Austin Journal of Clinical Pathology

Open Access

Austin Publishing Group

Review Article

Gallbladder Cancer: Approaches to Biomarker Discovery

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Received: September 04, 2015; **Accepted:** October 29, 2015; **Published:** November 05, 2015

Abstract

Gallbladder cancer is a relatively uncommon global health issue, affecting most commonly the middle-aged women. Due to lack of early diagnostic marker and late detection of tumor, survival of GBC patients is compromised. Surgical resection of gallbladder is the only option for treatment. Here, we present the updates on molecular mechanism of GBC, current technological strategies, limitations and future prospects of biomarker discovery. The association of genetic, cytogenetic and epigenetic mechanisms with GBC has brought tremendous alteration in the expression of various protein coding genes. The recent advancements in high throughput technologies have replaced the conventional methods and now being considered as major tools for biomarker discovery. The earlier efforts on the identification of chromosomal loci harboring loss of heterozygosity alleles to use of both conventional and advanced technologies all, have identified some 4000 plus molecules from various biological sources of GBC (tissue, blood and cell lines). In this review, we have updated the list of molecules identified by major high throughput as well as conventional methods, which are currently the main targets in the discovery of diagnostic and or therapeutic biomarkers for GBC.

Keywords: Gallbladder; Cancer; Biomarker

Introduction

Gallbladder cancer is an uncommon cancer and is a female biased health issue, affecting the middle age group more often. In 2015, 10910 new GBC cases and 3700 estimated deaths were predicted in the United States [1]. The highest incidence of GBC is reported 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) [2]. Our epidemiological study in north central Indian region showed significantly high incidence of GBC (7.8/1,00,000) [3]. The Indian Council of Medical Research (ICMR) Population Based Cancer Registry (ICMR-PBCR 2003-04) [4] reported the highest incidence of GBC in female to be 10.2 per lakh population, in Kamrup, Assam, India. Variations in the geographical distribution of the incidence of GBC reflect distinct ethnic (genetic) association. Gallstone disease is a potential risk factor of GBC. Increase in the number and size of the stones is directly related to the risk of developing GBC [5]. Reports have shown that 60% [6] to more than 80% GBC cases possess stone (multiple/single) [7]. Hundreds of genes have been identified to be significantly associated with GBC and having potential to be developed as useful diagnostic/prognostic and even therapeutic biomarkers. Discovery of useful non-invasive biomarkers have always been desirable for diagnostic purposes, which are supposed to be safe and rapid [8]. Although our effort to dissect out the mechanism of gallbladder tumorigenesis is in progress, there are many challenges at various fronts. Hence, detailed investigations are needed in well classified group of samples, employing advance high through put technologies to understand the role of those genes, showing association with the GBC pathogenesis. This review is an attempt to summarize our current understanding on gallbladder tumorigenesis, use of technological strategies in search of clinically relevant biomarkers for early diagnosis, limitations and future prospects.

Mechanisms of gallbladder tumorigenesis

Various reports claimed Gall Stone Diseases (GSD) to be genetically modulated [9]. Upcoming information on family based investigations has replaced the quote as "GSD to be genetically associated" [10-11]. Formation of gallstone and Anomalous Pancreato-Biliary Duct Junction (APBDJ) are two pathological states for gallbladder tumor formation [12-14]. As GSD is an early pathological event, probably towards GBC, it is interesting to reveal its possible genetic association. We can call GSD as driver of GBC. Here, our effort is to present an update on the mechanisms that lead to GBC.

Genetic basis: Genetic association of GBC has become clear from various reports. Initially, the use of PCR based Loss of Heterozygosity (LOH) method could identify the loci, which harbor susceptibility genetic markers. Some of the earlier studies using PCR based allelotyping method detected more than 10 LOH-loci in GBC from Chile [15-16]. Restriction Fragment Length Polymorphism-PCR (RFLP-PCR) or gene sequencing is one of the most conventional methods used for identification of genetic variants [17-18]. Genome-wide allelotyping identified 21 hot spot loci, including *RNF4, SH3BP2, AF6q21, CD24, p73, DUTT1, FHIT, RAR-β, BLIMP1, CCNC, SMOH, PDGFR b-like, N33, FEZ1, p16Ink4/CDKN2, p15INK4b/CDKN2, NBCCS, DEC1, TSC1, CACNB2, MEN-1, TP53, STK11/LKB1* and *NF2*, distributed on 16 different chromosomes in GBC [19]. In a genome wide study in Japanese population, a genetic variant of *Deleted in Colon Cancer* (*DCC*) was found linked with Gallbladder Cancer [20]. Earlier studies claimed mutations to be responsible for their loss of expression of *K-RAS* [21], *TP53* [22] and *CDKN2A* [15] in GBC, although later other mechanisms were also suggested. Hundreds of genes are expressed or regulated as genetic variants in GBC. Srivastava et al., (2011), in a meta-analysis, showed possible risk

Citation: Tekcham DS and Tiwari PK. Gallbladder Cancer: Approaches to Biomarker Discovery. Austin J Clin Pathol. 2015; 2(3): 1036.

