Original Research Article

Correlation of *GSTP1* polymorphism with severity of prostate cancer in an Eastern Indian population

Running title: *GSTP1* polymorphism and prostate cancer

ABSTRACT :

Background: *GSTP1* is one of the Glutathione-S-Transferases (GSTs) which restrict tumorogenesis by detoxifying toxic carcinogens and reactive oxygen species (ROS). Prostate cancer is related to several mutationsaffecting the expression of *GSTP1*. A single nucleotide polymorphism (SNP: Ile105Val) in the *GSTP1* gene results insignificant reduction in its anticancer activity. The current case control study was conducted to ascertain the risk of association of *GSTP1*polymorphism with risk of cancer prostate in an Eastern Indian population.

Materials and methods: During a study period of 2 years, DNA was isolated using the phenol chloroform extraction method from the blood of 225 histopathologically diagnosed prostate cancer patients and 120 matched controls. The *GSTP1* polymorphism was assessed by PCR amplification of the gene followed by restriction digestion with Alw261. Histopathological grading in the case group was performed using Gleason's scores and ISUP grading.

Results: Comparison of the distribution of different *GSTP1* alleles between the case and control groups was performed by chi square test and odds ratio analysis. A $\chi 2$ value of 18.56 suggested significantly higher number of *G* alleles in the case group. An odds ratio of 2.25 with a confidence interval of 1.52 to 3.34 for 95% CI showed that the *G* allele in *GSTP1* gene were linked with greater risk of prostate cancer. Post hoc ANOVA and logistic regression suggested that cases having *G* alleles had more progressive form of diseases as evident from ISUP grades.

Conclusions: From our study we can conclude that *GSTP1* polymorphism is not only significantly associated with risk of prostate cancer but also with its severity in our Eastern

Indian population. *GSTP1* polymorphism should be considered as a prognostic indicator for prostate cancer patients along with planning for more aggressive management of the disease.

Key words: Prostate cancer, *GSTP1* polymorphism, ISUP grading, Single nucleotide polymorphism, Restriction digestion.

1. INTRODUCTION:

Prostate cancer is the third leading cause of cancer death among men in the United States (1).It has higher mortality rate among African-Americans compared to Caucasians (2). However, in India, it has been reported to be the second killer cancer in large metro cities and among the top ten cancers in the rest of the country according to the population based cancer registries in India(3). Recurrence rate and mortality rate of prostate cancer depend on Gleason´s grading and a higher serum prostate specific antigen (PSA) level (4) even after radical prostatectomy(5).

Initiation of cancer is attributable to several genetic disarrangements including chromosomal deletion, translocation, changes in DNA methylation and point mutations (6, 7). These genetic changes become particularly important when they affect the expression of tumour suppressor proteins. Glutathione-S-Transferases (*GSTs*) belong to one of such tumor suppressor proteins which restrict the initiation and progression of tumorogenesis by detoxifying different toxic carcinogens and reactive oxygen species (ROS). The *GSTP1* gene is approximately 4 kb in length, comprises 7 exons and 6 introns and codes for a 715 base mRNA. *GSTs* have several isozymes with almost similar functions in different tissues. They are responsible for metabolism and biosynthesis of various metabolites including detoxification of exogenous carcinogen chemicals like polycyclic aromatic hydrocarbon which are abundant in diesel fuels, cigarette smoke and grilled meats. Overall, they detoxify several carcinogenic xenobiotics by conjugation with glutathione during the phase II of detoxification process of the electrophonic carcinogenic compounds (8, 9). Specific *GST* isoforms in the (M1), (T1) and p (P1) classes are highly expressed in the prostate tissues (10). Amongthe large family of their isoenzymes, the pi class enzyme *GSTP1*-1 is the most widely distributed and sowell studied in different types of cancers. *GSTP1- 1*expression has been found to be associated with resistance to cytotoxic drugs in breast cancer cells also (11).Therefore any alteration in the genetic polymorphic profile of *GSTP1*may

