHBA*) and four and a half LIM domains 1 (*FHL1*). Immunohistochemical labeling with anti-stanniocalcin 2 antibody demonstrated its overexpression in 132/140 (94%) cases, *IGFBP7* showed overexpression in 127/140 (91%) cases and overexpression of *INHBA* was observed in 62/105 (59%) of ESCC cases. In contrast, *FHL1* expression was observed only in 12/143 (8%) of ESCC cases suggesting its possible involvement in tumor suppression. These data suggest that *IGFBP7*, *INHBA*, *STC2* and *FHL1* might play an important role in ESCC tumorigenesis, which can be explored in future studies. Overall, our findings open up new avenues for development of novel therapeutics and/or diagnostic approaches in ESCC.

Keywords: Invasion, tumor suppression, esophageal adenocarcinoma, extracellular matrix

*Corresponding author: H.C. Harsha, Institute of Bioinformatics, International Technology Park, Bangalore, 560066, India. E-mail: harsha@ibioinformatics.org.
[1]Current address: Moores Cancer Center, University of California San Diego, La Jolla, CA 92093-0960, USA.
[2]Current address: Centre for Molecular and Biomolecular Informatics, and Nijmegen Centre for Molecular Life Sciences, Radboud, University, Nijmegen Medical Centre, Nijmegen, The Netherlands.

ISSN 1574-0153/12/13/$27.50

1. Introduction

Cancer of the esophagus is among the top ten cancers worldwide and constitutes 7% of total gastrointestinal cancers [1]. Tobacco and alcohol are important risk factors for development of esophageal squamous cell carcinoma (ESCC). In the past, we have carried out DNA microarrays as well as quantitative proteomics as a high-throughput screening tool to identify differentially expressed genes. These high-throughput studies have resulted in identification of several genes that show differential expression pattern. Although these studies provide potential markers, it requires extensive validation in large number of clinical specimen to truly establish its expression pattern, distribution as well as its potential utility as a marker. This often is done on a limited set of molecules based on the availability of appropriate antibodies or probing reagents and also the broader focus of the manuscript itself. This way, several potential markers that do get identified in high-throughput studies remain as part of large datasets without appropriate validation to reflect their utility and significance.

In the current study, we have selected four such molecules – INHBA, IGFBP7, STC2 and FHL1 that were identified in our previous high-throughput study and demonstrated their protein expression pattern in a large set of ESCC tissue sections, using tissue microarray platform [2]. These molecules were selected because they showed differential expression pattern in most of the ESCC cases we had studied using DNA microarrays, their biological importance based on the information from other cancers as well as availability of antibodies suitable for immunohistochemical labeling. Most importantly, the expression pattern of these molecules has not been systematically investigated in ESCC in the past.

2. Materials and methods

2.1. Tissue samples

For validation of potential candidates from the gene expression study, we used tissue microarrays (TMAs). The details of TMAs has been described in our earlier study [5]. Ethical clearance was obtained for use of archived samples from the Kidwai Memorial Institute of Oncology (KMIO), Bangalore. Immunohistochemical labeling was done as described earlier [2]. Correlation of expression of molecules and the differentiation status of the tumors was assessed by an experienced pathologist (RVK) [5].

2.2. Antibodies

Anti-IGFBP7 antibody was used at a dilution of 1:50 (catalog # HPA002196, the Human Protein Atlas, Stockholm, Sweden). Anti-STC2 was used at a dilution of 1:500 (catalog # 10314-1-AP, Proteintech Group, Inc., Chicago, IL). Anti-FHL1 was used at a dilution of 1: 50 (catalog # 10991-1-AP, Proteintech Group, Inc., Chicago, IL). Anti-INHBA was used at a dilution of 1:50 (catalog # 10651-1-AP, Iowa, Iowa City, IA). An isotype control anti-serum or PBS was used as negative controls in all immunohistochemical labeling experiments. The tissue microarrays were scored by an experienced pathologist (RVK). The staining intensity was scored as negative (0), mild (0.5+ to 1.5+), moderate (2+), or strong (3+). A comparison between the intensity of the staining of normal esophageal epithelium and that of carcinoma cells was made.

2.3. Statistical analysis

The statistical analysis for association between the distribution of expression pattern of *STC2, IGFBP7*, *INHBA* and *FHL1* with ESCC and normal tissues was performed using χ^2-test.

