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Identification of prosaposin and transgelin as potential biomarkers for gallbladder cancer using quantitative proteomics

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ABSTRACT

Gallbladder cancer is an uncommon but lethal malignancy with particularly high incidence in Chile, India, Japan and China. There is a paucity of unbiased large-scale studies investigating molecular basis of gallbladder cancer. To systematically identify differentially regulated proteins in gallbladder cancer, iTRAQ-based quantitative proteomics of gallbladder cancer was carried out using Fourier transform high resolution mass spectrometry. Of the 2575 proteins identified, proteins upregulated in gallbladder cancer included several lysosomal proteins such as prosaposin, cathepsin Z and cathepsin H. Downregulated proteins included serine protease HTRA1 and transgelin, which have been reported to be downregulated in several other cancers. Novel biomarker candidates including prosaposin and transgelin were validated to be upregulated and downregulated, respectively, in gallbladder cancer using tissue microarrays. Our study provides the first large scale proteomic characterization of gallbladder cancer which will serve as a resource for future discovery of biomarkers for gallbladder cancer.

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1. Introduction

Gallbladder cancer (GBC) is a relatively uncommon but aggressive malignancy with a high mortality rate. It is the fifth most common cancer of gastrointestinal tract and the most common malignancy of the biliary tract. More than 80% of gallbladder cancers are adenocarcinomas of epithelial origin [1]. Marked geographical variation is observed in the incidence of GBC. Countries such as Chile, India, Japan and China have a high incidence of GBC. The highest incidence is reported in women from north India (21.5/100,000), while the highest mortality is associated with GBC reported in southern Chilean inhabitants (35/100,000) [1,2]. Ethnicity is one of the key factors associated with incidence of GBC [3]. Incidence of GBC is higher in women than men and it increases with age [1]. Major risk factors for GBC include gallstones which are reported to be associated with 60–90% of GBC cases [4].

Molecular pathogenesis of GBC is poorly understood. Limited studies have investigated altered protein expression in GBC using

Abbreviations: GBC, gallbladder cancer; PSAP, prosaposin; TAGLN, transgelin.

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immunohistochemistry. Gene alterations of K-ras, p53, and p16 have been reported to be important steps in dysplasia [5]. Few global expression studies on GBC were reported in literature. Alvarez et al. used serial analysis of gene expression and identified connective tissue growth factor as a prognostic marker for GBC [6]. Gene expression analysis carried out by Kim et al., revealed that early and advanced GBC show genetic similarity [7]. A proteomic study by Wang et al., investigated the expression profiles of low and high metastatic cell lines identifying 24 proteins to be upregulated in highly metastatic cell lines including chloride intracellular channel 1, ezrin and vimentin [8]. Recently, S100A10 and haptoglobin were identified to be significantly upregulated in serum of gallbladder cancer and associated with poor prognosis [9].

Elevated levels of serum carcinoembryonic antigen (CEA), CA 19-9 and CA-125 are proposed markers for GBC. However, they have limited utility owing to low specificity and sensitivity [10,11]. Thus, there is a lack of sensitive and specific biomarkers for gallbladder cancer for early detection of the disease. Currently, surgical resection is the only curative modality for early stages of GBC. In the majority of cases, the tumor has already metastasized at the time of diagnosis. The five year survival for GBC is only 1–10% in Indian patients, most being diagnosed at an advanced stage [3]. Proteomic studies to understand altered proteome profile of the disease are warranted due to lack of diagnostic markers and limited understanding of the molecular basis of the disease.

In this study, we employed iTRAQ-based quantitative proteomic approach to identify differentially regulated proteins in GBC. Technical replicate analysis was carried out using high resolution mass spectrometry leading to identification of 2575 proteins. Of these, 286 proteins were upregulated ($\geqslant$1.5-fold), which included several lysosomal proteins including prosaposin and cathepsins. The downregulated proteins included transgelin and neurofilament heavy polypeptide. These proteins have been previously reported to be downregulated in various cancers including hepatobiliary malignancies. We constructed tissue microarrays for validation of prosaposin and transgelin, which were found to be significantly upregulated and downregulated, respectively. Our study provides the first high resolution quantitative proteome profile of gallbladder cancer. Quantitative proteomics coupled with tissue microarray based validation provides a robust pipeline for discovery and preliminary validation of potential biomarkers for GBC.