be associated with severity as well as the risk of recurrence of prostate cancer which has been strongly suggested by the present research works that found significant link between GSTP1 polymorphism and need for a repeat biopsy to evaluate a progression of prostate cancer(12).
GSTP1 is mainly expressed in the basal layer of normal prostate epithelium. Its expression has been found to be significantly down-regulated in the initial stages of majority of adenocarcinoma including the cancer prostate(13). The potential *GSTP1* gene promoter site remains unmethylated and an adenine (A) at the 303 position. Previous studies have shownthat the CpG-rich promoter region of the p-class *GSTP1*is variably methylated producing multiple restriction sites in themajority of prostate cancers(7).Another important single nucleotide polymorphism (SNP) in the *GSTP1* gene was found to be Ile105Val (rs1695 *A* > *G*) that replaced valine by isoleucine at the 105thpostion of the *GSTP1* protein causing sifnificant reduction in the detoxifying capability of this important *GST* isoenzyme(14).

However, in line with all polymorphic studies, outcomes of different studies reporting this SNP have been contradicting for prostate cancer risk varying indifferent regions of the world significantly. Some studies have reported strong association between it andprostate cancer whereas others reported their association to be negligible or nil. Two studies earlier reported more significant association of this SNP with prostate cancer in Caucasian people in comparison to the Asians and Americans(15, 16), while recent meta-analyical studies have suggested a stronger association of prostate cancer with Ile105Val among the Asian population(17). Keeping these factors in mind we hypothesized that prostate cancer is linked with this SNP of Ile105Val polymorphism (SNP rs1695 A > G) in our region and undertook the present study to ascertain its risk of association with the severity of prostate cancer in an Eastern Indian population.

2. MATERIALS AND METHOD:

The present study was a hospital based cross-sectional observational study conducted in the and Department of Biochemistry and Department of Urology over a period of 2 years from November 2016 to October 2018.

*2.1.Selection of case subjects:*Patients suffering from adenocarcinoma of the prostate gland diagnosed on the basis of clinical investigations, histopathology and prostate specific antigen were selected. At first, cases were selected provisionally on the basis of clinical

investigation at the Dept. of Urology by the method of convenience that was followed by their final inclusion by histopathology and PSA measurement. During this period all patients suffering from prostate cancers were selected irrespective of the tumor stage and their localization status. Thus, patients with both localized and metastatic disease were considered which were further given appropriate Gleason's score and ISUP grading based on histopathology. Patients with any other malignancies, metabolic disorder, smoking and alcohol addiction or any other drug addiction were excluded.

2.2.Selection of control subjects: Control subjects were selected from those patients attending urology OPD for ailments other than prostate cancers. Before their final inclusion,prostate cancer were ruled out in them by clinical investigation and PSA estimation. Subjects, suffering from any chronic inflammatory disorders, malignant diseases, metabolic diseases and addiction to smoking, alcohol or any drug were excluded.

Both case and control population were selected from the same geographical area in age matched manner with more or less similar nutritional and socioeconomic status.

2.3.Ethical considerations: The study was conducted following the guidelines and criteria for human studies as laid on by Helsinki declaration 1975 revised in 2000, and International Committee of Medical Journal Editors (ICMJE). Both informed and written consents were obtained in local language from all study participants in appropriately approved consent forms. The complete proposal was submitted to the Institutional Ethical Committee for the final approval and permission. The study was undertaken only after obtaining the written permission from the institutional ethical committee (vide CNMC/4, dated 26.10.16).

*2.4.Study technique:*3 ml of venous blood was collected in aseptic way from the participants. 1.5 ml will be stored in EDTA vial for DNA separation and rest will be stored in clotted vial for serum separation. The EDTA blood was stored at minus 20 degree centigrade till DNA isolation from which DNA was isolated within a maximum period of seven days.

