

# Frequent Promoter Hypermethylation and Down Regulation of BNIP3: An Early Event During Gallbladder Cancer Progression

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## Research Article

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## Abstract

Epigenetic alterations have been reported as one of the risk factors of Gallbladder cancer (GBC). Promoter hypermethylation is associated with high incidence and poor prognosis of GBC. Bcl-2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) is a pro-apoptotic protein member of Bcl-2 family. Present study was aimed to investigate expression profile and promoter methylation status of BNIP3 in GBC and its correlation with clinico-pathological parameters. We demonstrate down regulation of BNIP3 in 56% of the GBC samples. BNIP3 promoter is also frequently hypermethylated (69%) in GBC samples. Interestingly, we found that 69% (40/58) of the BNIP3 promoter hypermethylated samples had also reduced expression of BNIP3. Our data demonstrate significant correlation of the mRNA expression and promoter hypermethylation with late stage and nodal metastasis. Hypermethylation of BNIP3 promoter is associated with low overall survival period. Our results suggest that promoter hypermethylation is an early event and can be a frequent mechanism for down regulation of BNIP3 in GBC. BNIP3 can be a good prognostic and diagnostic marker of GBC.

## Introduction

Gallbladder cancer (GBC) is one of the rare cancers, however, it is the most common malignancy of biliary tract[1]. The incidence of GBC is 10–22/100,000 in North Indian population which is similar to high incident countries like Chile, Bolivia, and Columbia [2]. Tumor suppressor genes (TSGs) play critical role in various cellular processes including DNA repair, cell cycle inhibition, induction of apoptosis, and suppression of metastasis; therefore, loss of function of these genes have been predicted to be involved in initiation and progression of cancer[3]. Inactivation of TSGs by CpG promoter hypermethylation has been demonstrated as one of the common mechanisms in various cancers[4]. Bcl2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), a mitochondrial pro-apoptotic protein which belongs to the BH3-only subfamily, contains a single Bcl-2 homology 3 (BH3) domain[5, 6]. BNIP3, a putative tumor suppressor gene, is transcriptionally regulated mainly by HIF1 and NF- $\kappa$ B[7]. Over expression of HIF1 $\alpha$  and NF- $\kappa$ B has been reported in GBC[8, 9]. BNIP3 up regulation can induce cell death by reducing loss of mitochondrial membrane potential and ROS generation at the mitochondria. In contrast BNIP3 may also play role in cell survival by inducing selective mitochondrial degeneration[10].

In a previous study it has been reported that human hepatoma and colon carcinoma cells show BNIP3 promoter hypermethylation, and its expression is restored after treatment with 5-aza, 2'-deoxycytidine, a potent inhibitor of DNA methylation. Verticillin A can serve as a potent apoptosis sensitizer through increasing BNIP3 expression either by inhibitor of DNA methylation or DNA demethylation inducing factor. Verticillin A is a promising adjuvant agent to overcome drug resistance in human colon cancer therapy[11].

Earlier reports have demonstrated that restoration of BNIP3 with DNMT inhibitor results in increased induction of mitochondria mediated apoptosis in pancreatic cancer[5]. It has been shown that DNMT1 mediate promoter methylation of BNIP3 in pancreatic cancer via MEK pathway[12].

Studies have shown that regulation of gene expression via promoter hypermethylation is related with high incidence of GBC and its poor prognosis in Asian countries [13]. Therefore, in this current study we have investigated the mRNA expression profiles and promoter methylation status of BNIP3 in GBC. We have further attempted to correlate the expression level and promoter methylation status of BNIP3. We have also correlated expression and methylation status of BNIP3 with clinico-pathological indices of GBC patients.

