RESEARCH ARTICLE

Platelet Derived Growth Factor-B and Human Epidermal Growth Factor Receptor-2 Polymorphisms in Gall Bladder Cancer

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Abstract

Gall bladder cancer (GBC) is a gastro-intestinal cancer with high prevalence among north Indian women. Platelet derived growth factor-B (PDGFB) and human epidermal growth factor receptor-2 (HER2) may play roles in the etiology of GBC through the inflammation-hyperplasia-dysplasia-carcinoma pathway. To study the association of *PDGFB* and *HER2* polymorphisms with risk of GBC, 200 cases and 300 controls were considered. *PDGFB* +286A>G and +1135A>C polymorphisms were investigated with an amplification refractory mutation system and the *HER2* Ile^{655}Val polymorphism by restriction fragment length polymorphism. Significant risk associations for *PDGFB* +286 GG (OR=5.25) and *PDGFB* +1135 CC (OR=3.19) genotypes were observed for GBC. Gender wise stratification revealed susceptibility for recessive models of *PDGFB* +1135A>C (OR=3.00) and HER2 Ile^{655}Val (OR=2.52) polymorphisms among female GBC cases. GBC cases with gall stones were predisposed to homozygous +286 GG and +1135 CC genotypes. Significant risk associations were found for ACIle (OR=1.48), GAVal (OR=1.70), GAIle (OR=2.00) haplotypes with GBC cases and GCIle haplotype with female GBC cases (OR=10.37, P=<0.0001). Pair-wise linkage disequilibrium revealed negative associations among variant alleles. On multi-dimensional reduction analysis, a three factor model revealed significant gene-gene interaction for *PDGFB* +286A>G, *PDGFB* +1135A>C and *HER2* Ile165Val SNPs with GBC. Protein-protein interaction showed significant association of *PDGFB* and *HER2* with the epidermal growth factor receptor signaling pathway.

Keywords: Gallbladder cancer - *HER2* - *PDGFB* - single nucleotide polymorphisms

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Introduction

Gall bladder cancer (GBC) is an uncommon etiology with late diagnosis, limited treatment options and poor prognosis with overall five year survival rate of less than 10%. Remarkable variation in the incidence of GBC has been reported across the globe with low prevalence in the United States, United Kingdom and western Europe, and higher frequency in central and south America, central and eastern Europe, Japan (Randi et al., 2006) and China (Qu et al., 2012). Chile in south America has one of the highest incidence rates of GBC in world and GBC is the major cause of cancer deaths in females (Randi et al., 2006). It is reported as a disease of elderly females (Hamdani et al., 2012). In context to India, GBC is more prevalent among north Indians in comparison to their southern counterpart. Its incidence is to the tune of nine affected female cases per one lac population in north India (NCRP, 2002). The peculiar geographical and racial variations in its incidence suggest the importance of genetic factors in etiology of GBC. Earlier association of single nucleotide polymorphisms (SNPs) with GBC has been reported (Mishra et al., 2013).

Angiogenesis is a key factor for tumor growth and metastasis. Platelet derived growth factor (PDGF) is a critical mediator of tumor angiogenesis. *PDGFB* expression may result into uncontrolled replication of neoplastic cells leading to the progression of GBC. *PDGFB* gene expression has been studied in various cancers e.g. lung cancer, esophageal cancer, gastric cancer, pancreatic cancer, etc (Bravo et al., 1991; Chung et al., 1992; Wong et al., 1994; Yamamoto et al., 1996). However, genetic association of *PDGFB* +286 A>G and +1135 A>C SNPs have been studied first time in hepatitis C infection case (Ben-Ari et al., 2006).

Human epidermal growth factor receptor-2 (*HER2*/c-erbB-2) is a proto-oncogene member of the epidermal growth factor receptor (EGFR) family. Structural and functional alteration of *HER2* has been reported in different steps of carcinogenesis including initiation,

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promotion and progression (Marmor et al., 2004). SNP at codon 655 of *HER2* gene has been identified which encodes either isoleucine (Ile: ATC) or valine (Val: GTC) in the transmembrane domain-coding region (Papewalis et al., 1991; Xie et al., 2000). It has also been showed that *HER2* can be successfully used as a tumor marker for breast cancer (Xie et al., 2000). To assess *HER2* association with other cancers like hepato-cellular, gastric, ovarian, bladder, colo-rectal, prostate etc. various studies have been conducted (Nakajima et al., 1999; McKay et al., 2002; Wang et al., 2002; Kuraoka et al., 2003; Quaye et al., 2009; Karaca et al., 2012; Zahir et al., 2014). *PDGFB* and *HER2* belong to a family of receptor tyrosine kinases that are thought to participate in signal transduction mediating tumor cell proliferation and/or motility (Nakajima et al., 1999) that is why we studied both together.

