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MINIREVIEWS

Identification of genes associated with gall bladder cell carcinogenesis: Implications in targeted therapy of gall bladder cancer

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Abstract

Gall bladder cancer (GBC) is becoming a very devastating form of hepatobiliary cancer in India. Every year new cases of GBC are quite high in India. Despite recent advanced multimodality treatment options, the survival of GBC patients is very low. If the disease is diagnosed at the advanced stage (with local nodal metastasis or distant metastasis) or surgical resection is inoperable, the prognosis of those patients is very poor. So, perspectives of targeted therapy are being taken. Targeted therapy includes hormone therapy, proteasome inhibitors, signal transduction and apoptosis inhibitors, angiogenesis inhibitors, and immunotherapeutic agents. One such signal transduction inhibitor is the specific short interfering RNA (siRNA) or short hairpin RNA (shRNA). For developing siRNA-mediated therapy shRNA, although several preclinical studies to evaluate the efficacy of these key molecules have been performed using gall bladder cells, many more clinical trials are required. To date, many such genes have been identified. This review will discuss the recently identified genes associated with GBC and those that have implications in its treatment by siRNA or shRNA.

Key Words: Gall bladder cancer; Gene biomarker; Targeted therapy; siRNA mediated therapy; Prognosis; Advanced therapy of gall bladder cancer

Core Tip: The frequency of gall bladder cancer (GBC) in India has increased. The survival of GBC patients is also poor. In this context, some genes have been recognized which are involved in the carcinogenesis of GBCs. In this review, we have discussed such genes which could be aimed for the development of targeted therapy for GBC.

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INTRODUCTION

Gall bladder cancer (GBC) is the most common among hepatobiliary cancers. In India every year approximately 19570 new cases are detected and approximately 14736 death occurs due to GBC. According to the Cancer Registry, the rate of GBC is highest in Bolivia (14/100000) and Chile (9.3/100000)[1]. Recently, the occurrence of GBC in India is comparable and close to these countries. In 2001-2004, 6-7 individuals suffered from GBC among 100000 individuals, but in 2012-2014, 10-11 individuals suffered from GBC among 100000 individuals[2]. The total number of GBC cases in India is about 10% of the total GBC cases in the world. The rate of GBC is increasing in India in both males and females. If only the north and northeast are considered, the rates are even higher, which are 11.8/100000 and 17.1/10000 respectively (National Cancer Registry Program, 2012-2014). GBC is found to have a poor prognosis. Among the patients diagnosed with GBC, the average 5-year survival rate is as low as 19%. Unfortunately, most of the GBCs are detected after malignant cells have spread to other organs. In that case, chemotherapy and palliative treatment are the only options (Figure 1). Other approaches of tailored treatment are necessary for the treatment of this disease (Figure 1). Next-generation sequencing, whole exome sequencing, RNA sequencing, single cell isolation, and proteomics are some of the approaches to tailored treatment. This has been applied to specific target treatment, immunotherapy, vaccine therapy, and treatment with nanoparticles, which are already in clinical trials[3]. Therefore, a detailed study of the key molecules involved in the process of carcinogenesis of GBC is necessary for the tailored treatment of this disease.

Short interfering RNA (siRNA) is a small double-stranded RNA interfering molecule whereas, short hairpin RNA (shRNA) is a single-stranded RNA molecule folded into a hairpin or stem-loop-like structure. siRNA can be endogenous, exogenous, or artificial in origin for transient expression. Primarily, siRNA is to provide viral/bacterial defense and genomic stability. It is very similar to microRNA (miRNA), which mainly functions as an endogenous regulator of gene expression through gene silencing. ShRNA is an artificial molecule integrated into genomic DNA for long-term gene silencing of a specific gene expression. The shRNA is processed by DICER to generate siRNA. These siRNAs are then untwisted by helicases into two single strands, one is called the passenger strand and the other is called the guide strand. The guide strand combines with other components to form the RNA-induced silencing complex (RISC). The other main components of RISC are transactivation response RNA binding protein, protein activator of protein kinase R, and Argonaute2 (Ago2). The guide strand binds to its complementary mRNA strand. Ago2 cuts and removes the targeted mRNA. This mRNA becomes inactive and then cannot be translated and expressed. So, when a specific siRNA is introduced into the cell, it stops the translation of the corresponding mRNA.

