

# Do Expression Profiles of Cytokines VEGF, TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 Correlate with Gallbladder Cancer?

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**Citation:** Chaturmohta A, Dixit R, Narayan G, Gupta P, Prasad SB, et al. (2015) Do Expression Profiles of Cytokines VEGF, TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 Correlate with Gallbladder Cancer? J Cancer Sci Clin Oncol 2(2): 202. doi: 10.15744/2394-6520.2.202

**Received Date:** February 25, 2015 **Accepted Date:** June 08, 2015 **Published Date:** June 10, 2015

## Abstract

**Aim:** Gallbladder carcinoma is a multifactorial disease with a complex interplay at molecular levels. Here we look at the expression of specific cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and VEGF) in GBC patients to develop them as biomarkers of gallbladder cancer.

**Methods:** This study consisted of newly diagnosed cases of Gallbladder Cancer (GBC) (n = 25) and cholelithiasis (n = 25) as control. Total RNA was isolated from tissue by using TRI-reagent and mRNA expression levels of five above mentioned cytokines were seen by Reverse Transcriptase PCR (RT-PCR). The degree of over expression is correlated with tumour size, staging, differentiation, serosal invasion and metastasis of the disease by application of Chi-square and Fischer T tests.

**Results:** We found that IL-1 $\beta$ , TNF- $\alpha$ , IL-8 and VEGF mRNA expression was upregulated in gallbladder cancer cases as compared with control. IL-1 $\beta$  overexpressed in 65.3% cases and the statistical difference was significant (p = 0.0012). TNF- $\alpha$  was also upregulated in 69.6% (p = 0.002). Overexpression of IL-8 was seen in 47.8% of the malignant samples (p = 0.0082) and 52.17% (p = 0.034), thus significant. Overexpression of VEGF was observed in 52.17% of the malignant samples (p = 0.034) whereas IL-6 showed no expression.

**Conclusion:** These cytokines expression showed a higher expression level in gallbladder cancer and it also increase with the advance stage. Further studies are needed to establish the relationship between them.

**Keywords:** Cytokines; Gallbladder cancer (GBC); Cholelithiasis; IL-1 $\beta$ ; TNF- $\alpha$ ; IL-8

## Introduction

Carcinoma of the gallbladder (GBC) is the most common biliary tract tumour and is known as the fifth most common gastrointestinal tract cancer in India. The symptoms of GBC are non-specific and this non-specificity of symptoms is responsible for delayed diagnosis: in fact, this tumour is usually identified at an advanced stage when it has already become unresectable [1]. Therefore, prognosis of GBC is poor and only less than 5% of the patients survives five years postoperatively [2,3].

Cytokine or immunocytokine is a generic name used to describe a diverse group of soluble proteins and peptides which act as humoral regulators at nano to picomolar concentrations. They are known as the main culprit molecules in the pathway of progression from acute inflammation to chronic inflammation and from chronic to carcinogenesis [4]. Cancer cells can respond to host-derived cytokines that promote growth, attenuate apoptosis and facilitate invasion and metastasis. A more detailed understanding of cytokine-tumour cell interactions is the need of time as it provides new opportunities for improving cancer immunotherapy [5].

Our study investigated the behavior of five cytokines whose role in GBC has not yet been clearly established [6]. Studies which are available are confined with cholangiocarcinoma or biliary tract cancer. So far, not much work has been done in relation with these markers in GBC and small amount of data is available from India on the expression of these biomarkers in GBC. The present study was conducted to observe the expression levels of IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-8 and VEGF in GBC and to find their association with the clinicopathological profiles.

## Materials and Methods

### Samples

Newly diagnosed twenty five patients of GBC as cases and 25 patients of cholelithiasis as control group were included consecutively in the study from September 2011 to July 2013 after obtaining informed consent from the patients. All the cases were histopathol-

ogically proven. Tissue samples were collected from both the groups and snap frozen at -80 °C (deep freezer). The study was approved by the institute ethics committee.

### Isolation

Total RNA was isolated by using TRI Reagent® (Ambion® by life technologies) as given in the protocol. cDNA was synthesized by using high capacity cDNA synthesis kit (Applied Biosystems). cDNA was amplified with the help of PCR by using gene specific primers. PCR amplification samples were electrophoresed in agarose gel (Table 1).