## Materials And Methods

### Patient and tissue samples

A total of 84 tissue samples were collected from patients who underwent surgical resections for gallbladder carcinoma at the Institute of Medical Sciences, Banaras Hindu University (Varanasi, India) between February 2015 and March 2019 for the present study. A total of 29 gallbladder tissues obtained from patients operated for gallstone diseases were taken as normal controls. All the samples were histopathologically confirmed. The part of tissue for RNA isolation was immediately kept in RNA later and the tissue for DNA isolation was placed in plain vials which were then stored at -80 °C. Samples used in our study were free from preoperative chemotherapy and radiotherapy. Clinical and pathological characteristics of GBC cases were assessed on the basis of TNM staging according to the American Joint Committee on Cancer (AJCC) 7<sup>th</sup> edition, 2010. Written informed consent from all the patients were obtained according to the approved protocol by the Institutional Ethical Committee of Faculty of Science, Banaras Hindu University.

### RNA extraction and expression profiling

Total RNA was extracted according to manufacturer's protocol using TRizol reagent (Qiagen, Germany) from GBC and gallstone tissue samples stored in RNA later. High capacity cDNA reverse transcription kit (ABI, USA) was used in synthesis of the first strand cDNA followed by manufacturer's instructions. Expression of BNIP3 was analyzed using semi-quantitative RT-PCR and normalized with expression levels of β-actin used as internal control in gallstone and GBC tissue samples. Primers used in semi-quantitative RT-PCR are listed in (Supplementary Table 1). 2% agarose gel stained with ethidium bromide was used for electrophoresis of the PCR product. Differential expression of BNIP3 in GBC samples were calculated as mean of normal ± 2×SD[14, 15]. To validate the semi-quantitative RT-PCR results, real-time RT-PCR was performed in 12 samples with 2X SYBR Green PCR master mix (ABI, USA) followed by manufacturer's protocol. Relative mRNA expression was quantified by ΔΔCt method.

### Methylation-specific PCR (MSP)

Extraction of Genomic DNA from gallstone and GBC tissue samples were performed by standard phenol/chloroform extraction protocol. EpiTect Bisulfite Kit (Qiagen GmbH, Germany) was used for Sodium bisulfite modification of extracted DNA followed by manufacturer's instructions. MSP was performed using DreamTaq™ Hot Start PCR Master Mix (Thermo Scientific, US) and primer sets specific

for methylated or unmethylated sequences for promoter region of BNIP3 (Supplementary Table 1). Bisulfite converted lymphocyte DNA was used as positive control for unmethylated primer while same DNA treated with SssI methyltransferase (NEB, Beverly, MA) as positive control for methylated primer and water blank as negative control for each set of primers. Methylation-specific PCR was repeated in duplicate. To validate the MSP results and to confirm the methylation status, Sanger sequencing was done in one heterozygous methylated GBC sample and one unmethylated gallstone sample (Supplementary figure 1).

### Statistical analysis

Normalized expression values less than [mean of normal – SD] were considered to be down-regulated. All statistical analyses were done by using GraphPad Prism version 5 software (La Jolla, USA) and SPSS version 20 (SPSS, IBM Corporation, Armonk, NY, USA). Student's t-test was used for comparing two groups and One-way ANOVA test was applied for comparison between more than two groups. Two-tailed Fisher's exact test was used to determine the correlation between promoter methylation pattern with clinico-pathological characteristics and expression profile. Overall survival was calculated from the time of surgical resection till death of the patient or to last follow up. Patients were excluded who died within 15 days of surgery or lost to follow-up. Therefore, survival study comprised of 65 out of 84 GBC patients. Observed survival was estimated by Kaplan-Meier test and differences between the groups were analyzed by log rank test. All the data obtained from comparing two groups by parametric test are displayed as mean ± SEM (sum error mean). All the p-values <0.05 were considered as significant.

## Results

### Patient's clinical data and tissue samples

Patients sample were characterized on the basis of available clinico-pathological indices. Present study includes 21 male and 63 female patients (median age 50 yrs; range: 24-76 yrs). Majority of collected tumor samples were adenocarcinoma (69/84; 82.14%) whereas rest of the samples include 7 papillary adenocarcinoma, 5 adenocarcinoma with mucin and 3 adenosquamous carcinoma. Most of the patients who underwent surgery had stage II or III disease (54/84; 64.3%). 31 out of 84 patients had lymph node metastasis, while rest 40 were with absence of lymph node metastasis. Complete details of the patient's clinico-pathological indices are listed in Supplementary Table 2.