In this review, we will describe some siRNA-based gene therapeutic techniques that have yielded positive results for the implication of treatment of GBC. In the present review, we have identified some genes that are responsible for the spread of GBC cells. Approaches for silencing these genes could have the potential for the treatment of this disease. One approach to silencing these genes is by constructing and delivering specific short-interfering RNAs or siRNAs. Technology for the construction of specific siRNA has already been developed and is in practice.

TARGET GENES FOR siRNA-MEDIATED GBC THERAPY

Genes related to the development of gall bladder tumor

***KRAS* (Kirsten sarcoma viral oncogene homolog)**: The gene *KRAS* encodes a protein which is 21 kDa and occurs in the inner side of the plasma membrane of those cells which could bind GTP and have the ability to convert GTP into GDP. However, when KRAS is attached to GTP, it remains active and plays an important role in the cell signaling pathways[4]. When GTP is converted to GDP, KRAS becomes inactive. Mutations in the *KRAS* gene result in the loss of the ability of the KRAS protein to return to its inactive form. This affects cell signaling pathways[5]. It was evident from a set of experiments that mutation of *KRAS* causes the development of gall bladder adenoma[6]. The possible mechanism is that KRAS activates the NOTCH signaling pathway and leads to tumor development.

***PIK3CA* (Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha)**: The gene *PIK3CA* encodes a protein (p110), which is the stimulating subunit of the enzyme PI3K. It is well known now that PI3K participates in many events of cell signaling and is well-linked to cell growth and proliferation[7]. In a set of experiments examining the role of PI3K-AKT in gall bladder tumorigenesis, it was observed that the PI3K-AKT pathway is disturbed when *PIK3CA* is mutated. The gall bladder epithelial cells are directed towards mesenchymal transition and lead to tumor formation[8].

Genes related to invasive metastasis

***CD44* (Cluster of differentiation 44):** CD44 is a glycoprotein which is found in the lipid bilayer. It is encoded by the gene *CD44* present on chromosome 11[9]. This glycoprotein is required in the interplay between the cells, mainly in cell attachment and migration. It is found to have a function in the activation of lymphocytes, formation of blood cell

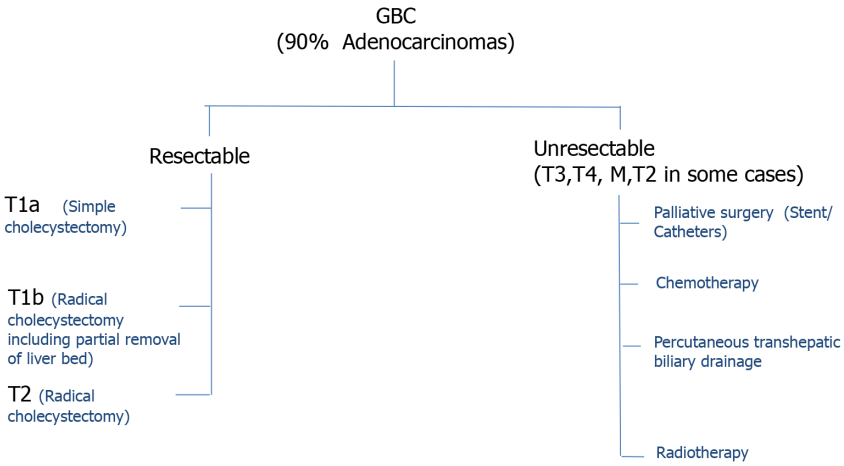

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Figure 1 Current scenario of gall bladder cancer treatment strategies. T1a, T1b, T2, T3, and T4 stand for the different T stages of gall bladder cancer. Usually, the T1a, T1b, and T2 tumors could be removed by surgery but this should be confirmed only after a CT scan. T3 and T4 tumors usually cannot be removed by surgery and other approaches are taken to treat them. GBC: Gall bladder cancer.

components, cell proliferation, cell differentiation, and angiogenesis. Though, CD44 has a few other ligands, mainly hyaluronan binds to CD44 and activates it for the regulation of many pathways like the RAS, MAPK, and PI3K pathways which are associated with cell proliferation, cell differentiation, and angiogenesis[10,11]. Figure 2 shows how CD44 is linked to the MAPK pathway.