Table 1: Selected list of LOH/genes/SNPs identified by high throughput technologies for biomarker identification in GBC and GSD.

SL. No.	No. of Molecules Identified	Significant markers	Biopsy	Technology	Status	References
	LOH/Genes/SNP			*Genomics*		
1	-------	KRAS, TP53, ERBB3, EGFR, ERBB2, ERBB4	Tissue	WES & UDS	GBC	[54]
2	14	IDH1, KRAS, NRAS, PIK3CA, MET	Tissue	SMA	GBC	[57]
3	26	TP53, STK11, RICTOR, TSC2	Tissue	NGS	GBC	[57]
4	------	D1S1597 and D1S407	Tissue	GWAS	GSD	[58]
5	130	DCC	Tissue	GWAS	GBC	[20]
6	21 hot spots	p73, DUTT1; FHIT, RARβ, RNF4; SH3BP2, AF6q21, CD24, BLIMP1, CCNC, TSC1, SMOH, PDGFR b-like, N33, p16, p15, NBCCS, DEC1, CACNB2, MEN-1, TP53, STK11/LKB1, NF2, FEZ1	Tissue	Genome-wide allelotyping	GBC	[19]
7	1281	RRM2, PTTG1, TYMS, CDC2, CCNB2, RACGAP1, SHMT2, ANK2, ACACB, MYOM1, ITGA7, CDKN1C ALDH1A2, CNN1, CES1, DES	Tissue	array CGH & RTPCR	GBC	[59]
8	5	APC, CDKN2A, ESR1, PGP9.5 and SSBP2	Tissue	ELISA based & qMSPCR GBC & GSD	GBC & GSD	[60]

Table 2: Selected list of small RNAs/ miRNAs identified by high throughput technologies for biomarker identification in GBC and GSD.

Sl. No.	No. of Molecules identified	Significant markers	Biopsy	Technology	Status	References
	MicroRNAs			***Transcriptomics***		
1	---	miR-26, miR-34a	Tissue/cell line	Validation Assays	GBC	[41,62]
2	---	miR-146b-5p	Tissue/cell lines	Validation Assay	GBC	[40]
3	11	let-7a, miR-21, miR-187, miR-143, miR-202, and miR-335	Blood Serum	Micro Array	GBC	[56]
4	17	miR-210	Tissue	Illumina Sequencing & RTPCR	GBC	[63]
5	17/880	miR-20a	Tissue	miRNA Library Screening by High Content Screening	GBC	[64]
6	36/481	miR-1, miR-133, miR-143, miR-145	Tissue	Microarray & RTPCR	GBC	[42]
7	---	miR-21, miR-142-3p, miR-122, miR-142-5p,miR-223	Mice Tissue	Microarray & RTPCR	GBC	[65]

and association of various genetic variants with GBC [18]. Significant association of *Prostate Stem Cell Antigen* (*PSCA*) gene variants with GBC is reported in female patients in North Indian population [23]. In the same population group, increased risk of GBC was also found associated where genetic variants of *Matrix Metalloproteinase* (*MMP-2, 7, 9*), *tissue inhibitor of metalloproteinase* (*TIMP-2*) [24], *CYP1A1* and *CYP1B1* [25] were pre-dominant. The *ApoB-100* X+X+ genotype is also suggested to be associated with reduced risk of GBC [26]. Micro Satellite Instability (MSI), a common genetic mechanism of change in tandem repeats, is commonly observed in several genes, like *TGFβR-II* [27], *BAT25, BAT26, D2S123* and *D17S250* [28] in GBC. Loss of heterozygosity is one such mechanism suggested to play a key role in GBC [15,19,29] (Table 1).

