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EPIGENETICS OF GALLBLADDER CANCER: ROLE OF DNA METHYLATION AND HISTONE MODIFICATIONS

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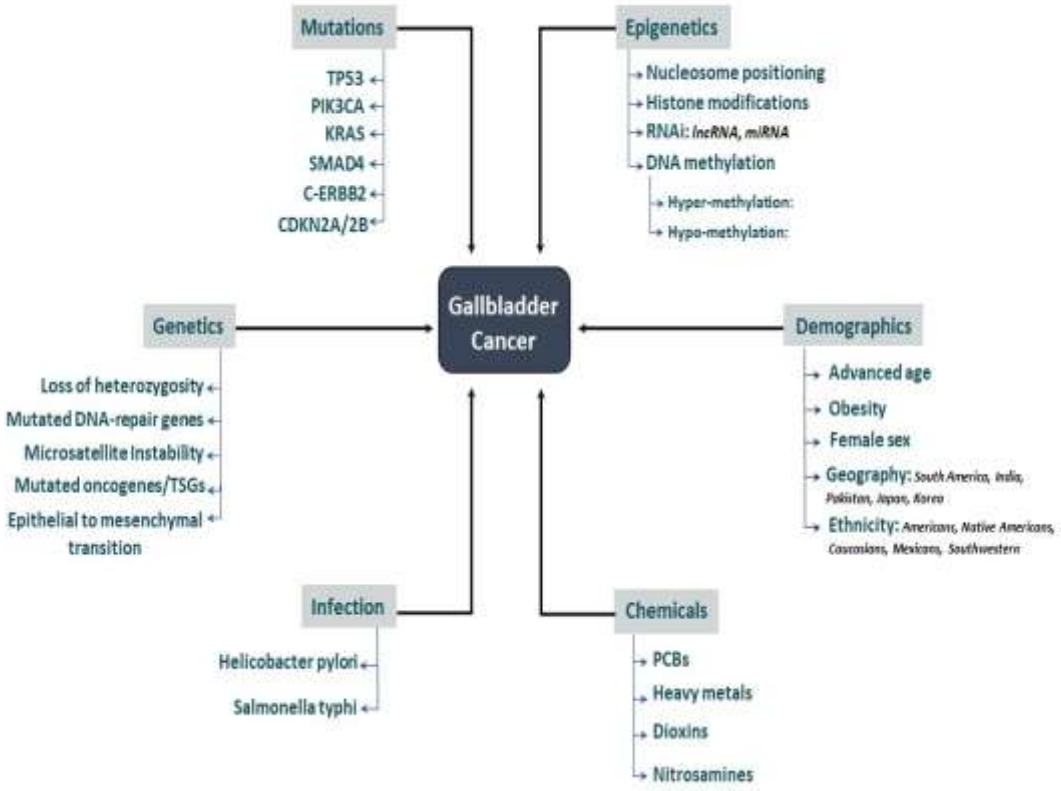
Abstract
Delayed diagnosis and inability to receive benefits from systemic treatment, gallbladder cancer has a worrisome prognosis and has become a major public health concern, worldwide. The precise cause of the occurrence of gallbladder cancer is concealed so far, however, several genetic (mutations), epigenetic (DNA methylation, histone modification), and environmental factors responsible for its causation have been identified. High-throughput techniques now permit an exhaustive study of genetic and epigenetic impairments linked to gallbladder cancer.

The subject of concern in this review is epigenetic regulation, particularly gene methylation, and histone modification, in gallbladder cancer. DNA methylation has evolved as a major molecular mechanism of gallbladder cancer. Different molecular markers have been identified as epigenetically modified in gallbladder cancer. The determination of DNA methylation profile would facilitate biomarkers to be developed for early identification, diagnosis, prognosis and selection of therapies for gall bladder cancer. This review provides an overview on the updated account of the studied made so far on the role of epigenetic modifications in the regulation of tumorigenesis of gallbladder.

Keywords: Gallbladder, Gallbladder cancer, Epigenetics, DNA methylation, Chromatin modification, Methylation specific PCR

1. Introduction

Gallbladder cancer is the most frequent malignant tumor of the biliary tract, with cholelithiasis and cholecystitis being its primary risk factors. The symptoms related to gallbladder cancer may be blurred, and not specific, which makes it even harder to detect early. It has been linked to a number of risk factors, such as age, bacterial infection, sex, ethnic origin, and obesity (1). The relationship between gallstones and gallbladder cancer has been quite disconcerting, just because the two are not directly related. However, gallstones are always a key risk factor for gallbladder cancer (2). A number of genetic, epigenetic and non-genetic factors are known to be responsible for this cancer.



(Fig. 1: Factors causing Gallbladder cancer).

The pathogenicity of gallbladder cancer is known little relative to other cancers (1) and the incidence varies widely from site to site depending upon ethnicity (3). Newly reported cases of gallbladders were 1,15,949 and the global fatality rate was 84,965 in 2020, in accordance with GLOBOCON (4). The prevalence of gallbladder cancer is especially high in South America and Asia, where cholecystitis and salmonellosis rates are also high. India also has a high rate of GBC incidence in its northern, north-eastern, central and eastern regions, however, significantly less prevalent in southern and western India (5).