It is overexpressed in many cancers including those of the liver and gall bladder. The movement and occupation of the stroma by the GBC cells are also decreased on CD44 inactivation. CD44/CD133 positive cells also behave as cancer stem cells. CD44, in union with NANOG (another cancer stem cell marker), is found to have stimulated some multidrug-resistant genes and thus tumor development[12]. When tested for cancer stem cells, they were found to be reduced in activation of CD44 by *CD44*-siRNA. The total life span of patients in whom CD44 was overexpressed was much less than those who did not express CD44[13].

In a set of experiments by He *et al*[13], *CD44*-siRNA constructs were prepared by Sangon Biotech. A GBC cell line (GBC-SD) was transfected with *CD44*-siRNA. The expression of CD44 decreased significantly in the *CD44*-siRNA transfected cells as revealed by immunohistochemical staining, Western blot, and real-time PCR. The apoptosis of these cells increased considerably, which was detected by flow cytometry, while the proliferation capacity of these cells was considerably decreased as observed by cell counting kit-8 (CCK-8) assay. In this study involving *CD44*-mRNA it was found that CD44 is unhindered in hepatobiliary tumors and CD44 protein is expressed much more in gall bladder carcinoma tissues than non-malignant tissues. The overall survival of these patients is also poor. Silencing of the *CD44* gene by delivering the corresponding siRNA in the cells has led to decreased expression of CD44.

***EGFR* (Epidermal growth factor receptor):** EGFR/ErBB1 is a member of the receptor tyrosine kinase family. It is a transmembrane protein that is turned by the binding of its ligand (epidermal growth factor or transforming growth factor). When EGFR is turned on, it forms a dimer with itself or with another member of the receptor tyrosine kinase family, leading to its phosphorylation. This phosphorylation, in turn, leads to the activation of its target proteins which are the components of either the MAPK or AKT, or JNK pathways, and ultimately cell proliferation occurs[14,15]. Figure 2 shows how EGFR could activate the MAPK pathway.

In a set of experiments by Iyer *et al*[16], exome analysis and sequencing were performed to detect tumor-specific mutations in GBC tissues. EGFR and ERBB2 were turned off using *EGFR*-specific shRNA and *ERBB2*-specific shRNA. Cell proliferation of the transfected cells was assessed by MTT colorimetric assay. Expression of various tumor markers in the transfected cells including EGFR was estimated by Western blot assay and Immunohistochemistry. Migration assays of the transfected cells revealed that there was a considerable decrease in migration and invasive abilities of the transfected cells.

***ERBB2* (erythroblastic oncogene B 2):** It is a tyrosine kinase, also known as HER2/neu or receptor tyrosine–protein kinase ERBB-2 or CD340, belonging to the Erb family of receptor tyrosine kinases. In humans this Erb family consists of Her1 (EGFR/ErbB1), Her2 (Neu/ErbB2), Her3 (ErbB3) and Her4 (ErbB4)[17,18]. HER2/neu is a protein encoded by the gene *ERBB2*, present in the long arm of chromosome 17. When a ligand binds to this receptor, it undergoes dimerization activates many signaling pathways, and finally leads to cell proliferation and growth[19]. It has been found that HER2/neu could initiate the PI3K/Akt[20,21] and also the MAP kinase pathway[22]. Figure 2 shows how HER2/neu could activate the MAP kinase pathway. In an experimental setup by Kiguchi *et al*[23], HER2 was overexpressed in Gall bladder adenocarcinoma of mice. Silencing of ERBB2 was found to curb the penetrating, relocating, and docking free growth properties of human GBC-SDs, provided that *KRAS* (G12V) is not mutated in these GBC-SDs[16], as visualized by proliferation and migration assays. It was also visualized that due to the silencing of *ERBB2*, it was unable to signal target proteins of the MAPK pathway.