2. Materials and methods

2.1. Tissue collection and sample preparation

Gallbladder tissue samples were collected after cholecystectomy from Cancer Hospital and Research Institute, Gwalior, India with approval from Institutional Human Ethics Committee and informed consent of the patients. The ten patient details are listed in Supplementary Table 1.

About 25 mg of each tissue sample was homogenized in 0.5% SDS using a Dounce homogenizer followed by sonication. Following protein quantification using Lowry's assay, equal amounts of protein lysates from 10 gallbladder adenocarcinoma were mixed to have a pooled cancer lysate. Similarly, equal amount of protein lysates from 10 adjacent non-tumor samples were mixed to have a pooled non-tumor lysate. The pooled lysates were split in equal amounts and used as technical replicates.

2.2. iTRAQ labeling and SCX fractionation

In-solution trypsin digestion and iTRAQ labeling were carried out essentially as described previously [12]. Peptides from technical replicate from adjacent non-tumor samples were labeled with iTRAQ labels 114 and 115 and gallbladder adenocarcinoma were labeled with iTRAQ labels 116 and 117. Labeling was carried out at room temperature for 1 h and was quenched using water.

SCX fractionation was performed as described earlier [12]. Pooled iTRAQ labeled samples were mixed with SCX solvent A (10 mM potassium phosphate buffer in 20% ACN, pH 2.8). PolySULFOETHYL A column (PolyLC, Columbia, MD) (200 Å, 5 μm, 200 × 2.1 mm) was coupled to 1200 Series LC (Agilent Technologies, Santa Clara, CA). Samples were washed for 10 min using 100% solvent A. Linear gradient of 5–50% solvent B (350 mM KCl in solvent A, pH 2.85) was applied for 40 min. Twenty-eight fractions were collected and desalted using stage-tips as described earlier [13].

2.3. LC–MS/MS analysis

Online LC–MS/MS analysis was carried out using a reversed phase analytical C18 column connected to 1200 Series Nanoflow LC (Agilent Technologies) interfaced with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany). Voltage of 2 kV was applied on the nanospray source fitted with an 8 mm emitter tip (New Objective, Woburn, MA). Samples were washed on trap column (75 mm × 10 mm) for 5 min with 3% solvent B (90% ACN in 0.1% formic acid). Linear gradient of 10–35% solvent B for 50 min at a constant flow rate of 0.4 mL/min was applied. Data were acquired using Xcalibur 2.1 (Thermo Electron). Data dependent acquisition was carried out for 20 most abundant ions in the scan range of m/z 350–1800. Fragmentation was carried out in HCD cell with normalized collision energy of 40. MS and MS/MS data were acquired with Orbitrap analyzer at the resolving power of 60,000 and 15,000 at 400 m/z, respectively. Lock mass was enabled and Polydimethylcyclosiloxane ions (m/z, 445.1200025) were used for internal calibration.

2.4. LC–MS/MS data analysis and data availability

Database searches against Human RefSeq 50 supplemented with common contaminants (33,947 entries) using Sequest and Mascot and quantitation were carried out through Proteome Discoverer 1.3. The parameters for database searching included fixed modifications as iTRAQ labeled at peptide N-terminus, iTRAQ labeled lysine residue and cysteine modification by methyl methanethiosulphonate (MMTS). Variable modification included oxidation of methionine. Mass error of 20 ppm and 0.1 Da was allowed for precursor and fragment ions, respectively. Trypsin was specified as a protease with maximum of 1 allowed missed cleavage. Reversed database was used as a decoy database. Peptides that did not match the contaminants and passed score cut-off for 1% FDR were considered for further analysis [14]. For quantitation, only unique peptides were considered. Data was normalized based on protein median. Data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [15] with the dataset identifier PXD000619.

Sub-cellular localization and biological processes of the identified proteins was also analyzed using Human protein reference database (HPRD: http://www.hprd.org) which is a GO compliant database [16].

