

MICROARRAY BASED GENE EXPRESSION PROFILING OF ADVANCED GALL BLADDER CANCER

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Background: Gall bladder cancer (GBC) is an aggressive cancer with specific predilection like female gender and specific geographical areas, however the molecular mechanisms and factors contributing to the clinical or biological behavior are not understood. **Aim:** The aim of this study was to perform a comprehensive analysis of differentially expressed genes in advanced GBC and chronic cholecystitis (CC) cases. **Materials and Methods:** Microarray was planned on fresh specimens of advanced GBC and CC cases using single color cRNA based microarray technique (8X60K format; Agilent Technologies, USA). Twelve advanced GBC and four CC patients were included in the study. **Results:** Of the total of 1307 differentially expressed genes, 535 genes were significantly upregulated, while 772 genes were significantly downregulated in advanced GBC vs CC samples. Differentially expressed genes were associated with biological processes (55.03%), cellular components (31.48%), and molecular functions (13.49%) respectively. The important pathways or key processes affected were cell cycle, DNA replication, oxidative stress, gastric cancer pathway. Using *in silico* analysis tools, three differentially expressed genes i.e. TPX2, Cdc45 and MCM4 were selected (for their significant role in DNA replication and microtubule function) and were further validated in 20 advanced GBC cohort by immunohistochemistry. Significant positive association of Cdc45 and MCM4 proteins was found in advanced GBC cases ($p = 0.043$), suggesting the probable oncogenic role of Cdc45 and MCM4 proteins in advanced GBC. **Conclusion:** Our data demonstrate the potential regulation of Cdc45-MCM4 axis in advanced GBC tumors. Additionally, our study also revealed a range of differentially expressed genes (e.g. TPX2, AKURA etc.) between GBC and CC, and further validation of these genes might provide a potential diagnostic or therapeutic target in future.

Key Words: gall bladder cancer, chronic cholecystitis, microarray, Cdc45, MCM4.

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Gall bladder cancer (GBC) is highly lethal malignancy. Its etiopathogenesis and the reason for its aggressive behaviour is poorly understood. GBCs are often diagnosed at late stages and are therapy resistant [1]. The incidence of GBC demonstrates marked geographic variation with female predominance; for example, it is the single largest cause of cancer death for women in Chile, but accounts for only < 0.5% of cancers in women in the United States. Chile and Bolivia (10 to 15 persons/100,000 population/year) have the highest incidence rate of GBC worldwide [2]. Much of the geographic variation correlates with the tendency to form gallstones, a well-known risk factor in GBC. Cholelithiasis refers to the presence of gall stones in gall bladder that might lead to cholecystitis (after cystic duct obstruction from cholelithiasis). It is a strong risk factor for GBC and is found in 70 to 94% of patients with GBC [2]. Not much work has been done that explain the genetic abnormalities in advanced GBC, however several oncogenes and tumor suppressor genes like *KRAS*, *BRAF*, *EGFR*, *CDKN2A* and *TP53* have been found to be mutated in GBC and cholangiocarcinoma [3, 4]. Study of differentially expressed genes using DNA

microarray technology has led to the finding of new molecules in GBC. Study by Kim *et al.* [5], though describe the comprehensive gene expression profile of GBC, but the study was not exclusively designed for advanced and incurable GBC. Apart from gene expression studies, there are various independent reports on deregulated protein profiles in GBC patients. In immunohistochemical study performed by Li *et al.* [6], authors demonstrated the aberrant expression of Skp-2 and p27^{Kip1} proteins thereby conferring aggressive behavior to GBC. In a similar immunohistochemical study, expression of p53, β -catenin and survivin proteins was found to be positively associated with progression of GBC, compared with chronic cholecystitis (CC) [7, 8]. In a recent study by Espinoza *et al.* [9], authors demonstrated increased mRNA and proteins levels of mucin 5B, carbonic anhydrase 9 and claudin 18 in GBCs and proposed these as theranostics markers.

Most of advanced GBC patients are inoperable and treated with palliative intent chemotherapy. By now we know that there is definite benefit of chemotherapy in advanced GBC, over best supportive care [10]. However, we still don't know if there are certain genes/ or proteins that might provide sensitivity, resistance or predict response to chemotherapy in GBC. That's why it will be helpful if such markers can be identified. Hence, the present study was planned to generate information pertaining to gene expression profile of advanced GBC.

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Abbreviations used: CC – chronic cholecystitis; GBC – gall bladder cancer; GO – Gene Ontology; RIN – RNA integrity number.