### Reduced expression of BNIP3 in gallbladder cancer biopsies

Expression profile of BNIP3 transcript was analyzed by semi-quantitative RT-PCR in 84 cases of surgically resected gallbladder carcinoma specimens and 29 gallstone tissues taken as control. Expression of BNIP3 was down-regulated in 56 % while 44 % of the tumor biopsies showed no significant change when compared with gallstone tissue (Fig. 1a). There is significant reduced expression of BNIP3 in GBC compared with gallstone ( $p < 0.05$ ) (Fig. 1b). There is sequential significant down regulation in late stage

tumors through early stage tumors (Fig. 1c). We have further analyzed 12 GBC and 12 gallstone tissue samples by using quantitative real time RT-PCR. Similar transcript pattern was observed as 9 out of 12 GBC samples shows down regulation and rest of 3 GBC samples had no change in expression. Clinical correlation of BNIP3 expression with patient age ( $\leq$ 50 years and  $>$ 50 years), gender, gallstone status (presence and absence), histological grade of tumor (differentiation: well, moderate and poor), tumor stages (I + II and III + IV), T-stage of tumor (T1 + T2 and T3), lymph node metastasis status of tumor (N0 and N1 + N2) and distant metastasis of tumor (M0 and M1) was analyzed. The mRNA expression levels show significant correlation with pathological staging, lymph node metastasis and T-stage of tumor (Fig. 1d-f) whereas no significant correlation was found with other clinical indices (Table-1).

BNIP3 promoter is frequently hypermethylated in gallbladder cancer biopsies.

Since there was reduced expression of BNIP3 in gallbladder cancer, we investigated the methylation status of the promoter region of BNIP3 in all 84 tumor biopsy samples. 69% (58/84) of GBC samples show promoter hypermethylation of which 11.9 % (N = 10) of tumor biopsies showed homozygous and 57.1 % (N = 48) of tumor biopsies showed heterozygous methylation. 31% of tumor biopsy samples (N = 26) were unmethylated. However, all gallstone samples were unmethylated (Fig. 2a, b; Supplementary Table 3). We also analyzed the correlation between methylation status of BNIP3 promoters and BNIP3 expression in tumor samples. 31% of (18/58) methylated gallbladder cancer cases exhibited no change in BNIP3 expression, while the remaining 69% (40/58) cases with reduced BNIP3 expression exhibited BNIP3 promoter methylation. These observations suggest that low BNIP3 expression is associated with BNIP3 promoter hypermethylation (Fig. 2e; Table 2). Clinical correlation of BNIP3 methylation status with patient age, gender, gallstone status, histological grade of tumor (differentiation: well, moderate and poor), tumor stages (I + II and III + IV), T-stage of tumor (T1 + T2 and T3), lymph node metastasis status of tumor and distant metastasis of tumor was analyzed. The BNIP3 promoter methylation status has significant correlation with pathological stage and lymph node metastasis ( $P < 0.05$ ) (Fig. 2c, d) whereas no significant correlation was found with other clinical indices (Table 1).

Table 1

Correlation between clinico-pathological parameters with the mRNA expression and promoter methylation status of BNIP3.