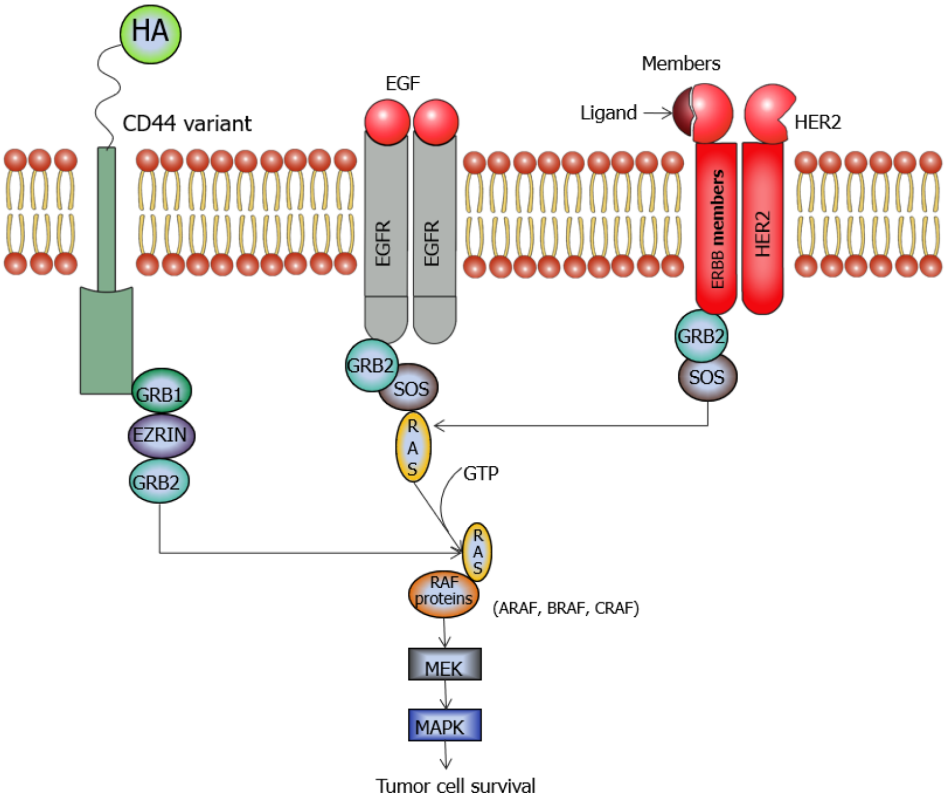

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Figure 2 Mechanism of MAP kinase activation by some of the target genes. ERBB members: EGFR/ Her2/ErbB3/ErbB4; GRB: A serine protease and shortened form of Granzyme B; EZRIN: A protein needed for activation of RAS; SOS: Guanosine nucleotide exchange factor helps in the activation of RAS, the name derived from Son of Sevenless gene; RAS: A G protein, the name is derived from rat sarcoma virus; RAF: Serine threonine protein kinases and shortened form of rapidly accelerated fibrosarcoma; MAP kinase: Protein kinases and shortened form of mitogen-activated protein kinase; EGFR, HER2/neu, and CD44 are receptors embedded in the phospholipid bilayer and A-RAF protein is present in the cytoplasm; EGFR, HER2/neu, CD44, and ARAF activate the protein kinase, MAPK which ultimately results in the survival of tumor cells; HA: Hyaluronic acid; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptors.

***ARAF*:** It is a serine-threonine kinase of the Raf-kinase family. There are three members of the Raf (rapidly accelerated fibrosarcoma) kinase family - ARAF, BRAF, and CRAF. These three enzymes have roles in the MAP kinase pathway (Figure 2). In humans, ARAF is encoded by the *ARAF* gene, located on the X chromosome (xp11.3)[24]. ARAF activates MEK proteins, which in turn activates extracellular regulated kinase (ERK) which is known to activate many transcription factors and drive the MAPK pathway toward cell proliferation[25,26]. In a set of experiments by Lin *et al*[27], the ARAF gene was found to be elevated in GBC cells. Silencing of ARAF by *ARAF*-siRNA hinders the proliferation of GBC cells in a GBC-SD, as studied by the CCK-8 assay. The drifting and intruding nature of GBC cells declined, as estimated from transwell and wound healing assays. When the GBC-SD was used for tumor formation in athymic nude mice, *ARAF*-siRNA was injected. On regular monitoring of tumor size and volume, it was found that *ARAF*-siRNA was able to reduce the tumor volume remarkably.