2.5. Construction of tumor microarrays and immunohistochemistry

Tumor microarrays were constructed with paraffin blocks from Cancer Hospital and Research Institute, Gwalior which consisted of 59 cases of gallbladder adenocarcinoma and 20 adjacent non-tumor tissues, the majority being cholecystitis. Two cores of 2 mm

size from each paraffin block were embedded in a recipient paraffin block.

Rabbit polyclonal anti-prosaposin (catalog # sc-32875, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and the mouse monoclonal anti- transgelin (catalog # sc-53932, Santa Cruz Biotechnology Inc., Santa Cruz, CA) antibodies were used at a dilution of 1:250 and 1:100, respectively. Immunohistochemical (IHC) staining was carried out using VECTASTAIN Universal Elite ABC reagents (Vector Laboratories, Burlingame, CA) essentially as described previously [12]. The immunohistochemical labeling was scored by the pathologist as negative (0), weak (2+), moderate (3+) and strong (4+). To determine statistical significance of difference of expression in tumor and non-tumor groups, Chi-square test was carried out using R version R-3.0.2.

3. Results and discussion

In the present study, we carried out iTRAQ-based quantitative proteomics using high resolution mass spectrometry to identify differentially regulated proteins in gallbladder cancer. Representative studies carried out previously on GBC using proteomics are listed in Supplementary Table 2. For an in-depth characterization of the GBC proteome, we selected 10 GBC and adjacent non-tumor tissues.

3.1. iTRAQ-based quantitative proteome profile of gallbladder cancer

To obtain relative quantitation of GBC proteome, iTRAQ labeling was carried out as depicted in Fig. 1. LC–MS/MS analysis of 28 fractions obtained post SCX fractionation using LTQ-Orbitrap Velos resulted in identification of 2575 proteins. With a cut-off of 1.5-fold, 286 proteins were observed to be upregulated and 226 proteins downregulated in GBC. Representative list of differentially regulated proteins in GBC is provided in Table 1.

3.2. Upregulated proteins in gallbladder cancer

To understand the dysregulated proteome, we first focused on the upregulated proteins in GBC. We also carried out a literature survey to check whether the upregulated proteins in GBC have been reported in other cancers. Mucin 5AC (3.3-fold) has been described previously to be elevated in mucinous carcinoma of the gallbladder, gallbladder adenocarcinoma and cholangiocarcinoma [17,18]. Novel upregulated proteins in GBC include nuclear casein kinase and cyclin-dependent kinase substrate 1 (NUCKS1) (3.3-fold) and deleted in malignant tumors 1 (DMBT1) (3.2-fold). Representative MS/MS spectra for the peptides of upregulated proteins are shown in Fig. 2(panels A and B). We considered biological processes and cellular localization of the upregulated proteins for further analysis. Supplementary Fig. 2A and B depict distribution of cellular localization and biological processes, respectively, of the upregulated proteins in GBC.

Several nuclear proteins were upregulated in GBC which have been previously described in association with cancer. NUCKS1 is a substrate of casein kinase and cyclin dependent kinase (CDKs) with an unknown function. It has a DNA binding activity which is under regulation of CDKs. It is observed to be upregulated in invasive ductal carcinoma of the breast and colorectal cancers [19,20]. Including DMBT1, several membrane and extracellular proteins were upregulated in gallbladder cancer. Some of these include mucin-13 (MUC13) (2.7-fold) and laminin subunit beta-3 (LAMB3) (1.9-fold). DMBT1 is a secreted scavenger receptor cysteine-rich protein. Increased expression of DMBT1 is reported in pancreatic cancer [21,22]. MUC13 is a transmembrane mucin that

