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Gallbladder carcinoma (GBC) is a chronic malignancy of the gall bladder and intrahepatic and extrahepatic common bile ducts with a high mortality rate and forms the fifth common cancer of gastrointestinal tract globally. Women remain at higher risk than men and recent studies have reported the highest rate of incidence in women from Delhi, India. GBC treatment suffers from the disadvantage of lack of suitable biomarkers for early diagnosis of the disease. Different proteomic approaches including (i) 2D gel electrophoresis (ii) Mass spectroscopic studies (iii) Isobaric tags for relative and absolute quantization (iTRAQ)-based quantitative proteomics studies are being employed for detection of biomarkers in order to undertake early diagnosis of the disease. In this review we focus on (i) risk factors in GBC, (ii) diagnosis and treatment, (iii) molecular markers, and (iv) proteomic studies in GBC. The future scope of this review lies in the identifying biomarkers of GBC, and may provide directions to unraveling future implications in disease treatment.

Study of Gallbladder cancer in the light of proteomics

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Abstract

Gallbladder carcinoma (GBC) is a chronic malignancy of the gall bladder and intrahepatic and extrahepatic common bile ducts with a high mortality rate and forms the fifth common cancer of gastrointestinal tract globally. Women remain at higher risk than men and recent studies have reported the highest rate of incidence in women from Delhi, India. GBC treatment suffers from the disadvantage of lack of suitable biomarkers for early diagnosis of the disease. Different proteomic approaches including (i) 2D gel electrophoresis (ii) Mass spectroscopic studies (iii) Isobaric tags for relative and absolute quantization (iTRAQ) -based quantitative proteomics studies are being employed for detection of biomarkers in order to undertake early diagnosis of the disease. In this review we focus on (i) risk factors in GBC, (ii) diagnosis and treatment, (iii) molecular markers, and (iv) proteomic studies in GBC. The future scope of this review lies in the identifying biomarkers of GBC, and may provide directions to unraveling future implications in disease treatment.

Running title: Proteomic studies in Gallbladder Cancer: Recent Developments

Key words: Gallbladder, cancer, proteomics

Introduction

Gallbladder carcinoma (GBC) is an adenocarcinoma of epithelial tissues of gall bladder and involves a chronic biliary tract malignancy with a high mortality rate. Reported as one of the most aggressive carcinomas (Sahasrabuddhe et al., 2014), it is also reported as the fifth common cancer of gastrointestinal tract (Huang et al., 2014). Globally, occurrence of GBC have been reported to be higher in East Asia including Korea and Japan and Eastern Europe extending across Slovakia, Poland and Czech Republic (World Cancer Report, 2008). Women are at 5 folds higher risk as compared to men in high-risk zones encompassing Pakistan, India, Spain and Colombia (World Cancer Report, 2008). It is also reported to be the most common cause of cancer related mortality in Northern and North-eastern parts of India (Barbhuiya et al., 2008; Singh et al., 2014). According to recent report, the highest rate of incidence of GBC has been reported in women from Delhi, India (21.5/1,00,000) followed by South Karachi, Pakistan (13.8/1,00,000) and Quito, Ecuador (12.9/1,00,000) while the highest mortality has been reported from Chile with higher mortality rate in men (7.8/1,00,000) as compared to that of women (16.6/1,00,000) (Barbhuiya et al., 2008; Singh et al., 2014). The major challenges associated with this disease are the lack of suitable specific markers for its early detection and diagnosis. Although several markers have been tested, there still remains a dearth of specific effective markers for early diagnosis in GBC. Therefore the study of gall bladder cancer finds importance in the current day across the globe. Recent proteomics approaches are now being employed and act as promising tools towards early diagnosis. In this review we focus on the recent developments in the field of GBC with reference to its (i) risk factors in GBC, (ii) diagnosis and treatment, (iii) molecular markers, and (iv) proteomic studies in GBC.