S. No.	Gene	Primer sequence	Ta	Cycles	Product
1.	IL-8	F5'-CTGCGCCAACACAGAAATTA-3' R5'-CATCTGGCAACCCTACAACA-3'	53 °C	32	234 bp
2	IL-6	F5'-TGAACCTCTTCTCCACAAGC-3' R5'-CTGAAGAGGTGAGTGGCTGT-3'	51 °C	36	150 bp
3	VEGF	F5'-GAGACCCTGGTGGACATCTT-3' R5'-CTCCTATGTGCTGGCCTTG-3'	54 °C	30	190 bp
4	TNF-α	F5'-CCTGGAAAGGACACCATGAG-3' R5'-GCCAGAGGGCTGATTAGA	57 °C	40	236 bp
5	IL-1β	F5'-AGGCTGCTCTGGGATTCTCT R5'-CCCTTGCTGTAGTGGTGGTC	56 °C	37	236 bp

**Table 1:** Primer sequences of genes including Ta, number of cycles of PCR and product size

### Statistical Analysis

Densitometric value of gene of interest was normalized with densitometric value of housekeeping gene β-actin by taking the ratio of gene of interest and β-actin. Mean and standard deviation of control sample were calculated. The normalised value of tumor was compared with 'Mean±2xSD'. Sample showing value more than 'Mean+2xSD' were supposed to be up regulated, less than 'Mean-2xSD' down regulated and rest were treated as showing no significant change. The degree of over expression is correlated with tumour size, staging, differentiation, serosal invasion and metastasis of the disease by using Chi-square and Fischer T tests.

## Results

### Patients Demographics

The mean age in the GBC group of our patients was 52.08±12.8 years (range 20-72 years) whereas in the cholelithiasis was 47.96±13.4 years (range 22-70 years). The study comprised of 17 (68%) females and 8 (32%) males in the GBC group while 19 (76%) female and 6 (24%) male patients in the cholelithiasis group. Thus, the male to female ratio was 2:1 and 3:1 in the GBC and cholelithiasis group, respectively (Table 2).

Characteristics	Gallbladder Cancer (%) n = 25	Cholelithiasis (%) n = 25
<b>Symptoms</b>		
Pain	22 (88%)	21 (84%)
Jaundice	6 (24%)	0
Lump abdomen	16 (64%)	0
Fever	4 (16%)	4 (16)
Vomiting	5 (20%)	4 (16)
Anorexia	15 (60%)	0
Weight loss	15 (60%)	0
Hepatomegaly	16 (64%)	0
<b>TNM stage</b>		
T1	0	NA
T2	4 (16%)	
T3	12 (48%)	
T4	9 (35%)	
N0	5 (20%)	
N1	20 (80%)	
M0	20 (80%)	
M1	5 (20%)	

Characteristics	Gallbladder Cancer (%) n = 25	Cholelithiasis (%) n = 25
<b>Stage</b>		
Ia	0	NA
Ib	4 (16%)	
IIa	1 (4%)	
IIb	11 (44%)	
III	4 (16%)	
IV	5 (20%)	
<b>Histology</b>		
Adenocarcinoma	25 (100%)	0
Chronic cholecystitis	0	22 (88%)
Acute cholecystitis	0	1 (4%)
Xanthogranulomatous cholecystitis	0	2 (8%)
<b>Differentiation</b>		
Well differentiated	15 (60%)	NA
Moderately differentiated	6 (24%)	
Poorly differentiated	4 (16%)	

**Table 2:** Clinical characteristics of gallbladder cancer and cholelithiasis group

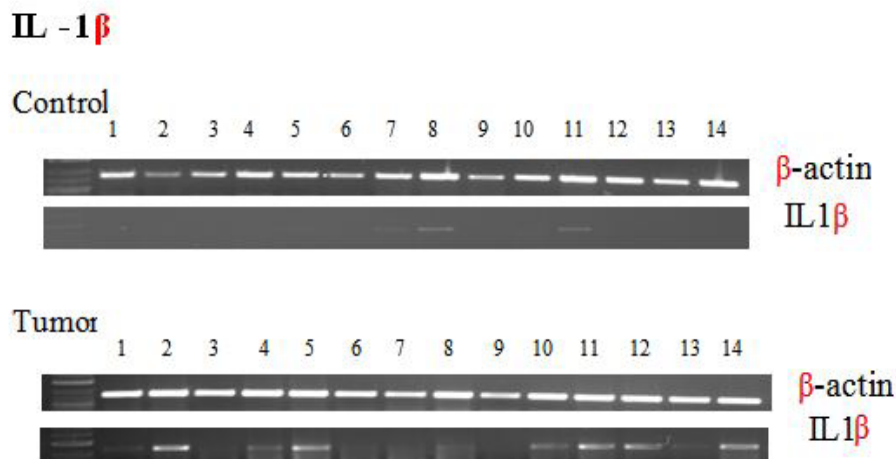
### Gene Expression Analysis

Out of total 50 tissue samples (25 each from GBC and cholelithiasis) collected, 23 cases and 22 controls RNA was considered good for RT-PCR and expression analysis (Table 3).

