5. Discussion

Gall bladder cancer (GBC) is the most common biliary tract cancer worldwide; it often goes undiagnosed until advanced stage of disease and is associated with poor prognosis. Epidemiological studies have indicated a peculiar ethnic and geographic distribution of GBC. Northern India has one of the highest incidence rates of GBC in the world (4.5/100,000 in males and 7/100,000 in females). Far East Asian nations like Korea and Japan also have disproportionately high incidence rates of GBC. There are two remarkable distinct features between India and East Asian countries about GBC - in India female/male ratio is very high as compared to Korea and Japan where female/male ratio is almost equal (Raniet et al., 2006); Gall bladder cancer has a particularly high incidence in Chile, Japan, and northern India (Kawahara and Nagakawa., 2007; Chaurasia et al., 1999; Dhir, et al., 1999). It is considered as one of the leading causes of death due to cancers among Chilean women. GBC is the leading cause of cancer-related mortality in the northern parts of the Indian subcontinent also (Mohandas et al., 2006), especially in the Indo-Gangetic planes (Baskaran, 2001; Kumaret al., 2006). GBC shows a dismal picture in India (Batra et al., 2005). As the incidence of GBC in north and central India is very high, it has been described as a north Indian disease (Kapoor et al.,2003). Gall stones (GS) are a major risk factor for GBC in India and are present in 70-80% of GBC whereas GS are not a major risk factor for GBC in East Asian countries (Brett, 1976). The other risk factors for GBC include gender, age, obesity, reproductive factors, chronic infections, and environmental exposure to certain chemicals.
Although it is the most common cancer of the biliary tract, GBC still remains an uncommon disease in the West. As a result, many clinicians rarely encounter it and there is uncertainty regarding its proper management. But most of the patients are asymptomatic at first clinical presentation while a few present with clinical features suggestive of benign gall stone disease such as right upper abdominal pain interspersed with occasional attacks of nausea and vomiting. In many cases, the diagnosis of GBC is made after a cholecystectomy has been performed for gall stone (GS) disease and an incidental tumor is identified in the specimen (Miller and Jarnagin, 2008). Unfortunately, therapy for advanced and metastatic GBC is ineffective. It is clear that prevention or early detection is the best way to prevent rising death rates from this fatal tumor. Therefore, advances in cellular and molecular pathogenesis may provide innovative means for the early diagnosis and treatment of GBC.

In spite of considerable progress having been made regarding the molecular pathogenesis of human neoplasms such as colorectal carcinoma, pancreatic carcinoma and breast carcinoma, very limited information is available about the genetic changes involved in gall bladder carcinogenesis (Rashid et al., 2002; WistubaII et al., 1999). Recently some reports suggested association of polymorphisms in GSTs (GSTP1) (Pandey et al., 2006), chemokine receptors (CCR5 delta 32) (Srivastava et al., 2008), TNFA and IL6 (Vishnoi et al., 2007), cytochrome P450 1A1 (CYP1A1) (Pandey et al., 2008), Epidermal growth factor (EGF) and transforming growth factor β1 (TGF β1) (Vishnoi et al., 2008) cholesterol 7alpha-hydroxylase (CYP7A1) (Srivastava et al., 2008), Lipoprotein receptor associated protein (LRPAP1) (Pandey et al., 2006), cholecystokinin receptor A (Srivastava et al., 2007) and APOB (Pandey et al., 2007), ABCG8 D19H
Discussion

(Srivastava et al., 2009), Cholesterol 7-alpha hydroxylase (CYP7A1) (Srivastava et al., 2010), DNA (cytosine-5-) methyltransferase 3 Beta (DNMT3B), Toll like receptors (TLRs)-TLR4 polymorphisms was associated with increases risk of GBC in females (Srivastava et al., 2010), Caspace-8 (CASP8)-CASP8 gene variants may be susceptible for GBC (Srivastava et al., 2010), Matrix metalloproteinase (MMP-2, 7, 9)-MMP-2,7,9 were risk associated with GBC (Sharma et al., 2012), CYP17 polymorphism- CYP17 was associated with increase risk in GBC (Rai et al., 2014), Death receptor (DR4) haplotypes are associated with increased susceptibility of gallbladder cancer (Rai et al., 2014) in north Indian populations.