Variables (n; %)	Normalized mRNA expression	p value	Promoter methylation status		p value
			Methylated, n (%)	Unmethylated, n (%)	
Age	0.1871 ± 0.014	0.5973	29(34.5%)	18(21.4%)	0.1532
≤ 50 (47; 56%)	0.1757 ± 0.015		29(34.5%)	8(9.6%)	
> 50 (37; 44%)					
Gender	0.1871 ± 0.024	0.7753	16(19%)	5(6%)	0.5867
Male (21; 25%)	0.1800 ± 0.011		42(50%)	21(25%)	
Female (63; 75%)					
Gallstone	0.1889 ± 0.014	0.5375	29(34.5%)	15(17.9%)	0.6376
Present (44; 52.4%)	0.1755 ± 0.015		29(34.5%)	11(13.1%)	
Absent (40; 47.6%)					
Histological Grade	NA	0.9545	19(22.6%)	10(11.9%)	0.8245
Well (29; 34.5%)			24(28.6%)	9 (10.7%)	
Moderate (33; 39.3%)			15(17.9%)	7 (8.3%)	
Poor (22; 26.2%)					
Stage	0.2100 ± 0.018	0.0257	20(23.8%)	16(19.1%)	0.0312
I + II (36; 42.9%)	0.1619 ± 0.012		38(45.2%)	10(11.9%)	
III + IV (48; 57.1%)					
Extent of tumor	0.2095 ± 0.016	0.0171	27(33.3%)	15(18.5%)	0.3479
T1 + T2 (42; 51.9%)	0.1574 ± 0.013		29(35.8%)	10(12.4%)	
T3 (39; 48.1%)					

Variables (n; %)	Normalized mRNA expression	p value	Promoter methylation status		p value
			Methylated, n (%)	Unmethylated, n (%)	
Lymph node metastasis Present (31; 43.7%)	0.1484 ± 0.013	0.0422	25(35.2%)	6 (8.5%)	0.0450
	0.1895 ± 0.013		23(32.4%)	17(23.9%)	
Absent (40; 56.3%)					
Distant Metastasis Present (13; 16.2%)	0.1685 ± 0.020	0.5281	12(15%)	1(1.2)%	0.0539
	0.1876 ± 0.012		43(53.8%)	24(30%)	
Absent (67; 83.8%)					

Table 2  
Correlation between BNIP3 methylation status and BNIP3 mRNA expression.

Methylation status of BNIP3	mRNA expression		p value
	Down	No change	
Methylated	40	18	0.0039
Unmethylated	7	19	

## Prognostic and Diagnostic value of BNIP3 expression and methylation

To assess the prognostic value of BNIP3 at mRNA level, expression of BNIP3 was divided into two groups: low and no change in expression, no significant difference was observed in survival between two groups of expression although patients with reduced BNIP3 expression show low survival (Fig. 3a). Similarly, at promoter methylation level, data was dichotomized as: methylated and unmethylated, patients with methylated allele of BNIP3 showed shorter period of survival than with unmethylated BNIP3 allele (Fig. 3b). The mean survival was 17 months for patients with methylation of BNIP3, and 31 months without methylation (Table 3).

Table 3  
Correlation of BNIP3 mRNA expression and promoter methylation status with GBC patient's survival.

<b>BNIP3</b>	<b>Total patients (n)</b>	<b>Deceased patients (n)</b>	<b>Mean survival ± SEM (95% CI), months</b>	<b>p value</b>
BNIP3 (mRNA)	35	19	16.026 ± 2.048 (12.013–20.039)	0.224
Low expression	30	16	26.289 ± 3.632 (19.171–33.407)	
No change in expression				
BNIP3 (methylation status)	21	8	31.383 ± 4.530 (22.505–40.262)	0.034
Unmethylated	44	27	17.713 ± 2.298 (13.210–22.217)	
Methylated				

ROC curve was plotted for analysis of diagnostic utility of BNIP3 mRNA and promoter methylation status. The area under curve for BNIP3 mRNA and promoter methylation status was 0.8452 and 0.7778 respectively (Fig. 3c, d).