Genes related to apoptosis

***BMI1* (B-cell specific Moloney murine leukemia virus integration site 1):** BMI1 is a member of the polycomb group family proteins which suppresses transcription. To date, many such polycomb proteins are known[28,29]. Some of the common ones are PRC1, PRC2, PHC1, PHC2, HP1, BMI1, PCGF1, PCGF2, RYBP, RING1, SUV39H1, L3MBTl2, EZH2, EE2, SUZ12, JARID2, REST, RNF2, CBF-β and YY1. Basically, they organize the chromatin of the specific gene in such a way that they are suppressed for a long period of time and during cell division. The human *BMI1* gene is placed at the short arm of chromosome 10[30].

BMI1 gene has been found to interact with p16 and p19[31], which are inhibitors of cyclin-dependent kinases (CDK4 and CDK6). P16 is a protein that disallows the cell from the G1 phase to the S-phase. P19 is a protein that has been found to direct p53 to stop the cell cycle at G1 and apoptosis in fibroblast cells[32]. BMI1 has been found to downregulate these inhibitors[33] and thus plays an important role in the progression of the cell cycle and sustaining cell division[34]. In a set of experiments by Jiao *et al*[35], *BMI1*-siRNA constructs were prepared by Shanghai Biotech. A GBC-SD was transfected with *BMI1*-siRNA. Initially, BMI1 was found to be expressed in a majority (84%) of the GBC patients as compared to 40% of normal patients who were taken up for the study. The expression of BMI1 decreased significantly in the *BMI1*-siRNA transfected cells as revealed by immunohistochemical staining, Western blot, and real-time PCR. The apoptosis of these cells increased considerably, which was detected by flow cytometry, while the proliferation capacity of these cells was considerably decreased as observed by the CCK-8 assay. Silencing of *BMI1* by specific siRNA could induce apoptosis of

the GBC cells and decrease the proliferation of GBC cells, which could hinder the growth of GBC cells.

***BCL2* (B-cell lymphoma 2):** BCL2 belongs to the Bcl-2 family of proteins. It is present in the outer mitochondrial membrane, embedded in it, and plays an important role in the suppression of the pro-apoptotic proteins BAX and BAK [36,37]. Thus, it plays a role in cell growth and proliferation. Overexpression of this gene with the upregulation of the proto-oncogene *MYC* has been found to be associated with some cancers[38]. In a set of experiments, GBC-SD were transfected with *BCl2*-siRNA according to the standard procedure by Lipofectamine[39]. The cells were cultured in media. The growth and the morphology of the resultant cells were observed. After a certain period of culture, expression of BCL2 was measured in the resulting cells by quantitative real time PCR (qRT-PCR) and Western blot. Expression of the *BCL2* gene was found to be significantly lower in the *BCL2*-siRNA transfected group. On silencing of this gene, the GBC cells were found to be more responsive to chemotherapy drugs. In the nude mouse model experiments, the tumor volume also significantly decreased in the experimental group where this gene was silenced.

***JAB1* (c-JUN activation domain binding protein 1):** The gene *JAB1* is positioned on chromosome 8. JAB1 is a co-activator of c-JUN, which is a well-known oncogene[40,41]. JAB1 is a member of a complex called constitutive photomorphogenic-9 signalosome (CSN) complex, which positively controls cell proliferation and transcription of various genes[42]. It is essential in the advancement of the cell cycle from the G1 phase to the S-phase, as c-JUN controls the level of Cyclin D1. If c-JUN is not activated, the expression of p53 and p21 could be upregulated to prevent the cell cycle from advancing farther[43,44]. Activating factor AP1 binds to the promoter region of *JUN* and transcription of JUN begins[45]. AP1 is a transcription factor consisting of mainly four groups of proteins *i.e.* Jun (C-Jun, Jun-B, and Jun-D), Fos (FosC, FosB, Fra1, and Fra2), ATF/cyclic AMP responsive element, and Maf family proteins. Thus, c-JUN transcription is also controlled by c-JUN itself. JUN transcription could also be started with active ERK[46]. In a set of experiments by Pandey *et al*[47], increased levels of JAB1 were found in GBC tissues. *JAB1*-siRNA constructs were prepared and used to transfect GBC cells. JAB1 expression, cell growth, and apoptosis of these GBC cells were studied by qRT-PCR, Western blot, MTT, reactive oxygen species, Hoechst, and FITC/Annexin-V staining. In the cells in which JAB1 is expressed highly, proliferation is turned on in these cells. GBC cells when transfected with *JAB1*-SiRNA decreased this proliferation considerably and programmed cell death was initiated. The cell cycle was found to halt at the G1 phase. Expression of the apoptotic gene CASP 3 and apoptotic regulatory genes p27, P53, and BAX were evident.