Our study group also performed several molecular and genetic association studies in GBC. These included Cox (Ghosh et al., 2000), K-ras (Singh et al., 2004), MUC 1 core protein (Ghosh et al., 2005), FHIT and p53 (Priya et al., 2009), loss of hetrozygosity (LOH) in E-cadherin (CDH1) gene (Priya et al., 2010), immuno-histo-chemical (IHC) expression of tumor markers e.g. carcino-embryonic antigen (CEA) and carbohydrate antigen 19.9 (CA-19.9), tumor suppressor gene p53 (Agrawalet al., 2010) and C-erbB2 expression (Kumari et al., 2012).

In the present study we have investigated the genetic association of VEGF, PDGFB and HER2SNPs with GBC and compared it with healthy normal controls as well as with other two gallbladder benign diseases viz. CC and XGC.

5.1. VEGF polymorphisms

Our study revealed that some genotypes, alleles and haplotypes of VEGF were either risk associated or risk protective for GBC when compared with healthy normal controls and with benign inflammatory conditions of gall bladder viz. CC and XGC.
We found -1154GA genotype and -1154A allele of -1154G>A polymorphism to be risk associated and -1154GG genotype to be risk protective for GBC. In line with previous studies, we found -2578CA genotype to be risk protective for GBC. To the best of our knowledge this is first such study involving north Indian population. There is only one study so far, reported in Chinese population (Hsing et al., 2008) showing role of VEGF polymorphism in GBC. They studied VEGF +936C>T polymorphism in 237 patients with GBC (out of 411 biliary tract cancers), 845 biliary stones and 786 controls. Their findings are, however, contrary to our findings. They found T allele (CT and TT genotype) to confer reduced risk (OR=0.70, 95% CI=0.50 - 0.97) of GBC. They, however, studied only one variant of VEGF whereas we have studied four variants. In a study of 154 Korean stomach cancer patients, genotype CT and CT+TT were more frequent (OR=3.05, 95% CI=1.57-5.93; OR=3.13, 95% CI=1.64-5.99) in women than in man (Bae et al., 2008). GBC is more common in women than in men and our study has also shown significant association of TT genotype and T allele with GBC.

Angiogenesis plays a central role in tumor growth, progression, invasion and metastasis of solid tumors. VEGF is an important growth factor which is involved in angiogenic process. VEGF expression has been confirmed to play a prominent role in vascularization in many solid tumors. Jain et al., have described in detail the distribution the VEGF SNP’s among several forms of cancer. (Jain et al., 2009) The distributions of the studied VEGF SNPs across the global control population have been shown in Table 2.1 (Chapter 2). VEGF induced tumor angiogenesis plays a significant role in tumor progression as well as in metastasis (Tian et al.,2006). Ferrara et al., (2002) also suggested
that enhancement in the VEGF expression eventually leads to tumor growth and metastasis while suppression in tumor growth is observed with decreased VEGF level. Neutralization of VEGF leads to a marked inhibition of angiogenesis, tumor growth and metastasis, interfering with VEGF or downplaying the expression of VEGFR-2 may lead to inhibition of tumor growth (Ferrara, 2003). VEGF positivity has also been reported to resist radiotherapy, chemotherapy and endocrine therapy (Ferrara et al., 2002; Koukourakis et al., 2004). Even prognosis and survival has been shown to be related to VEGF expression. Ozdemiret et al., (2006) reported that the survival rates of the VEGF positive tumors is less than the VEGF negative tumors. Carriers of homozygous +936TT genotype were reportedly having a poorer survival as compared to the wild type +936CC genotype in gastric cancer (Kim et al., 2008), on the contrary, in another study variant allele T of +936C>T was linked with improved survival as compared to wild type genotype +936CC in esophageal cancer patients (Bradbury et al., 2009).