3. Results and discussion

We have previously reported a total of 2,235 differentially expressed genes in a transcriptomic analysis of ESCC [2]. Of these, 881 genes were upregulated and 1,354 genes were downregulated. In this study, we selected genes involved in cell communication based on their biological importance in different biological processes, presence of signal peptide motif and the availability of suitable antibodies for IHC labeling. Of the validated genes, IGFBP7, INHBA and STC2 contain signal peptides and are involved cell communication process. FHL1 is an adaptor protein involved in cell growth and/or maintenance. These genes were differentially expressed between normal and tumors of the same patients. The log2 fold values for twenty such cases are shown in Fig. 1. Immunohistochemical labeling for the selected proteins was done using tissue microarrays.

Table 1
Summary of immunohistochemical evaluation of STC2, FHL1, IGFBP7 and INHBA

	STC2		FHL1		IGFBP7		INHBA	
	Normal	ESCC	Normal	ESCC	Normal	ESCC	Normal	ESCC
Negative (0)	60	8	26	131	26	13	1	43
Mild (1+)	34	17	73	10	51	29	8	21
Moderate (2+)	9	46	21	2	14	74	31	31
Strong (3+)	0	69	0	0	03	24	20	10
Total	103	140	120	143	94	140	60	105
p-value	7.26E-019		4.60E-03		0.0004341		1.12E-007	

Distribution of staining pattern and statistical analysis for STC2, IGFBP7, FHL1 and INHBA in esophageal squamous cell carcinoma.

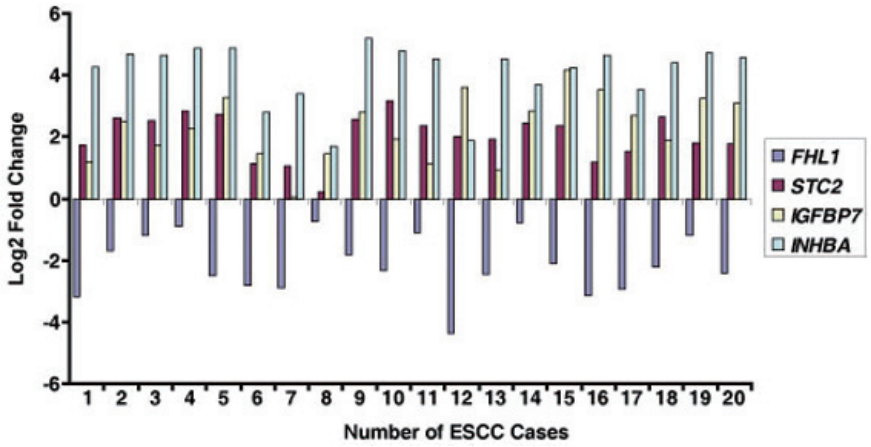

Fig. 1. Log2 distribution of fold change among different ESCC patients. The figure shows the log2 distribution of mRNA expression levels of *STC2*, *IGFBP7*, *INHBA* and *FHL1* among different ESCC patients. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/CBM-120289)

3.1. Validation of selected candidate genes

3.1.1. Stanniocalcin 2 is overexpressed in majority of esophageal squamous cell carcinomas

In our gene expression studies, STC2 was found to be overexpressed in 19 of the 20 ESCC cases. STC2 encodes a 32 kDa extracellular matrix protein bearing a signal peptide [7]. It is known to have important roles in many physiological processes including bone development, reproduction, wound healing, angiogenesis, and modulation of inflammatory response. Elevated expression of STC2 has been reported in various cancers including renal cell carcinoma [8], breast cancer [9], gastric cancer [10], prostate cancer [11] and neuroblastoma [12]. In addition, Kita et al., have reported STC2 as a predictive marker for lymph node metastasis in ESCC based on mRNA measurements in 70 clinical samples [13]. In the same study, immunohistochemical labeling of STC2 was carried out on 10 cases that showed significantly high STC2 expression. It was reported that STC2 showed positive staining in both cytoplasm as well as nuclei of cancer cells.