2.5.Isolation of DNA: DNA were isolated from the EDTA blood by phenol chloroform extraction method as described by Stafford and Blin(18). The quality and integrity of isolated DNA were checked by gel electrophoresis in 0.7 % agarose gel and spectrophotometric quantification at 260 and 280 nm.The *GSTP1* Ile105Val (rs1695 A >

G) polymorphism were assessed by amplification of the gene by PCR technique followed by restriction digestion.

2.6.PCR technique for the GSTP1 gene: For PCR we used the PCR mastermix from Thermofisher,USA. The forward and reverse primers selected were 5′-*GTCT*CT*CATCCTTCCACGCA*-3′.and 5′-*CTGCACCCTGACCCAAGAA*-3′ respectively. We used 10 pmol of each primer in the final PCR mixture of 25µl. The PCR protocol was as follows. The initial preheating was at 95ºCfor 2 minutes, followed by 30 cycles of denaturation at 95ºC for 30 seconds,annealing at 60ºC for 30 seconds and extension at 72ºC for 1minute. After completion of 30 cycles the final extension was programmed at 72ºC for 5min. the PCR process was performed using Veriflex PCR Thermocycler (Proflex^{TM}) obtained from the Applied Biosystems, Thermofischer Scientific, USA.PCR products obtained were run in 1.2% agarose gel against 100 bp DNA ladder (Bangalore Genie, India) and were identified as 365 bp using the Gel Doc system obtained from Applied Biosystem, Thermofischer Scientific, USA.

2.7.Restriction digestion: PCR products obtained were digested using the restriction enzyme Alw261 obtained from Thermofischer USA. Restriction digestion products were identified on3% agarose gel against 100 bp DNA ladder using the gel doc system.

2.8.ISUP grading: The grading system proposed by the International Society of Urological Pathology (ISUP) have improved the overall Gleason grading system(19). Accoringly, the prostate cancer patients in our study were divided into five distinct ISUP grades; Grade 1: Gleason's score ≤6, Grade 2: Gleason's score 3 + 4 = 7, Grade 3: Gleason's score 4+3 = 7, Grade 4: Gleason's score 4+4 = 8, and Grade 5: Gleason's score 9 and 10.

2.9.Statistical Analysis:

Comparison of the distribution of different *GSTP1* alleles between the case and control groups was performed by chi square test and odds ratio analysis. Statistical comparison of ISUP grade distribution between the Ile/Ile, Ile/Val and Val/Val was done by post hoc ANOVA with Bonferroni correction. Dependence of severity of prostate cancer as indicated by ISUP grading was done by logistic regression analysis. All statistical analyses were done using SPSS software version 20 for windows.

3. RESULTS AND ANALYSIS:

Following the inclusion and exclusion criteria, finally 225 cases and 120 control subjects were selected for data analysis for statistical interpretation.

Independent t test suggested that case (and control groups were age matched in our study (mean ± SD for case and control group were 69.3 ± 1.9 and 70.2 ± 5.2 respectively, P = 0.06, data not shown in Tables).

The pattern of digestion of the PCR products of different genotypes of *GSTP1* gene and distribution of the digested fragments according to their base pair lengths through agarose gel electrophoresis is shown in the Figure 1. When compared against 100 bp DNA ladder fragments, the PCR product of *GSTP1* gene was reflected by the undigested wild *AA* genotype and was found to be of 365 bp as expected for the given set of primers (e.g. lane no. 1,6,8,9,11-15). The mutant *GG* genotype showed two digested products of 140 bp and 225 bp (e.g lane no. 3). On the other hand, the heterozygotes showed three bands of 365, 225 and 140 bp (e.g. lane nos. 2,4,5,10,16).

