Epigenetic Changes in Carcinogenesis of Gallbladder

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Abstract Gallbladder cancer (GBC) is a lethal and a common malignancy affecting mostly females. There are restricted high incidence pockets across the world and in northern India highest incidence of GBC is reported from the Gangetic belt. The etiology of this disease remains largely unknown though several risk factors have been stated. The genetic aberrations in GBC involving mutations in tumor suppressor genes and oncogenes have been reported in literature. However, there is scarcity of data regarding epigenetic changes that may also be involved in gallbladder carcinogenesis. This review attempts to summarize our current understanding of the epigenetic changes in GBC.

Keywords Epigenetic changes · Gall Bladder Cancer · DNA methylation · Histone modification

Introduction

Gall Bladder Cancer (GBC) is the third most common cancer of gastrointestinal tract and the most common malignancy of the biliary system in northern Indian females [1]. In India, GBC is most prevalent in northern and north-eastern states of Uttar Pradesh, Bihar, Orissa, West Bengal and Assam [2]. Six cancer registries of the Indian Council of Medical Research (ICMR) (1990–96) show a ten times

lower incidence of GBC per 1,00,000 in South India compared with the North, the age-adjusted incidence rate (AAR) for females being 0.8 in Chennai in the south and 8.9 in Delhi in the north [3]. GBC ranks amongst the first ten cancers in the ICMR registries (2006–2008) of Delhi, Dibrugarh, Kolkata, Bhopal and Mumbai [4]. Besides Northern India, GBC is a very common disease in countries such as Chile (16–27/100,000), Japan (7/100,000), central Europe—Poland (14/100,000), Israel (5/100,000) and south Pakistan (11/100,000); however, it is uncommon in United States (1.5/100,000) [5]. Overall, the rates are three times more frequent in women than in men with a gender discrepancy even more pronounced in India and South America. The incidence of GBC increases after the age of 45 years and is maximum at the age of 65 years [6]. Thus, the worldwide incidence shows striking gender, geographic and ethnic variations, suggesting a strong influence of genetic and environmental factors in its causation.

GBC is notoriously known for vague presenting symptoms mimicking symptomatic cholelithiasis and is often diagnosed only in advanced stage. The mortality from GBC remains high due to its aggressive and silent nature [7]. The overall mean survival rate for patients with advanced GBC is 6 months: with a 5-year survival rate of 5 % [8].

The well-recognized risk factors for GBC are gallstones and cholecystitis. Gallstones are present in 74–92 % of patients with GBC [9]. Duration of gallstones more than 20 years is also a risk factor (RR 6.2) [10]. However, contribution of silent/asymptomatic gallstones in causation of GBC remains enigmatic [11].

Chronic bacterial infections also cause irritation and inflammation in the gallbladder. *S. typhi* carriers have a RR of 12.7 to 167 with 6 % developing GBC [12–14]. *H. pylori* DNA too has been detected in the gallbladder of patients with GBC [15]. *H. pylori* is found 3.5 times more frequently in presence of gallstones and 9.9 times more frequently in presence of GBC in comparison to normal control [16] but it

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is uncertain if *H. pylori* has a role in the pathogenesis of gallbladder disease [17]. Gallbladder polyps [18], dietary factors [10, 19, 20], obesity [21], smoking and alcohol use [22, 23], environment water pollution by chemicals and pesticides have also been implicated in the causation of GBC [24–26]. It has been observed that GBC patients here in northern India often belong to low socioeconomic status, consume open mustard oil and water from hand pump [27].

The research in GBC will be greatly helped if populations with significantly lower or higher rates of disease could be identified and categorized as high risk and low risk zones of GBC. This will help not only in focused research but also in planning prophylactic measures such as cholecystectomy in selected patients with asymptomatic gallstones in high risk zones [28]. The high incidence of GBC in the Gangetic belt is a big challenge for us to explore the association of risk factors for GBC at the genetic level. We have published a number of original basic research papers in the subject [29–35]. However, not much work has been done on epigenetic changes that could be involved in the etiopathogenesis of GBC. This is especially important because epigenetic changes are emerging as a mechanism involved in toxicant-induced malignant transformation for environmental and occupational carcinogens such as arsenicals, cadmium 1,3-butadiene, pesticides, pharmaceutical and biological agents [36–39]. Epigenetic alterations have been reported in cancer of the breast [40, 41], lung [42], prostate [43], cervix [44], colorectal [45, 46], esophagus [47], stomach [48], head and neck squamous cell carcinoma [49], urinary bladder [50] and osteosarcoma [51]. This article intends to provide a brief overview of epigenetic changes in GBC.