MATERIALS AND METHODS

Sample collection. This study was conducted at All India Institute of Medical Sciences, New Delhi after approval by the Institute ethics committee (Ref IEC/NP-245/2010). Period of study was from March 2012 to February 2014. A total of 18 samples of GBC and 6 samples of CC were collected. The advanced stage GBC tissues were obtained by core biopsy and gall bladder tissues for control were obtained from the cholecystectomy specimens. Samples were collected in RNeasy lysis buffer (Qiagen, USA) and then they were snap frozen using liquid nitrogen and stored in Trizol reagent (Invitrogen, USA) at -80°C till further processing.

RNA isolation. Total RNA was isolated from all the samples using the mirVana™ miRNA isolation kit (Ambion, USA). RNA quality and quantity were checked using NanoDrop-1000 spectrophotometer and Agilent 2100 Bioanalyzer, using an Agilent RNA 6000 Nano Kit. Only samples with RNA integrity number (RIN) value ≥ 7.0 were processed for gene expression studies, and these included 12 GBC samples out of 18 GBC and 4 control out of 6 samples of CC.

cRNA microarray and hybridization. Double-stranded cDNA was generated from 200 ng total RNA using the low input quick amp labelling kit (Agilent Technologies, USA), T7 primer, dNTPs and Affinityscript RNase block. *In vitro* transcription of cDNA to cRNA was performed using T7 RNA polymerase and NTP mix and labelled with Cyanine3 using Cy3-CTP. The labelled cRNA was purified according to manufacturer's protocol using RNeasy extraction kit (Qiagen, Germany). The efficiency of cRNA synthesis and dye incorporation was measured using NanoDrop-1000 spectrophotometer. These values were then used to calculate specific activity of Cy-3 using the following formula: Conc. of Cy3/Conc. of cRNA $\times$ 1000 and expressed as pmol Cy3 per μg of cRNA. For hybridization, 600 ng of Cy-3 labeled cRNA was mixed with $10\times$ blocking agent and fragmentation buffer and incubated at 60°C for exactly 30 min to fragment RNA followed by addition of hybridization buffer to stop further fragmentation. The labeled cRNA mixture was then applied to a microarray slide (8X60K format; Agilent Technologies, USA), assembled in a hybridization chamber fitted with a gasket slide and incubated for 17 h in a hybridization oven at 65°C and 10 rpm. After the incubation period, the microarray and gasket slide were dissociated inside a staining dish containing hybridization wash buffer, washed again in fresh wash buffer followed by a second wash with gentle agitation from a magnetic stirrer. The slides were scanned using specific scanning protocols for gene expression microarrays in a microarray scanner (Agilent Technologies, USA). Fluorescent intensities from raw microarray image files were obtained using Feature Extraction Image Analysis Software.

Microarray data analysis. A supervised analysis was performed using GeneSpring 12.6 GX-PA software (8X60K format; Agilent Technologies, USA) [11]. For normalization of the data, threshold raw signals were

set to 1.0 and quantile normalization was implemented. Unpaired *t*-test was executed to define the genes differentially expressed between the two groups; a significance level of $p < 0.05$ and a fold change cut off ± 2 was taken into account. Gene Ontology (GO) tool and pathway analysis tool of gene spring were used to predict biological function and pathways represented by the differentially regulated genes [12].

Immunohistochemical validation of differentially expressed genes. The archival formalin-fixed, paraffin-embedded tissues blocks of gall bladder patients were cut into $5\ \mu\text{m}$ sections. After routine deparaffinization and rehydration steps, exogenous peroxidase activity was blocked using 0.1% of H_2O_2 in methanol for 20 min, followed by citric acid (10 mM) buffer antigen retrieval step in microwave. After blocking, slides were incubated with primary antibodies i.e. respectively at 4°C overnight [Primary antibodies: MCM4 (sc-28317; dilution: 1:100), TPX2 (sc-271570; dilution: 1:100) and CDC45 (sc-55569; dilution: 1:200)]. All primary antibodies were procured from Santa Cruz Biotechnology Inc. (Texas, USA)]. After O/N incubations, slides were washed and treated with CRF-Anti-Polyvalent HRP Polymer Kit (SkyTek laboratories, UT, USA) at room temperature for 60 min, followed by developing with diaminobenzidine (available with kit). Finally, the sections were counterstained with hematoxylin and mounted.

Microscopic scoring. The percentage of the immunostained tumor cells was determined semi-quantitatively by assessing the whole section and classified into 7 groups: 0 ($< 10\%$ positive cells); 1 (11–20% positive cells); 2 (21–40% positive cells); 3 (41–60% positive cells); 4 (61–80% positive cells) and 5 ($> 80\%$ positive cells). The intensity of staining was further graded as 1 (absent/very faint), 2 (strong) and 3 (very strong). To calculate H score, scores from each section were multiplied together and a total score greater than 2 was designated as a positive result.