The present cross sectional study was designed to investigate genetic associations of *PDGFB* +286A>G (rs#1800818), *PDGFB* +1135A>C (rs#1800817) and *HER2* Ile^{655}Val (rs#11362000) polymorphisms and their haplotypes with GBC. Previously no genetic association studies of *PDGFB* and *HER2* polymorphim have been done in gallbladder cancer.

Materials and Methods

Ethics Statement

The study was approved by the Institutional Ethical Committee (IEC) of the Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGIMS), Lucknow India. All the participants were provided with and signed written informed consent for the study. The recruitment of subjects was carried out according to norms of the Declaration of Helsinki.

Subjects

The study included 200 GBC cases of which five cases were excluded due to erroneous data. The demographic characteristics were evaluated based on gender, 119 (61%) females and 76 (39%) males were present in out of 195 GBC cases. The mean age with standard deviation of GBC cases was 52.7±11.2. Gall stone (GS) was present in 122 (62.6%) whereas GS was absent in 73 (37.4%) GBC cases. All the cases were registered in the Department of Surgical Gastroenterology, SGPGIMS, Lucknow, a tertiary care hospital in north India. Gender matched controls, 200 (67%) females and 100 (33%) males were included in this study, as GBC is predominantly found among females .The mean age with standard deviation of controls was 43.6±11.0. Blood samples for controls with no prior history of cancer were collected from two previous studies (Aggarwal et al., 2011; Prakash et al., 2012). The controls had similar ethnic background to that of the cases. GBC was confirmed by fine needle aspiration cytology (FNAC) and/or histopathology. Presence or absence of GS in GBC was confirmed by ultrasonography (US) or at the time of surgery. DNA was extracted from venous blood by using QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA).

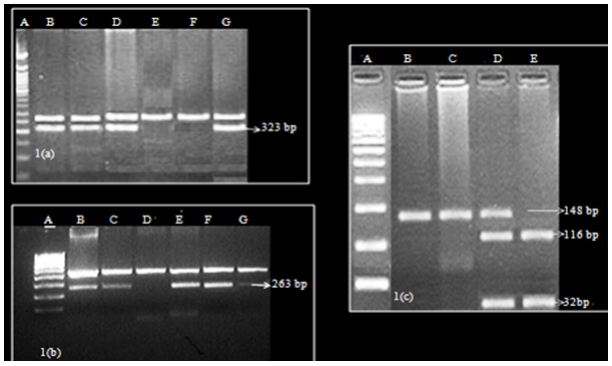

Figure 1. Representing Gel Images Showing: 1(a) PDGFB 286A>G Polymorphism, lane B+C-AG, D+E-AA and F+G-GG genotype; 1(b) PDGFB 1135A>C Polymorphism, Lane B+C-AC, D+E-CC and F+G-AA genotype; 1(c) HER2 Ile^{655}Val Polymorphism, Lane B+C-Ile/Ile, D-Val/Val and E-Ile/Val genotype. In lane A-100bp DNA ladder showed in 1(a) and 1(b) and a 50bp ladder showed in 1(c)

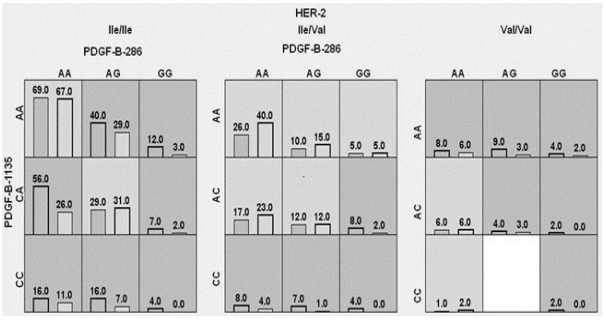

Figure 2. The Best Model in MDR Analyses was Composed of PDGFB +286A>G-PDGFB +1135 A>C-HER2 Ile655Val GB. In each cell of genotype combination, the left bar represents score for GBC and the right bar for Normal Control. High-risk combinations for GBC are shaded dark grey, while low-risk combinations are shaded light grey