What are Epigenetic Changes?

Our DNA (deoxy-ribonucleic acid) is a right handed double helix structure formed on the backbone of nucleotides Adenine (A)—Thymine(T) and Cytosine(C)—Guanine(G) that are bound together by phosphodiester bonds. A gene though originally defined as a “unit of inheritance”, now has evolved to mean a DNA-based unit that can exert its effects on the organism through RNA (ribonucleic acid) or protein products. Some genes contain instructions for cell growth and division. Genes that promote cell division and proliferation are called *oncogenes*. Genes that slow down cell division or cause cells to die at the right time are called *tumor suppressor genes* (TSG). Cancers can be caused by a number of mutations (deletion, frame shift mutation, substitution, e.t.c.) that can turn on oncogenes or turn off the TSGs resulting in over-expression of the gene. Changes in several different genes are usually needed for a cell to become cancerous.

A typical protein coding gene has regulatory regions called ‘promoters’ which act as a gateway to the gene and

play an important role during transcription (Fig. 1). Changes may also occur in the promoter regions that have a direct bearing on gene expression.

Epigenetic changes are defined as heritable changes in gene expression activity and expression that occur without alteration in DNA sequences but which are sufficiently powerful to regulate the dynamics of gene expression [52]. The key processes that are responsible for epigenetic regulation are DNA methylation, modifications in chromatin (covalent modification of core histones), nucleosome positioning (physical alteration), and posttranscriptional gene regulation by noncoding RNA or micro-RNAs (miRNAs) [53]. The promoter regions are especially rich in the CpG islands and hence prone to epigenetic changes (Fig. 2) [54].

DNA Methylation

DNA methylation occurs at the 5' position of the cytosine ring within CpG dinucleotides via addition of a methyl group to form 5-methyl cytosine. The modification at 5-methyl cytosine is catalyzed by enzymes known as DNA methyl transferases (DNMTs). Although DNA methylation regulates gene expression in normal tissues through genomic imprinting and female X-chromosome inactivation, these processes are significantly altered in cancer due to loss of imprinting (LOI). In addition to DNA methylation, methylated DNA binding proteins (MECP2, MBD1, MBD2, MBD3, MBD4, and Kaiso) can bind to methylated cytosine, forming a complex with histone deacetylase (HDAC) that leads to chromatin compaction and gene silencing [55].

Histone Modification

Chromatin proteins serve as building blocks to package eukaryotic DNA into higher order chromatin fibers. Each nucleosome encompasses ~146 bp of DNA wrapped around an octamer of histone proteins. These octamer consist of double subunits of H2A, H2B, H3, and H4 core histone proteins. The histone proteins coordinate the changes between tightly packed DNA (heterochromatin) that is inaccessible to transcription and exposed DNA (euchromatin) that is available for binding to and regulation of transcription factors [56]. These changes occur due to structural characteristics of the nucleosome that are known as “histone tails,” which extend from the core octamer. These tails consist of N-termini of the histone proteins and are the major sites for posttranslational modifications. The list of

Fig. 1 A typical protein coding gene

Fig. 2 Epigenetic inactivation of a DNA repair gene promoter. Promoters are often embedded within CpG islands. These CpG-rich sequences are usually unmethylated in normal tissues and are associated with the active histone mark H3K4me3. H3K4me3 prevents DNA methylation. During tumorigenesis, the CpG island becomes methylated, is associated with inactive chromatin marks (e.g. H3K9me3), and the gene becomes silenced [54]

potential modifications includes acetylation, methylation, phosphorylation, ADP-ribosylation, ubiquitination, sumoylation, and biotinylation. The majority of these modifications take place at lysine, arginine, and serine residues, and within the histone tails of H3 and H4 core proteins. These modifications are reversible and are controlled by groups of enzymes. Histone deacetylases (HDACs) and histone acetyl transferases (HATs) are the key enzymes responsible for these reversible modifications [57].

Noncoding RNAs

Noncoding RNAs participate in the epigenetic phenomenon of posttranscriptional gene modification. The miRNAs are 21–23 nucleotides in length. Although miRNA are vital to normal cell physiology their misexpression has been linked to carcinogenesis [58].

In normal cells, CpGs within repetitive DNA elements and coding regions of the genes are methylated, whereas in tumor cells LINE-1 repeats, satellite DNA, and moderately repeated DNA sequences become unmethylated, whereas genes containing CpG clusters become hypermethylated, rendering them transcriptionally silent [59].