Statistical analysis. Pearson χ^2 or Fisher's exact test was used to examine the association between the proteins. A *p*-value of less than 0.05 was considered statistically significant. Statistical analysis was performed using IBM SPSS 22.0 version.

RESULTS

Of the 18 patients of unresectable advanced and metastatic GBC, 12 samples have RIN value ≥ 7.0 , so they were evaluated using gene expression array. Median age of patients was 54 years with an average duration of symptoms of 4.3 months. All the patients have stage 4 disease with pain being the most common symptom. The control samples were taken from patients with CC who have underwent planned cholecystectomy. None of these control patients had personal or family history of malignancy. The clinicopathological parameters of GBC patients as well as controls are provided in Table 1.

A total of 18240 genes were differentially expressed between samples from advanced GBC and CC of which 1307 genes satisfied $p < 0.05$ by unpaired *t*-test and a fold change cut off of ± 2 . Out of these 1307 genes,

535 genes were upregulated and 772 genes were downregulated in advanced gall bladder carcinoma, compared to CC samples (Data available online at <http://lms.snu.edu.in/micore/gbc.php>). To unravel the biological functions of gene expression signatures, these gene lists were subjected to the GO browser and the categories of GO which were statistically overrepresented among the obtained gene lists were extracted. A total of 58 GO terms satisfied $p < 0.05$ and fold change cut off ± 2 . The distribution of these 58 GO terms into molecular functions, cellular components and biological processes. The pathway analysis tool demonstrated that a total of 24 pathways were affected by the differentially regulated genes, out of 24, top 10 pathways that were statistically significant are shown in Table 2. The important pathways which

were affected were cell cycle pathway (Table 3), DNA replication pathway (Table 4), oxidative stress pathway (Table 5) and gastric cancer network (Table 6). Briefly, the cell cycle regulatory genes significantly upregu-

Table 1. Clinical parameters of advanced GBC patients (A) and controls (B) A.

Parameters	Cases (n = 12)
Age: Median (Range)	54 years (42–65)
Female: Male	3:1
Average duration of symptoms	4.3 months
Pain	91%
Icterus	33%
ECOG PS	
1–2	9
3	3
Albumin < 3.5	2
Stage	4

B.

Parameters	Cases (n = 4)
Age: Median (Range)	41 yrs (34–63) yrs
Sex (Male: Female)	2:2
Gall stones	Nil
F/S/O of chronic cholecystitis	4

Table 2. Important pathways affected

Pathway affected	p-value
1 Superoxide radical degradation	0.003
2 Integrated cancer pathway	0.00006
3 Cell cycle pathway	0.0000001
4 RB in cancer	0.002
5 DNA replication	0.0003
6 Oxidative stress	0.04
7 Vitamin B ₁₂ metabolism	0.04
8 Selenium pathway	0.02
9 Gastric cancer network	0.0001
10 G1 to S cell cycle control	0.0002

Table 3. Important genes deregulated in cell cycle pathway

Gene	Fold change	Chromosome number	Function
UPREGULATED GENES			
<i>Skp2</i>	4.87	5	Acts as proto oncogene and negatively regulates p27Kip1
<i>BUB1</i>	12	15	Phosphorylates mitotic check point complex and activates spindle check points
<i>MAD2L1</i>	22.33	4	Acts as a mitotic assembly checkpoint
<i>ESPL1</i>	12.07	12	Separation of the sister chromatids during anaphase
<i>E2F1</i>	10.95	20	Controls apoptosis
<i>PTTG1</i>	7.8	5	Prevents spearing from sister chromatids separation
<i>PTTG2</i>	5.44	4	
<i>CHEK1</i>	9.15	11	Acts as cell cycle checkpoints, cell cycle arrest and DNA repair
<i>CDC20</i>	9.55	1	Acts as a regulatory protein at different steps in cell cycle. Activate anaphase promoting complex
<i>CDK2</i>	2.63	12	Helps in G1 to S phase transition
<i>CDC2</i>	2.76	1	Activates Cyclin dependent kinase 1
<i>CCNB1</i>	6.32	5	Helps in G2/M phase transition
<i>CCNB2</i>	10.94	15	Controls cell cycle at G2/M transition
<i>CDC45</i>	9.7	22	Required for DNA replication
<i>PKMYT1</i>	7.99	16	Negatively regulates G2/M phase transition
<i>RBL1</i>	5.23	20	Tumor suppressor protein that controls cell cycle
<i>PASK</i>	3.35	2	Regulates insulin gene expression
<i>CCNE1</i>	5.92	10	Interact with CDK2 and are thought to trigger cell cycle activity in carcinogenesis
<i>CCNB1</i>	6.32	5	Regulatory protein involved in mitosis
DOWN REGULATED			
<i>CCND2</i>	4.83	12	Forms complex with CDK 4/6 and inhibits RB gene
<i>TGFB3</i>	5.96	1	Leading to recruitment and activation of SMAD family transcription factors that regulate gene expression