A significantly greater association of the *GG* genotype of the *GSTP1* gene with the prostate cancer patients in comparison to the control group was reflected by the data in Table 1 with a chi square value of 18.56 ($P < 0.001$) against a degree of freedom (d.f) of 2. This observation was strengthened by an odds ratio of 16.9 (range of 1.5 to 3.3 at 95% CI) for *G* allele in the case group (Table 2).

ISUP grades were validated in our present study by correlating them with the individual Gleason's score. The stratified grade group of ISUP showed a high degree of association with individual Gleason's score (Pearson's correlation coefficient = .923, $P < 0.001$ (Figure 2). Data in the Table 3 showed that the ISUP grading was significantly higher in those prostate cancer patients who had *G* alleles of *GSTP1* gene which suggested that the patients with valine substitution for isoleucine were more posed to the risk of advanced stages of this cancer. The higher trend of Gleason score in the *G* allelotype was also evident from the boxplot shown in the Figure 3 and 4, where the overall distribution of Gleason's scores was shown according to the different genotypic variation among the case group.

Both simple one way and Post hoc ANOVA results from the Table 3A and 3B respectively showed the individual comparison of different genotypes among the prostate cancer patients. A significantly high ISUP grade score in the mutant *GG* against the wild *AA* genotype (P = .04,

Table 3B) suggested higher risk of progressive tumour with the *GG* homozygosity. An insignificant difference between the Gleason's score values between the genotypes of *GG* and *GA* as well of *GA* and *AA* suggested that the mutant homozygote variety posed a greater risk of progressive cancer of prostate against the non mutant *AA* homozygotes. Furthermore, results of the logistic regression analysis in Table 4 suggested that the G allele has a significant contributory role in progression of the disease in contrast to the *A* allele both in its homozygous heterozygous form.

4. DISCUSSION:

The current study was conducted to detect the association of *GSTP1* genetic polymorphism with the risk of prostate cancer as well as its severity in an Eastern Indian population. A chi square value of 18.56 for a d.f of 2 in the Table 1 suggested that number of *GG* phenotypeswere significantly higher in the case group. A significantly higher association of prostate cancer with the *G* allele in *GSTP1* gene was further strengthened by an odds ratio of 2.25 with a confidence interval of 1.52 to 3.34 for 95% CI as shown in the Table 2. Our findings and outcomes are in well congruence with findings of some other studies where prostate cancers have been found to be associated with the G alleles or substitution of valine for isoleucine in the *GSTP1* gene(20).Expression of *GSTP1* is regulated mainly at the transcriptional level. It has been suggested that replacement of isoleucine with the less bulkier but more hydrophobic valine in the protein results in the alteration in substrate binding capability of its catalytic site and hence reduction in its detoxifying capability of the pro-oxidant heterocyclic amine carcinogens(21). Loss of detoxifying capability due to mutant *GSTP1* gene has been reflected by a direct association between the lipid peroxidation product 4-hydroxynonenal and prostate cancer in some recent studies(22). Results of our study not only support this view but in addition the one way (Table 3A) and post hoc ANOVA (Table 3B) suggest that the prostate cancer patients having the *G* alleles have more progressive form of the disease as evident from their higher ISUP grade. To find out the contribution of the mutant G allele on the ISUP grades in prostate cancer patients, we carried out logistic regression analysis considering the ISUP grade as a dependent variable on both A and G alleles of all three genotypes (Table 4). Results showed a significant predictive role of only G alleles on the ISUP score (regression coefficient 0.50, P = 0.0068).

Polymorphic changes from A to G not only alter the substrate binding property of this enzyme, but also alter its signal transduction process related to the regulation of cell growth. *GSTP1* enzyme protein is also closely linked to the signal transduction process involving Jun N terminal kinase (JNK pathway)(23). Moreover, its specific inhibitor TER 199 has been found to stimulate the growth of granulocytes(24) suggesting an inhibitory effect of *GSTP1* mediated intracellular signal transduction pathway on abnormal cell growth. These cellular mechanisms provide plausible biochemical explanations for the inducing effects of Ile105Val SNP (313 A to G) on prostatic carcinogenesis and its progression to more severe disorder.Hence, from our study we can conclude that *GSTP1* polymorphism in the form of Ile105Val isnot only significantly associated with risk of prostate cancer but also with its severity in our Eastern Indian population study group.