Epigenetic Changes in GBC

Major epigenetic mechanism for silencing of TSG is aberrant methylation of 5' gene promoter regions in many cancers. DNA methylation of the promoter regions is emerging as the major mechanism of inactivation of TSGs. It is proposed that aberrant DNA methylation of the CpG island genes provides an alternative pathway to gene deletion or mutation for the loss of TSG function. In GBC, there is very limited information regarding abnormal methylation of cancer-related genes.

The existence of methylation in the promoter gene areas has been related to the cellular proliferation, invasion and metastasis and also in cases of chronic cholecystitis, suggesting that this epigenetic phenomenon represents a crucial early event in gallbladder carcinogenesis [60].

There is a report of methylation analysis of six candidate TSGs (*P16*, *APC*, *MGMT*, *hMLH1*, *RARβ2*, and *p73*) [61] that showed significant differences in gene methylation patterns between 54 GBC cases from Chile and the United States. Aberrant methylation of any of the six candidate TSGs was detected in 72 % of the GBC, 28 % of the cases of chronic cholecystitis, and in only 1 of the 15 normal gallbladder controls. Genes *p16* (56 %), *p73* (28 %), *APC* (27 %), and *hMLH1* (14 %) were found most commonly methylated in GBC. Gene methylation significantly differed GBC samples from the United States and those from Chile. *APC* ($p=0.028$) and *p73* ($p=0.034$) methylation was present in 42 % and 13 % respectively of the United States GBC samples compared to 14 % and 40 % respectively of the Chilean tumors. This led the authors to state that the acquisition of hypermethylation at multiple TSG-promoter sites may contribute to tumor formation and progression within the chronically inflamed gallbladder. The apparent differences in methylation patterns among the Chilean and United States gallbladder cases may indicate a unique biology associated with this cancer in different parts of the world.

Yet another study from Chile [62], investigated methylation profile of 14 genes in 35 chronic cholecystitis (separated according to the presence or absence of metaplasia), 19 early GBC (mucosa or muscularis propria invasion) and 48 advanced GBC with invasion of the gallbladder subserosa (25 cases) and serosa (23 cases). A progressive significant ($p \leq 0.05$) increase in their methylation status from chronic cholecystitis without metaplasia to advanced GBC was seen in four genes (*DAPK1*, *DLC1*, *TIMP3*, and *RAR-beta2*). This did not differ with the age of the patient. A methylated *DLC1* gene signified poor prognosis ($p=0.04$), whereas a methylated state *MGMT* gene correlated with better patient survival ($p=0.006$). The authors concluded that aberrant hypermethylation of promoter regions is an early, progressive and cumulative event in gallbladder carcinogenesis. Furthermore, the methylation levels seem to accumulate in the progression of chronic cholecystitis without metaplasia to chronic cholecystitis with metaplasia, a fact that could provide new evidence to consider this morphological adaptation of gallbladder mucosa as a premalignant lesion. Finally, the methylation status of some individual genes could be useful biomarkers with potential clinical application in diagnosis or prognosis of GBC if they are validated in a greater number of clinical samples [62].

Roa JC et al. from Chile [63], analyzed the methylation pattern of *CDKN2A* (*p16*), *MLH1*, *APC*, *FHIT*, and *CDH1* (*E-cadherin*) genes in 20 GBC samples and further

examined the immunohistochemical expression of proteins, clinical features, and patient's survival. 95 % of tumors were moderate or poorly differentiated. Methylation of the promoter area of genes was observed in 5 %, 20 %, 30 %, 40 %, and 65 % of cases, and an altered immunohistochemical pattern (AIP) in 5 %, 35 %, 21 %, 25 %, and 66 % for the MLH1, CDKN2A, FHIT, APC, and CDH1 genes, respectively. The Kappa concordance index between methylation of the promoter area and AIP for the MLH1 and CDH1 genes was very high ($K > 0.75$) and substantial for APC ($K > 0.45$). There was no correlation between the methylation of the genes and survival [63]. Again, these findings suggest that the promoter methylation and subsequent inactivation of TSGs and genes related to the control of cellular proliferation is important in gallbladder carcinogenesis.