At the present time, the epigenetic basis of cancer, particularly DNA methylation in cancer, is the most studied domain. Epigenetics first came into existence in 1942, when Waddington was struggling to interpret the genotype versus phenotype connection (6). The epigenetics phenomenon is heritable, reversible gene regulatory forms, which do not rely on the sequence of DNA. The Key epigenetic changes include methylation of DNA and alteration of histones. Epigenetic mechanisms as a novel approach to therapy have received a great deal of attention in recent research over the last two decades. With the emergence of new scientific technologies and an epigenomic study, another epigenetic branch, known as 'Pharmacoepigenomic', is on the rise and may be used to investigate epigenetic profiles to study molecular pathways for drug susceptibility assessment in people with cancer and determine the most appropriate treatment options available (7).

2. Epigenetics and cancer

From inception, it has been believed that cancer is caused by both genetic and environmental factors. However, soon it was discovered that apart from these two factors, epigenetic factors also play equally responsible role in modifying the normal process (8). Some well-characterized epigenetic alterations are related to abnormal gene functions and modified gene expression that are crucial to the pathobiology of cancer, such as DNA methylation, covalent modification of histone proteins and RNA based mechanisms (8). DNA methylation is a critical component of the epigenetics mechanism regulating the gene expression. In eukaryotes, DNA methylation takes place by the addition of methyl

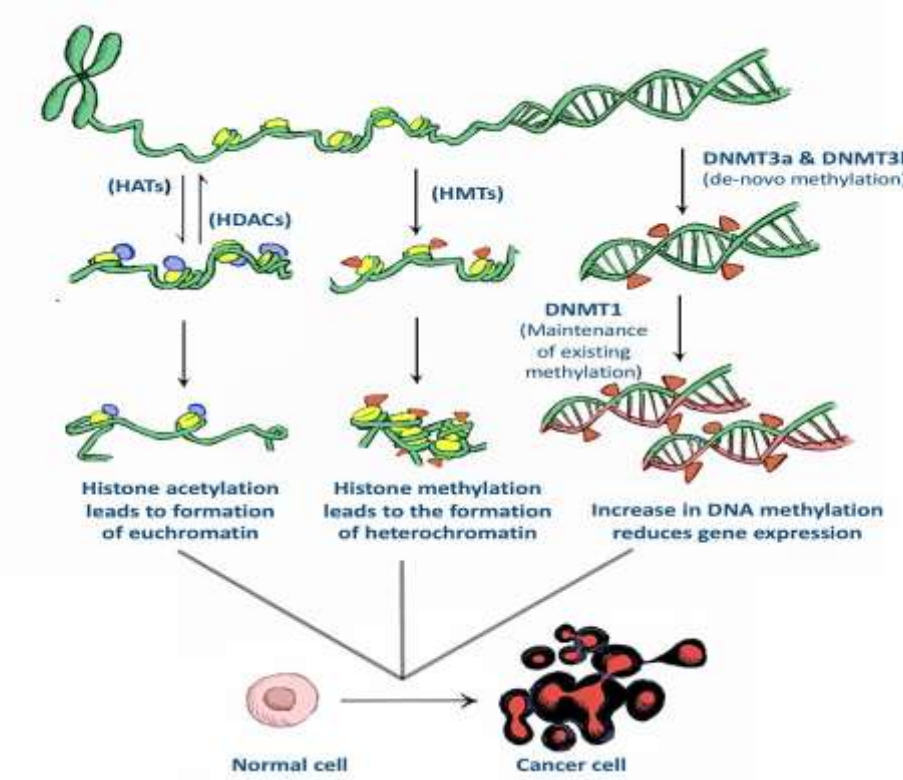
group to the 5' position of the cytosine in the CpG dinucleotide to form a 5-methyl cytosine (9). This modification of DNA methylation is essentially performed by different DNMTs, i.e. DNA methyltransferases. DNMT1 maintains existing methylation profiles after DNA replication by adding methyl groups to hemi methylated CpG site, while DNMT3a and DNMT3b are de novo methyltransferases. Different processes exist that lead to hypomethylation of DNA, such as altered DNMT activity, histone modification, altered exogenous insult, non-coding RNA, and defective DNA repair. These altered methylations lead to abnormal gene expression, loss of imprinting, and microsatellite instability (10). Hypermethylation suppresses the transcription of the tumor suppressor gene that leads to gene inhibition, which is a critical step towards cancer development, while overall hypomethylation leads to oncogenesis. To date, multiple genes have been reported in several cancers that undergo methylation. Not extensively studied as DNA methylation, the posttranslational modification of histone proteins is critically required to organize the chromatin structure.

By modulating the structure of chromatin, the histone protein acts on various essential processes, such as gene transcription, DNA replication, recombination and repair. Depending on the type and position of the attached functional groups and amino acid residues that are modified, the gene could be activated or repressed (11). Aberrant change in histone can be caused by inappropriate targeting of histone-modifying enzymes, such as HDACs (Histone deacetylases)), HATs (Histone acetyl transferases), HMTs (Histone methyl transferases) and HDMs (Histone demethylases), which, in turn, controls the transcription activation and repression. These changes are associated with the cause of multiple cancers. The balance of Histone acetyltransferase (HAT) and histone deacetylases (HDAC) adjusts histone modification, while the unbalanced activity of these two enzymes causes various cancers (12).