To determine STC2 protein expression pattern in a larger cohort of ESCC cases, we carried out immunohistochemical labeling in 140 cases. STC2 expression was found to be elevated in 132/140 (94%) of ESCC cases. In 115 cases, the intensity of the staining was $> 2+$ as compared to the normal cases where it was only seen in 8 cases with similar intensity. The staining pattern for STC2 in representative ESCC and normal sections is shown in Fig. 2. The architecture of STC2 protein is shown as described in the Human Protein Reference Database (HPRD; www.hprd.org). *STC2* is reported to be a target gene of HIF-1 transcription factor and is known to promote cell proliferation in hypoxic conditions [14]. STC2 was also found to promote epithelial-mesenchymal transition (EMT) and invasiveness in hypoxic human ovarian cancer cells [9]. STC1, a related gene had also been recently reported to be overexpressed in ESCC [5]. In cell line based studies, marked increase in STC1 expression was observed in hypoxic conditions. In the same study, STC1 has been proposed as a potential prognostic marker in esophageal cancer patients who have undergone curative surgery.

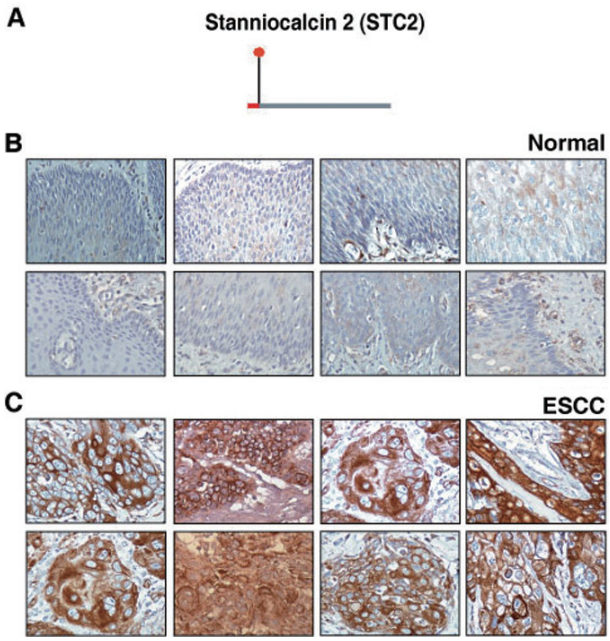

Fig. 2. STC2 expression in a group of normal and ESCC sections. Panel A show the domain architecture of STC2 protein as annotated in the Human Protein Reference Database (HPRD). The red color indicates the signal peptide and the racket shape structure shows phosphotyorsine site at Tyr28 residue. Panel B shows expression of *STC2* in normal, where the staining is either absent or very weak in the basal epithelium. Panel C shows expression of *STC2* among different ESCC tumors and its expression in stromal region and epithelial carcinoma cell as well. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/CBM-120289)

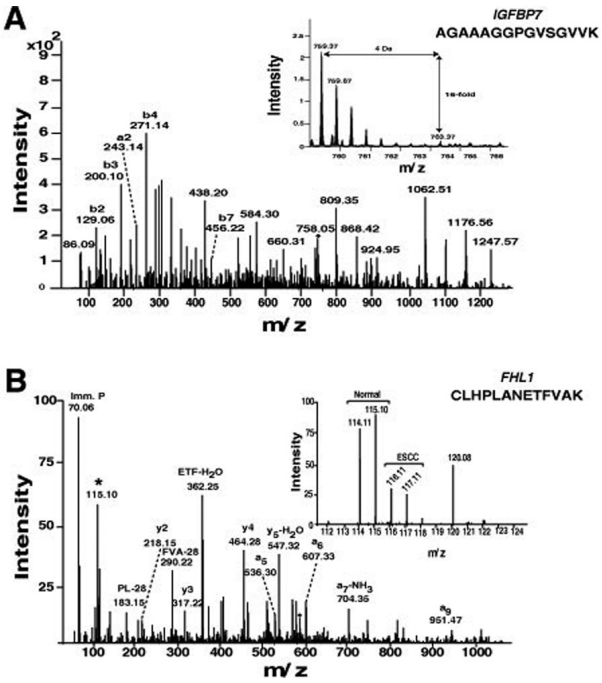

Fig. 3. MS and MS/MS for Insulin Growth Factor Binding Factor 7 and Four and a half LIM domains 1. Panel A shows MS and MS/MS for *IGFBP7* where it was 16-fold upregulated in ESCC derived secretome as compared to normal in a SILAC based experiment. Panel B shows MS and MS/MS for *FHL1* where it was 4-fold downregulated in ESCC as compared to the pooled adjacent normal epithelial in an iTRAQ based experiment. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/CBM-120289)