The major limitation of the present study is a relatively modest number of sample size of prostate cancer patients that could be included in the study according to the stipulated inclusion and exclusion criteria. Although, this limitation can be circumvented using larger study group, but keeping in mind several other studies performed worldwide, out study sample size was not at a lower level that much. Hence, based on our statistical calculations and result output we suggest that *GSTP1* polymorphism at Ile105Val level should be explored in much wider areas involving different regions so that it can be considered as a prognostic indicator for prostate cancer in those study populations. Assessment of this polymorphism at the earlier stages of the disease may also help in planning more aggressive management of the disease for preventing further spread and its lethal outcome.

5. REFERENCES:

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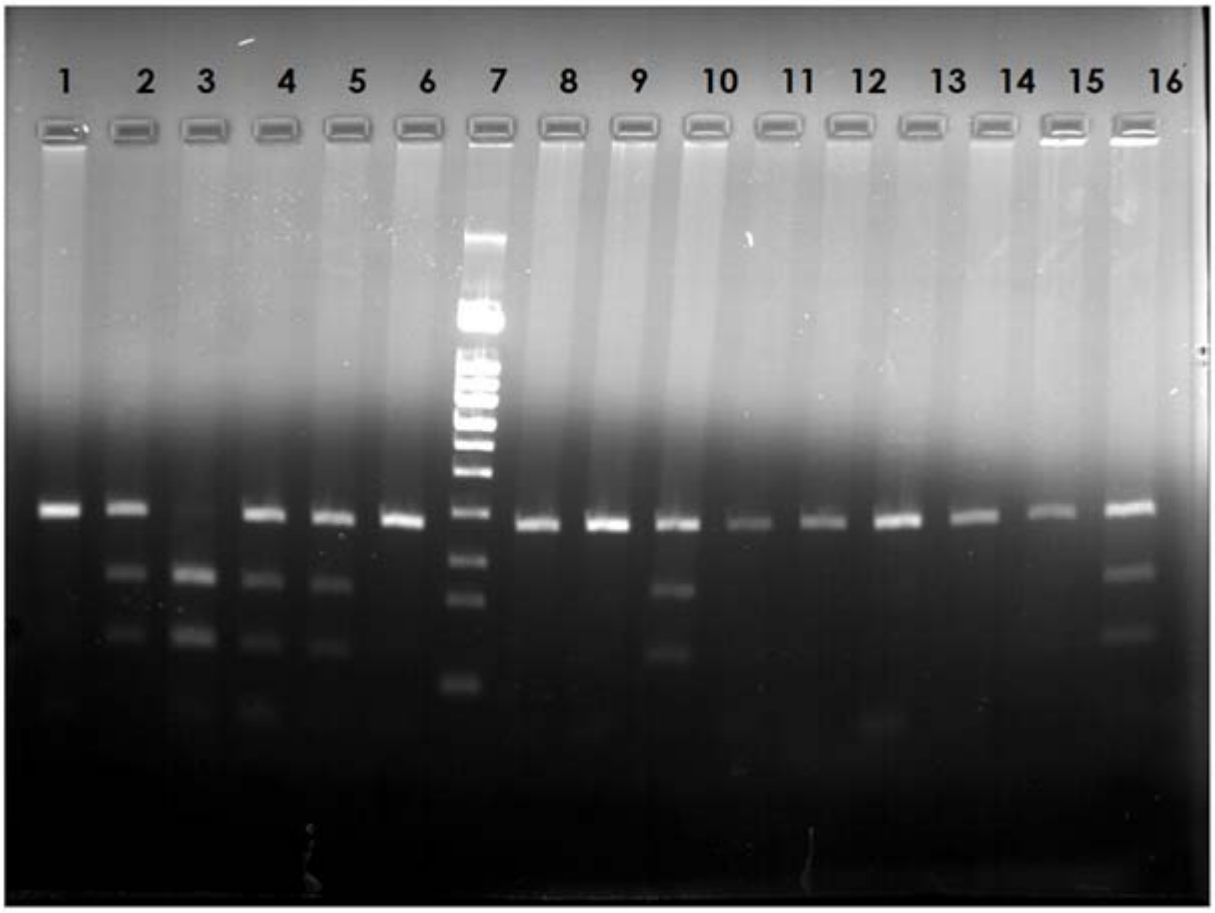
Figure 1: RFLP pattern of the polymorphic genotypes in 3 % agarose gele electrophoresis.