3. Epigenetic Regulation of gene expression

The epigenetic mechanism enables the organism to react to the environment by a change in gene expression. This is either the post-synthetic modification of the DNA or the protein that interacts with the DNA. This is brought about by the genes that has the modification of particular type that effect the signalling downstream and cause changes in the normal process. Epigenetic processes responsible for alteration of gene expression, chiefly include DNA methylation, posttranslational modification of histone proteins, remodelling of chromatin and RNA-based mechanisms (13). DNA methylation occurs most frequently on the CpG island, most often in the 5' promoter region of the gene (14). The gene silencing via DNA methylation is through the covalent addition of methyl groups from S-adenosylmethionine (SAM) to the 5th position of the cytosine base pair. The formation of 5-methyl cytosine structure may prevent binding of transcription factors (TFs) to gene promoters resulting in the repressive gene expression (15). The hypermethylation of the gene promoter region directly blocks the binding of key transcription factors critical to gene expression, thereby causing silencing of gene expression (16). Hypermethylation of other genes, in addition to tumor suppressor genes can also be highly associated with early and progressive cancer.

Apart from DNA methylation, histone alterations also play an important role in the regulation of gene expression by affecting the normal process leading to carcinogenesis. Histone modification regulates the transition from the state of transcriptionally active or silent chromatin to the regulation of coded genetic information in DNA. In chromatin, DNA is packed in a very compact structure around histone proteins, forming nucleosomes that makes it easier to control the accessibility of DNA sequence to various regions. Histone globular regions form the nucleosome, while N terminal tails extend beyond nucleosomes and contains the site for the variety of posttranslational modifications, such as methylation, acetylation, phosphorylation, ubiquitinylation and SUMOylation at specific amino acids (17). Among all the modifications of histones, acetylation and methylation of lysine at H3 and H4 are most often investigated. Histone acetylation is usually greater among promoters of active genes and affectsgenetranscription



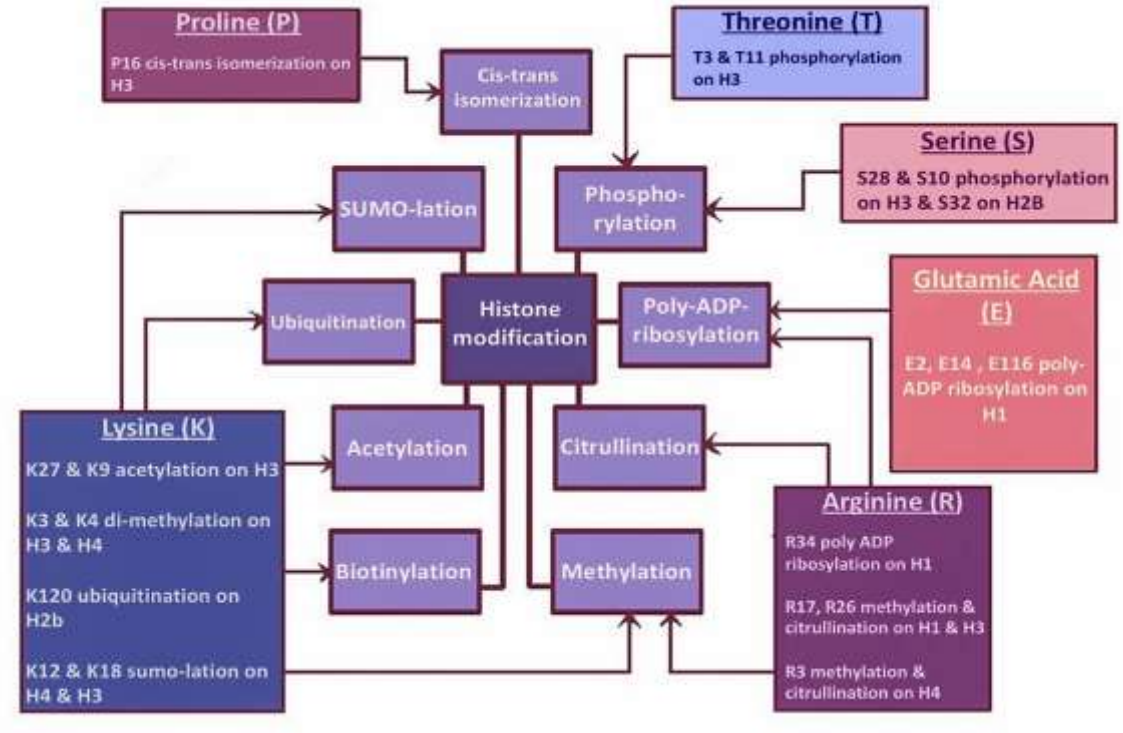
(Figure 2: Mechanism of DNA methylation and histone modification in carcinogenesis).

The addition of the acetyl group with the help of enzymes such as histone acetyltransferases and histone deacetylases allow the binding of transcription factors, while removal tightens the configuration. Overall, maintaining the equilibrium of histone acetylation alterations is critical for regulating gene expression and to prolong normal cellular conditions. The methylation process is somewhat complicated because lysine methylation at H3 leads to an increase in transcription, whereas at H4, results in transcription deceleration (18). The modification of these processes may lead to an unbalanced expression of genes and is widely observed in human cancers. By analysing the genomic profiles of histone modifications and gene expression, three distinct types of 'repressed' configurations were identified, viz repressed i.e., the closed configuration, 'active', i.e. the open configuration and 'bivalent', having both repressive and active histone markers.

In this era of high-throughput data, a number of new technologies are developed to measure the state of DNA methylation and chromatin structure at the genomic level, such as microarray-based and sequencing technologies. Thus, oncologists have access to world-class measurement technologies that evaluate DNA methylation at genome level, chromatin changes and protein interactions.