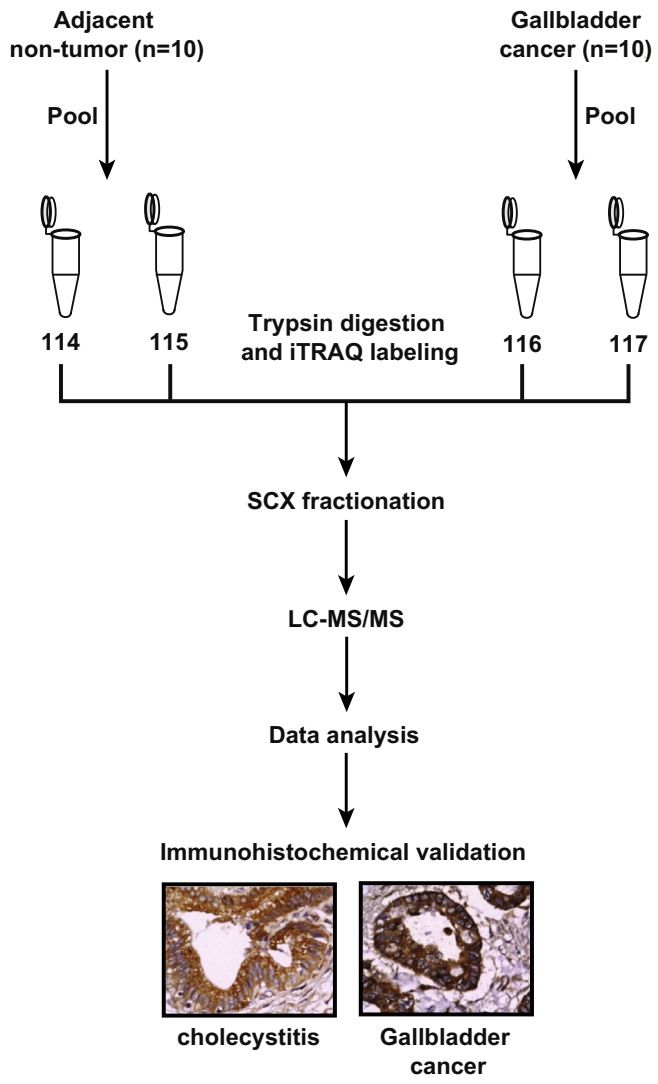

Fig. 1. Schematic workflow to identify differentially regulated proteins in GBC using iTRAQ. Work flow for quantitative tissue proteomics using iTRAQ labeling and validation of potential biomarkers for gallbladder cancer using immunohistochemistry. Proteins were isolated from ten tumor and adjacent non-tumor tissues. Proteins were subjected to trypsin digestion followed by iTRAQ labeling. Post labeling, the tumor and adjacent non-tumor tissue derived peptide mixture was pooled and fractionated using strong cation exchange (SCX) chromatography, followed by liquid chromatography tandem mass spectrometry on a LTQ-Orbitrap Velos mass spectrometer. The data was searched using SEQUEST and Mascot search algorithms. Some of the dysregulated proteins that were not previously described were validated using IHC labeling on tissue microarrays.

is reported to be upregulated in pancreatic, colon and gastric cancers [23–26].

3.3. Upregulation of lysosomal proteins in gallbladder cancer

Prosaposin (PSAP) (2.7-fold) is primarily localized in lysosomes and is localized in membrane. It is reported to be a secretory protein as well. It is a precursor protein and its cleavage products; saposin A–D have diverse functions. Prosaposin has been reported to be upregulated in multiple cancers including prostate and breast [27,28]. Along with prosaposin, several other lysosomal proteins were upregulated in gallbladder cancer including cathepsin Z (CTSZ) (2.2-fold), ganglioside GM2 activator (GM2A) (1.9-fold), cathepsin H (CTSH) (1.9-fold), gamma-glutamyl hydrolase (GGH) (1.7-fold) and alpha-N-acetylgalactosaminidase (NAGA) (1.7-fold). Cathepsin Z and H are associated with metastasis and migration [29,30].

Table 1
A partial list of differentially regulated proteins in gallbladder cancer.

	Gene symbol	Protein	Gallbladder cancer/ adjacent non-tumor	Previously described disease association
1	*NUCKS1*	Nuclear ubiquitous casein and cyclin-dependent kinases substrate	3.4	Colorectal and breast cancer [19,20]
2	*DMBT1*	Deleted in malignant brain tumors 1	3.2	Pancreatic cancer [22]
3	*HMGB2*	High mobility group protein B2	2.9	Hepatocellular carcinoma and bladder cancer [39,40]
4	*PSAP*	Prosaposin	2.7	Prostate cancer and breast cancer [27,28]
5	*NEFH*	Neurofilament heavy polypeptide	0.3	ESCC [31]
6	*S100A8*	S100-A8	0.3	Pancreatic cancer, ESCC [41,42]
7	*TAGLN*	Transgelin	0.5	Prostate and breast cancer [33,43]

This table lists representative proteins that are differentially regulated in GBC along with the gene symbols, fold-change and previously described disease association.