S.No.	Genes	Groups		p-value
		GBC (%)	Cholelithiasis	
1	IL-1 $\beta$	15/23 (65.3)	No	0.0012
2	TNF- $\alpha$	16/23 (69.6)	No	0.002
3	IL-8	11/23 (47.8)	No	0.0082
4	VEGF	12/23 (52.17)	No	0.034
5	IL-6	Nil	No	-

**Table 3:** Expression analysis of five cytokines [IL-1 $\beta$ , TNF- $\alpha$ , IL-8, IL-6 and VEGF] in both groups [GBC and cholelithiasis]

**IL-1 $\beta$ :** IL-1 $\beta$  over expression has been observed in 65.3% (15/23) of the malignant samples ( $p = 0.0012$ ). A strong association was observed between IL-1 $\beta$  and differentiation, liver metastasis and a linear correlation between IL-1 $\beta$  and omental metastasis of gall bladder cancer (Table 4) (Figure 1).



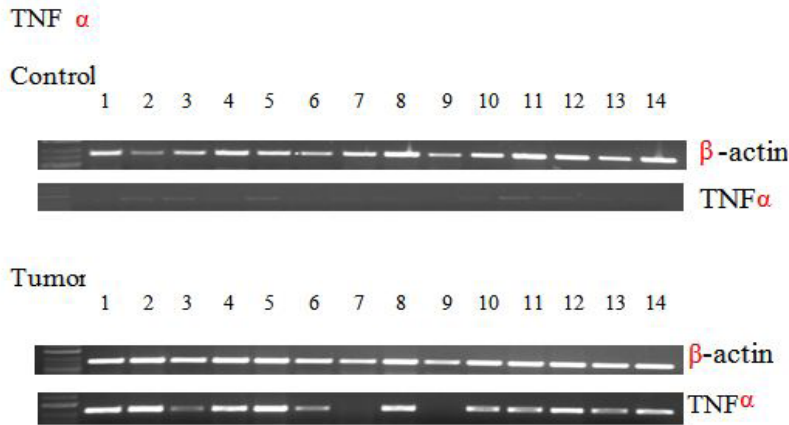
**Figure 1:** Image showing the positive expression of IL-1 $\beta$  in cholelithiasis (control) and GBC (tumour) samples