3.1 Chromatin modification

Eukaryotic genomic DNA is wrapped in chromatin by two major types of small, positively charged proteins, the core histones (H2A, H2B, H3 and H4) and the linker histone (H1). It essentially controls all DNA-based nuclear processes, including transcription, DNA replication and DNA repair. Approximately 146bp of DNA envelopes around an octamer of histone proteins (H1, H2A, H2B, H3 and H4) to form a nucleosome. In chromatin, heterochromatin has tightly packed DNA that cannot transcribe, while in euchromatin it is loosely packed DNA that can bind and regulate transcription. Changes in chromatin structure are caused by the histone tail present in the core octamer. N termini present at the tail of the histone proteins are the major site for posttranslational modifications. Modifications of the chromatin landscape often occur at the same time as alterations of DNA methylation. Acetylation and methylation at the lysine residues are the most common modifications taking place at the N terminal of the histone tail (19). Other includes phosphorylation, Ubiquitinoylation, SUMOylation and glycosylation(20)



(figure 3: Types of histone modification and histone modified sites).

As a result, changes in gene expression are regulated dynamically by chromatin alterations, frequently encountered in many human cancers. This may be either by functional gain or loss, suppression or overexpression by altered methylation level, mutations in histone complexes, or even in the modification site of the histone.

3.2 Histone alteration and its association with cancer

3.2.1. Histone methylation

Histone methylation is a process that plays a significant role in the development and differentiation. Any deterioration in their level causes cancer (21). Findings on histones methylation are quite contextual and may be related to differential gene expression. Histone methylation is closely linked to transcription regulation by impacting chromatin architecture. Generally, it occurs on lysine and arginine residues, primarily on histone H3, followed by H4.

EZH2 (Zest Enhancer homolog 2), a polycomb group protein, is a widely investigated histone-lysine N-methyltransferase enzyme, reported in many cancers (22). EZH2, following trimethylation of histone H3K27, transcriptionally supress the distinctive clusters of genes downstream. In the case of GBC, EZH2 is expressed significantly higher in GSD (gallstone diseases) (23). An over-expressed EZH2 and expressional loss of PTEN are associated with invasiveness, metastases and low progression of gallbladder carcinoma. (24). Histone de-acetylase inhibitor (suberoylanilide hydroxamic acid) and suppression of EZH2 synergistically inhibit the growth of gallbladder cancer (25).

LSD1 is a histone demethylase which functions as an epigenetic co-regulator of transcription and plays an important role in carcinogenesis, proliferation and metastasis (26). Overexpression of LSD1 promotes the development of GBC and can be a predictor of a deteriorating prognosis (27).

3.2.2. Histone acetylation

Histone acetylation is the extensively studied reversible posttranslational histone modification. It occurs on the ε-amino group of lysine residues and is regulated by the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (28). Acetylation of lysine amino acid is a post-translation alteration that modifies gene transcription through targeting histones and various

transcription factors of the nucleus (29). The type A histone acetyltransferases may be divided into three or more distinct groups according to homology of amino acid sequence and conformation framework, such as the GNAT, MYST and p300 families (30). KAT5 (lysine acetyltransferase 5) is a histone acetyltransferase enzyme in humans encoded by the KAT5 gene. Analysis of Microarray data showed that KAT5 RNAi can cause upregulation of cleavage casp9 by activating p38MAPK into GBC-SD cells. The expression of KAT5 mRNA is highly regulated in GBC tissues compared to adjacent normal tissues (31).
TCF12 promoter deacetylation is induced by HDAC1, leading to increased cellular invasion and migration. In addition, TCF12 targets HDAC1 to reduce regulation of the EMT marker (32). The development of cholangiocarcinoma and gallbladder carcinomas in vitro and in vivo may be avoided by trichostatin A (TSA), a histone deacetyalse inhibitor (33).

3.2.3 Histone phosphorylation- Phosphorylation of histone occurs predominantly on residues of tyrosine, serine and threonine and control gene expression by regulating cell cycle and proliferation (34). Phosphorylation interferes with histone-DNA interaction assigned to unstable chromatin structure that is required for structurally recombinant chromatin agglutination into chromosome for mitosis (35). It is important to take into consideration the different types of phosphorylated sites in histone that are highly related to the different chromatin functions. Furthermore, histone phosphorylation along with other alterations, such as histone acetylation and histone methylation, is involved in a range of cellular processes (36,37).

3.2.4. Histone ubiquitination- Both histone ubiquitination and histone deubiquitination are a part of metabolic reprogramming in tumour cells and are multi-stage enzymatic processes, implicated in various cellular activities (38). Cases of dysregulation of histone ubiquitin and histone deubiquitin have been observed in various types of cancers (39).

3.2.5. Histone sumoylation- Histone sumoylation is the covalent addition of small ubiquitin type modifier to a target protein and mitigates transcription activity via negative regulation. Although sumoylation has been detected primarily in histone H4, it also occurs partially in the other four core histones (40).

3.3 DNA methylation

While there are many ways to modify gene expression in epigenetics, DNA methylation is a usual phenomenon of silencing gene expression. It is not the same as histone methylation. This modification is essential for human development and diseases (41); however, the understanding of regulation is limited. An addition of a methyl group at the 5' carbon of cytosine residue in CpG island in the genomic DNA is DNA methylation. Methylation is controlled by DNA methyltransferases, e.g., DNMT1, DNMT3A, DNMT3B, DNMT3L (42). Methylation of DNA can affect the packaging of DNA into nucleosomes, followed by chromatin condensation into heterochromatin, inhibiting transcription (43). The majority of CpG islands are found in the gene promoter regions (44), and some of these undergo methylation during development, causing gene silencing. Abnormal DNA methylation at the promoter, enhancer, and sites regulating chromatin level can convert normal genes into oncogenes or tumor suppressors (45). DNA hypermethylation of a gene promoter usually results in gene silencing (42). On the other hand, DNA hypomethylation often occurs in cancer, subsequent to which chromatin becomes less dense and DNA transcription can take place. It is worth noting that inhibition of tumor suppressor genes by hypermethylation and activation of protooncogenes by hypomethylation are important mechanisms for promoting cancer progression.