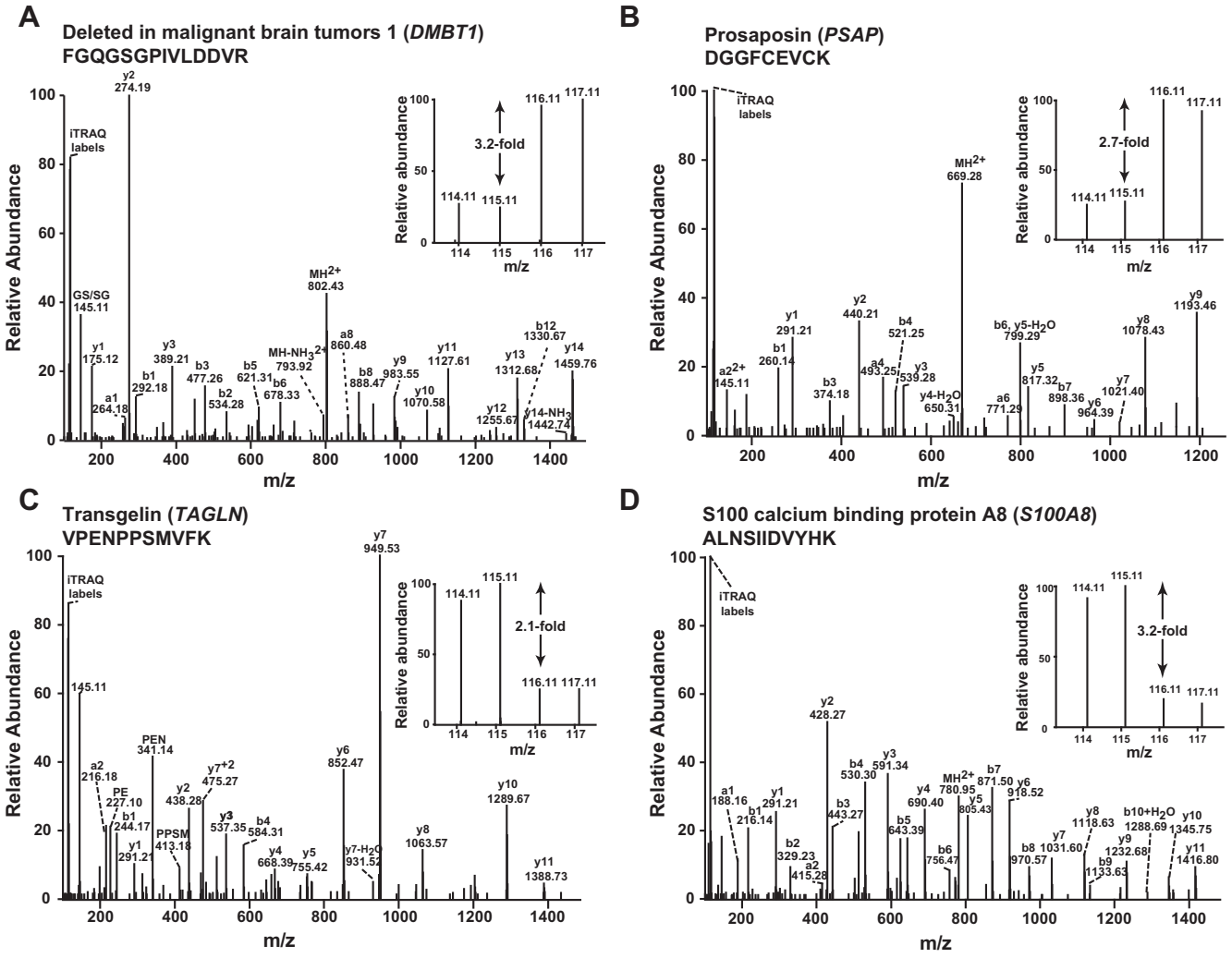

Fig. 2. Representative MS/MS of the peptides from upregulated and downregulated proteins in GBC. MS and MS/MS spectra of peptides from representative differentially regulated proteins identified in this study. (A) Deleted in malignant brain tumors 1 (*DMBT1*); (B) Prosaposin (*PSAP*); (C) Transgelin (*TAGLN*); and (D) S100 calcium binding protein A8 (*S100A8*).