Table 4. Important genes deregulated in DNA replication pathway

Gene	Fold change	Chromosome number	Function
UPREGULATED			
<i>CDK2</i>	2.63	12	Helps in G1 to S phase transition.
<i>PASK</i>	3.35	2	Regulates insulin gene expression.
<i>GMNN</i>	3.6	6	Inhibits DNA replication
<i>MCM4</i>	4.6	8	Helicase and uncoiling of DNA strands
<i>CDC45</i>	9.72	22	Helps in replication.
<i>RPA3</i>	2.01	7	Stabilizes single stranded DNA formed during replication
<i>POLE</i>	3.99	12	DNA replication and repair
<i>RFC4</i>	3.64	2	loading of DNA polymerase on DNA to synthesize leading and lagging strands

Table 5. Important genes up-regulated or down regulated in oxidative stress pathway

Gene	Fold change	Chromosome number	Function
UPREGULATED			
<i>SOD2</i>	3.46	6	Binds to the superoxide by-products of oxidative phosphorylation and converts them to hydrogen peroxide and diatomic oxygen
<i>CUL2</i>	2.32	10	Component of multiple cullin-RING-based ElonginB/C-CUL2/5-SOCS-box protein E3 ubiquitin-protein ligase complexes, which mediate the ubiquitination of target proteins
<i>SKP2</i>	4.87	5	Limit DNA damage and apoptosis triggered by oxidative stress.
<i>STAT1</i>	3.44	2	Transcription factor induced during oxidative stress.
<i>TNF</i>	4.95	6	Regulator of ROS during oxidative stress
DOWNREGULATED			
<i>COX4I2</i>	5.06	20	Encodes isoform 2 of subunit IV. Cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, catalyzes the electron transfer from reduced cytochrome c to oxygen
<i>COX7A1</i>	6.57	19	Encodes polypeptide 1 (muscle isoform) of subunit VIIa. Cytochrome c oxidase reduced cytochrome c to oxygen
<i>CKMT2</i>	29.32	5	Transfer of high energy phosphate from mitochondria to the cytosolic carrier, creatine

Table 6. Important genes up-regulated or down regulated in gastric cancer pathway

Gene	Fold change	Chromosome number	Function
UPREGULATED			
<i>ECT2</i>	8.7	3	Acts as an oncogene
<i>KIF20</i>	3.7	10	Enhances microtubular binding and microtubular motor activity.
<i>MCM4</i>	4.6	8	Helicase and uncoiling of DNA strands
<i>CENPF</i>	12.47	1	Helps in cell division.
<i>AURKA</i>	2.84	13	Regulates spindle activity
<i>TPX2</i>	16.71	20	Protein coding gene.
DOWNREGULATED			
<i>SMOC2</i>	21.82	6	Controls angiogenesis in tumor growth
<i>FGF2</i>	8.00	4	Signals through four receptor tyrosine kinases and acts in a variety of developmental processes, including angiogenesis.
<i>WNT10B</i>	2.58	12	Transcription androgen receptor nuclear signaling and Wnt signaling pathway and pluripotency.

lated in the advanced GBC were *E2F1*, *CDK2*, *CCNE1*, *CDC20*, *CDC45*, while *CCND2* and *TGFB3* were down-regulated. In the DNA replication pathway, genes like *MCM4*, *POLE*, *CDC45* and *RCF-4* were upregulated suggesting the fact that enhanced replication drives GBC proliferation and progression. In advanced GBC, oxidative stress pathway genes were found to be up-regulated, i.e. *SOD2*, *CUL2* and *SKP2*, suggesting alteration in cancer cell metabolism is one of the factors responsible for GBC progression. Significant downregulation was reported in mitochondrial genes, i.e. *CKMT2*, *COX4I2* and *COX7A1*, might suggest the impaired mitochondrial state in advanced GBC. Some of the genes associated with gastric cancer pathway i.e. *ECT*, *CENPF*, *AKURA* and *TPX2*, were found to be upregulated, while *SMOC-2* and *FGF-2* were downregulated in advanced GBC samples, compared to CC.