(I) Risk factors associated with Gallbladder Cancer

A positive correlation of gallstone expression together with increased incidences of gallbladder cancer (GBC) diagnosis has been reported (Wistuba et al., 2004). Formation of gallstones and prolonged cholelithiasis are the major potential risk factors associated with GBC, along with obesity and chronic infection of the gallbladder (World Cancer Report, 2008). The association of GBC with obesity is stronger in women than in men (Randi et al., 2006; Larsson et al., 2007). High prevalence of gallstones was found among the women of American Indian community in the USA and Mapuche Indians in Chile, where high incidence of GBC is reported (World Cancer Report, 2008). The highly female skewed pattern of GBC incidences suggest that there could be a hormonal component (Borena et al., 2014) involved adding to the risk factor for occurrence of GBC for women. Formation of cholesterol gallstones have also been attributed to presence of excess of cholesterol in the bile (Randi et al., 2006; Larsson et al., 2007) adding to the risk factor in gallbladder cancer.

Diagnosis and Treatment

The appearance of symptoms is progression-stage-dependent, and mostly appears during advanced metastasis. Therefore it is difficult to detect the cancer at an earlier stage and treat them. However, current diagnostic tools for GBC include Liver function test, carcino-embryonic antigen (CEA) assay, CT scan, ultrasound scan, percutaneous trans-hepatic cholangiography (PTC), endoscopic retrograde cholangiopancreatography, biopsy, heparoscopy (Singh et al., 2012; Lurie et al., 1975; Strom et al., 1990; Rodriguez-Fernandez et al., 2004; Chattopadhyay et al., 2005; Rao et al., 2005; Okuda et al., 1974; Neoptolemosa et al., 1988; Cox et al., 1993). The different treatment methods currently include both invasive methods

involving surgical removal of the small sized tumor which are not yet metastatic (Donohue et al., 1998), radiation therapy in advanced stages where surgical removal of specific organs/organ components is not possible (Donohue et al., 1998) and chemotherapy and external beam radiation (Pandey and Chandramohan, 2004). Due to the non-availability of diagnostic marker at the earlier stages, complete treatment of GBCs face a bottleneck (World Cancer Report, 2008) and remains a major challenge. Therefore search for biomarkers in GBC finds extreme importance in modern day clinical research.

(II.) Molecular markers

The progressive stages of GBC are characterized by chronic cholelithiasis with inflammation, metaplasia, dysplasia, *in situ* carcinoma and invasive carcinoma. The causes of GBC widely studied include mutations, microsatellite instability, gene methylation and loss of heterozygosity, altered expression of proteins, (Saetta et al., 2001; Maurya et al., 2010; Wang et al., 2014; Kawasaki et al., 2014; Jain et al., 2014; Moy et al., 2015; Dwivedi et al., 2015) singly or in combinations and the study of these alterations and mapping their regulation finds importance in improved prognosis of GBC.

(i) Genetic Markers

Gene methylation and microsatellite instability (MSI) are reported to be important prognostic markers (Walawalkar et al., 2015) in gallbladder carcinoma. GBC is characterized by low level of microsatellite instability (Yoshida et al., 2000). Studies reveal that MSI bears a positive correlation with gallbladder disorders including gallbladder cancer (Yanagisawa et al., 2003). Abnormal DNA methylation bears a strong correlation with gallbladder cancer. Relatively high frequency of abnormal methylation of the following genes including SHP1 (80%), 3-OST-2 (72%), CDH13 (44%), P15INK4B (44%), CDH1 (38%), RUNX3 (32%), APC (30%), RIZ1

(26%), P16INK4A (24%), and HPP1 (20%) (Letelier et al., 2012; Takahashi et al., 2004) are observed in GBC and finds importance in indicating their diagnostic and prognostic potential.