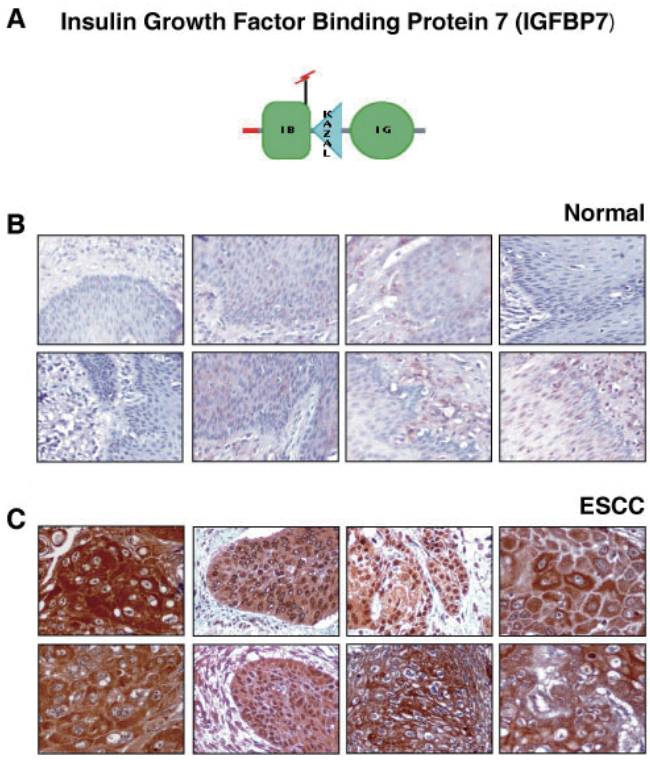

Fig. 4. IGFBP7 expression in a group of normal and ESCC sections. Panel A shows the domain architecture of IGFBP7 from Human Protein Reference Database (HPRD). This protein consists of red color indicates the signal peptide while the green colored rectangular and round structures indicate Insulin growth factor-binding protein homologues and Immunoglobulin domains, respectively. The blue colored triangular structure indicates the Kazal type serine protease inhibitors domain. The green rectangle indicates insulin growth factor-binding protein homologues (IB) domain and the other green circle indicates the immunoglobulin (IG) domain. Panel B showing expression of *IGFBP7* in normal, where the staining is either absent or very weak in the basal epithelium. Panel C is showing expression of *IGFBP7* among different ESCC tumors, and its nuclear localization in the epithelial carcinoma cell. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/CBM-120289)

3.1.2. IGFBP7 expression in ESCC

IGFBP7 belongs to the IGFBP family, whose functions include binding to insulin-like growth factors (IGF) and regulating their bioavailability, as well as a range of IGF-independent biological activities. IGFBP7 is a secreted protein. It has been reported as a novel marker for acute leukemia [16]. In acute myeloid leukemia (AML), IGFBP7 was found to be co-expressed with the BAALC (brain and acute leukemia, cytoplasmic) gene. Furthermore, high IGFBP7 level was associated with stem cell features and treatment failure in T-ALL [16]. There are contradictory studies in the literature regarding the role of IGFBP7: In some studies, it has been reported as a tumor suppressor [17,18], while in others as an oncogene. In our previous study, IGFBP7 was found to be upregulated in the secretome derived from ESCC cell lines as compared to normal esophageal epithelial cells as shown in Fig. 3 [4]. IGFBP7 stained positive in 127/140 (90.7%) ESCC cases. In our study the localization of IGFBP7 protein was found to be cytoplasmic and nuclear. IGFBP7 has been reported to be upregulated greater than 4-fold in Barrett's esophagus and esophageal adenocarcinoma as compared to normal tissues [19]. Expression of IGFBP7 showed weak immunoreactivity in tumor tissue as compared to the adjacent normal. The localization of IGFBP7 was found to be cytoplasmic and in $>$ 50% of the cells [20]. Some reports suggest that expression of IGFBPs is regulated by hypoxia [21]. In hypoxic condition, IGFBP3 & IGFBP1 expression was reported to be induced but IGFBP4 was unaffected [22]. In the case of IGFBP7, not much has been explored in ESCC so far. There is certainly a need to delineate the detailed functional and pathological aspect of IGFBP7 in ESCC specially in light of the recent study where IGFBP3 (another member of the same family) overexpression has been reported to be associated with prolonged overall survival [23]. The staining pattern for IGFBP7 is shown in representative ESCC and normal sections in Fig. 4.