4. Epigenetic modification in Gallbladder cancer-

In the case of gall bladder cancer, information about abnormally methylated cancer genes are highly restricted. Methylation of promoter region of the gene has been associated with cell proliferation, invasiveness and metastasis, as well as chronic cholecystitis, implying that epigenetic is a critical

early event in the cause of gallbladder cancer (46). The ongoing investigations to identify the methylation status of several candidate genes associated with gallbladder cancer are giving promising insight into the mechanism of Gallbladder tumorigenesis.

4.1 Epigenetic Biomarkers of Gallbladder Cancer: Protein Coding Genes and their Association with Gallbladder cancer

Studies carried out till date have projected several candidate genes in Gallbladder cancer undergoing significant changes in their methylation status, with consequent alterations in function as a result of altered promoter methylation. A brief description of some of these candidate genes may be worth here (Tables 1 and 2).

Table 1- Genes with histone modification in gallbladder cancer

Gene	Type of enzyme	Expressivity (GBC)	Other cancers	References
EZH2	**Histone methyltransferase**	**High**	**Prostate cancer, gastric cancer**	**50,51,52,53**
LSD1	**Lysine specific histone demethylase**	**High**	**Bladder cancer, Prostate cancer**	**140,141,142**
SMYD3	**Histone lysine methyl transferase**	**High**	**Gastric cancer, breast cancer**	**143,144,145**
EP300	**histone acetyltransferase**	**High**	**pancreatic cancer, Breast cancer, Prostate cancer**	**146,147,148**
BAP1	**de-ubiquitinase**	**Low**	**Breast cancer, Pancreatic cancer**	**149,150,151**
NSUN2	**Sun RNA methyltransferase 2**	**High**	**Head and neck squamous carcinoma**	**152,153**

Table 2- Hyper and hypo-methylated genes reported in gallbladder cancer

Gene	Full name	Functions	Pathway	Expression (GBC)	Methylation status (GBC)	Hyper methylation (Other cancer)	Hypo methylation (Other cancer)	References
MASPIN	Serpin family B member 5	Tumour suppressor	P53 pathway	Low	Hypomethylation	Thyroid, breast cancer	Pancreatic, hepatobiliary	57,58,59, 60,61,62
CDO1	Cysteine dioxygenase type 1	Tumour suppressor	MEK/ARK pathway	High	Hypermethylation	IPMN, Pancreatic cancer		64,65,66
WIF1	Wnt inhibitory Factor 1	Tumour suppressor	a Wnt/β-catenin signalling	High	Hypermethylated	NSCLC. Endometrial adenocarcinoma		67,72,73
DKK3	Dickkopf-related protein 3	Tumour suppressor	Wnt signalling, Beta-catenin signalling	Low	Hypermethylation	Hepatocellular, Ovary granulosa tumor		74,75,76, 77
PTEN	Phosphatase and Tensin Homolog	Tumour suppressor	PI3 Kinase pathway	Low	Hypermethylation	Breast cancer, cervical cancer		111,112, 113
APC	Adenomatous Polyposis Coli	Tumour suppressor	Wnt signalling	Low	Hypermethylation	Breast cancer, colorectal cancer		114,115, 116,117, 118
14-3-3-sigma	Stratifin		P38 and Wnt signalling	low	Unmethylated	Breast cancer, Urinary bladder carcinoma	Endometrial cancer, lung cancer	80,81,82, 83,84
CDH13	Cadherin 13	Tumour suppressor	Canonical Wnt signalling	low	hypomethylation	Pancreatic cancer		85,86

3-OST2	Heparin-Glucosamine 3-O-Sulfotransferase		Circadian rhythm related	low	hypermethylation	Breast, lung, pancreatic cancer		87,88,89
Myc	Myc protoncogene	Proto-oncogene	IL2 pathway				Lung and Prostate cancer	90,91,92, 93
FHIT	Fragile Histidine Triad		Metabolic pathway	low	Hypermethylation	NSCLC, liver cancer		96,97,98, 99
RASSF1	Ras Association Domain Family Member 1	Tumour suppressor	K RAS pathway	low	Hypermethylation	Colorectal cancer, Breast cancer		100,101, 102,103

4.1.1 EZH2 (Enhancer of zeste homolog 2)-

EZH2 modify the expression of downstream target genes by trimethylating Lys-27 in histone 3 (H3K27me3) and also takes part in cellular proliferation, apoptosis and senescence (47). Due to its functions in various biological processes, it is linked to many diseases, including cancer. EZH2 is currently in its early clinical trial stage for many cancers. Some small molecules such as S-adenosylmethionine-competitive inhibitors, are reported to effectively supress its activity (48). Various EZH2 methyltransferase inhibitors are used to inhibit metastases in Luminal B breast cancer where targeted treatment options are limited (49). The metastatic property of the gene has been reported in many cancers, including gastric cancer and prostate cancer (50,51). In gallbladder cancer, it functions as a transcriptional repressor by trimethylating H3K27me3. The MINCR /miR-26a-5p/ EZH2 axis is involved in propagating, invading and apoptosis of gallbladder cancer cells (52). Hence, EZH2 plays a crucial role in the progression of gallbladder cancer and can be a potential therapeutic biomarker in Gallbladder cancer (53).