There are very few studies reporting association or aetiology of cancer with VEGF. One study linked causation of gastro-intestinal adenocarcinoma to VEGF expression (Brown et al., 1993) but several other studies have reported no genetic risk to be caused due to -1154G>A, -2578C>A or +936C>T polymorphisms among colo-rectal cancer cases (Hofmann et al., 2008 and Dassoulas et al., 2009). McCarron et al., (2002) found that VEGF -1154AA genotype was decreased (6.3% vs. 12.9%) in 247 prostate cancer patients as compared to 263 controls (OR=0.45, p=0.01, 95% CI=0.24-0.86). In another study, Howell et al., (2002) found a trend towards higher frequency of VEGF -2578CC (40.3 vs. 30.8%) and -1154GG (54.6 vs. 45.6%) in 152 patients with cutaneous malignant melanoma than in 266 controls.
In a case control study of 154 Korean stomach cancer patients, Bae et al., (2008) showed significant association of 936T allele with stomach cancer. They reported significant differences in frequencies of VEGF polymorphism between control and patients (+936CT – AOR=2.007, 95% CI=1.277 - 3.156), (+936TT – AOR=4.790, 95% CI=1.174 - 19.539), (CT + TT - AOR=2.147, 95% CI=1.382 - 3.337). VEGF SNPs have been extensively studied in colonic cancer. In another case control study, Bae et al., (2008) reported higher frequencies (OR=1.52, 95% CI=1.03-2.25) of T allele of +936C>T polymorphism in 262 cases vs. 229 controls, the authors concluded that VEGF +936C>T polymorphism may be a genetic determinant for colonic cancer in Korean population. But in another study in Korean population again, no difference was found in genotype frequencies of VEGF -2578C>A polymorphism between 246 colon cancer patients and 203 controls but differences were found when data were stratified by gender (Park et al., 2007). The risk association between polymorphism and cancer may vary with ethnicity from population to population. In another study of 427 colo-rectal cancer patients and 427 controls, no risk association was found between +936C>T and -2578C>A polymorphism and colon cancer in Austrian population (Hofmann et al., 2008). In a meta-analysis of several studies, Jain et al., (2009) found only VEGF -1154G>A polymorphism to be associated with cancer risk. VEGF haplotypes have previously been correlated with susceptibility to cancer. Some haplotypes (-2578A/-1154A/-634G and -1154A/-634G) have been found to be associated with reduced risk of breast cancer (Katoka et al., 2006) and prostate cancer (Sfaret et al., 2006). A haplotype analysis in 445 colorectal cancer patients, the -2578A/-634G/+936T haplotype showed a significantly worse survival when compared with
the wild -2578C/-634G/+936C haplotype (OS: HR, 3.866; P < 0.001) (Kim et al., 2008). There is no study comparing these VEGF haplotypes in GBC and GS associated inflammatory diseases i.e. CC and XGC. We found ICAC haplotype to be risk protective (OR=0.23, 95% CI=0.10-0.48) for GBC and ITGC haplotype as a risk protective for CC (OR=0.18, 95% CI=0.03-0.60) as compared to controls. These are important findings and if this is confirmed in other populations and with larger sample sizes, these might become useful markers to screen patients with GS for their risk of GBC.