Lane nos. 1,6,8,9,11-15: 365 bp uncut homozygous AA
Lane no. 3: Cut homozygous GG of 225 and 140 bp
Lane nos. 2, 4, 5, 10, 16: heterozygote AG of 365, 225 and 140 bp
Lane no. 7: 100 bp ladder.

Figure 2 : Scatter plot showing the association between stratified grade grouping as indicated by ISUP grading and stage of prostate cancer as indicated by Gleason's score.

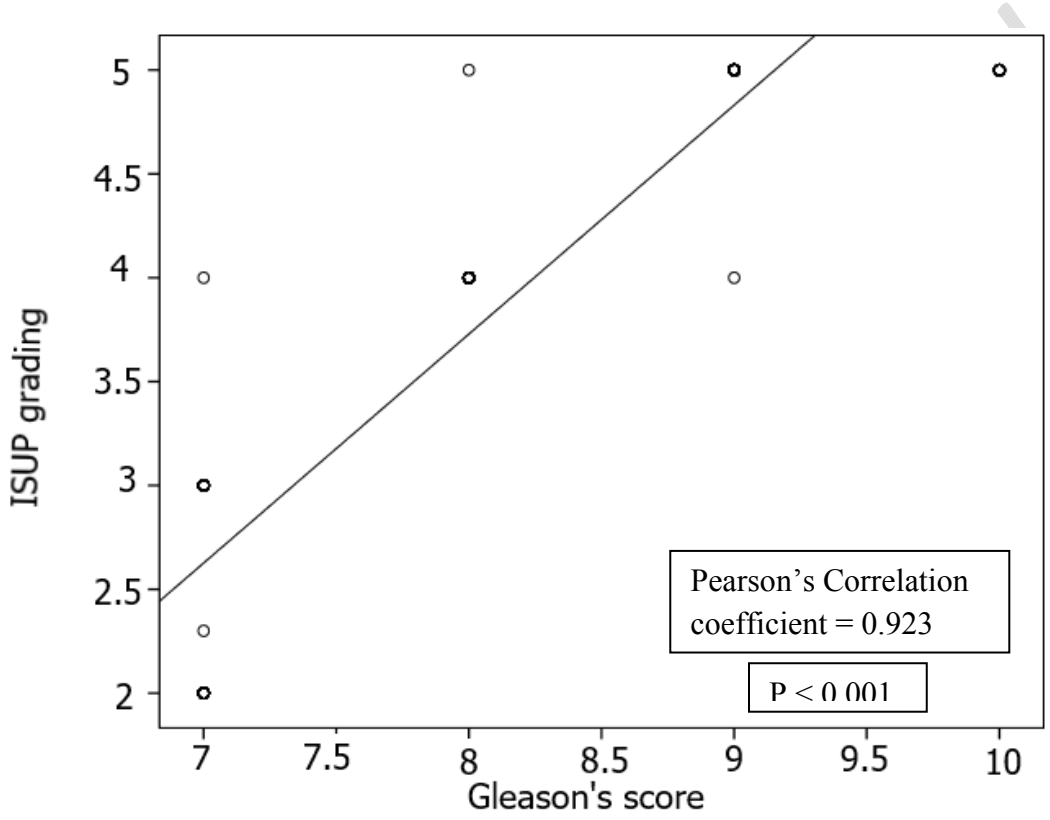


Figure 3: Boxplot showing the distribution of Gleason's score between the *II* and *VV* allele in the prostate cancer patients.