3.4. Downregulated proteins in gallbladder cancer

We identified 226 proteins that were downregulated ⩽1.5-fold in GBC. Some of the downregulated proteins include neurofilament heavy polypeptide (NEFH) (3.9-fold), serine protease HTRA1 (HTRA1) (2.8-fold), transgelin (TAGLN) (2.1-fold) and ras suppressor protein 1 (RSU1) (1.7-fold). Representative MS/MS spectra for representative peptides of two downregulated proteins are illustrated in Fig. 2(panels C and D). NEFH downregulation has been reported to result in the activation of Akt/beta-catenin pathway in ESCC [31]. Serine protease HTRA1 is an extracellular protease which is a member of the human HtrA family proteins of serine proteases. It is reported to bring about proteolytic cleavage of TGF-beta and is reported to be downregulated in multiple cancers

Table 2
Summary of immunohistochemical staining of prosaposin and transgelin.

	Staining pattern	Prosaposin	Transgelin
1	Strongly positive (tumor)	49	0
2	Weakly positive to negative (tumor)	10	49
3	Strongly positive (non-tumor)	8	7
4	Weakly positive to negative (non-tumor)	12	13
5	*p*-Value	2.06E-04	1.25E-05
6	Subcellular location of staining	Predominantly cytoplasmic	Predominantly cytoplasmic

Staining pattern, *p*-value and cellular localization of prosaposin (PSAP) and transgelin (TAGLN).

including hepatocellular carcinoma [32]. Transgelin is an actin binding protein, which is required for maintenance of normal morphology of the cells. It is reported to be a sensitive marker for early transformation and has been reported to be downregulated in several cancer including prostate [33].

3.5. Validation of prosaposin and transgelin as novel candidate biomarkers using immunohistochemical staining

We used immunohistochemistry as an orthogonal method to validate our findings and assess if the selected proteins can serve as potential biomarkers for GBC. We selected prosaposin and transgelin as such candidate molecules which were, upregulated and downregulated, respectively, in our iTRAQ data.

3.5.1. Prosaposin

In the discovery proteomics study, prosaposin was found to be 2.7-fold upregulated in GBC. The immunohistochemistry analysis of prosaposin shows 83% (49/59) strong positivity in tumors as given in Table 2. Chi-square exact test was performed to ascertain the significant level of difference between the groups, which showed high significant upregulation of prosaposin ($p < 0.001$) in GBC at confidence level of greater than 95%. Strong staining for prosaposin was observed predominantly in the cytoplasm. Staining pattern for prosaposin in cholecystitis and tumor tissues are represented in Fig. 3A and B.

As mentioned above, prosaposin is a lysosomal protein that is localized in the membrane and is also secreted. Proteolytic cleavage of prosaposin by cathepsin D results in four cleavage products (saposins A–D) which act as activators of glycosphingolipid hydrolases. Reduced levels of prosaposin result in accumulation of ceramides which are known pro-apoptotic agents [34]. Prosaposin is also known to be secreted and has pleiotropic growth factor activity. Serum levels of prosaposin are elevated in advanced prostate cancers [27]. It is known to promote growth of breast cancer and increase ERα levels through MAPK signaling pathway [28]. Overall, elevated prosaposin levels could result in increased degradation of ceramides providing a survival advantage to the cancer cells, cater to energy needs and potentially serve as a biomarker for GBC.

3.5.2. Transgelin

Amongst the downregulated proteins, transgelin was 2.1-fold downregulated in GBC. In the immunohistochemistry analysis of transgelin, none of the GBC cases showed strong positivity (0/59), with a statistically significant level of difference ($p < 0.001$) at higher than 95% confidence interval as given in Table 2. Representative staining pattern of transgelin in cholecystitis and tumor tissues are shown in Fig. 3C and D.