Epigenetic basis: Introduction of methylation specific PCR to cancer research in 1996 opened the new area of **"epigenetics"** in cancer [30]. The large number of reports published so far has demonstrated epigenetics to be equally important as genetic mechanisms in the process of tumorigenesis. DNA methylation, histone modification (acetylation, phosphorylation, ubiquitination, sumoylation, methylation, etc.,), RNAi, etc., are a few examples of the epigenetic mechanisms. Much less is known about the role of epigenetics in GBC. Promoter hypermethylation of *p16, p73, APC, MGMT, hMLH, RAR* [31], *SHP1, 3-OST-2, CDH13, p15, CDH1, RUNX3, RIZ1, p16INK4A* and *HPP1* genes [32] are commonly reported in GBC. We also screened the methylation pattern of several target tumor associated genes in the GBC patients of north central India, which showed *APC* [33] and *PTEN* (manuscript submitted) to be epigenetically down regulated in GBC. Among other genes analyzed, *Maspin* and *14-3-3 sigma* genes also showed promoter methylation in most of the GBC cases [34]. Recently, Feng et al. demonstrated regulation of proliferation of GBC cells by histone acetyl transferase enzyme genes, *ATF2*, *KAT5* and *EPC1* [35]. A list of methylated genes in GBC may be found in Letelier et al [36]. The role of histone modification in GBC is still not fully known. Recent reports have implicated the role of small non-coding miRNAs, like miR-215-8p [37], miR-130a [38], miR-138 [39], miR-146b-5p [40], miR-26a [41], miR-1, miR-145 [42] and miR-20a [43] in GBC. More extensive investigation is required to understand fully the role of epigenetics in GBC (Table 2).

Alterations in Gene Expression in Gallbladder Cancer

The above mentioned epigenetic mechanisms alter the pattern of expression of many genes in GBC. The bile proteomic study by our group demonstrated more than 2500 proteins in human bile in cholecystitis [44]. Tan, et al., [45-46] also identified few dozens of proteins in GBC serum and tissue, and demonstrated Annexin A3, S100A10 and Haptoglobin as promising molecular targets of GBC. Increased expression of Phospho-mTOR [47], CLIC1 [48], ER1 and VEGF-A [49] and lower expression of Equilibrative

Table 3: Selected list of proteins identified by high throughput technologies for biomarker identification in GBC and GSD.

Sl. No.	No .of Molecules identified	Significant markers	Biopsy	Technology	Status	References
	Proteins			***Proteomic***		
1	2550	MARCKS, BSG, EPCAM, ICAM1, AXL, PAK1, ERBB2, NDRG1, CIAPIN1, ALCAM	Human bile	LC-MS/MS	Cholecystitis	[44]
2	544	S100A8	Tissue	2D & MS	GBC	[66]
3	495	S100A8	Tissue	2D & MS	Cholecystitis	[66]
4	24	S100A10, Haptoglobin, Cystatin-B, Profilin-1 and superoxide dismutase	Serum	MALDI-TOF-MS	GBC	[46]
5	46	Annexin A3, PEBP1	Tissue	2D & MS	GBC	[45,67]
6	26	CLIC1, Ezrin, Vimentin, Annexin A3, WD repeat domain 1, Triosephosphate isomerase, C1-tetrahydrofolate synthase, Rho GDP-dissociation inhibitor 1, T-complex protein 1, Heterogeneous nuclear ribonucleoprotein K, Glutamate dehydrogenase 1, Proteasome activator complex subunit 3 and Rab GDP-dissociation inhibitor beta	Cell line	2D & MS	GBC	[57]
7	6	ANXA4, HSP90 beta, Dync1h1, ACTA2, Serum albumin	Tissue	MALDI-TOF-MS	GBC	[68]
8	2575	NUCKS1, DMBT1, Mucin 5A, MUC13, LAMB3, PSAP, HMGB2, NEFH, S100AS, TAGL	Tissue	LC-MS/MS	GBC	[51]
9	26	Vimentin	Cell line/*in vivo*	2D & MS	GBC	[69]

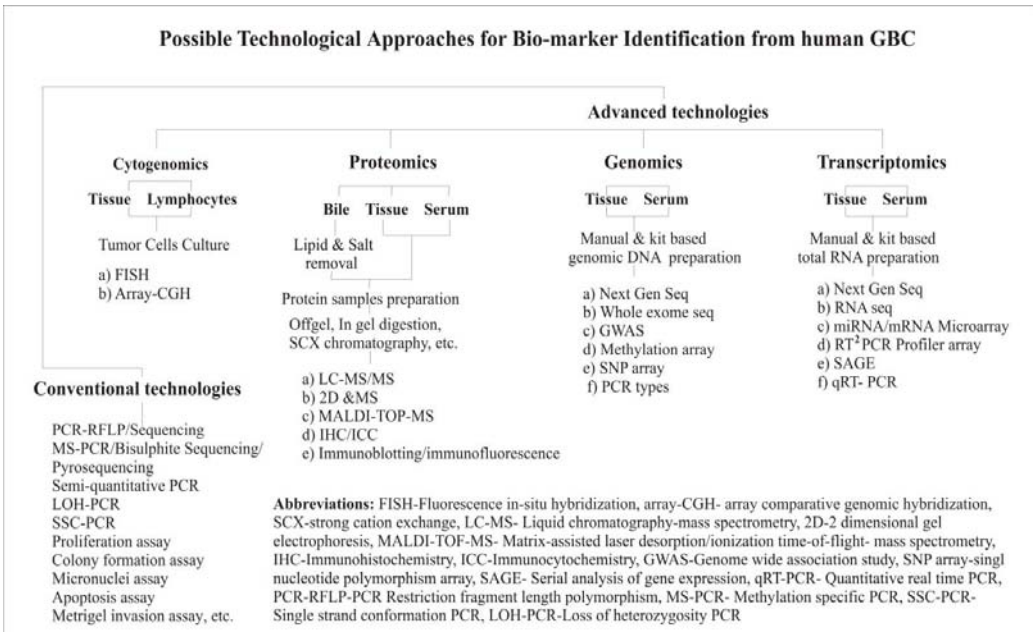

Figure 1: Flowchart showing various conventional and advanced technological based biomarker identification approaches available for GBC.