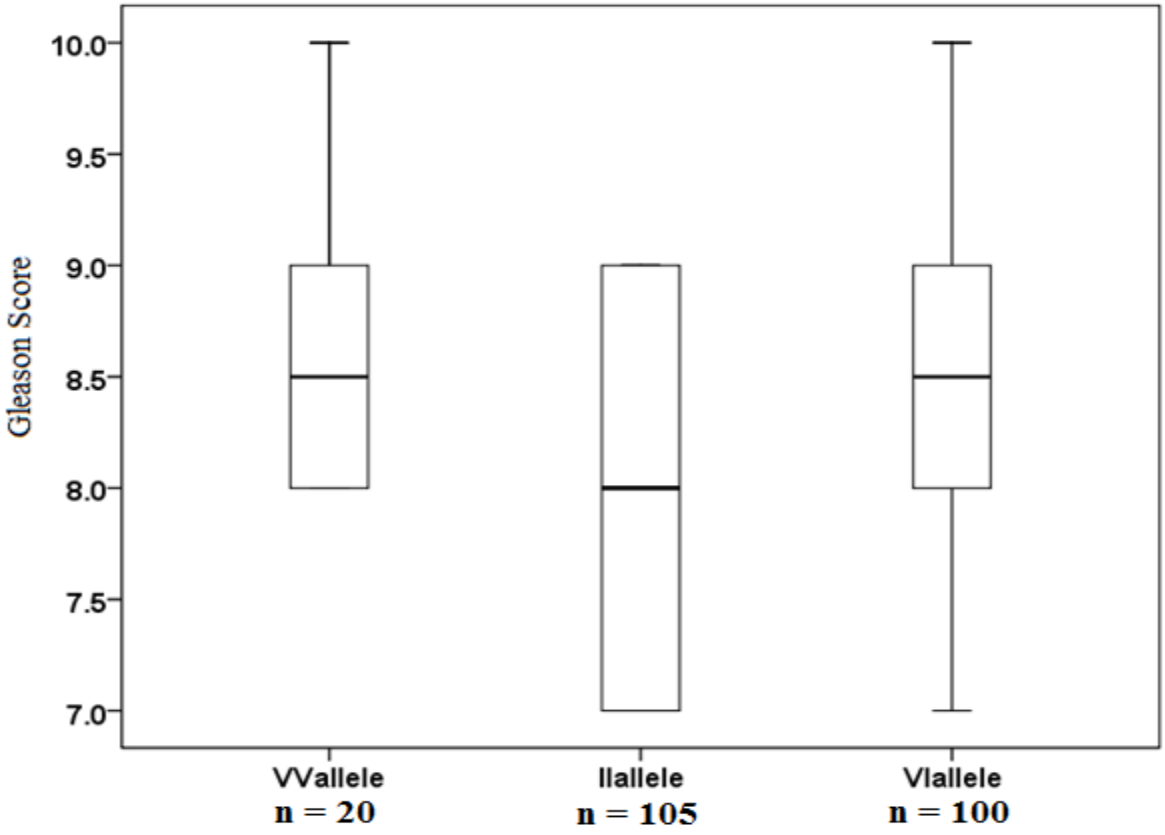


Figure 4: Distribution of Gleason score in G and A allele containing prostate cancer patients.