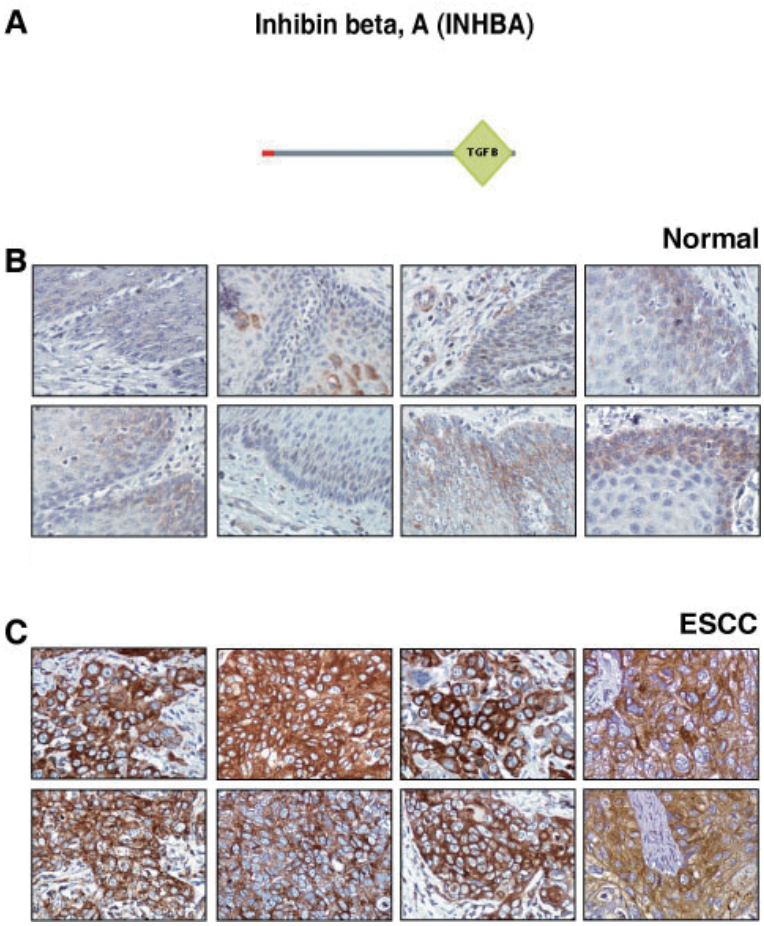

Fig. 5. INHBA expression in ESCC as compared to adjacent normal epithelial. Panel A shows the domain architecture of the protein INHBA. The red color indicates the signal peptide motif. The green square shape structure indicates the presence of the Transforming growth factor-beta (TGF-beta) domain. Panel B showing expression of INHBA in normal, where the staining is strong in the epithelial squamous cells. Panel C showing expression of INHBA among different ESCC tumors, and its expression was either weak or absent in majority of the sections. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/CBM-120289)

3.1.3. Inhibin beta A is overexpressed in a subset of esophageal squamous cell carcinomas

Inhibin beta A (*INHBA*) was found to be overexpressed in 19 of 20 ESCC cases in our gene expression profiling study [2]. INHBA is a subunit of the complex that inhibits pituitary FSH secretion. Overexpression of INHBA is reported in various cancers including gastric cancer [20,24,25], glioblastoma [26], lung adenocarcinoma [27], esophageal adenocarcinoma [28], tongue squamous cell carcinoma [20], head and neck squamous cell carcinoma [29] and pancreatic adenocarcinoma [30]. Overexpression of INHBA is associated with poor survival in gastric cancer [27], lung adenocarcinoma [27] and head and neck squamous cell carcinomas [29].

Immunohistochemical labeling showed positive staining for INHBA in 62/105 ESCC cases. The staining pattern in majority of the cases was cytoplasmic and membranous. Figure 5 shows representative tissue sections stained for INHBA.

3.1.4. FHL1 protein expression is suppressed in majority of esophageal squamous cell carcinomas

FHL1 is a putative tumor suppressor gene known to negatively regulate tumor growth. Decreased expression of FHL1 is reported in various cancers including gastric [31,32], lung [33], thyroid [34], breast, kidney and prostate cancer [35]. Silencing of FHL1 gene expression in most tumors is reported to be mainly through promoter DNA methylation [36,37]. FHL1 including other members of the same protein family are