4.1.2 MASPIN (Mammary serine protease inhibitor) -

MASPIN is a serum protease inhibitor that inhibits the motility and pervasive metastasis of breast cancer cells through tumor suppression (54). Originally, MASPIN gene was detected in the normal mammary epithelium using subtractive hybridization (55). In some studies, the gain of function in MASPIN is related to malignant behavior (56). A study suggested that p53 governs the expression of maspin, a tumor suppressor gene (57). In gallbladder cancer, over-expression of the MASPIN protein is suggested to play a significant role in malignant progression and is associated with an adverse prognosis for the patient (58). A change in gene expression has been reported in thyroid, breast, pancreatic, and hepatobiliary cancers (59,60,61,62).

4.1.3 CDO1 (Cyclin dioxygenase type 1)

CDO1 is a cyclin dioxygenase type 1 gene with tumour suppressor activity. It increases apoptosis by increasing reactive oxygen species (ROS) by eliminating the generation of glutathione in breast cancer cells (63). CDO1 is hypermethylated in xanthogranulomatous cholecystitis. Hypermethylation of CDO1 promoter is found significantly associated with the malignancy of gallbladder tumour (64). Gene hypermethylation has also been diagnosed in both IPMN and pancreatic cancer (65,66).

4.1.4 WIF1 (Wnt inhibitory factor1)

The Wnt inhibitory factor1 (WIF1) acts as a tumor suppressor gene. Expression of WIF-1 is reduced in many different types of cancers. Methylation of Wif1 promoter is a common feature throughout cancer progression, resulting in the silencing of its transcription and the concurrent upregulation of

Wnt signalling (67). Wif1 can interact directly with a variety of Wnt ligands and inhibit their binding to membrane receptors (68). It is reported that during the developmental process, in mice and zebrafish, Wif1 autoregulates its expression in a similar way that suggests its importance in embryonic development (69). In Gallbladder cancer and cholecystitis tissues also, hypermethylation of the WIF-1 promoter was detected by bisulfite sequencing and mmethylation-specificPCR (70,71). Hypermethylation of the gene has also been reported in other cancers including non-small cell lung cancer and endometrial carcinoma (72,73)

4.1.5 DKK3 (Dickkopf-related protein 3)

Abnormal activation of canonical WNT signalling due to impairment of DKK3 (Dickkopf-related protein 3) expression is suggested to cause cancer (74). In gallbladder cancer, cell invasion, proliferation and colony forming capacity are found to be reduced due to increase in DKK3, suggesting its role in gallbladder cancer tumorigenesis (75). Hypermethylation and altered gene expression are also associated with hepatocellular carcinoma and ovarian granulosa cell carcinoma. (76,77).

4.1.6. 14-3-3-sigma

The 14-3-3-sigma protein participates in various biological activities, including cell cycle control and signal transduction (78). According to the particular context and interactions of the protein route, 14-3-3 sigma seems to have certain tumor suppressing or oncogenic properties (79). In case of gallbladder carcinoma 14-3-3-sigma was found to be overexpressed, with an improved prognosis (80). The 14-3-3 sigma has also been shown to be hypermethylated in breast and urinary cancers, while it is hypomethylated in endometrial and lung cancers (81,82,83,84).

4.1.7 Cadherin 1 (CDH1)

CDH1 is a member of a family of genes directly associated with tumour invasion and cytoskeletal destabilization processes. The expression of CDH1 was studied in lesser differentiated tumours, with a generally negative prognosis. In gall bladder cancer, reduced E-cadherin expression was significantly associated with an adverse prognosis (85). Decreased gene expression also occurs in pancreatic cancer (86).

4.1.8. 3-O-sulfotransferase 2 (3 OST2)

3-O-sulfotransferase 2 belongs to the group of biosynthetic enzymes and modifies the proteoglycosaminoglycan chain of heparan sulfate. Modified expression of this gene was also reported in breast, lung and pancreatic cancers (87). The elevated expression of the gene is an indication of its role in the evolution of cancer. In case of lung cancer, the exogenous expression of OST2 blocks proliferation, migration and invasion, while reversal reinvents all of these functions (88). It is observed that the methylation frequency of the 3OST2 gene increases from 12% in chronic cholecystitis to 72% in gall bladder cancer, indicating its role in gallbladder cancer progression (89).

4.1.9. MYC Proto-Oncogene (MYC)

Myc proteins take part in a variety of functions, such as cell proliferation, cell cycle progression, cell growth, differentiation and tissue remodelling. Overexpression of MYC occurs in 30-50% of high-grade mammary tumours (90). The MYC gene gain in the copy number was found in 86.7% of gall bladder carcinoma (91). The deregulation of an oncogenic myc gene is seen in variety of cancers, including lung cancer and prostate cancer (92,93).

4.1.10 Fragile Histidine Triad (FHIT)

Methylation of the FHIT promoter results in decreased expression in breast and lung cancers (94). Decline in the expression of FHIT protein is also seen in primary and cervical cancer cell lines, implying the significance of FHIT in cervical cancer (95). Hypermethylation of FHIT (66%) is positively correlated with reduced expression, suggesting its potential role in gallbladder cancer patients (96). Also, there is evidence of reduction in FHIT protein expression (45%) in gallbladder

cancer (97). Deregulation in FHIT gene expression also occurs in non-small cell lung cancer and liver cancer (98,99).