Nucleoside Transporter member 1 (ENT1) [50] are suggested to lower the prognosis of GBC. Our recent proteome profiling of GBC tissues identified Prosaposin and Transgelin as potential diagnostic biomarkers for GBC [51]. SOX4 and HSPgp96 were recently reported to have independent prognostic values, strongly correlated with the survival of GBC patient [52-53]. Positive expression pattern of Maspin, IMP3, and S100P and the negative expression of pVHL are suggested to be useful in discriminating tumor tissues of GBC from normal ones [54] (see Table 3).

Technological Strategies in Biomarker Identification

Advancement in the high throughput technologies has brought tremendous improvement in the ideological development to identify biomarkers during the last decade. Biomarker may be any molecule having prognostic, diagnostic and therapeutic properties. Although, conventional technologies are still useful for the purpose, the novel high throughput technologies have significantly added a number of significantly altered molecules to the list of biomolecules of diagnostic/ prognostic utility, already identified in GBC. Next generation sequencing, exome sequencing, RNA sequencing, Genome-Wide Association Study (GWAS), Proteomics, microarray, etc., are some of the major high throughput technologies currently being employed in the discovery of biomarkers in GBC (see Figure 1). Besides, selection of biopsy samples needs meticulous planning. Gallbladder tissues, bile, blood and urine are the main sources for the discovery

of biomarkers, which may add to the specificity of the identified candidate genes. Exome sequencing based analysis have identified several mutations in *TP53, KRAS* and *ERBB3*. Extensive mutations were reported in the *ErbB* signaling pathways [55]. Similarly, a recent genome-wide association study in GBC cases from Japanese population identified *DCC* to be associated with GBC [20]. Our lab is currently validating the methylome data on GBC tissue samples obtained from Illumina's Infinium 450K Beadchip array technology. So far, no published report is available on global methylation array and RNA seq in GBC. Microarray analysis of miRNA has updated the list of miRNAs in GBC and has drawn miRNA specific signatures in GBC [55]. Small RNAs, like miR-187, miR-143 and miR-202, are found to be key molecules having clinicopathological significance in GBC [56]. The other most promising strategy for biomarker identification could be the comparison of tissue, bile and serum proteomics together. Any altered molecule present in tissue may likely be released into body fluid during the course of tumorigenesis. Hence, simultaneous identification of molecules in all three media shall reveal the potential of differentially expressed molecules as a candidate biomarker in GBC. So far, no discovery has been made on the ultimate molecules with specificity and efficacy for diagnosis of GBC at an early stage. Identification of Annexin A3 [45], Prosaposin and Transgelin in tumor tissue [51], S100A10 and Haptoglobin in serum [46] and Chloride Intracellular Channel 1 (CLIC1) in GBC cell lines [48,57] are the part of this venture, however, having different prognostic values.

Limitations of strategies

There are certain common problems arise in such investigations. Availability of all types of biopsies is the main issue. Due to patient's health issues, blood samples are often missed. Different technologies also have their own limitations. The whole genome or high through put technologies have higher chance of erroneous incorporation of some faulty data, which always needs validation by conventional technologies to confirm and correlate the findings. Early stages of the discovery, i.e., collection, preparation and processing of biological samples, are some of the important tasks to be taken care of in such investigations.

Future perspectives

The technological advancement always has a "to-go" approach to get more reliable and specific markers of GBC. Global methylation, miRNome and RNA sequencing analyses are the key technologies, which will have strong platforms to provide support and meaning to the currently available data obtained from proteomics and other methods in near future. We need to focus on all possible approaches to reach our goal. We have to wait till we identify molecules/markers with altered expression in tissue and blood (and hopefully, urine), and their subsequent confirmatory tests in other cancers too. Arriving at this stage may take some more time, but is not too far.

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Austin J Clin Pathol - Volume 2 Issue 3 - 2015
ISSN : 2381-9170 | www.austinpublishinggroup.com

Citation: Tekcham DS and Tiwari PK. Gallbladder Cancer: Approaches to Biomarker Discovery. Austin J Clin Pathol. 2015; 2(3): 1036.