In the present study, we further validated three genes for their protein expression in advanced GBC, i.e. *TPX2*, *CDC45* and *MCM4*, mainly for two reasons. Firstly, all these genes share same interactome generated using GIANT tissue specific prediction [13] server demonstrating their direct role in DNA replication and microtubule polymerization & depolymerization (Fig. 1). Secondly, these three (*TPX2*, *CDC45* and *MCM4*) genes might have interdependence or association with each other in advanced GBC (as demonstrated using String-protein interaction; Fig. 2). Additionally, *TPX2*, *CDC45* and *MCM4* are regulated by p53-DREAM (p53-p21-DREAM-E2F/CHR) pathway, responsible for p53-mediated cell cycle arrest [14]. Three differentially expressed upregulated genes in advanced GBC, compared with CC were picked (*TPX2* (fold change = 16.71), *Cdc45* (fold change = 9.7) and *MCM4* (fold change = 4.6) for validation for their protein expression in small cohort of 20 advanced GBC and 4 CC patients (Table 7A). For *TPX2* (microtubule nucleation factor), 11/20 (55%) cases of GBC showed positive staining in the nuclei (Fig. 3a), however 2/4 (50%) cases of CC showed positivity too. Similarly, *Cdc45* (cell division cycle 45), 10/18 (55%) cases of GBC showed positive staining

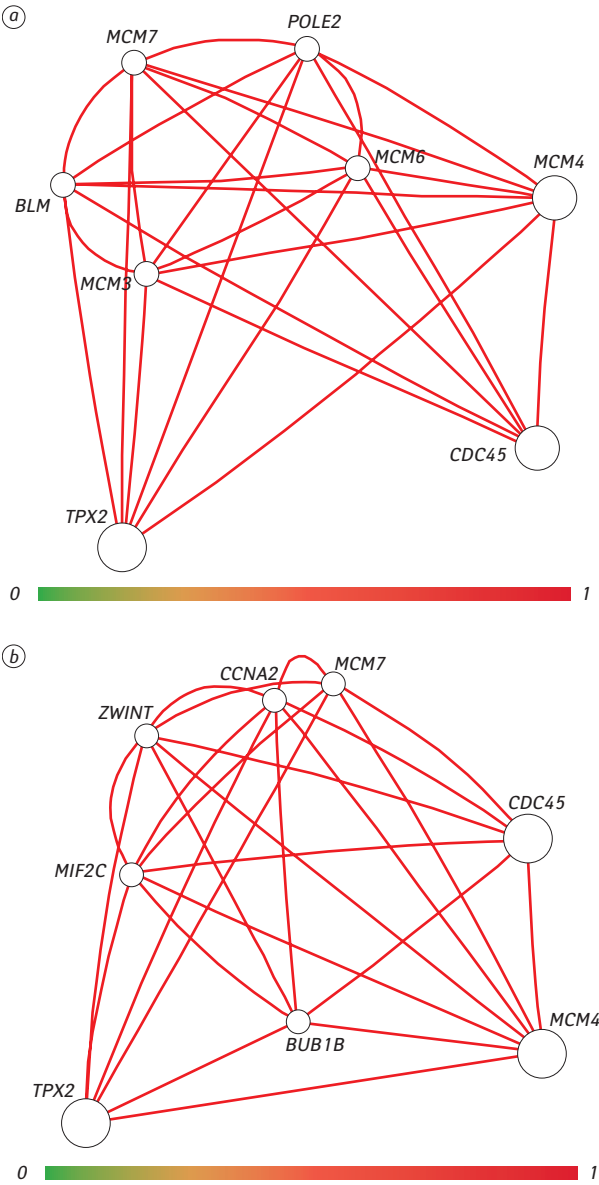


Fig. 1. Schematic of the GIANT tissue-specific interaction prediction server. GIANT is queried with three genes *TPX2*, *CDC45* and *MCM4* in the (a) biological process (DNA replication)-specific interaction and (b) biological process (microtubule polymerization and depolymerization)-specific interaction. The predicted interactions to *TPX2*, *CDC45* and *MCM4* are shown as a network visualization where edges are predicted posterior probabilities of three genes functionally interacting during the processes of DNA replication and microtubule function

in the cytoplasm (Fig. 3b), however 2/4 (50%) cases of CC showed positivity too. A clear-cut demarcation was found in *MCM4* (mini-chromosome maintenance complex component 4) protein expression, where 6/20 (30%) cases of GBC showed positive staining in the nuclei (Fig. 3c), however none of cases of CC showed positivity for *MCM4*. Significant positive association was observed between *Cdc45* and *MCM4* protein expression ($p = 0.043$; OR=1.67 [95% CI = 1.005–2.765]). All the four cases positive for *MCM4* expression were found to be *Cdc45* positive (Table 7B). However, no such association was observed between *TPX2* and *MCM4* expression ($p = 0.058$).