Parameters	IL-1 $\beta$ (15/23)	TNF- $\alpha$ (16/23)	IL-8 (11/23)	VEGF (12/23)
<b>T Stage</b>				
T1	-	-	-	-
T2	6.7%	6.25%	9%	16.7%
T3	46.7%	43.8%	36.3%	25%
T4	46.7%	56.25	54.6%	58.3%
	( $\rho = +.46464$ ; $p = 0.04$ )	( $\chi^2 = 10.977$ ; $p = 0.004$ ) ( $\rho = +.654$ ; $p = 0.001$ )	NS	( $\rho = +.43264$ ; $p = 0.004$ )
<b>M Stage</b>				
M0	66.7%	68.8%	63.6%	78.3%
M1	33.3%	31.2%	36.4%	21.7%
	NS	NS	NS	( $\chi^2 = 5.856$ ; $p = 0.016$ ) ( $\rho = +.534$ ; $p = 0.001$ )
<b>Stage</b>				
Ia	0%	0	0	0
Ib	6.7%	0	9.1	16.7
IIa	6.7%	0	9.1	0
IIb	33.3%	43.8	36.4	16.7
III	20%	25	9.1	25
IV	33	31.2	36.4	41.7
	NS	( $\chi^2 = 13.081$ ; $p = 0.011$ ) ( $\rho = +.684$ ; $p = 0.001$ )	NS	( $\chi^2 = 10.91$ ; $p = 0.028$ ) ( $\rho = +.464$ ; $p = 0.024$ )
<b>Differentiation</b>				
Well	67%	62%	72.7%	63%
Moderately	6%	18.75%	9%	19%
Poorly	27%	18.75%	18.2%	18%
	( $\chi^2 = 9.153$ ; $p = 0.010$ )	NS	NS	NS
<b>Serosal invasion</b>				
Absent	6.7%	0		
Present	93.7%	100%		
	NS	( $\chi^2 = 7.886$ ; $p = 0.005$ ) ( $\rho = +.586$ ; $p = 0.03$ )	NS	NS
<b>Lymph nodes</b>				
Absent	13.3%	0	18.2%	16.7%
Present	86.7%	100%	81.8%	83.3%
	NS	( $\chi^2 = 11.068$ ; $p = 0.001$ ) ( $\rho = +.694$ ; $p = 0.001$ )	NS	NS
<b>Liver metastasis</b>				
Absent	26.7%	18.8%	36.4%	33.3%
Present	73.3%	81.2%	63.6%	66.7%
	( $\chi^2 = 2.813$ ; $p = 0.094$ )	( $\chi^2 = 9.168$ ; $p = 0.002$ ) ( $\rho = +.631$ ; $p = 0.001$ )	NS	NS
<b>Omental metastasis</b>				
Absent	26.7%	25%	45.5%	16.7%
Present	73.3%	75%	54.5%	83.3%
	( $\chi^2 = 4.960$ ; $p = 0.026$ ) ( $\rho = +.426$ ; $p = 0.02$ )	( $\chi^2 = 7.304$ ; $p = 0.007$ ) ( $\rho = +.564$ ; $p = 0.02$ )	NS	( $\chi^2 = 7.340$ ; $p = 0.007$ ) ( $\rho = +.565$ ; $p = 0.005$ )

Where  $p > 0.05$  has been taken as significant;  
( $\rho$  = Spearman's rho correlation co-efficient and shows linear correlation);  
NS = not significant.

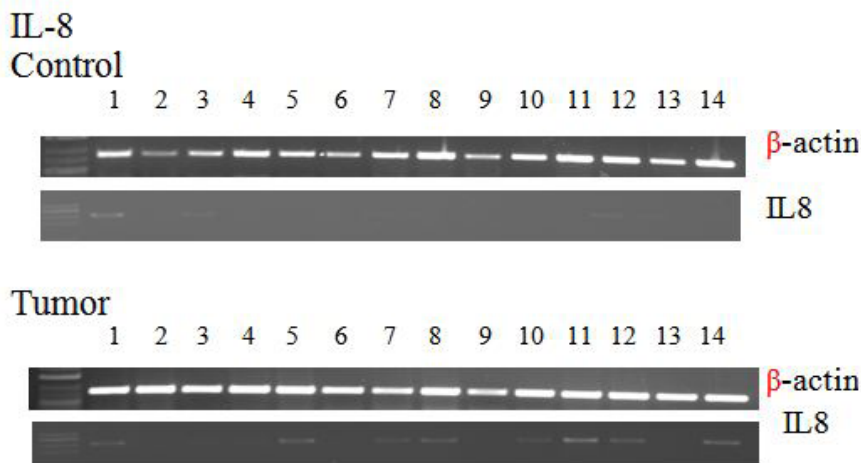
**Table 4:** Comparison between gene expressions of genes with different parameters

**TNF- $\alpha$ :** Out of 23 GBC samples, 69.6% (16/23) showed an upregulated expression of TNF- $\alpha$  as compared to cholelithiasis samples (p=0.002). Thus the association is statistically significant with increase in stage, serosal invasion, lymph node involvement, liver metastasis and omental metastasis (Table 4) (Figure 2).