## Discussion

Complex interplay at the epigenomic, genomic, transcriptomic, and proteomic levels have earlier been reported in GBC[4]. BNIP3 belong to Bcl-2 protein family member which contain only Bcl-2 homology 3 (BH3) domain. Unlike BH3 only proteins, BNIP3 pro-death activity is controlled by both the BH3 domain and transmembrane (TM) domain of BNIP3[5]. It has been reported during hypoxic and ischemic condition BNIP3 can play different roles including apoptosis, programmed necrosis, autophagy and mitophagy[16]. In uveal melanoma BNIP3 expression detection by IHC reveals downregulation in 68.1% (32/47) samples while 31.9% (15/47) were upregulated[17]. BNIP3 is reported as upregulated in breast cancer[18, 19], prostate cancer[20], lung cancer[21, 22], cervical cancer[23] and endometrial cancer[24] whereas studies demonstrated its reduced expression in colorectal cancer[25], pancreatic cancer[5], gastric carcinoma[26], ccRCC[6] and hematopoietic tumors[27]. However, BNIP3 expression profile has not been correlated with its promoter methylation status in GBC. In the present study we have demonstrated through semi-quantitative RT-PCR that 56% (58/84) of the GBC samples show down regulation of BNIP3 at transcript level. Interestingly, the lower expression in GBC show significant correlation between BNIP3 mRNA expression and clinico-pathological parameters of GBC patients including pathological late stage and nodal spread. Similar correlation has also been demonstrated in invasive breast cancer and pancreatic cancer[5, 28]. Significant reduced expression in late stage and GBC with presence of nodal metastasis suggests its role in GBC initiation and progression. Although not significant, the patients with reduced BNIP3 expression have lower survival. Significant correlation of reduced expression of BNIP3 with low survival has been demonstrated in pancreatic cancer[5, 29]. We have also found similar correlation of BNIP3 expression with patient overall survival, however, it did not

reach the significance level. In the present study, we examined sensitivity and specificity of BNIP3 for GBC. Our diagnostic utility analysis indicates high diagnostic potential of BNIP3 at mRNA level.

It has been reported from Indian population that there is increased promoter methylation of tumor suppressor genes in late stages of GBC[30]. Promoter hypermethylation may be directly related to high incidence and poor prognosis of GBC in Asian countries[30]. Thus, we further investigated whether the promoter hypermethylation of BNIP3 is a possible mechanism of its reduced expression. BNIP3 promoter hypermethylation is reported in colorectal cancer[25, 26, 31], pancreatic cancer[5, 12, 32, 33], gastric cancer[26, 34] whereas hypomethylation of BNIP3 in prostate cancer is also observed[20]. Shao et al. (2019) demonstrated that inactivation of BNIP3 is through histone deacetylation but not promoter methylation in clear cell renal cell carcinoma[6] while a few reports depicts that DNA methylation as well as histone deacetylation play key role in silencing of BNIP3 in hematopoietic tumors[27] and colorectal cancer[31]. In our study 69% of GBC samples show promoter hypermethylation. Further, correlation of methylation status of BNIP3 with late stage and lymph node metastasis reveals promoter hypermethylation may play a critical role in initiation of GBC. Sugita et al. (2011) and Shimizuet al. (2010) demonstrated association of BNIP3 methylation with poor overall survival in gastric and colorectal cancer respectively [25, 34]. Similarly, our study also demonstrates that hypermethylation of BNIP3 promoter is associated with reduced overall survival and is correlated with its transcription suggesting its prognostic utility in GBC.

In conclusion our study suggests that promoter hypermethylation is a major mechanism of BNIP3 downregulation in GBC, suggesting its role in initiation and progression of the disease. Since the promoter hypermethylation of BNIP3 is more frequent in late stage tumors and there is significant decreasing expression of its transcripts from gallstone to late stage of GBC, promoter hypermethylation and down regulation of BNIP3 are early events in GBC progression. This study also suggests that BNIP3 may serve as diagnostic and prognostic marker in GBC. Further studies are needed to explore the functional role of BNIP3 for its therapeutic potential in GBC.

## Declarations

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Conflicts of interest: Authors declare that there is no competing conflict of interest

Availability of data and material: Not Applicable

Code availability: Not Applicable

Authors' contributions: GN and SS designed and supervised the study; AB carried out the experiments; MT, and MAA provided the tumor and normal biopsies along with the clinical information; DS and AB did the statistical analysis of the data; Manuscript preparation was done by AB and GN and overall editing was done by SS.