5.2. PDGFB and HER2 Polymorphisms

Autocrine pathway involving PDGFB/PDGFR signaling establishes self-sufficiency in growth for cancer cells. PDGFs are frequently produced by tumor cells and affect tumor growth and dissemination in several different ways. Amplification and overexpression of PDGFB and HER2 are usually involved in the growth, progression and metastasis of established tumors and may play a role in the etiology of and susceptibility to tumor also. Expression of PDGF gene has been widely studied in many cancers including pancreas, colo-rectal, breast, lung, skin, prostate, ovarian, and others (Heldin et al., 1999). Genetically prominent associations of the PDGFB gene polymorphisms with GBC were found in the present study. The mutant homozygous genotypes +286GG (OR=5.25) and +1135CC (OR=3.19) along with mutant alleles +286G (OR=2.02) and +1135C (OR=1.81) showed increased risk association with GBC. +286GG genotype and +286G allele were found to be risk associated with GBC (OR=4.32 and 1.98 and OR=3.58 and 1.71) when compared with CC and XGC, respectively. +1135CC genotype and +1135C allele also showed risk association with GBC (OR=2.58 and 1.66) when compared with CC. Since PDGF belongs to
the same family as VEGF; it was justified to investigate the possible clinical significance of SNPs in the related PDGF system also. There is no existing literature available to correlate our results with other genetic association studies of PDGF SNPs in GBC or other cancers. However, there are some reports suggesting the association of PDGF B markers in hepatitis C and chronic pancreatitis, have reported that PDGF-B may play an essential role in the development and progression of hepatic fibrosis in hepatitis C. They have found the AA genotype of +1135 A>C SNP to be increased predominantly in patients with recurrent HCV infection. However, they did not find any association between +286 A>G SNP with the studied liver etiology (Ben-Ari et al., 2006). Muddana et al., (2010), in a study on recurrent acute pancreatitis (RAP) and chronic pancreatitis (CP) patients found no difference in genotypic frequencies of +286A>G and +1135A>C SNPs among RAP, CP and controls.

A study showed that co-expression of PDGF-B and VEGFR-3 was strongly associated with lymph node metastasis and poor survival in non squamous cell lung cancer (NSCLC) (Donnemet et al., 2010). Another similar study reported the prognostic significance of PDGF-BB expression in esophageal squamous cell carcinoma, suggesting a key role in lymphangiogenesis and tumor growth (Matsumoto et al.,2007). PDGFs and PDGF Rs not only promote angiogenesis and direct tumor cell growth but also play an important role in lymphangiogenesis (Cao et al., 2005). GBC is highly metastatic disease and lymph node metastasis is very common; PDGF may therefore, be playing a role.

Alterations of HER2 encodes the receptor tyrosine kinase have been implicated in carcinogenesis and are frequently observed in a variety of tumors. A Japanese study of 234 gastric cancer patients and 287 control
subjects showed that the frequency of Ile/Val and Val/Val genotype was significantly higher in patients than in controls (p=0.005 and 0.033, respectively). Val/Val genotype revealed a significantly higher risk (OR=3.25) compared to Ile/Ile genotype. This study concluded that HER2 SNP could be associated with risk for development of gastric cancer and may be a predictor marker for gastric cancer (Kuraoka et al., 2003). In our study, frequency of Val/Val genotype was higher in GBC (12.8%) than controls (8%) but we did not find any significant association with HER2 SNP in GBC and CC; however, dominant model was risk associated (OR=2.11, p value=0.0370) and Val allele showed protective association (OR=0.52, p value=0.036) when XGC was compared with controls. McKay et al., (2002) also did not find any significant association with HER2 SNPs when 249 colorectal cancer patients were compared with 257 normal healthy control subjects. They found same Ile allele and Val allele frequency (80% and 20%, respectively) in colorectal cancer and controls and suggested that HER2 is not a prognostic marker for colorectal cancer. Nakazawa et al., (2005) studied amplification and overexpression of HER2 in 221 biliary tract carcinomas (BTC), of which 89 was GBC, 28 intrahepatic bile duct cancer, 78 extrahepatic bile duct cancer, and 26 of ampulla of Vater. Overexpression of HER2 was found in 15.7% GBC patients which was higher than other BTC patients, 79% HER2 gene amplification was also observed. An immune-histo-chemical study at our center reported 80% overexpression of HER2 in GBC (Kumari et al., 2012).

We performed haplotype analysis to see the combined effect of alleles of all three studied polymorphisms in GBC, CC and XGC. We found that three haplotypes i.e. ACIle (OR=1.48, p value=0.0005), GAVal (OR=1.70, p value=0.0444) and GAIle (OR=2.00, p value=0.004) were
risk associated with GBC as compared to controls. There is no study to compare haplotypes in GBC and GS associated inflammatory diseases i.e. CC and XGC. This is, thus, a very important finding but our results need further confirmation in a larger sample size.