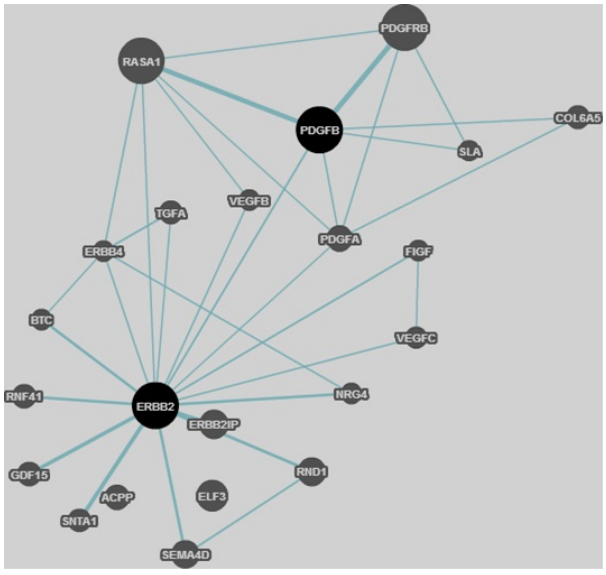

Figure 3. Protein-protein Network Analysis Showing Major Genes Linked with PDGFB-HER2 Associated Biological Pathways. Prominence of genes in the network is evidenced from the weights assigned basing on number of interactions involved with a particular gene

Genotyping

Amplification of *PDGFB* +286A>G, *PDGFB* +1135A>C and *HER2* Ile^{655}Val SNPs were carried out in a thermal cycler (Mastercycler gradient; Eppendorf, Hamburg, Germany). Genotyping was done for *PDGFB* +286A>G and +1135A>C polymorphisms by amplification refractory mutation system (ARMS) PCR using a common set of primers as mentioned by (Ben-Ari et al., 2006) and *HER2* Ile^{655}Val by restriction fragment length polymorphism (PCR-RFLP) using a common set of primers and PCR conditions as described before (Papewalis et al., 1991; Xie et al., 2000). PCR products of *HER2* Ile^{655}Val polymorphisms were digested using BsmAI. The 148 bp PCR product was cut by BsmAI into two fragments of 116 and 32 bp if the Val allele was present, whereas the product of Ile allele was uncut and produced a single fragment of 148 bp (Figure 1). PCR and RFLP products were run by gel electrophoresis on 2% agarose gel stained with ethidium bromide.

Statistical Analysis

Statistical power of the study and sample size estimation were carried out using G*Power version 2 (Heinrich Heine Universitat, Dusseldorf, Germany). Differences in the *PDGFB* and *HER2* genotype, allele frequencies and haplotypes between the study and control groups were analyzed with Fisher exact test and p values ≤ 0.05 were considered statistically significant. Odds Ratio (OR) was used to measure strength of association between genotypes, allele frequencies and haplotypes between GBC. Analysis for the genotypes was done under additive, recessive and dominant models of inheritance. Haplotypes were generated using Arlequin software (University of Geneva, Switzerland) and statistical analysis was done using SPSS software version 16 (IBM Corporation, New York, NY USA). The degree of pair wise linkage disequilibrium (LD) was calculated for each pair of SNPs taking into consideration the GBC cases using the SNP Stats program (Catalan Institute of Oncology, IDIBELL, Epidemiology and Cancer Registry L'Hospitalet, Barcelona, Spain) (Sole et al., 2006). Conventional LD was calculated using Lewontin's principle. We have also calculated correlation coefficient (r) using Cramer's V statistic and corresponding p-value for each pair wise LD measure. The multi dimensional reduction (MDR) software version 2.0 beta 8 (Vanderbilt University Medical School, Nashville, TN, USA) (Hahn et al., 2003) was used to identify high-order gene-gene interactions associated with GBC. The nonparametric MDR was used to overcome limitations of logistic regression (i.e., sample size limitations) for the detection and characterization of gene-gene interactions. The MDR results were validated through cross-validation and permutation testing. Protein-protein interaction network involving *PDGFB* and *HER2* genes was constructed using Gene MANIA (Warde-Farley et al., 2010) which effectively predicted hypothesis about gene function, analyzed gene lists and prioritized genes for constructing interaction networks.