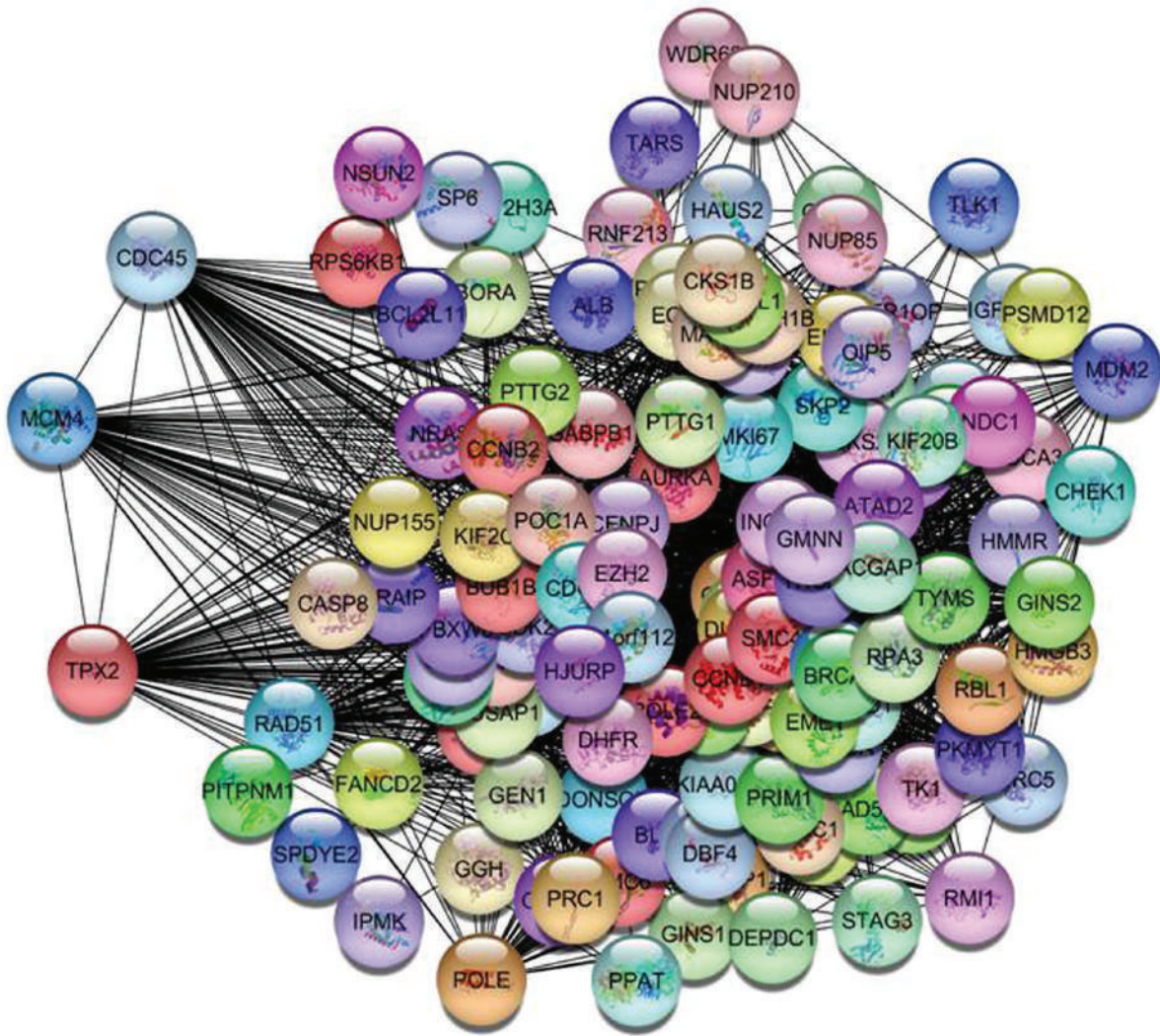


Fig. 2. The protein-protein interaction network. Protein-protein interaction network demonstrating differentially expressed genes in advanced GBC regulated by three protein TPX2, Cdc45 and MCM4 in an interactome. The shortest path proteins were retrieved from the shortest paths between every protein pair coded by the top 535 genes selected

Table 7.