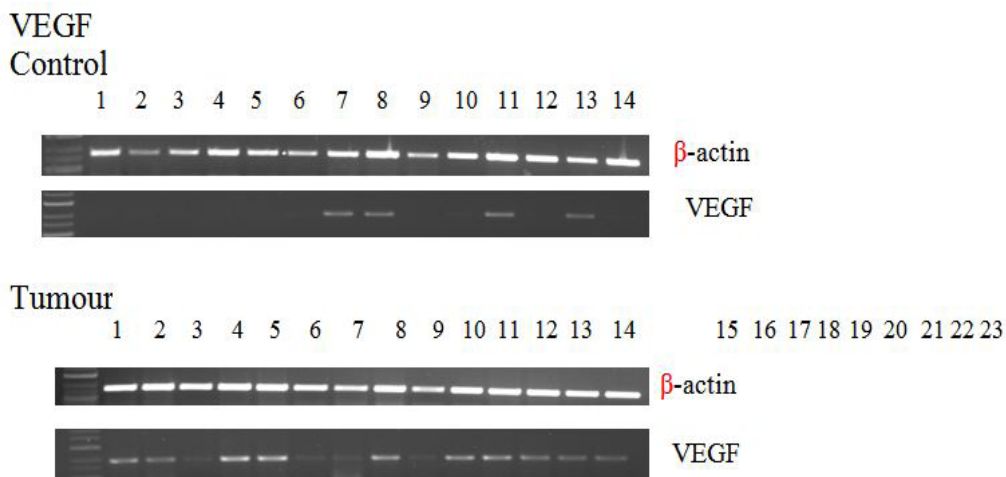
**Figure 2:** Image showing the positive expression of TNF- $\alpha$  in cholelithiasis (control) and GBC (tumour) samples

**IL-8:** IL-8 over expression has been observed in 47.8% (11/23) of the malignant samples (p = 0.0082). Up regulation of IL-8 was significant (p-value = 0.0082) (Table 4) (Figure 3).



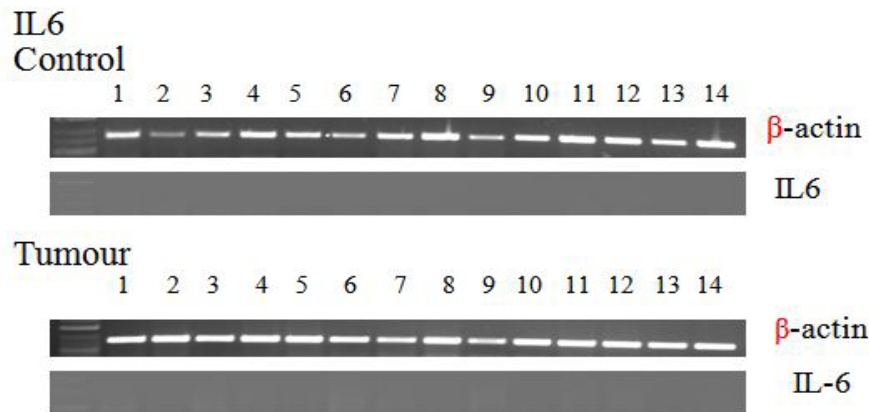
**Figure 3:** Image showing the positive expression of IL-8 in cholelithiasis (control) and GBC (tumour) samples

**VEGF:** VEGF over expression was seen in 52.17% (12/23) of the malignant samples (p = 0.034). A positive linear co-relationship of VEGF with liver and omental metastasis of gallbladder carcinoma has been demonstrated in the present study (Table 4) (Figure 4).