Table 1. Comparison of PDGFB and HER2 Genotype and Allele Frequency Distribution in GBC with Controls

Genotype	Controls (n=300) n (%)	GBC (n=195) n (%)	OR (95% CI)	P-Value
PDGFB+286A>G (rs#1800818)		GBC vs Controls		
GG (additive model)	14 (4.5)	37 (18.9)	5.25 (2.70-10.2)	<0.0001†
AG (additive model)	101 (33.5)	65 (33.4)	0.72 (0.46-1.14)	0.1792
AA	185 (62)	93 (47.7)		
GG *vs* AA+AG (Recessive model)			4.78 (2.51-9.11)	<0.0001†
AA *vs* GG+AG (Dominant model)			0.56 (0.39-0.81)	0.003*
Allele frequency				
286G	129 (21.5)	139 (35.6)	2.02 (1.52-2.68)	<0.0001†
286A	471 (78.5)	251 (64.4)		
PDGFB+1135A>C (rs#1800817)				
CC (additive model)	25 (8.5)	39 (20)	3.19 (1.81-5.63)	<0.0001†
AC (additive model)	105 (35)	73 (37.4)	1.42 (0.95-2.11)	0.0846
AA	170 (56.5)	83 (42.5)		
CC *vs* AA+AC (Recessive model)			2.75 (1.60-4.71)	0.0003†
AA *vs* CC+AC (Dominant model)			0.56 (0.39-0.81)	0.0024*
1135C	155 (25.8)	151 (38.7)	1.81 (1.37-2.38)	<0.0001†
1135A	445 (74.2)	239 (61.3)		
HER2 Ile655Val (rs#1136200)				
Val/Val (additive model)	24 (8)	25 (12.8)	1.51 (0.82-2.77)	0.2119
Ile/Val (additive model)	102 (34)	50 (25.6)	0.71 (0.47-1.07)	0.1228
Ile/Ile	174 (58)	120 (61.5)		
Val/Val *vs* Ile/Ile+Ile/Val (Recessive model)			1.69 (0.93-3.05)	0.0907
Ile/Ile *vs* Val/Val+Ile/Val (Dominant model)			1.15 (0.80-1.67)	0.4546
Allele frequency				
Val allele	150 (25)	100 (25.6)	1.03 (0.77-1.38)	0.8227
Ile allele	450 (75)	290 (74.4)		

*Statistically significant and risk-protective genotype/allele; †Statistically significant and risk-associated genotype/allele; Additive Model: comparing mutant homozygous and heterozygous genotypes individually with wild homozygous type; Recessive Model: comparing mutant homozygous genotype with wild homozygous and heterozygous genotype taken together; Dominant Model: comparing wild homozygous genotype with mutant homozygous and heterozygous genotype taken

Results

PDGFB +286A>G (p-value=0.9533), *PDGFB* +1135A>C (p-value=0.1690) and *HER2* Ile^{655}Val (p-value=0.1803) were in Hardy-Weinberg equilibrium at one degree of freedom among controls. This indicated no genotyping errors, inbreeding, genetic drift, and mutation or population substructure. Significant deviations from the expected proportions of homozygote and heterozygote classes among patients may be due to association with the disease allele. Significant statistical power was obtained against GBC and control group combinations (0.99) which justified sample size for the present study.

Genotype and allele frequency

Genotype and allele frequencies of GBC compared with controls are shown in Table 1. Homozygous GG genotype and G allele along with the recessive model of *PDGFB* +286A>G SNP were found to be risk associated with GBC. CC genotype and recessive model of *PDGFB* +1135A>C polymorphism revealed susceptibility whereas dominant model showed protective association with GBC. However, no significant association was found for *HER2* Ile^{655}Val SNP with GBC.

Gender-wise comparison of genotype and allele frequencies of GBC with controls is given in Table 2. Homozygous GG genotype and G allele of *PDGFB* +286 A>G along with CC genotype and C allele of *PDGFB* +1135 A>C SNPs were observed to be risk associated in both male and female GBC cases. Recessive models of *PDGFB* +286A>G, *PDGFB* +1135 A>G and *HER2* Ile^{655}Val SNPs also showed predisposing associations in female GBC cases. Similarly homozygous Val/Val genotype of *HER2* Ile^{655}Val SNP also revealed susceptibility with female cases.

Comparison of GBC with and without gall stone (GS)

GS was observed among 62.6% of GBC cases in our study. GBC cases were categorized into GBC with and without GS and were compared with normal controls (Table 3). Risk association among GBC cases with and without GS for recessive models of *PDGFB* +286 A>G and *PDGFB* +1135 A>C SNPs were evident. Similarly both GBC with and without GS cases were predisposed to GG genotype and G allele of *PDGFB* +286 A>G polymorphism along with CC genotype and C allele of *PDGFB* +1135 SNP. Heterozygous Ile/Val genotype of *HER2* Ile^{655}Val polymorphism showed risk protective association in GBC with GS whereas recessive model was risk associated in GBC without GS.