Transgelin is an actin stress fiber-associated protein. It is known to be elevated with differentiation and localize along stress fibers. Transgelin is observed to be downregulated in multiple types of cancers including breast, colon and prostate. Its expression is

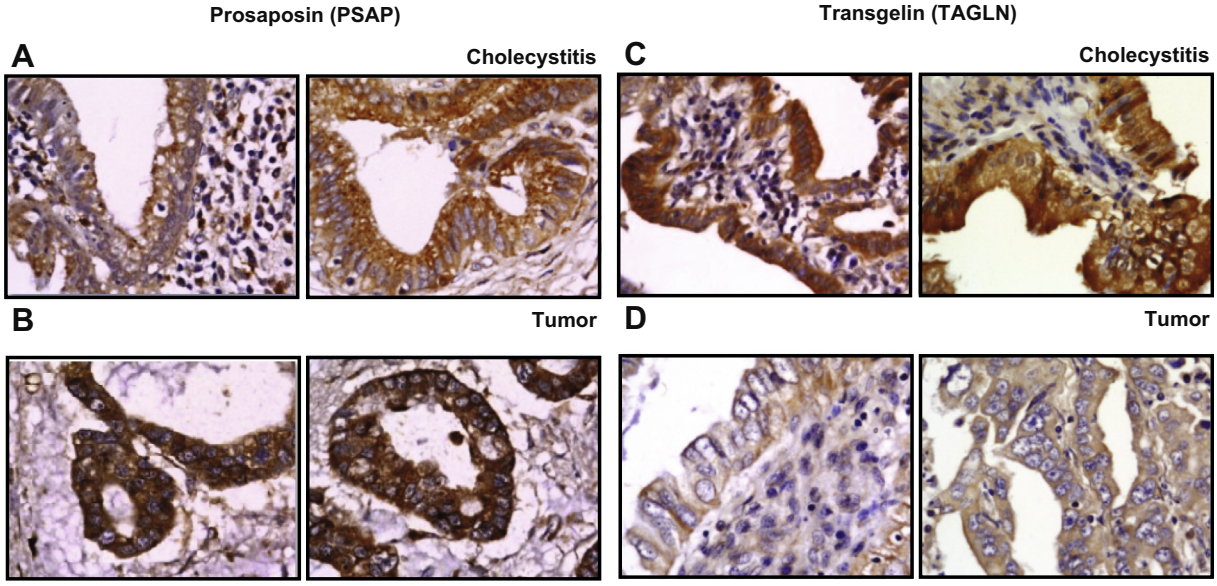

Fig. 3. Immunohistochemical staining of prosaposin and transgelin in GBC and cholecystitis tissues using tissue microarrays. Validation of prosaposin and transgelin using immunohistochemical staining. Representative sections at the magnification of 40X from tissue microarrays stained with anti-prosaposin is shown (A) expression pattern of prosaposin in representative cholecystitis mucosa and (B) expression of prosaposin in GBC. Representative sections from tissue microarrays stained with anti-transgelin is shown (C) expression pattern of transgelin in representative cholecystitis mucosa and (D) expression pattern of transgelin in GBC.

downregulated by oncogenic Ras [35]. It is known to be an early marker for transformation. Reduced levels of transgelin disrupt the normal actin architecture and contribute to invasive property of cancer cells. Transgelin acts as a repressor of MMP-9, a crucial protease for metastasis [36]. Transgelin is also reported to interact with p53, induce apoptosis and inhibit AR pathway in prostate cancer cells [37,38]. These studies show possible tumor suppressor activity of transgelin. Functional significance of downregulation of transgelin in GBC needs to be investigated further.

Further studies investigating expression levels of these proteins in a larger cohort of GBC patients are warranted. Promoter methylation studies on the downregulated proteins such as transgelin may lead to identification of epigenetic markers for gallbladder cancer. Further functional studies are required to understand role of upregulated lysosomal proteins including prosaposin. As prosaposin is also secreted, it needs to be investigated further in body fluids such as bile, urine and blood from gallbladder cancer, cholecystitis patients and healthy controls to assess its utility as an early diagnostic biomarker.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.017.

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