Genes related to the immune system

***MIF* (Macrophage migration inhibitory factor):** MIF is a protein that plays a role in inflammatory responses. It has been found that though inflammation is a mechanism of the body's defense if it occurs for a prolonged period, which occurs in the case of chronic inflammation could lead to cancer in some cases. One way, that chronic inflammation could be linked to cancer is thought to be through MIF[48,49]. MIF binds to its receptor CD74 and activates it and it then activates PI3K-AKT, ERK, and NF-kappa B pathways[50,51]. It has been found to subdue the anti-inflammatory cytokines and enhance the other inflammatory cytokines[52]. MIF has been found to be upregulated in many cancers including GBC[53,54]. Inactivation of the *MIF* gene using *MIF*-siRNA reduces the proliferation and intruding properties of cancer cells as observed in the colony formation assays.

***CD73*:** CD73 resists the proper functioning of the T-cells by interfering with their clonal expansion, their activation, and their cytolytic activities. It plays an important role in the process due to which tumors can escape the immune system. CD73 is a cell surface enzyme that induces the dephosphorylation of AMP into adenosine[55]. Adenosine activates the G-protein coupled receptors (A2AR and A2BR) and plays a role in the escape of tumor cells by the immune system[56,57]. NOZ cells, a human GBC-SD were transfected with specific *CD73*-siRNA by Cao *et al*[58], and the expression of CD73 was found to be reduced in transfected cells as observed by quantitative reverse transcription PCR and Western blot. Adherent cells and spherical aggregates of cancer cells were found to decrease when CD73 was silenced. When CD73 was silenced by *CD73*-siRNA, epithelial to mesenchymal transition was found to reduce in GBC cells. It could be one of the important reasons for the opposition to drugs by cancer stem cells. The growth of GBC cells in single layers and attached to the surface and also those which can grow without embankment were retarded. The migrating nature of the GBC-NOZ cells was found to be reduced as detected by trans well assays.

***PDL1* (Programmed cell death protein 1 ligand):** PDL1 binds to its receptor programmed cell death protein 1 (PD1) which is found on activated T-cells' and B-cells' surfaces[59]. The interplay between PD1 and PDL1 hinders the proper functioning of the T-cells[60]. PDL1 is expressed on the surface of many cancer cells[61]. The interaction of PD1 with PDL1 interrupts major histocompatibility complex and thus obstructs the interaction of PDL1 with its receptor PD1, as a result, antigen presentation to cytotoxic T-lymphocytes[3]. PDL1 is overexpressed in the GBC-NOZ cells. When PDL1 was silenced with *PDL1*-siRNA cell growth and transportability of GBC-NOZ cells were diminished. This was revealed by proliferation as well as wound healing assays[58].

Other genes: miRNA is short (about 20 nucleotides), single-stranded, intrinsic, and non-coding sequences of RNA found in all tissues or blood. They could silence a gene by binding it to the specific RNA, complementary to it. Their mechanism of action is similar to that of siRNA. However, miRNAs are procured from different regions of DNA than siRNA. About more than 2500 miRNAs have been found in humans. In GBC several miRNAs have been found to be uncontrolled.