Haplotype analysis

Seven haplotypes (ACIle, GAVal, AAIle, GAIle, GCIle, AAVal and ACVal) were found to be common

Table 2. Gender wise Comparison of PDGFB and HER2 Genotype and Allele Frequency Distribution in GBC with Controls

Genotype	Male				Female			
	Control (n=100)	GBC (n=76)	OR (95% CI)	P-Value	Control (n=200)	GBC (n=119)	OR (95% CI)	P-Value
	n (%)	n (%)			n (%)	n (%)		
PDGFB+286A>G (rs#1800818)			GBC vs Controls					
GG (additive model)	5 (5)	18 (23.8)	7.57 (2.55-22.4)	<0.0001†	9 (4.5)	19 (15.9)	4.09 (1.75-9.55	0.0014†
AG (additive model)	34 (34)	29 (38.1)	1.79 (0.92-3.48)	0.0927	67 (33.5)	36 (30.2)	1.04 (0.62-1.72)	0.8978
AA	61 (61)	29 (38.1)			124 (62)	64 (53.9)		
GG vs AA+AG (Recessive model)			5.89 (2.07-16.74)	0.0005†			4.03 (1.75-9.24)	0.0008†
AA vs GG+AG (Dominant model)			0.39 (0.21-0.72)	0.0037*			0.71 (0.45-1.13)	0.1593
Allele frequency								
286G	44 (22)	65 (42.8)	2.64 (1.66-4.21)	<0.0001†	85 (21.2)	74 (31.1)	1.62 (1.16-2.40)	0.0061†
286A	156 (78)	87 (57.2)			315 (78.8)	164 (68.9)		
PDGFB+1135A>C (rs#1800817)								
CC (additive model)	8 (8)	13 (17.1)	2.98 (1.11-7.99)	0.0458†	17 (8.5)	26 (21.8)	3.32 (1.66-6.65)	0.0007†
AC (additive model)	35 (35)	32 (42.1)	1.68 (0.87-3.21)	0.1382	70 (35)	41 (34.4)	1.27 (0.76-2.11)	0.3657
AA	57 (57)	31 (40.8)			113 (56.5)	52 (43.6)		
CC vs AA+AC (Recessive model)			2.37 (0.92-6.06)	0.0986			3.00 (1.55-5.82)	0.0011†
AA vs CC+AC (Dominant model)			0.51 (0.28-0.95)	0.0476*			0.59 (0.37-0.94)	0.0284*
Allele frequency								
1135C	51 (25.5)	58 (38.2)	1.80 (1.14-2.84)	0.0144†	104 (26)	93 (39.1)	1.82 (1.29-2.57)	0.0007†
1135A	149 (74.5)	94 (68.8)			296 (74)	145 (60.9)		
HER2 Ile655Val (rs#1136200)								
Val/Val (additive model)	8 (8)	6 (7.9)	0.85 (0.27-2.62)	1.0	14 (7)	19 (15.9)	2.31 (1.09-4.92)	0.0335†
Ile/Val (additive model)	34 (34)	19 (25)	0.63 (0.32-1.24)	0.2369	68 (34)	31 (26.2)	0.77 (0.46-1.30)	0.3645
Ile/Ile	58 (58)	51 (67.1)			118 (59)	69 (57.9)		
Val/Val vs Ile/Ile+Ile/Val (Recessive model)			0.98 (0.32-2.97)	1.0			2.52 (1.21-5.24)	0.0135†
Ile/Ile vs Val/Val+Ile/Val (Dominant model)			1.47 (0.79-2.75)	0.2728			0.95 (0.60-1.520	0.9066
Allele frequency								
Val allele	50 (25)	31 (20.4)	0.76 (0.46-1.27)	0.371	96 (24)	69 (28.9)	1.29 (0.90-1.85)	0.1905
Ile allele	150 (75)	121 (79.6)			304 (76)	169 (71.1)		

*Statistically significant and risk-protective genotype/allele; †Statistically significant and risk-associated genotype/allele; Additive Model: comparing mutant homozygous and heterozygous genotypes individually with wild homozygous type; Recessive Model: comparing mutant homozygous genotype with wild homozygous and heterozygous genotype taken together; Dominant Model: comparing wild homozygous genotype with mutant homozygous and heterozygous genotype taken

Table 3. Comparison of PDGF-B and HER-2 Genotype Frequency Distribution in GBC (with and without GS) with Controls