### Compliance with ethical standards

Ethics approval: The study was approved by the Institutional Ethical Committee of Faculty of Science, Banaras Hindu University vide ref. no. F.Sc./Ethics Committee/2015-16/3.

Consent to participate: Written informed consent from all the patients were obtained according to the approved protocol by the Institutional Ethical Committee of Faculty of Science, Banaras Hindu University.

Consent for publication: All authors have consented for the publication.

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## Figures

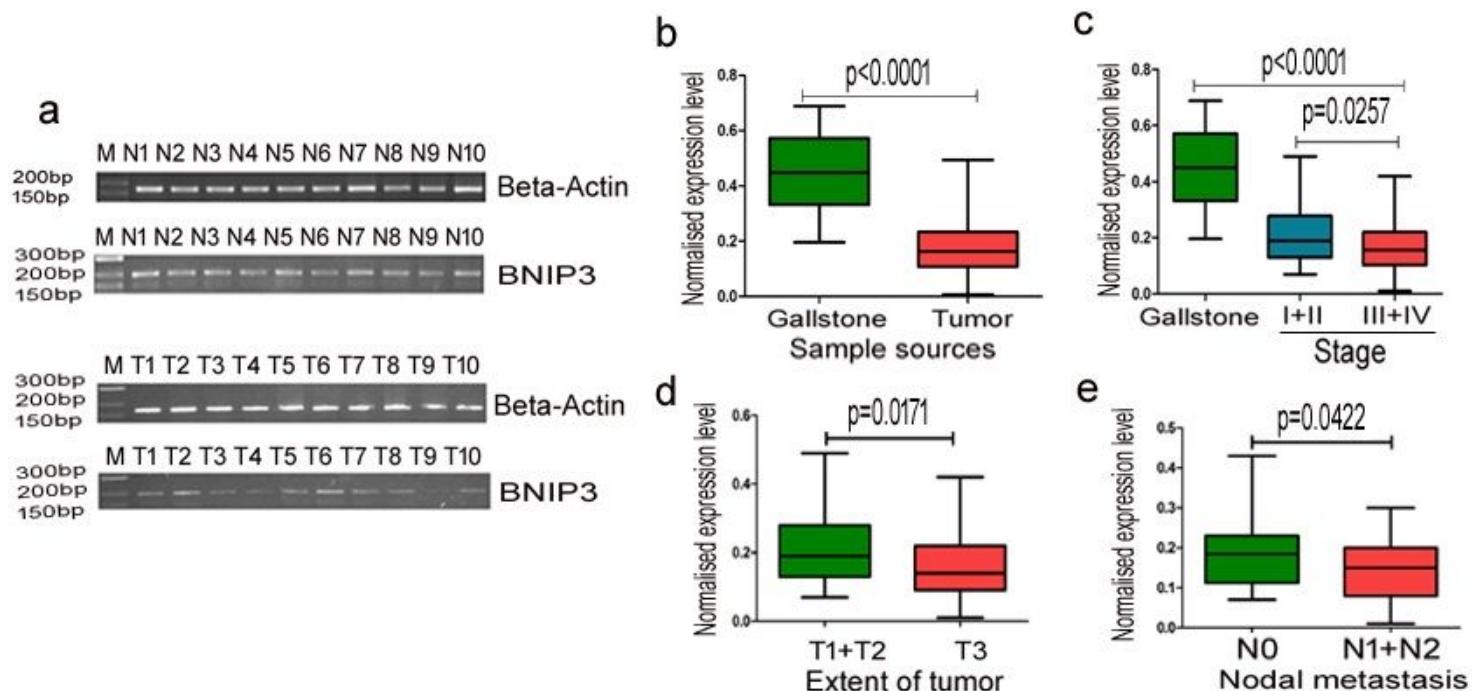
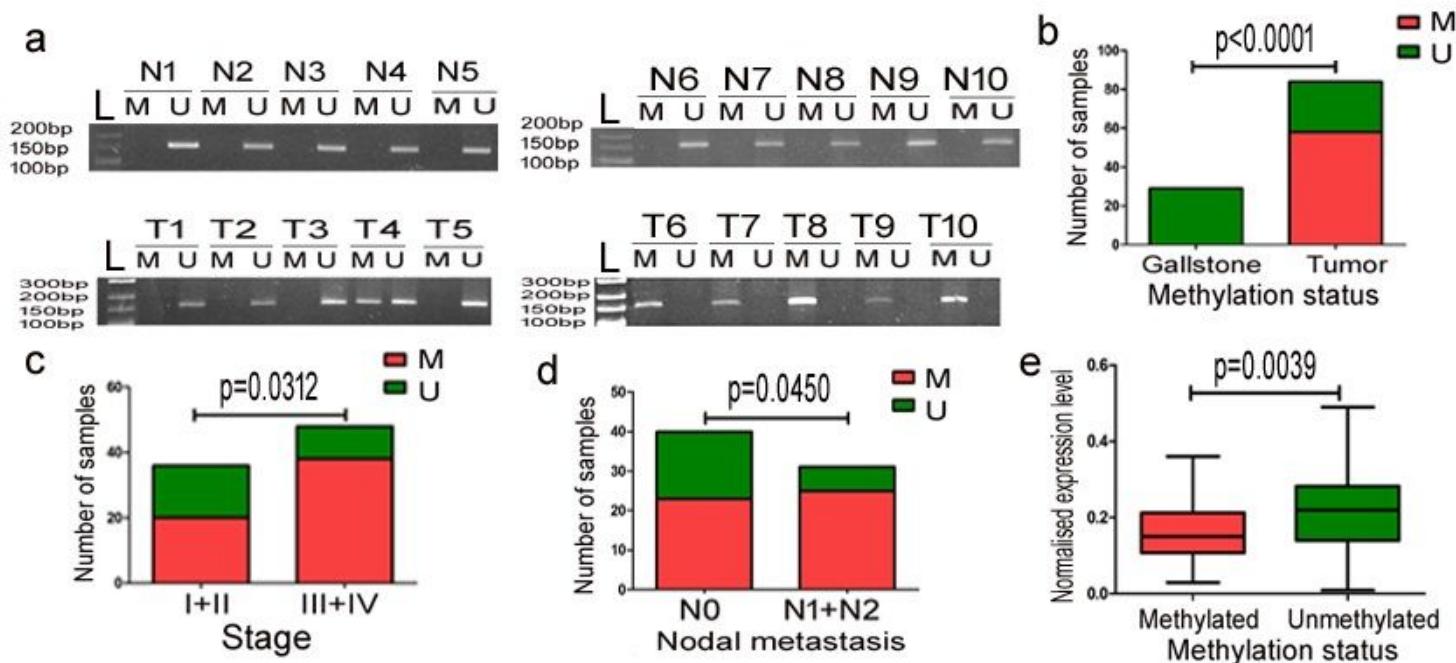