**Figure 4:** Image showing the positive expression of IL-8 in cholelithiasis (control) and GBC (tumour) samples

**IL-6:** IL-6 showed no expression in both the cholelithiasis and GBC samples (Figure 5).



**Figure 5:** Image showing no expression of IL-8 in cholelithiasis (control) and GBC (tumour) sample

## Discussion

Cytokines modulate the functional activities of individual cells and tissues both under normal and pathogenic condition. Cytokines affect nearly every biological process; these include embryonic development, disease pathogenesis, non-specific response to infection, specific response to antigen, changes in cognitive functions, and degenerative processes of ageing [7,8]. Recently many clinical studies have looked into the role of cytokines in the pathogenesis of cancer. Cytokines such as IL-1 $\beta$ , IL-6 and IL-8 are now being regarded as main culprit molecules in the pathway of progression of chronic inflammatory process to carcinogenesis [9]. Therefore, we have evaluated the expression of different cytokines (IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-8 and VEGF) in GBC. TNF- $\alpha$  is released in response to infection and inflammation and can inhibit tumor development and progression [10]. TNF is a multifunctioning lymphokine produced by activated macrophages and lymphocytes [11]. We have found overexpression of TNF- $\alpha$  in 69.5% GBC samples compared to cholelithiasis. The expression of TNF- $\alpha$  is increased with the advance stage ( $p = 0.004$ ) and lymph nodal metastasis of the cancer ( $p = 0.001$ ). Hence highest TNF- $\alpha$  expression was seen in T<sub>4</sub>N<sub>2</sub> tumour groups. TNF- $\alpha$  showed a positive linear correlation ( $p = 0.005$ ) with serosal invasion of the disease with over expression seen in 100% of serosal invasion cases. In a previous study, Kang et al [12] found a higher plasma level of TNF- $\alpha$  as compared to control in colorectal adenomas. IL-1 $\beta$  is known to have an association with tumor staging and metastasis [13]. mRNA TNF- $\alpha$  and VEGF expression were also elevated in chronic liver disease specimen [14]. IL-1 $\beta$  acts indirectly on cancer cells by stimulating lymphocytes to kill cancer cells and it is approved for treating metastatic kidney cancer and metastatic melanoma. It is also being investigated for the treatment of leukemia, lymphoma and cancers of the brain, colon, breast and prostate [15]. Other studies have also reported that IL-1 $\beta$  protein is produced in solid tumours like breast, colon, lungs and head and neck cancer [9]. In our study, we found a significant association between the expression of interleukin IL-1 $\beta$  and histological differentiation of cancer ( $p = 0.010$ ). Of the 65.3% samples showing significant over expression, 67%, 6%, 26% were well, moderately and poorly differentiated respectively ( $p = 0.0012$ ). Carcinoma with liver metastasis showed a high IL-1 $\beta$  expression (73.3% cases,  $p = 0.094$ ). Those with omental metastasis showed a positive linear correlation ( $p = 0.026$ ).

There is an accumulating evidence linking IL-6 to colon cancers. IL-6 was also found to play a regulatory role on the proliferation of intestinal epithelial cells [16]. In colon cancer patients, serum levels of IL-6 were found to be highly elevated and positively correlated to tumor load [17]. In our study, IL-6 showed low expression in both tumour and cholelithiasis samples which indicates that it has no significant role in carcinogenesis of GBC. Another study reported that altered gene expression of IL-6 enhances tumor growth in cholangiocarcinoma. But a similar study by Vishnoi et al acclaimed that IL-6 polymorphism does not influence the susceptibility for GBC [18].

Although IL-8 showed a moderately increased expression in GBC, no definite association could be documented with any parameters in our study. IL-8 mediates the recruitment and activation of neutrophils in inflamed tissue [13]. IL-8 can be detected in synovial fluid from patients with various inflammatory rheumatic diseases and mucosal levels of IL-8 are elevated in patients with active ulcerative colitis [5]. Kheirelseid et al also found a progressively increased expression of IL8 in tumour-associated normal, to polyps, to tumours in colorectal cancer by doing quantitative PCR [19].

VEGF is a signal protein produced by cells that stimulate vasculogenesis and angiogenesis [4], and has been found to play a major role in angiogenesis and metastasis [20]. VEGF has been found to be highly expressed in a broad range of human tumours [21]. We observed an equal expression of VEGF genes in tumour and cholelithiasis samples ( $p < 0.05$ ). Overexpression was seen in 52.17% cases of GBC. The amount of angiogenesis increases in a linear fashion with invasion of the disease ( $p = 0.006$ ). Expression was strongly positive in metastatic samples. VEGF also showed a linear correlation with staging of the disease ( $p = 0.02$ ). Petrenko et al [22] suggested a 76% and 26% increased expression of VEGF in GBC and cholelithiasis respectively. Similar observation about

significantly higher VEGF expression has been made in breast cancer [23]. Results of other study also suggested that VEGF-A was highly expressed in GBC and correlated with poor prognosis [24]. Doval et al observed an overexpression of VEGF in about 34% of the cases. But they observed a decreased rate of overexpression and were found to be statistically associated with alive cases [25]. We reported a significant relationship between the expressions of TNF- $\alpha$ , IL-1 $\beta$ , IL-8 and VEGF and GBC stage, grade and metastasis. However, no correlation was obtained between expression of IL-6 and gallbladder carcinoma suggesting that its involvement in the pathogenesis and progression of the disease is unlikely.

## Conclusion

In conclusion, these cytokines expression showed a higher expression level in gallbladder cancer and it also increase with the advance stage. Thus the results suggest that there is a role of these cytokines except IL-6 in gallbladder cancer progression.

The study has limitations in terms of its small sample size. However further studies with larger sample size are needed to substantiate association, causation between the two.

## Acknowledgement

The Authors thank Dr. Sen Pathak and Dr. S Basu for critically reviewing this manuscript.

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