GBC patients expressing ALDH1A3 exhibited poor survival rate (Yang et al., 2013). The methylation frequency of 44% was reported in the p15 gene (Garcia et al., 2009), which correlated with poor survival rate in GBC patients. CDH13 gene has been reported to be highly (44%) methylated in gallbladder carcinoma, as compared to 8% methylation observed in cholecystitis patients (Garcia et al., 2009). Since this gene plays an important role in cell-cell adhesion, its methylation would significantly increase the metastasis thus leading to GBC (Imai and Yamamoto, 2008). GPX proteins are known to play an important biological role as antioxidant enzyme. The loss of GPX3 expression has been reported to correlate significantly with metastasis, invasion, tumor node metastasis (TNM) stage, and poor prognosis in both squamous cell/adenosquamous carcinomas (SC/ASC) and adenocarcinoma (AC) patients. (Yang et al., 2013). Patients diagnosed with negative GPX3 expression showed poor survival rates (Yang et al., 2013) and bears correlation indicating prognosis in GBC. The important role of APC is that it regulates cell migration, adhesion and apoptosis. Its methylation frequency is 30% and shows higher methylation frequency in GBC as compared to cholecystitis (Garcia et al., 2009) leading to poor survival rates (Miyamoto et al., 2003).

SHP1 gene playing role in cell growth, mitotic cycle and differentiation was reported to be methylated to a frequency of 88% in case of cholecystitis and around 80% in GBC patients wherein methylation sets in early in GBC (Garcia et al., 2009). RUNX3 plays an important role in TGF- β signal pathway. The RUNX3 transcription factor interacts with TGF- β -activated SMAD proteins to mediate transforming growth factor- β (TGF- β) signaling (Hanai et al., 1999). Its methylation frequency was reported to be 32% (Garcia et al., 2009) in GBC patients.

Environmental factors such as tobacco smoking and *Helicobacter pylori* infection can accelerate the process of DNA methylation (Letelier et al., 2012; Lazcano-Ponce et al., 2001). P16, acyclin dependent kinase has been reported of methylation frequency of 24% in GBC patients (Garcia et al., 2009) with poor chances of survival (Lazcano-Ponce et al., 2001) promising as a significant prognostic biomarker in GBC. MGMT, belonging to the methyltransferase family was reported to be a significant prognostic marker for GBC (García, et al., 2009). DLC1, a GTPase activating protein with methylation gene frequency of 39% reported from Chile (Miyamoto et al., 2003), revealed correlation of hypermethylation with poor survival rates and increased metastasis in GBC. Some other genes, which can serve as an important prognostic marker, are P73, RAR2, DAPK, TIMP3 and P14 genes in GBC hypermethylation of which results in poor survival (Walawalkar et al., 2015). Serial analysis of gene expression studies from both neoplastic GBC patients and non-neoplastic gallbladder mucosa revealed that (Alvarez et al., 2008) the alternative lengthening of telomeres (ALT) phenotype in gallbladder carcinoma holds promise as a significant prognostic marker in ALT positive GBC patients (Heaphy et al., 2011).

Other genetic markers that show promises in diagnosis of GBC patients include K-ras, p53, p16, Rb genes. Mutations in the K-ras gene, belonging to the Ras family has been detected in 20% of GBC patients (Kim et al., 2001) serving as a potential biomarker for GBC patients (Takahashi et al., 2004; Zhang et al., 2014). KRAS normally remains associated with cell membrane through its C-terminal isoprenyl group (Kranenburg, 2005) which on activation, binds to GTP and aids to cleave the terminal phosphate of the nucleotide converting it to GDP (Kenichi Suda and Tetsuya, 2010). The mutation in K-ras gene leads to a sequence alteration of GGT (Glu) in codon 12 to GAT (Asp). K-ras mutations were significantly found in GBC

patients but were absent in adenomas and dysplasia (Kim et al., 2001) indicating the disease specific nature of mutation specific to GBC.

p53 gene playing an important role in apoptosis, genomic stability and angiogenesis is also considered as an important factor in ageing has been reported from 35.7% gall bladder carcinoma cases contributing to tumor above stage 2 (Reid et al., 2007). Five out of fourteen GBC patients with p53 mutation (Kim et al., 2001) revealed that of these five, three mutations occurred in codon 248 of exon 7 on single-strand conformation polymorphism (SSCP) analysis. While two of them CGG (Arg) has been reported to mutate to TGG (Trp), the third one revealed CGG (Arg) mutation to CAG (Gln) (Beier, 1993). The other two mutations reported in GBC were observed in exon 8 at codon 282 where CGG (Arg) altered to TGG (Trp) and at codon 285 where GAG (Gln) had been mutated to GCG (Asp)(7). Immunohistochemical analysis revealed that people with p53 gene mutation showed an over expression of p53 protein (Kim et al., 2001).