4.1.11 RAS association domain family protein 1a gene (RASSF1A)

RASSF1A acts as a tumour suppressor protein positioned at chromosome 3p21, which performs variety of cellular functions (100). There is frequent evidence that RASSF1A is inactivated by hypermethylation in many types of cancers. RASSF1A was hypermethylated in colorectal cancers (up to 12% to 81%) (101). In breast cancer, RASSF1A has (56%) increased promoter methylation as compared to normal tissue (8%) (102). An extremely low frequency of methylation of RASSF1A is observed (8%) in gallbladder cancer in the Chilean population (96). RASSF1A is reported methylated in about 22.7% of gallbladder cancer patients and 12.5% of adenoma patients (103).

4.1.12 Thrombospondin 1 (THBS1)

THBS1, a glycoprotein of the extracellular matrix has been identified as an antiangiogenic factor (104). The alteration in the expression of THBS1 is responsible for causing colorectal and liver metastasis through epithelial–mesenchymal transition (105). The elevated expression of THBS1 is reported in the desmoplastic component of the invasive ductal carcinoma of the breast (106). Further dysregulated expression of THBS1 is also found in several other cancers such as oral squamash cell, prostate tumour and gastric cancer (107-109). Altered methylation and overexpression (44%) of THBS1 is also reported in gallbladder cancer patients (110).

4.1.13 Phosphatase and tensin homolog (PTEN)

PTEN works as a gene suppressing tumor, any alteration in its functions clarifies its role in several cancers. Tekcham et al. reported hypermethylation of PTEN promoter region in patients with gallbladder cancer (111). Expression analysis at the transcription level indicates weak expression in patients with gallbladder cancer, but not in bile disease. Breast and cervical cancer patients also reported a modification in gene expression (112,113).

4.1.14 Adenomatous polyposis coli (APC)

The functional significance of APC methylation has been thoroughly investigated in colorectal carcinoma as well as in gallbladder carcinogenesis. The Wnt signaling pathway is activated and the levels of transcription factors of β-catenin in the nucleus are also increased due to the functional loss of APC, leading to cellular proliferation (114). Alteration in APC expression due to DNA methylation in gallbladder cancer and bile disease was initially observed in the Chilean population (30%, n=30 GBC and 25 GSD) (115). Following this, significant changes in APC gene expression were noted in the Northern Indian population also (116). Decline in the expression due to hypermethylation, in a different population, clarifies its involvement in the cause of gall bladder carcinogenesis, and its loss of expression is also found in breast and colorectal cancer (117,118).

5. Therapeutics: Epigenetics markers as drug targets

Epigenetic dysregulation has the potential to become pathological, causing heart and neurological illnesses, metabolic perturbations and the development of cancer (119). Therefore, there is significant clinical interest in identifying the drugs that supress these epigenetic alterations. The need for epitherapy arises for treating gallbladder cancer because of the difficulty in treating and late diagnosis of the disease. Understanding the biology of the disease is needed to improve clinical outcomes and develop new targeted therapies for patients with advanced gallbladder carcinogenicity. As there are currently few treatment options for patients with gallbladder cancer. New therapeutic approaches, such as targeted cancer therapy should be explored to guide rational therapies towards better outcomes. Targeted cancer therapy can enhance therapeutic effectiveness, particularly in cases of resistance to conventional chemotherapy. Gene therapy for cancer promises to enable greater distribution of therapeutic products and the possibility of targeting a specific pathway. Research have helped us identify certain genes that should be a potential biomarker and, therefore, can be used in

targeted therapy. This therapy is based on the alteration of epigenetic phenomenon, occurring in cancer and establishing a sound epigenome (120).

Some compounds, similar to nucleoside, approved by FDA, are the earliest form of the methylation inhibitant. 5-Azacytidine (nitrogen instead of carbon in position c5), which is similar to cytidine is recognized by DNMT1, forms an irreversible binding that leads to degradation of the enzyme DNMT1 (121-123). It has been demonstrated that nanaomycin A, one of the unique inhibitors of DNMT3B, reactivates silent "RASSF1A", tumour suppressor genes (124).

HDAC inhibitors are the first successful anti-cancer therapy based on epigenetics (125). BET inhibitor JQ1, HDAC inhibitor SAHA and most importantly, their combined treatment, has exerted high levels of anti-cancer activity in vitro and in vivo against gallbladder cancer cells (126). Another study reported the role of mTOR inhibitor INK-128 and HDAC inhibitor JNJ-26481585 in inhibiting the growth of cancer cells in gallbladder cancer (127). Garcinol, a natural histone acetyltransferase inhibitor could significantly inhibit the growth of GBC cells in a dose- and time-dependent manner with the suppression of Stat3 and Akt signalling pathways. It inhibits their downstream targets, such as MMP2 and MMP9 (128). Other than the role of HDACi, there has been significant interest in DNMT inhibitors (DNMTis) in recent years to modulate abnormally methylated DNA in a reversible manner. KIF11 (promoter enriched with H3K27ac) promotes the carcinogenesis of the gallbladder through increased expression, but when treated with the inhibitor C646 (H3K27ac inhibitor), showed a lowered expression in gallbladder cancer and can be used in the epitherapy (129). Similarly, PCI-24781 (histone deacetylase inhibitor), which inhibits ErbB2 (overexpressed in cancer) and reduces expression in cancer of the biliary tract, can be used as a powerful therapeutic agent (130).