A. Immunohistochemical staining results of TPX2, Cdc45 and MCM4 in the 20 cases of GBC and 4 cases of CC						
Number of positive cases/total number of cases (%)						
	TPX2			CDC45		
CC	2/4 (50%)			2/4 (50%)		MCM4
GBC	11/20 (55%)			10/18 (55%)		6/20 (30%)

B. Correlation of MCM4 expression with TPX2 and Cdc45 in gall bladder carcinomas						
MCM4	TPX2				Cdc45	
	Positive	Negative	Total		Positive	Negative
Positive	5	1	6	4*	0	4
Negative	6	8	14	6	8	14
Total	11	9	20	10	8	18

DISCUSSION

The majority of patients with GBC in India have advanced and unresectable disease. Due to non-specific symptoms, GBC diagnosis often occurs at late stage and has been associated with poor survival, i.e. 5-year survival is less than 5% [15]. Its precursor event is a chronic process, i.e. CC that goes for a long period of time and is associated with gall stone disease [15]. Infection with *Salmonella Typhi* has also been implicated as inciting event for GBC with varying proportion [16].

Previously, many research groups have identified differential expression of proteins in GBC, compared

with CC, among them p53 immunoreactivity has been shown to significantly increased in GBC compared to either CC or normal gallbladder [17–19]. However, proliferation marker like Ki67 demonstrated no change in GBC compared to CC [17]. Significant up-regulation in the protein levels of CEA [18], nuclear β -catenin [19] and survivin [20] was reported in GBC, compared with CC. Apart from protein expression, several gene expression studies has also been performed on GBC. The seminal gene-based microarray study in GBC was conducted by Kim *et al.* [5], but they have included both early and advanced GBC as cases, and gall bladder from early bile duct cancers as control.

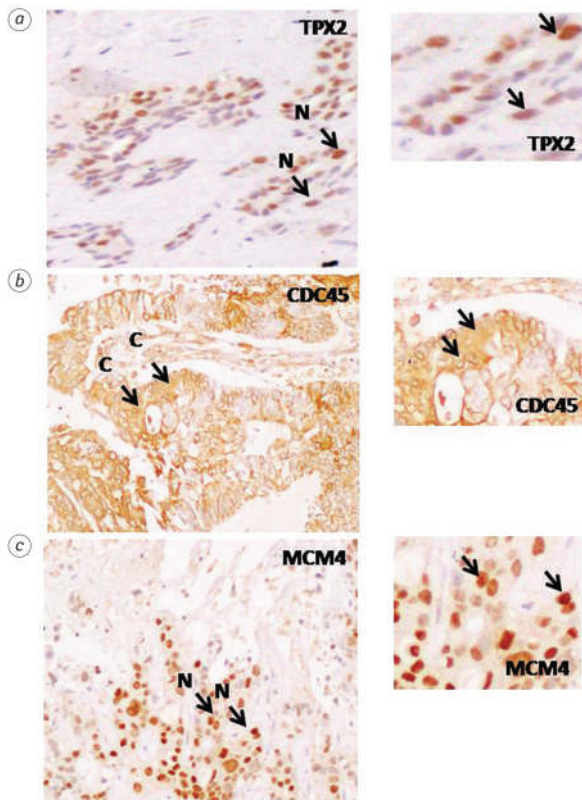


Fig. 3. Expression of TPX2, CDC45 and MCM4 in GBC. Representative images demonstrating the expression patterns of TPX2 protein (a), CDC45 (b) and MCM4 (c) in advanced GBC (20X). Arrows show nuclear “N” and cytoplasmic “C”. Magnified images are also presented as cut-outs to show specific localization of the proteins

Compared with the study performed by Kim *et al.* [5] (2,270 upregulated and 2,412 down regulated genes), we found overexpression of 535 genes and underexpression of 772 genes (in present study). Important genes which were upregulated and found to be common in the present as well as study by Kim *et al.* [5] were *BCL2*, *CDC2*, *BUB1B*, *FGFR*, *POLE* and important downregulated genes in both the studies were *RB1*, *DSP*, *VWF* and *CCND2*. The reason behind the less numbers of genes identified in our study is due to induction of CC as control, since inflammation plays pivotal role in both CC as well as GBC. In all, we found that deregulation of 18,000 genes and about 1300 genes had > 2 folds change with p -value < 0.05. Alteration of these genes lead to significant differential regulation of 24 pathways. In present study, some of the affected pathways in advanced GBC were associated with apoptosis, cell cycle, DNA replication, oxidative stress and gastric cancer pathway. Apart from these, other important pathways found to be involved in GBC progression are RB pathway and integrated cancer pathways. Among various apoptotic genes found to be upregulated in advanced GBC were *BCL2A1* (fold change: 2.29), *BIRC5* (fold change: 8.16), *E2F1* (fold change: 10.95), and *TRAF2* (fold change: 2.2). Early stage GBCs express significantly high Bcl-2 protein, and its inhibition sensitizes gallbladder tumors to cisplatin [21, 22]. Anti-apoptotic protein BIRC5 or survivin and E2F1 expression has also been associated with