p16genes, coding for p16 proteins being a tumor suppressor protein (Nobori et al., 1994; Stone et al., 1995), are known to play an active role in controlling the G1-S phase of the cell cycle. Studies on the p16 genes revealed that its reduced expression leads to increased rate of proliferation and metastasis and correlated with the increased the rate of GBC. The p16 protein is reported to have been mutated in 30.7% of GBC patients. 3 mutations among 14 were found on exon 2 and the other one was in exon 1 (Kim et al., 2001). Reduced expression of protein also correlated the Immunohistochemical studies (Kim et al., 2001).

The Rb gene coding for the retinoblastoma, tumor suppressor protein (Murphree and Benedict, 1984) that prevents excessive cell growth by inhibiting cell cycle progression until a cell is ready to divide in normal cells. But in the dividing cells, phosphorylated Rb allows cell cycle

progression. Mutation in Rb gene was correlated to the progress of cancer thus proving an effective biomarker for GBC (Ma et al., 2005).

Quantification of GBC global methylome by ELISA based methods and promoter DNA methylation of eight tumor suppressor genes (APC, CDKN2A, ESR1, MCAM, PGP9.5, RARB and SSBP2) (Kagohara et al., 2015; House et al., 2003; Yamashita et al., 2006; Konishi et al., 2009; Vasiljevic et al., 2011; Liu et al., 2008) by quantitative methylation-specific PCR methods together with the calculation of global DNA methylation Index (GMI) and promoter methylation revealed that GMI would serve as an effective biomarker for early detection of Gallbladder carcinoma (Kagohara et al., 2015).

(ii) Protein markers

Up regulation of ANX4 has been reported from primary gallbladder cancer tissues by two-dimensional electrophoresis (2-DE), MALDI-TOF and mass spectroscopic (MS) studies (Huang et al., 2014) and has been reported as a significant diagnostic biomarker in Gallbladder carcinoma (Huang et al., 2014). ANXA4 (Annexin IV) a member of the Annexin family located on membrane surfaces play an important role in regulating the membrane proteins and has been reported to show structural properties to form ion channels (Garke and Moss, 2002). Over expression of ANXA4 proteins results in the regulation of carcinogenesis-associated proteins such as RHAMM, AKT, p21, PBK, and CDK1 (Lin et al., 2012). The ANXA4 has been reported to binds to the plasma membrane in a Ca^{2+} -dependent manner and induce downstream signal transduction, which up regulates RHAMM protein, and subsequently regulates carcinogenesis-associated proteins (Lin et al., 2012) in gastric cancer.

In normal individuals Hsp90B, functions as a chaperone protein that stabilizes proteins against heat stress and is required for the function of a number of conditionally expressed signaling proteins (Huang et al., 2014). It is also involved in proteostatic maintenance of oncoproteins that promote tumor cell growth, survival and maintenance. Down-regulation of Hsp90B protein has been reported from patients with primary gallbladder cancer with increased metastasis (Xu and Neckers, 2007). Dync1h1, a large protein of $>3.191 \times 10^{26}$ KDa is a crucial subunit of cytoplasmic dynin complex required for retrograde axonal transport in neurons. Dync1h1 protein is reported to be down regulated in the GBC patients as compared to the normal volunteers (Huang et al., 2014). Surface-enhanced laser desorption/ionization (SELDI) [44] confirmed that upregulated expression of ANXA4 and decreased expression of Hsp90 β and Dync1h1 is a significant biomarker in GBC carcinoma (Huang et al., 2014).