In a microarray analysis of GBC tissues from patients who survived for a long period after diagnosis and those who survived for a short time[62], changes in the expression of miRNA were identified. It was found that only two miRNAs (hsa-miR-30a-3p and hsa-miR-660-5p) were suppressed in patients who survived long. However, 11 miRNAs were suppressed and 11 were stimulated in patients who did not survive long. This recommended the possible roles of these

miRNAs in GBC. In some other microarray analyses, changes in the expression of many other miRNAs have been detected in GBC. The table below (Table 1) lists the important ones and the genes associated with these miRNAs.

Some of the miRNAs including the miR-55 and miR-20a have been considered as oncogenic miRNA or onco-miR as when they were expression levels were increased, the cell proliferation and intruding capabilities of the Gall bladder cells also increased[63]. The status of the important miRNAs in the GBC tissue of a particular patient could determine his response to a particular kind of therapy and prognosis.

TRANSLATIONAL RESEARCH AND CLINICAL TRIALS RELATED TO GBC

Mutations of *KRAS*, *INK4A* (p16), *TP53*, and *HER 2*/neu have been commonly noticed in GBC[5,64-66]. Mutations of *PIK3CA* in GBC are also not rare[65]. *BRAF* and *PI3K* mutations also have been detected in GBC but they are not as common as KRAS[8,67,68]. There have been attempts to develop drugs against KRAS but the chemical nature of these drugs was such that they could not be controlled, so they were not approved. Some therapeutic drugs, targeting some genes are under development for the treatment of GBC. They are in different phases of clinical trials (Table 2). Many immune checkpoint inhibitors against PDL1 and CTLA4 for the treatment of GBC are also under various stages of clinical trial[69,70].

CONCLUSION

The combined chemotherapy regimens have been found to increase the overall survival of patients in clinical trials, but still, they have been found to have toxic effects, and resistance is developed later[3,71,72]. Whenever clinical trials were performed some unforeseen hurdles were found, including the side effects and development of resistance to a particular drug. The cause of this resistance to a particular drug could be diversity within the same tumor and genetic differences among the patients[73].

The siRNAs have been found to be capable of silencing a specific gene in experimental models. However, there are certain constraints for the siRNA-mediated therapy. These are mainly the firmness and delivery of siRNA. Choosing the gene to be silenced is another issue with siRNA-mediated therapy. All these issues have to be answered before starting clinical trials in patients. As the siRNA molecules are negatively charged and have an inflexible structure, their diffusion across the membrane becomes difficult. So, they are taken up by the cell through endocytosis. In this case, there is a huge chance of the collection of many molecules in the endosomal compartments rather than reaching the cytosol where it can form the RISC. In the past few years, scientists have attempted to prepare strategies for the endosomal escape of these molecules, and a few have been reported[74]. Few endosomolytic media have been obtained from natural substances or manufactured and many other agents like polymers, liposomes, nano-particles, and other coupling agents have also been developed for the successful carriage of siRNA. Some recent clinical trials have disclosed the safety of nano-particle-based delivery of siRNA in some other cancers[62]. Still, there have been issues regarding the delivery of shRNA[75,76]. However, various attempts have been made to optimize the delivery of shRNA by viral vectors. Many vectors have been studied for their efficiency in the delivery of shRNAs within the target cell[77,78]. These viral vectors have improved the delivery of shRNAs but the safety of these vectors is in question as they are based on viruses. Consequently, these approaches are not applicable to clinical trials. Selection of the gene to be silenced is another important aspect of these siRNA-based technologies. It is suggested by experts to select such a combination therapy for patients which targets the important molecular pathways governing cancer metastasis and also would have less toxicity. To date, many genes have been identified which are responsible for the cells being able to avoid senescence and those which are responsible for metastasis. Genes that are linked to multiple pathways and have been expressed in many cancers are perhaps suitable for targeting. In the case of GBC, a recent study suggests that there are predominantly mutations of the genes *ARID1A, ARID2, ATM, CTNNB1, ERBB2, ERBB3, KMT2C, KMT2D, KRAS, PIK3CA, SMAD4, TERT, TP53*, and *ZNF521*[65]. High expression of the gene *GLI2* has also been found to increase the number of GBC cells and their aggressive property[79].