5.3. Gallstone analysis

GS are present in majority of patients with GBC – more than 90% in Chile, 60-70% in India and 50-60% in Japan. The incidence of GBC is higher in populations with high GS prevalence rates. Patients with GS have 7 fold higher risk of GBC than normal population (Levin, 1999). Risk of GBC has been reported to increase with increasing size of GS; patients with larger (>3 cm) GS have about 10 times higher risk of having GBC than those with smaller (<1 cm) GS (Diehl., 1983 and Lowenfels et al., 1989). The type of GS may also be important – >80% of GS in northern India, where GBC is common, are cholesterol GS (Choudhuri et al., 1995). In southern India, where GBC is much less common, majority (>60%) of GS are pigment and only 5% are cholesterol GS (Jayanthi et al., 1998). VEGF polymorphism is usually involved in the growth, progression and metastasis of established tumors but may also play a role in the aetiology and susceptibility of tumor. GS are major risk factor for GBC and majority (60-90%) of GBC cases have a history of GS. In our study GS was present in 63% of GBC cases. Family history of gallstones has been reported in several studies (Sarin et al., 1995; Kratzer et al., 1998; Miquelet al., 1998), but there is only one study that assessed the role of family history of gallstones in GBC. A case-control study was carried out by Strom et al., in Bolivia and Mexico, they reported that family history of gallstones were associated with a risk of GBC (Strom et al., 1995). The mechanisms are unclear, but it is possible that some yet unidentified susceptibility mechanism may further elevate the risk of GBC associated
with stones. We compared GBC with and without GS with normal healthy controls and found significant association of GS with GBC. Hsing et al., (2008) also find a strong association between inflammation-related genes and biliary stones in biliary tract cancer (BTC). In our study -1154GA genotype of VEGF-1154 G>A genotype and -1154A allele were risk associated with GBC with GS (OR=1.87 and p=0.0058), (OR=1.64 and p value=0.0031). Mutant -2578AA genotype and +936T allele were also found risk associated (OR=1.90 and p value=0.0434) and (OR=1.61 and p value=0.0194) among GBC with GS. In case of PDGFB and polymorphisms, mutant +286GG genotype of +286A>G (OR=5.52 and p value=<0.0001) and mutant +1135CC genotype of +1135A>C (OR=2.89 and p value=0.0014) were found to be significantly risk associated in GBC with and GBC without GS cases. We hypothesized that polymorphisms may play a role in the etiology of GBC though the inflammation-hyperplasia-dysplasia-carcinoma pathway as some SNPs were risk associated with CC and XGC also when compared with controls. -1154 GA genotype and -1154A allele of VEGF -1154G>A were found to be risk associated for CC (OR=1.68, p value=0.016 and OR=1.70, p value=0.001 respectively). +936TT genotype and T allele of +936C>T polymorphism were also risk associated with CC (OR=3.48 p value=0.0033 and OR=2.63, p value=<0.0001 respectively). This strengthens the fact that these polymorphisms may play a role in the etiology of GBC through the inflammation-hyperplasia-dysplasia-carcinoma pathway. Hsing et al., (2007) also stated that the combination of GS and CC increases the risk of GBC.

5.4. Survival analysis

VEGF, PDGFB and HER2 SNP’s were correlated with clinico-pathological data like gall stone, tumor depth, lymphnode metastasis,
distant metastasis and tumor stage in 80 GBC out of 195 patients who were operated on or before 2010 (to ensure at least 5 years survival) and in whom follow up data is available. There was no association of found of these SNPs with tumor depth, lymphnode metastasis, distant metastasis and tumor stage. VEGF-2549ID genotype was significantly risk associated (OR=6.20 and p value=0.011) with GBC with stone. Dassoulas et al., (2009) also did not found association between VEGF SNP’s and tumor characteristics like size, histological grading, positive regional lymph node metastases or tumor stage, however, the -2578AA, -634CC, and +936TT genotypes found to be associated with a significantly lower overall survival in this study. A Turkish study in 25 gastric carcinoma, found HER2 Ile/Val genotype was associated with stage IV gastric cancer but they also did not find any correlation with histological type, tumor invasion, lymph node metastasis, and distant metastasis (S-Tufan et al., 2006). McKay et al., (2002) also did not find any correlation with clinico-pathological data in 249 colorectal cancer patients. In a study of 100 gastric cancer patients, -2578AA (p value=0.025), -634CC (p value=0.013), +936TT (p value=0.0001) genotypes was found to be significantly associated with larger tumor size while the -2578AA genotype was found to be significant (p value =0.01) in poorly differentiated and advanced stage of disease. The patients with +936TT genotype was significantly associated with metastatic disease (p value=0.0035) (Tzanakis et al., 2006).