	Control (n=300)	GBC with GS (n=122)			Control (n=300)	GBC without GS (n=73)		
	n (%)	n (%)			n (%)	n (%)		
PDGFB+286A>G (rs#1800818)								
GG (additive model)	14 (4.5)	23 (18.9)	5.52 (2.66-11.4)	<0.0001†	14 (4.5)	14 (19.2)	4.68 (2.14-11.0)	0.0002†
AG (additive model)	101 (33.5)	44 (36.0)	1.46 (0.92-2.33)	0.1182	101 (33.5)	21 (28.8)	1.01 (0.56-1.81)	1.0
AA	185 (62)	55 (45.1)			185 (62)	38 (52.0)		
GG vs AA+AG (Recessive model)			4.74 (2.35-9.58)	<0.0001†			4.84 (2.19-10.7)	0.0002†
AA vs GG+AG (Dominant model)			0.51 (0.33-0.78)	0.0023			0.67 (0.40-1.12)	0.1445
Allele frequency								
286G	129 (21.5)	90 (36.9)	2.13 (1.54-2.95)	<0.0001†	129 (21.5)	49 (33.6)	1.84 (1.24-2.73)	0.0033†
286A	471 (78.5)	154 (63.1)			471 (78.5)	97 (66.4)		
PDGFB+1135A>C (rs#1800817)								
CC (additive model)	25 (8.5)	23 (18.9)	2.89 (1.52-5.51)	0.0014†	25 (8.5)	16 (21.9)	3.75 (1.78-7.87)	0.0007†
AC (additive model)	105 (35)	45 (36.9)	1.34 (0.84-2.14)	0.2321	105 (35)	28 (38.4)	1.56 (0.88-2.77)	0.1387
AA	170 (56.5)	54 (44.2)			170 (56.5)	29 (39.7)		
CC vs AA+AC (Recessive model)			2.55 (1.38-4.70)	0.0036†			3.08 (1.55-6.15)	0.0027†
AA vs CC+AC (Dominant model)			0.60 (0.39-0.92)	0.0239*			0.50 (0.29-0.84)	0.0126*
Allele frequency								
1135C	155 (25.8)	91 (37.3)	1.70 (1.24-2.34)	0.0011†	155 (25.8)	60 (41.1)	2.00 (1.37-2.92)	0.0005†
1135A	445 (74.2)	153 (62.7)			445 (74.2)	86 (58.9)		
HER2 Ile655Val (rs#1136200)								
Val/Val (additive model)	24 (8)	13 (10.6)	1.17 (0.57-2.43)	0.7069	24 (8)	12 (16.4)	2.17 (1.00-4.71)	0.0729
Ile/Val (additive model)	102 (34)	29 (23.8)	0.61 (0.37-1.01)	0.0569*	102 (34)	21 (28.8)	0.89 (0.50-1.60)	0.77
Ile/Ile	174 (58)	80 (65.6)			174 (58)	40 (54.8)		
Val/Val vs Ile/Ile+Ile/Val (Recessive model)			1.37 (0.67-2.79)	0.4475			2.26 (1.07-4.77)	0.0439†
Ile/Ile vs Val/Val+Ile/Val (Dominant model)			1.37 (0.88-2.13)	0.1557			0.87 (0.52-1.46)	0.6929
Allele frequency								
Val allele	150 (25)	55 (22.5)	0.87 (0.61-1.24)	0.4795	150 (25)	45 (30.8)	1.33 (0.89-1.98)	0.172
Ile allele	450 (75)	189 (77.5)			450 (75)	101 (69.2)		

*Statistically significant and risk-protective genotype/allele; †Statistically significant and risk-associated genotype/allele; Additive Model: comparing mutant homozygous and heterozygous genotypes individually with wild homozygous type; Recessive Model: comparing mutant homozygous genotype with wild homozygous and heterozygous genotype taken together; Dominant Model: comparing wild homozygous genotype with mutant homozygous and heterozygous genotype taken

among GBC and normal control groups. 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 predisposed to GBC while AAIle (OR=0.14, p-value=<0.0001) and AAVal (OR=0.53, p-value=0.0051) showed protective association. In gender wise stratification of GBC cases, GAVal (OR=2.82, p-value=0.0255), AAIle (OR=2.07, p-value=0.0349), GAIle (OR=2.90, p-value=0.0025), and AAVal (OR=2.82, p-value=0.0255) haplotypes showed risk association in male GBC cases. GCIle (OR=10.3, p-value=<0.0001) haplotype showed risk association while AAIle (OR=0.39, p-value=0.0008) haplotypes showed protective associations among females GBC cases.

Linkage disequilibrium (LD) analysis

The pair wise analysis revealed weak and negative LD in our study. Significantly weak LD were noted among all the variant allele pairs like *PDGFB* +286 G/+1135 C (D=0.12, r=0.53), *PDGFB* +286 G/*HER2* Valine (D=0.11, r=0.48) and *PDGFB* +1135 C/*HER2* Valine (D=0.12, r=0.51).