S100A10, a member of the S100 (SP) family is a multifunctional signaling protein involved in numerous cellular functions such as protein phosphorylation, enzyme activation, calcium homeostasis and interaction with cytoskeletal component (Donato, 2003). It is also reported to regulate cellular processes like cell growth, cell cycle progression, differentiation, transcription and secretion (Salama et al., 2008). Over expression of this protein has been shown to result in increased metastasis (Tan et al., 2011). Increased expression of SA100A10 in the patients with GBC were indicative of patients their poor prognosis (Tan et al., 2011). Haptoglobin is the acute phase proteins and primarily synthesized in the liver (Baumann et al., 1990). Its expression level is regulated by several cytokines including IL-1, IL-6, TNF- α and TGF- β . Haptoglobin is known to bind to the free haemoglobin in blood plasma with high affinity thus inhibiting its oxidative activity. The importance of the Sialyl-Lewis X determined in cell adhesion suggests that dysregulated haptoglobin glycosylation could interfere with metastasis (Kannagi et al.,

2004). Haptoglobin also shows some evidence of being a pro-angiogenic factor, thus its up regulation could lead to stimulation of tumor angiogenesis (Fosslien, 2001). Up regulation of this protein could significantly increase tumor invasion and poor prognosis in GBC (Tan et al., 2011).

Prosaposins, lysosomal protein localized in the membrane, secreted and acts as a pleiotropic growth factor is reported to be elevated in patients of gallbladder carcinoma (Sahasrabuddhe et al., 2014). Their up regulation has been reported to correlate with the increased degradation of ceramides, thus leading to a survival advantage to cancer cell, which in turn leads to increased invasiveness (Sahasrabuddhe et al., 2014). Transgelin, an actin stress fiber-associated protein on the contrary is reported to be down regulated in gallbladder carcinoma (Sahasrabuddhe et al., 2014) and is reported to be an effective biomarker in GBC. The down regulation disrupts the normal actin architecture and results in increased invasiveness of cancer cells. It also acts as a repressor of MMP-9, which is a crucial protease for metastasis (Nair et al., 2006). Quantitative proteomics method have shown that both transgelin and prosaposin are important biomarkers for diagnosis of GBC.

(III.) Proteomic- based studies in GBC

The various proteomic methods that have been used for determination of essential biomarkers are two-dimensional gel electrophoresis (2-DE), western blot, immunohistochemical assay, quantitative real-time PCR, iTRAQ based proteomic analysis, LC MS-MS Analysis, MALDI-TOF-MS (matrix-assisted laser desorption ionization time-of-flight mass spectroscopy), SELDI-TOF MS Analysis etc. These proteomic studies that have been used to screen potential biomarkers for GBC as summarized in the Figure 1.

Two-dimensional gel electrophoresis (2-DE) followed by gel scanning for profiling of protein obtained from GBC patients, cholecystitis patients and normal gallbladder tissues (Huang et al., 2014) indicated the differential expression of proteins in patients suffering from GBC as compared to patients suffering from cholelithiasis and those of normal healthy individuals. (Huang et al., 2014).

MALDI-TOF was used for identification of differentially expressed proteins. Three upregulated proteins (serum albumin, ANXA4, ACTG) and three down regulated proteins (Hsp90B, ACTA2, Dync1h1) were identified by using MALDI-TOF (Huang et al., 2014) from patients suffering from gall bladder cancer. In order to validate the results of 2-DE and to confirm whether differentially expressed proteins could behave as potential diagnostic marker proteomic techniques used were western blot, immunohistochemical assay and quantitative real-time PCR. Western blot analysis showed that there was no significant change in the levels of ACTG and ACTA2, whereas ANXA4 was significantly over expressed while Hsp90B and Dync1h1 were decreased (Huang et al., 2014). The same result was obtained through immunohistochemical study and quantitative real-time PCR. In order to further validate the down regulation of Hsp90B and Dync1h1 SELDI-TOF-MS analysis was used (Huang et al., 2014). SELDI-TOF-MS analysis is a variation of MALDI-TOF that is used for quantifying and detecting proteins differentially expressed in carcinoma. It is highly sensitive, efficient and is able to profile a large number of samples quickly (Huang et al., 2014).