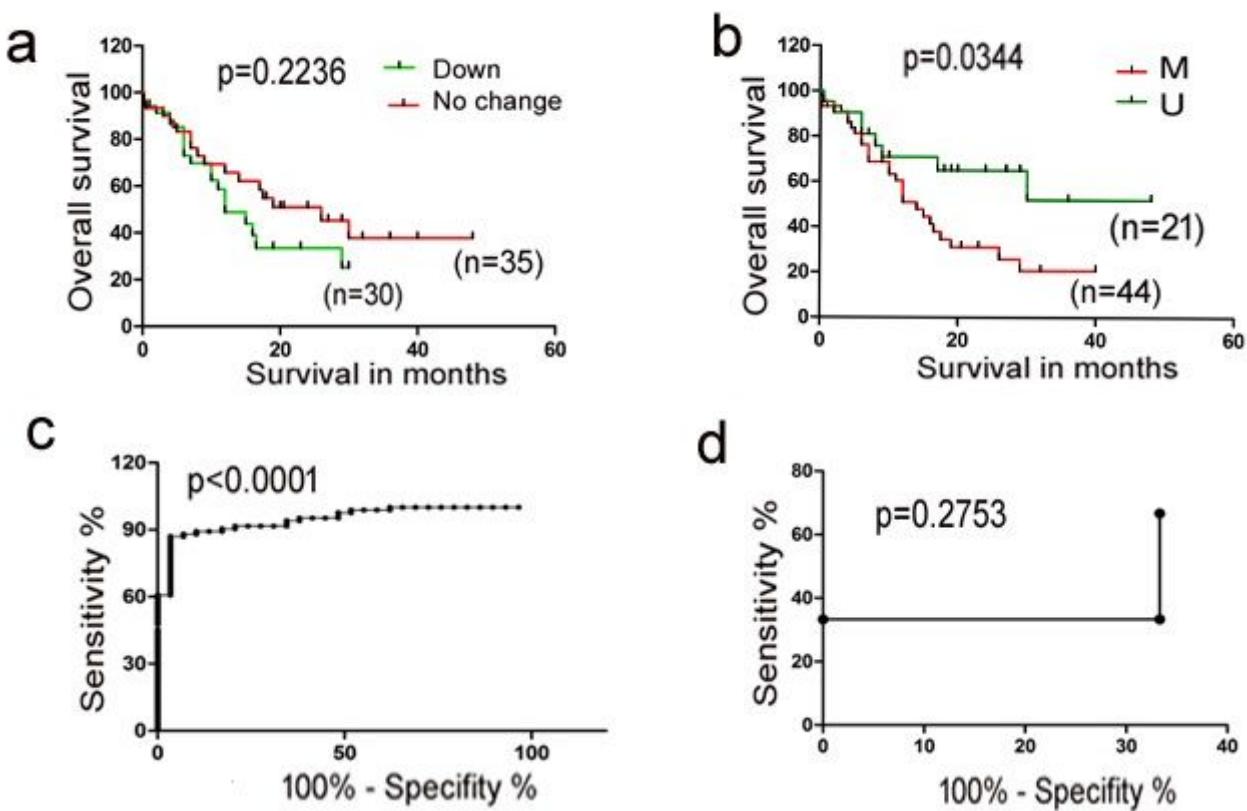
Figure 1

Correlation of deregulated mRNA expression of BNIP3 with clinicopathological indices in GBC; (a) Semi-quantitative RT-PCR showing mRNA expression of BNIP3 in gallstone tissues and tumor biopsies (N = gallstone tissues, T = Tumor biopsies). (b) Scatter plot showing overall change in expression of BNIP3 in tumor biopsies (N = 62) compared to gallstone tissues (N = 28). (c) Reduced expression of BNIP3 from gallstone to late stage of GBC. (d-f) Correlation of deregulated mRNA expression of BNIP3 with stage, extent of tumor, nodal metastasis



**Figure 2**

Correlation of promoter hypermethylation BNIP3 with clinico-pathological indices; (a) Gel image of MSPCR in gallstone and GBC tissue samples. b) Correlation of methylation status with gallstone and GBC. (c) and (d) Correlation of promoter hypermethylation BNIP3 with stage and nodal metastasis. (e) Correlation of reduced expression of BNIP3 at mRNA level with promoter hypermethylation



**Figure 3**

Detection of prognostic value and diagnostic utility of BNIP3 in GBC; (a) Percent survival of patients with differential mRNA expression of BNIP3. (b) Percent survival of patients with promoter methylation state of BNIP3. (c) ROC curve of BNIP3 at mRNA level. (d) ROC curve of BNIP3 at promoter methylation level

## Supplementary Files

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- SupplementaryFig.1MSPsequencing.tif
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- SupplementaryTable2.docx
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