Gene-gene interaction analysis

To carry out MDR analysis GBC cases and normal controls were considered. Results so obtained were validated through cross-validation and permutation testing. Results revealed best one-factor model for *PDGFB* +1135 A>G (testing accuracy=0.5015, cross-validation consistency (CVC)=6/10, permutation (P)=0.023), two-factor model for *PDGFB* +286 A>G and *PDGFB* +1135 A>C (testing accuracy=0.5191, CVC=7/10, P=0.0001). The three-factor model revealed significant gene-gene interaction for three SNPs namely *PDGFB* +286A>G, *PDGFB* +1135 A>G and *HER2* $Ile^{655}Val$ with GBC (testing accuracy=0.5484, CVC=10/10, P≤0.0001). There were sixteen genotype combinations involving these SNPs which have showed risk association for GBC (Figure 2).

The MDR analysis revealed presence of higher frequencies and mutant homozygous or heterozygous genotype combinations of *PDGFB* and *HER2* alleles (Ile/Val-CC-AG=12%, Ile/Ile-AA-GG=6.1%, Ile/Val-AC-GG=5.8%, Ile/Ile-AC-GG=5.1%, and Val/Val-AA-AG=4.6%) increase the risk of GBC.

Impact of PDGFB and HER2 on biological pathway

Protein-protein interaction datasets were generated by taking into account the genes that interact in close proximity with *PDGFB* and *HER2* (Figure 3). Genes falling in the network were assigned with weights depending on their association with *PDGFB* and *HER2* in various biological pathways. Network analysis revealed 111 prominent biological functions to be associated with *PDGFB*, *HER2* associated pathways of which most prominent ones are: epidermal growth factor receptor signaling pathway (P=1.62E-12), ERBB signaling pathway (P=1.62E-12), phosphatidylinositol-mediated signaling (P=6.52E-10), inositol lipid-mediated signaling

(P=6.52E-10) and fibroblast growth factor receptor signaling pathway (P=2.82E-8).

Discussion

Autocrine pathway involving *PDGFB*/PDGFR signaling establishes self-sufficiency in growth for cancer cells. PDGF is frequently produced by tumor cells and affects tumor growth and dissemination by different means. Amplification and over expression of *PDGFB* and *HER2* are usually involved in the growth, progression and metastasis of established tumors. Genetically prominent associations of the *PDGFB* gene polymorphisms with GBC were found in the present study. Mutant homozygous genotypes +286GG (OR=5.25) and +1135CC (OR=3.19) along with mutant alleles +286 G (OR=2.02) and +1135 C (OR=1.81) of *PDGFB* showed increased risk association with GBC.

PDGF belongs to the PDGF/VEGF (vascular endothelial growth factor) family. An earlier study by our group has shown association of VEGF SNPs with GBC (Mishra et al., 2013). Since PDGF belongs to the same family as VEGF; it is justified to investigate possible clinical significance of SNPs in related PDGF system. There is no existing literature available to correlate our results with other genetic association studies of PDGF SNPs in the milieu of GBC. However, some reports have suggested the association of *PDGFB* markers in hepatitis C (Ben-Ari et al., 2006) and chronic pancreatitis (Muddana et al., 2010). The AA genotype of +1135 A>C SNP was in predominance among patients with recurrent HCV infection; however, no association was observed for +286 A>G SNP with the studied liver etiology (Ben-Ari et al., 2006). Muddana et al, 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 (Muddana et al., 2010). A study showed co-expression of *PDGFB* and VEGFR-3 to be associated with lymph node metastasis and poor survival in non squamous cell lung cancer (Donnem et al., 2010). Another similar study reported the prognostic significance of *PDGFB*B expression in esophageal squamous cell carcinoma, suggesting a key role in lymphangiogenesis and tumor growth (Matsumoto et al., 2007). PDGFs and PDGFRs not only promote angiogenesis and direct tumor cell growth but also play an important role in lymphangiogenesis (Cao et al., 2005). GBC is a highly metastatic disease and lymph node metastasis is very common. PDGF may therefore, be playing a role. GBC is associated with GS in majority (60-90%) of cases. GS causes inflammation of the gallbladder in the form of chronic cholecystitis (CC). The combination of GS and cholecystitis increases the risk of GBC (Hsing et al., 2007). In our study 62.5% of GBC cases had GS and we found significantly increased risk of the genotypes and alleles of *PDGFB* +286A>G (OR=5.52 and OR=4.68) and +1135AA>C (OR=2.89 and OR=3.75) in GBC with and without GS. This strengthens the fact that *PDGFB* may play a role in the etiology of GBC through the inflammation-hyperplasia-dysplasia-carcinoma pathway.