Takahashi T, et al. [64] investigated the TSG methylation profile in GBC using the 24 candidate genes frequently found silenced by aberrant methylation in a number of tumors. These 24 genes were chosen for study due to their presumed or known roles in various cellular functions related to cancer development such as cell cycle regulation, tissue invasion and metastasis, signal transduction, apoptosis, angiogenesis, putative cytokine, mitotic stress checkpoint, methyltransferase superfamily, and *O*-sulfotransferase. Analysis of the methylation frequencies in 50 GBCs from Chile were compared with the findings in 25 chronic cholecystitis specimens demonstrated a group of eight genes (*3-OST-2*, *CDH13*, *CDH1*, *RUNX3*, *APC*, *RIZ1*, *P16* and *HPPI*) that showed specificity for GBC. Of these genes, the gene with the highest frequency (72 %) of methylation in GBC was *3-OST-2*. This gene encodes an *O*-sulfotransferase that is involved in the final modification step of glycosaminoglycan chains of heparan sulfate proteoglycans (HSPGs), that are known to play major roles in cell growth, adhesion, and migration by interaction with a wide range of growth factors, morphogens, cytokines, and extracellular matrices [65]. Silencing and aberrant promoter hypermethylation of *3-OST-2* has also been demonstrated in several human cancers, including those of the breast (88 %), colon (80 %), lung (70 %), and pancreas (100 %) [66].

A study from Korea [67], examined the methylation status of the promoter of PGP9.5 gene and its expression in surgical biopsy samples of 22 GBC, eight adenomas, 26 normal epithelia. Protein gene product 9.5 (PGP9.5) is a neurospecific peptide that removes ubiquitin from ubiquitinated proteins and prevents them being targeted for degradation by proteasomes. Its expression is a potential marker of non-small lung cancer, invasive colorectal cancer and esophageal squamous cell carcinoma. PGP9.5 promoter was found methylated in 84.6 % (22/26) of normal gallbladder epithelium, 37.5 % (3/8) of adenomas and 27.2 % (6/22) of GBC. GBC samples were found to have an unmethylated PGP9.5 gene promoter and exhibited positive staining for

PGP9.5 in epithelial and neoplastic cells, but no PGP9.5 expression was observed in normal epithelia or in tumor tissues with a methylated promoter. PGP9.5 hypomethylation was found to be inversely correlated with the presence of a gallstone ($P = 0.015$). These results suggest that PGP9.5 promoter hypomethylation could be a reliable marker for GBC and that DNA hypomethylation might play an important role in re-expression of the PGP9.5 gene in GBC [67].

Another study from Korea [68], analysed the methylation status of tumor suppressor gene Ras association domain family 1A (RASSF1A), the expression of RASSF1A protein, and the correlation between these and the clinicopathological features of GBC in 22 GBC, 8 adenomas, 26 normal epithelia samples. RASSF1A promoter (region 1) and first exon (region 2) was found significantly methylated in 22.7 % (5/22) and 36.4 % (8/22) in GBC respectively as compared to 12.5 % (1/8) and 25.0 % (2/8) adenomas, 0 % (0/26) and 8.0 % (2/26) normal gallbladder epithelia respectively. Reduction or loss of RASSF1A expression was observed in most methylated adenocarcinomas suggesting that downregulation of RASSF1A expression by DNA hypermethylation may be involved in gallbladder carcinogenesis.

Loss of heterozygosity (LOH) involving polymorphic markers on the short arm of chromosome 3 is frequently detected in a variety of human epithelial tumors, including GBC. LOH is a surrogate marked for chromosomal deletions. Riquelme et al. [69] carried out analysis of methylation status of six candidate TSGs located in chromosome 3p, including DUTT1 (3p12), FHIT (3p14.2), BLU, RASSF1A and SEMA3B (3p21.3) and hMLH1 (3p21.3) in 50 GBC samples. A very high frequency of methylation was detected in SEMA3B (46/50, 92 %) and FHIT (33/50, 66 %), intermediate incidences in BLU (13/50, 26 %) and DUTT1 (11/50, 22 %) and very low frequencies in RASSF1A (4/50, 8 %) and hMLH1 (2/50, 4 %). Allelic loss at 3p21.3 was found in nearly half of the GBCs examined. Hence, epigenetic inactivation by abnormal promoter methylation is a frequent event in chromosome 3p candidate TSGs in GBC pathogenesis, especially affecting genes SEMA3B (3p21.3) and FHIT (3p14.2).

Conclusions

It is apparent there are limited studies exploring the possible role of DNA methylation in the etiopathogenesis of GBC. The epigenetic alterations affecting TSGs involved in regulation pathways, cell cycle control, cell adhesion and extracellular matrix degradation, seem to have an important role to play in gallbladder carcinogenesis. Further, determining DNA methylation patterns would allow them to be used as biomarkers for the early detection, diagnosis, prognosis and/or therapeutic selection in GBC and should be a subject of intense research [70].

Unfortunately, similar studies have not yet been published from India where GBC is very common. A research project funded by the Department of Biotechnology, New Delhi looking in to this aspect is currently underway at our department and we do hope to come up with results that may throw further insights in to the epigenetic molecular aberrations involved in gallbladder carcinogenesis in this part of the world.

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