In survival analysis of our study, GBC patients with VEGF -1154 GA and +936 CC genotype showed significantly longer survival (Chi-square=10.18, p value=0.0006 and Chi-square=7.332, p value=0.026 respectively). In 503 gastric cancer patients, +936 TT genotype of +936C > T polymorphism, showed worse overall survival (OS) (p value
compared with the C/C genotype (Kim et al., 2007). Another study by Kim et al., in 445 colorectal cancer patients the +936 C/T genotype (OS: HR, 12.809; P < 0.001) and TT genotype (OS: HR, 37.260; P < 0.001) was associated with a worse survival compared with the +936 C/C genotype (Kim et al., 2008). In a recent study in 2015, 89 CRC patients were included to study the association of VEGF 936C>T polymorphisms and efficacy of bevacizumab. The +936CC genotype was associated with a significantly better time to treatment failure (14.2 months) as compared to the CT and TT genotypes (6.0 months). Patients with +936T allele showed worse overall survival (p value= 0.016) and progression-free survival as compared to homozygous CC (p value = 0.044) (S-Blancet et al., 2015). In our study PDGFB+1135CC genotype showed significantly longer survival (Chi-square=7.424 and p value=0.024) but there was no significant survival results was obtained with PDGFB +286A>G and HER2Ile>Val polymorphisms. McKay et al., (2012) also did not found any association of HER2Ile>Val polymorphisms in colorectal cancer patients. There is no PDGFB SNPs study in cancer is available to compare our survival results.

5.5. Conclusion

Angiogenesis plays an important role in the pathogenesis of cancer. We have found significant risk association of VEGF -1154G>A, VEGF +936C>T, PDGFB +286A>G and PDGFB 1135A>C. On the basis of these finding we can say that these polymorphisms may be risk susceptibility markers for GBC. Gall bladder is a unique organ in that it provides an opportunity to study inflammatory conditions e.g. CC and XGC in addition to cancer i.e. GBC also. We hypothesised that polymorphisms may play a role in the aetiology of GBC though the inflammation - hyperplasia - dysplasia - carcinoma pathway. It is well
established that gall stones are present in 60-70% GBC patients and it causes the inflammation in GB wall that may leads to GBC. In this study we have found significant risk association of \textit{VEGF}-1154G$>$A, +936C$>$T SNP’s with CC as well as in GBC with gall stone cases. We found ICAC haplotype as a risk protective for GBC and ITGC haplotype as a risk protective for CC. ICAA haplotype was risk associated only in CC. We also found three risk associated ACIle, GAVal and GAIle haplotypes of \textit{PDGFB} and \textit{HER2} SNP’s with GBC. These are important findings and if this is confirmed in other populations and with larger sample sizes, these might become useful markers to screen patients with GS for their risk of GBC. Early detection of GBC is difficult, and this cancer can rarely be surgically resected at early stages because of late diagnosis. Targeted-therapies, which specifically inhibit growth factor receptors and their related signaling pathways, are promising approaches for the innovative medical treatment of GBC. Molecular targeted agents that inhibit angiogenesis and EGFR pathways are under clinical trials. More understanding about the molecular mechanism of gallbladder carcinogenesis coupled with more extensive genetic profiling of GBC patients will help to assess the therapeutic relevance of targeting a specific pathway. Amplification, expression, serum and plasma level estimation studies with \textit{VEGF}, \textit{PDGFB} and \textit{HER2} gene, will also further strengthen the findings of our study. At present, the study cannot be used for disease prediction or diagnosis; however, it may be used for screening of patients with GS to predict their risk for developing GBC.