A number of novel agents were also evaluated in Phase I and II clinical trial studies. The Phase II clinical trial with erlotinib showed positive results for the treatment of biliary tract cancer (131). Bevacizumab and sorafenib target the vascular endothelial growth factor (VEGF) receptor in metastatic gallbladder cancer and are in phase II epitherapy testing (132, 133). Associations between the MEK pathway (mutations) and gallbladder cancer pathogenesis is also reported. Selumitinib, an MEK inhibitor was used in a Phase II clinical trial against patients with advanced biliary tract cancer (134).

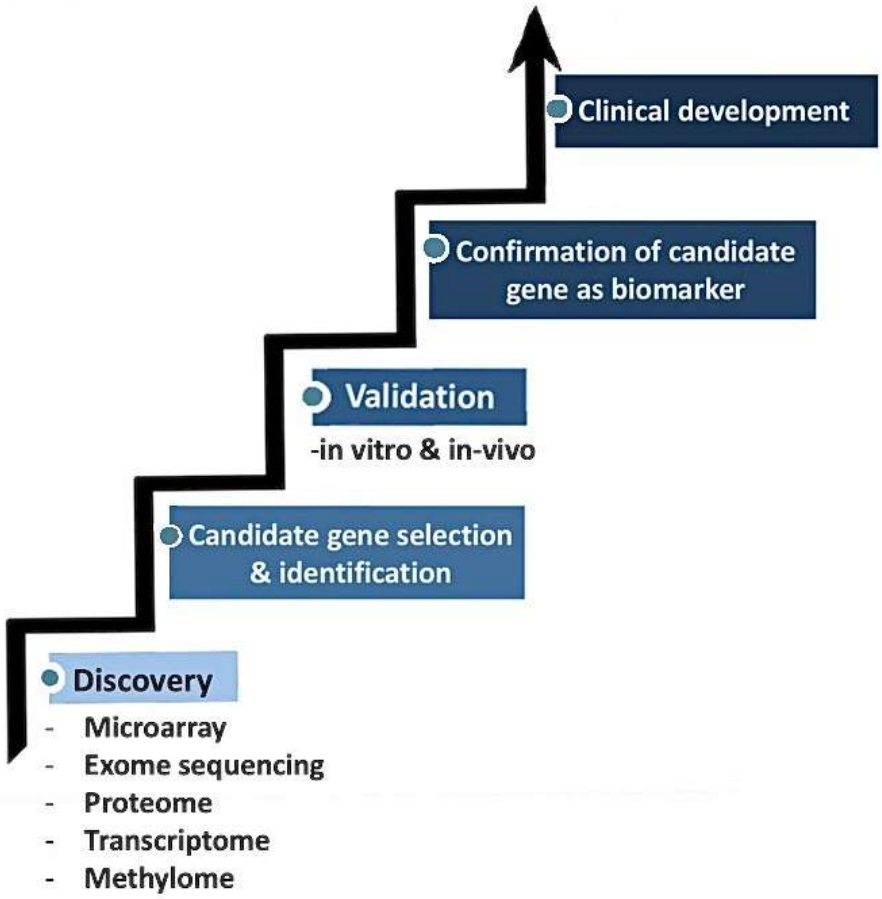
The Hedgehog pathway participates in many developmental processes, including determining cell fate, patterning, proliferation, and its role in the initiation and progression of cancer has recently been recognized. Although this pathway is silenced in most mature tissues, aberrant activation of this pathway has been documented in a variety of malignant tumors (135). Several small molecule inhibitors were developed against the hedgehog signaling pathway. Vismodegib and saridegib, a small molecule that binds to smoothed (SMO) and inhibits this pathway, are in phase I and II clinical trials (136,137).

Artemisinin upregulates the expression of p16, whereas down-regulates the pERK1/2, CDK4 and cyclin D1 expression, leading to inhibition of the ERK1/2 pathway, cause the G1 phase arrest of the cell cycle and promote the caspase 3 mediated apoptosis (138).

INK-128, an mTOR inhibitor and JNJ-26481585, an HDAC inhibitor, eliminates the growth of tumour and metastasis and can be a potential therapeutic for patients with gallbladder cancer. The mTORs and S6K1 phosphorylation (epigenetic alteration) can also be used as biomarkers to treat gallbladder cancer (139).

Furthermore, exploration at various clinical stages can provide a comprehensive picture of the disease process and offers new options for the development of biomarkers that will be useful for early diagnostics as well as for targeted treatment of gallbladder cancer (Figure 4: Steps of biomarker development).

Figure4: Steps of Biomarker Development



6. Conclusion

This review article is a subject of discussion about the different possible candidate genes and their epigenetic alteration in gallbladder cancer. The remarkable betterment in the curative rate of patient with gallbladder cancer in the last few years is because of the radical surgical method that has been endorsed, and the awareness towards it. Epigenetically engineered drugs already make up an important part of the clinical arsenal, and under certain refractories are the only active medications accessible to patients. Nevertheless, despite the initial successes, there are still challenges in future studies. The demands for the development of the biomarker for the prognostic and diagnostic purpose has increased due to increase in the incidence rate of gallbladder carcinogenesis. Some genes, such as APC, PTEN, RASSF1A, etc., whose expressions are altered due to alteration in the methylation level, can serve as useful possible biomarkers. The subject of concern of this article is to review the genes, in which methylation occur in the CpG island of the promoter region and their likely role in the cancer. As the present information is still limited, the involvement and the identification of new biomarker is essential for the treatment of GBC at its early stage. The present development of epigenetic biomarker in medical research and accumulation of validated molecular data provide an ample support to the fact that involving DNA methylation and histone modifications as an adequate remedy for the treatment of cancer patient, complementing the pathogenesis.

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