poor prognosis in GBC [20, 23, 24]. Among cell cycle regulatory genes, *CDK2*, *CCNB1*, *CDC20*, *CDC45* and *SKP2* were found to be upregulated in advanced GBC (as shown in Table 3). It has already been demonstrated that Cdk2, CDC45 and CCNB1 (cyclin B1) positively drives GBC progression [25, 26]. Similar to our findings, downregulation of CCND1 (cyclin D1) has also been demonstrated in gall bladder cell lines (SGC996 and NOZ cells) [26]. Another cell cycle protein, Skp2 is upregulated in GBC and emerged as independent adverse prognostic factor by IHC ($p = 0.004$) [27]. Overall, cell cycle proteins are significantly deregulated in advanced GBC suggesting the basis for therapeutic interventions through CDK inhibitors like AZD 5438 and NSC 693868.

DNA replication is a crucial process that is tightly regulated in normal cell and also an integral element in cancer cells. In present study, we also found several DNA replication genes, i.e. *MCM4*, *CDC45* and *RCF4* upregulated in advanced GBC with 4.6, 9.7 and 3.6 fold change respectively. All of these genes have been positively associated with cancer progression and metastasis [28–31]. MCM4 and CDC45 are part of the core complex of DNA helicases, i.e. CMG (Cdc45-Mcm2-7-GINS) complex. Presently, DNA-helicases are emerging as potential therapeutic targets of serious hyper-proliferative diseases like cancer [32].

Oxidative stress pathway genes were found to be modulated in advanced GBC patients. Upregulated genes like *CUL2*, *SKP2*, *STAT1* and *TNF* has been positively associated with aerobic glycolysis and tumor progression [33–36], while downregulation of mitochondrial associated genes, i.e. *COX4I2*, *COX7A1* and *CKMT2* suggest compromised oxidative phosphorylation in advanced GBC. Deregulation in gastric cancer pathway genes were also found in advanced GBC, genes like *ECT2*, *CENPF*, *AURKA*, *TPX2* were found to be upregulated while *SMOC-2* and *FGF-2* were downregulated. The guanine nucleotide exchange factor ECT2 and centromere protein F are established oncogenes [37, 38]. Similarly, TPX2/AURKA signaling axis has gained attention as they are overexpressed in neoplastic conditions and can regulate MYC, thereby making this axis a potential therapeutic target [39, 40]. In present study, SMOC-2 was found to be downregulated in advanced GBC, similar to breast, ovarian, hepatocellular carcinomas [41, 42] etc.

In the present study, we validated three differentially expressed genes that were found to be upregulated in advanced GBC, as compared with CC, i.e. *TPX2* (fold change = 16.71), *CDC45* (fold change = 9.7) and *MCM4* (fold change = 4.6), in an independent cohort of 20 GBC and 4 CC patients by immunohistochemistry. Of the three proteins, only MCM4 protein was not expressed in CC (0/4), however, expression of TPX2 and Cdc45 protein was observed in around 50% of CC. Pathologically the four cases analyzed for CC were highly inflamed and might be the reason why positive expression (in 50%) of the TPX2 and CDC45 proteins were observed in these cases, as in-

flammation plays important role in cancer development and progression (Table 7). In advanced GBC, positive expression of MCM4, TPX2 and CDC45 was found to be 30%, 55% and 55% respectively. Other important finding of the present study was the positive association between Cdc45 and MCM4 proteins in advanced GBC cases ($p = 0.043$) (Table 7) suggesting enhanced DNA helicase activity. As discussed, CDC45 forms complex with MCM and GINS (CMG), and is conserved component of DNA replication machinery and is required for DNA synthesis during genome duplication [32]. Our finding of co-existence of CDC45 and MCM4 proteins put-forward the notion of the pro-tumorigenic role of the complex in advanced GBC. Pathway enrichment (data not shown) showed that MCM4 and CDC45 fall under the same pathway then TPX2. Furthermore, TPX2 and another upregulated gene AURKA (Aurora-A kinase) can be an additional therapeutic target for advanced GBC [40]. The frequent upregulation of Aurora-A and TPX2 in cancers (including gall bladder, findings of present study) suggests that the complex act as a tumorigenic unit and small compounds that interfere with TPX2 binding to Aurora-A can be used as therapeutic intervention for advanced GBC [43, 44].

In the present study, we investigated differential gene expression profiles in advanced GBC patients, compared to CC. Among various pathways found to be deregulated were cell cycle, DNA replication, oxidative stress, gastric cancer pathway etc. Our present data propose the rational of using inhibitors for CMG and AURKA/TPX2 complexes as potential therapeutic options in advanced GBC patients. Future studies can be planned on GBC patients cohort (with follow-up) to decipher the prognostic relevance of these deregulated proteins.

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