From the protein-protein interaction analysis we have observed prominent associations of *PDGFB* and *HER2* genes with epidermal growth factor receptor and ERBB signaling pathways which have well evidenced role in the causation of GBC (Li et al., 2014). Approximately one-third of all human cancers exploit deregulated signaling by the ERBB family for growth, survival and other functions toward tumor perpetuation. The ERBB signaling pathway that includes *HER2* and its downstream genes has been reportedly the most extensive mutated pathway affecting nearly 36.8% of GBC cases in a Chinese cohort (Li et al., 2014). Significance for the fibroblast growth factor receptor signaling pathway was also observed in the GBC. The fibroblast growth factor activates a signaling pathway that positively regulates the PDGF receptors in oligodendritic progenitor cells which enhances the angiogenesis process leading to progression of different cancers.

Alteration of *HER2* encodes the receptor tyrosinekinase which has been implicated in carcinogenesis and is frequently observed in a variety of tumors. We have also found *HER2* to be significantly associated with positive regulation of protein tyrosinekinase activity among GBC. A Japanese study of 234 gastric cancer patients and 287 control subjects showed that the frequency of Ile/Val and Val/Val genotypes were 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 probable association of *HER2* SNP with risk for development of gastric cancer and may act as a predictor 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. (McKay et al., 2002) also did not find any significant association with *HER2* SNPs when 249 colorectal cancer patients were compared with 257 normal controls subjects. They found same frequencies for Ile allele (80%) and Val allele (20%) in colorectal cancer and controls and suggested that *HER2* is not a prognostic marker for colorectal cancer. Kara et al also found no evidence of over-expression of *HER2* on 34 colorectal cancer cases (Kara et al., 2012; Nakazava et al., 2005) studied amplification and over-expression of *HER2* in 221 biliary tract carcinomas (BTC), of which 89 were GBC, 28 intrahepatic bile duct cancer, 78 extrahepatic bile duct cancer, and 26 ampulla of Vater cases. Over-expression of *HER2* was found in 15.7% GBC patients which was higher than other BTC patients along with 79% *HER2* gene amplification. *HER2* deregulation was also observed to be a significant genetic event leading to non-small cell lung cancer (Panagiotou et al., 2012). It has also been suggested as a potential prognostic marker for targeted therapy of gastric cancer (Rakhshani et al., 2014). An immunohistochemical study at our center by (Kumari et al., 2012) reported 80% over-expression of *HER2* in GBC. These results suggested that *HER2* overexpression/amplification plays an important role in carcinogenesis.

Upon performing pair-wise linkage disequilibrium we have found significantly weak or negative D values for the studied *PDGFB* and *HER2* SNPs. This strengthens the fact

that these SNPs do not lie in LD (Ben-Ari et al., 2006).

Gender wise comparison revealed susceptibility for the *PDGFB* and *HER2* SNPs among female GBC cases. GBC is very common in females in north India; in our study 61% GBC cases were females. Recessive model of *PDGFB* +1135A>C SNP was found significantly risk associated (OR=3.00) in females with GBC. Val/Val genotype and recessive model of *HER2* $Ile^{655}Val$ polymorphisms was also found to be risk associated (OR=2.31 and OR=2.52) in females with GBC. *HER2* is an established diagnostic marker in breast cancer and it was thus justified to study this polymorphism. The haplotype analysis revealed three haplotypes i.e. ACIle (OR=1.48, P=0.0005), GAVal (OR=1.70, P=0.0444) and GAIle (OR=2.00, P=0.004) to be risk associated with GBC as compared to controls while GCIle haplotype was predisposed among female GBC cases (OR=10.37 and P=<0.0001) on gender-wise stratification. There is no study to compare haplotypes in GBC. This is, thus, a very important finding but our results need further confirmation on a larger cohort. The three-factor model, involving *PDGFB* +286A>G, *PDGFB* +1135A>C and *HER2* Ile>Val showed significant gene-gene interactions in GBC. Thirteen high-risk combinations involving *PDGFB* +286A>G, *PDGFB* +1135A>C and *HER2* Ile>Val SNPs showed higher frequency in GBC cases as compared to controls which signifies the probable role of the studied SNPs with GBC.

Our study suggests that *PDGFB* +286A>G and *PDGFB* +1135A>C SNPs may be susceptible markers for GBC. However, this needs further validation on larger and diverse populations along with gene expression analysis of *PDGFB* and *HER2* genes. 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.

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