To study serum biomarkers of GBC, comparative gel images from two-dimensional gel electrophoresis (2-DE) (Tan et al., 2011) of GBC patients and normal healthy volunteer were analysed to understand the differential expression of serum proteins. On comparison of corresponding spots in GBC and normal patients they received 64 differentially expressed

proteins. MALDI-TOF-MS was used to measure the peptide mass and to know the up or down regulation of differentially expressed proteins in GBC. MALDI-TOF-MS analysis identified twenty-four differentially expressed proteins among which twelve were up regulated and twelve were down regulated (Tan et al., 2011). Western blot analysis was performed in order to validate the protein identification and differential expression of serum S100A10 and haptoglobin protein in GBC. As compared to normal volunteers the GBC patients had higher serum S100A10 and haptoglobin protein. Immunohistochemical assay was further used to confirm differentially expressed protein. Positive immunostaining of S100A10 and haptoglobin in case of GBC indicate over expression of these proteins in case of GBC patient (Tan et al., 2011).

iTRAQ based proteomic study using high-resolution mass spectroscopy was used to identify differentially regulated proteins in GBC (Sahasrabuddhe et al., 2014). iTRAQ labeling was followed by SCX fractionalization and LC-MS/MS analysis. 286 up regulated proteins and 226 down regulated proteins were obtained. Prosaposin, up regulated protein and transgelin, down regulated protein were selected as novel candidate markers for GBC. Immunohistochemical staining was used to validate their finding and make sure if these two proteins would serve as efficient biomarkers (Sahasrabuddhe et al., 2014). The immunohistochemical analysis gave a high proportion of positive results in GBC tumors. Chi-square exact test showed significant up regulation of prosaposin and strong staining was obtained in cytoplasm. Transgelin was significantly down regulated and this gave a positive test for it to be considered as significant biomarker in GBC cases (Sahasrabuddhe et al., 2014).

Discussion

GBC is one of the most aggressive carcinomas, though rare it has a higher mortality rate among all other biliary tract malignancies. GBC also has a poor prognosis and poor survival rates and suffers from lack of markers capable of early diagnosis of the disease. Due to poor understanding of risk factors and biomarkers for early diagnosis, GBC still remains a major area of research. Although several genetic markers and protein marker are being tested of their potential in detection of GBC, no single marker has enabled its early detection. Thus, a study regarding the proteome analysis in patients suffering from GBC seems imperative. This finds importance in designing clinical strategies against the disease and to find out the novel candidate that can act as potential diagnostic and prognostic marker for GBC. Occurrence of GBC tends to be more in females than in males. Formation of gallstones and cholelithiasis are the potential risk factors of GBC, thus they are considered to be precancerous lesions for GBC (World Cancer Report, 2008). In this study, we have summarized recent proteomic studies performed in GBC and the importance of potential biomarkers for the diagnosis and prognosis of GBC. Protein markers such as ANXA4, Hsp90B, Dync1h1, S100A10, haptoglobin, prosaposin and transgelin were reported as novel biomarkers (Table 1) using different proteomic studies (Table 1).

This review summarizes on proteomics study performed to find out novel candidates that could effectively serve as prognostic and diagnostic biomarkers in gallbladder carcinoma. The future scope of this review remains in designating essential biomarkers in GBC with large-scale clinical applications for early detection and improved prognosis for GBC and targeting these biomarkers proving as essential therapeutics for treatment of GBC.