All the genes mentioned in this review are linked to important cell signaling pathways. Among them, CD44, EGFR, and MIF have been found to be linked to multiple pathways. CD44 has been found to activate the MAPK, PI3K-AKT pathways, MIF has been linked with the MAPK, PI3K-AKT, and the NF-κB pathways and EGFR has been found to stimulate the MAPK, PI3K-AKT, and JNK pathways. The genes ERBB2, ERBB3, KRAS, and PIK3CA were already known to have been linked with multiple pathways. So, these genes which could activate multiple pathways could be potential targets for si-RNA/shRNA mediated knockdown. siRNA and shRNA-mediated knockdown of the genes mentioned above has shown to have decreased the invasiveness of the GBC considerably. The question of delivery of specific siRNA/shRNA in patients is expected to be answered. If the question is answered, they seem to have the potential for the tailored treatment of GBC. In that case, the toxicity resulting from the knockdown of the selected gene has also to be tested in preclinical models. Another aspect is the high cost of this siRNA/shRNA-mediated therapy. Therefore, before it comes to regular clinical practice all these issues need to be resolved. All these challenges have made it quite far from commercialization.

Table 1 MicroRNAs identified to have changed expression in gall bladder cancer

miRNA	Status in gall bladder cancer	Target genes
miR-146b-5p	Suppressed	EGFR[80]
miR-124	Suppressed	CDK6, ROCK1[81]
miR20a	Stimulated	SMAD7[82]
miR155	Stimulated	[82]
miR182	Stimulated	CADM1[82]
miR122	Stimulated	BMI1[82]
miR34a	Suppressed	PNUTS[82]
miR335	Suppressed	[82]
miR130a	Suppressed	HOTAIR[82]
miR135a5p	Suppressed	VLDLR[82]
miR-145-5p	Suppressed	STAT1[83]
miR26a	Suppressed	HMGA2[82]
miR145	Suppressed	AXL[82]
miR143	Suppressed	[82]
miR2185p	Suppressed	BMI1[84]

miRNA: MicroRNA.

Table 2 Target genes for treating gall bladder cancer (under investigation and those under clinical trial)

Target genes silenced by siRNA (under investigation)	Target genes for GBC treatment (under clinical trial)
BMI1, CD44, CLIC1, JAB1, EGFR	EGFR and Her/2 together (Afatnib-NCT04183712, Apatinib-NCT03702491)
HER 2/neu, ARAF	HER/2 (Trastuzumab-NCT00478140)
MIF	MEK (Trametinib-NCT02042443)
CD73, PDL1	DNMT (Guadecitabine-NCT03257761)

GBC: Gall bladder cancer.

FOOTNOTES

Co-corresponding authors: Ruma Dey Ghosh and Soma Mukhopadhyay.

Author contributions: Ghosh I, Dey Ghosh R, Mukhopadhyay S conceived the idea and designed the manuscript; Ghosh I and Dey Ghosh R, collected information, analyzed the data, constructed figures and tables, and wrote the manuscript; Ghosh I drafted the manuscript; Dey Ghosh R critically reviewed, edited and corrected the manuscript; All authors were involved in the critical review of the results and have contributed to, read, and approved the final manuscript. Dey Ghosh R, and Mukhopadhyay S contributed equally to this work as co-corresponding authors. The reasons for designating Dey Ghosh R, and Mukhopadhyay S as co-corresponding authors are threefold. First, the research was performed as a collaborative effort, and the designation of co-corresponding authorship accurately reflects the distribution of responsibilities and burdens associated with the time and effort required to complete the study and the resultant paper. This also ensures effective communication and management of post-submission matters, ultimately enhancing the paper's quality and reliability. Second, the overall research team encompassed authors with a variety of expertise and skills from different fields, and the designation of co-corresponding authors best reflects this diversity. This also promotes the most comprehensive and in-depth examination of the research topic, ultimately enriching readers' understanding by offering various expert perspectives. Third, Dey Ghosh R, and Mukhopadhyay S contributed efforts of equal substance throughout the research process. The choice of these researchers as co-corresponding authors acknowledges and respects this equal contribution, while recognizing the spirit of teamwork and collaboration of this study. In summary, we believe that designating Dey Ghosh R, and Mukhopadhyay S as co-corresponding authors of is fitting for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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