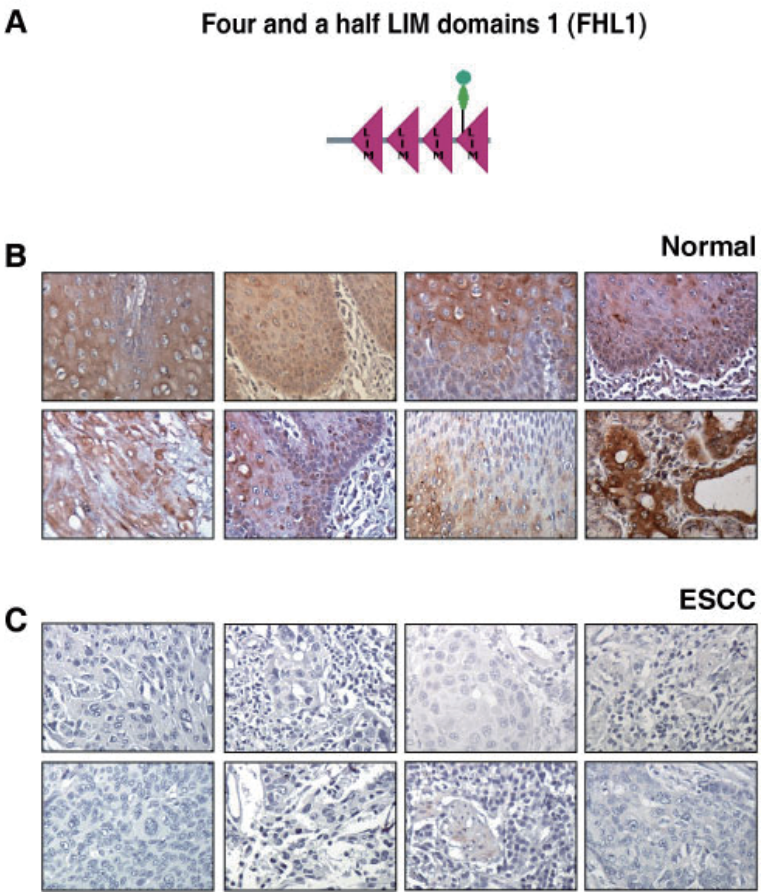

Fig. 6. FHL1 downregulation in ESCC as compared to adjacent normal epithelial. Panel A shows the domain architecture of FHL1 protein. The purple triangle denotes four LIM domains. The green and blue colored shapes indicate a post-translational modification (PTM) of lysine (K) residue at position 233. Panel B showing expression of *FHL1* in normal, where the staining is strong in the epithelial squamous cells. Panel C showing expression of *FHL1* among different ESCC tumors, and its expression was either weak or absent in majority of the sections. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/CBM-120289)

known to be negative regulators of HIF-1 activity [6]. This is thought to be a potential mechanism by which FHL1 suppresses tumor growth. FHL1 expression is inversely related to invasion and metastatic potential of cancer cells [32]. We observed downregulation of FHL1 in ESCC in both gene expression profiling as well as quantitative proteomics studies [3]. In quantitative tissue proteomics study, FHL1 was found to be 4-fold downregulated in ESCC as compared to the adjacent normals [3]. The MS and the MS/MS data is shown in Fig. 3. A total of 131/143 (91.6%) ESCC cases showed no staining for FHL1. Conversely, FHL1 staining was observed in 94/120 (78.33%) normal cases. Among ESCC tumors, only 12 out of 143 (8.4%) were positive for FHL1 staining. The staining pattern for FHL1 staining has been shown in Fig. 6. The staining pattern in majority of the cases was cytoplasmic.

In summary, it is possible that these newly discovered molecules provide enhanced sensitivity for detection of ESCC at an early stage or to serve as therapeutic targets for treatment. They could also be used for monitoring of disease if they can be detected in body fluids such as serum or urine. Further validation will be required to pursue these possibilities.

Acknowledgement

We thank the Department of Biotechnology (DBT), Government of India for research support to the Institute of Bioinformatics. MKK was supported with Independent Sr. Research Fellowship from the Indian Council of Medical Research (ICMR), New Delhi. T. S. K. Prasad and Rekha V. Kumar are supported by

a DBT grant (DBT/CSH/GIA/1583/2010-2011). Harsha Gowda is a Wellcome Trust/DBT India Alliance Early Career Fellow. Harsh Pawar and Jyoti Sharma are recipients of the Senior Research Fellowship from Council of Scientific and Industrial Research (CSIR), Government of India.

4. Conflict of interest

There is no conflict of interest associated with this study.

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