TABLE 1: Proteomic studies in GBC

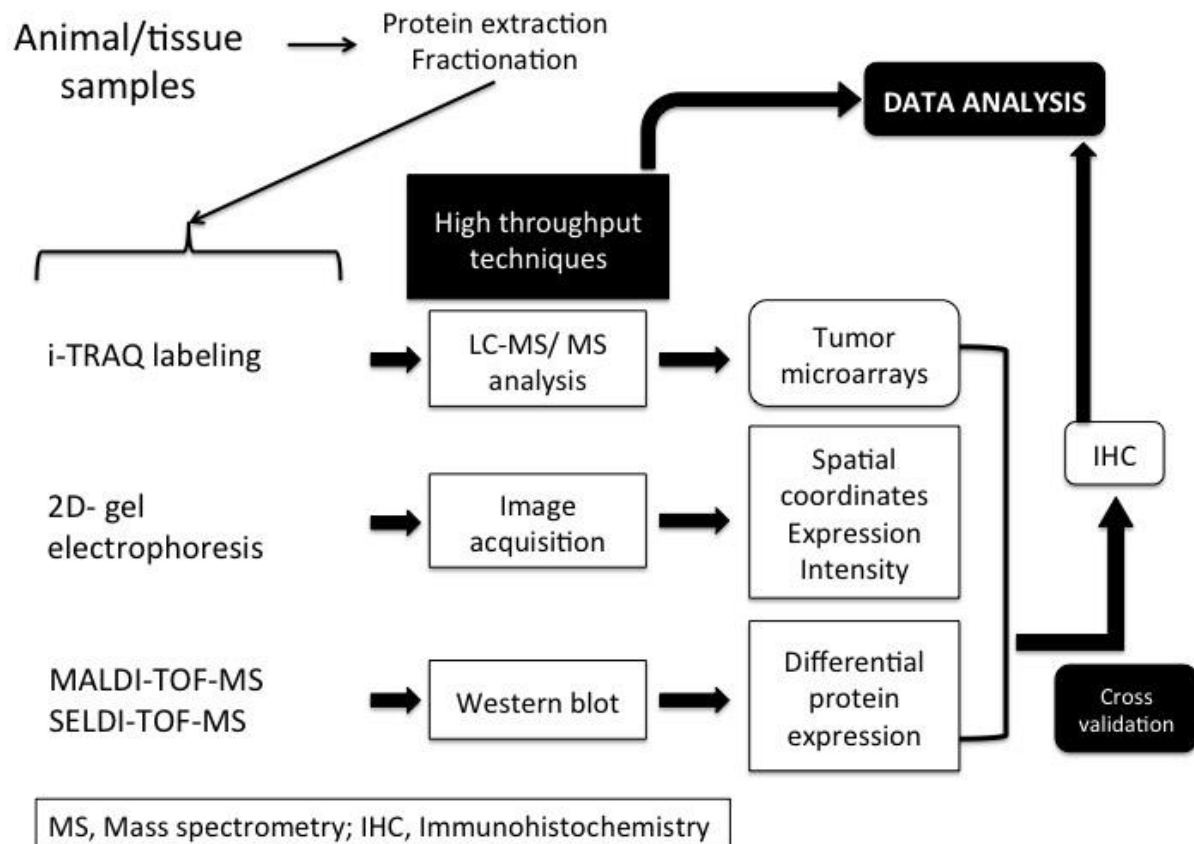
Protein markers	Proteomic studies	Expression status of Protein markers	Ref(s).
ANXA4	a) 2-DE b) WB c) MALDI-TOF-MS d) IHC e) qRT-PCR	Up regulated	Huang et al., 2014
Hsp90B	a) 2-DE b) WB c) SELDI-TOF-MS d) IHC e) qRT-PCR	Down regulated	Huang et al., 2014
Dync1h1	a) 2-DE b) WB c) SELDI-TOF-MS d) IHC e) qRT-PCR	Down regulated	Huang et al., 2014
S100A10	a) 2-DE b) WB c) MALDI-TOF-MS d) IHC	Up regulated	Tan et al., 2011
Haptoglobin	a) 2-DE b) WB c) MALDI-TOF-MS d) IHC	Up regulated	Tan et al., 2011
Prosaposin	a) iTRAQ b) LC MS/MS analysis c) IHC	Up regulated	Sahasrabuddhe et al., 2014
Transgelin	a) iTRAQ b) LC MS/MS analysis c) IHC	Down regulated	Sahasrabuddhe et al., 2014

Abbreviations used in Table 1:

2-DE, two-dimensional electrophoresis; IHC, Immunohistochemical analysis; iTRAQ, Isobaric tags for relative and absolute quantitation; LC MS, Liquid chromatography-mass spectrometry; MALDI-TOF-MS, Matrix-assisted laser desorption/ionization- time-of-flight mass spectrometer; qRT-PCR, Quantitative real time polymerase chain reaction; SELDI-TOF-MS,

Surface-enhanced laser desorption/ionization time-of-flight mass spectrometer; WB, Western Blot.

Figure 1: Different Proteomic techniques in study of gallbladder cancer.



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