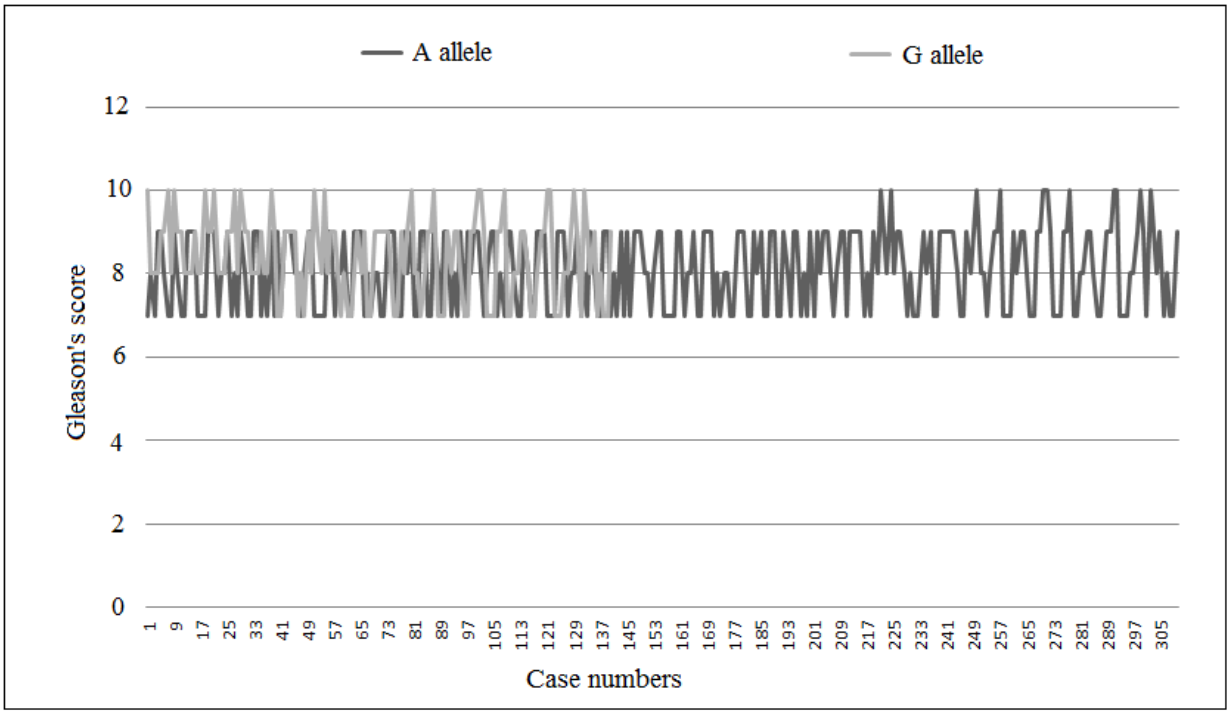


Table 1: Chi square test showing the distribution of wild and mutant variants of *GST P1* genotypes among the Case (n = 225) and Control groups (n = 120).

	Homozygote for AA (Ile/Ile)	Heterozygote AG (Ile/Val)	Homozygote GG (Val/Val)	Chi square ($\chi 2$) value	P value
Cases	105(46.6)	100(44.4)	20(9)	18.56	P< 0.001*

Controls	85(70.8)	30(25)	5(4.2)		

*P valueis significant at P < 0.05

Percentage for the respective values are shown in parenthesis.

Table 2: Odds ratio analysis for assessing the distribution of A and G alleles as risk factors between the case and control groups.

	A alleles	G alleles
Cases	310(68.8)	140(31.2)
Controls	200(83.3)	40(16.7)

P < 0.001. OR = 2.25, Range = 1.52 to 3.34 at 95% confidence interval.

Percentage for the respective values are shown in parenthesis.

Table 3A: Simple one way ANOVA test to show the overall distribution of ISUP grading among all three genotypes of prostate cancer in the present study.

All genotypes					
	Sum of squares	d.f	Mean square	F value	Sig (P value)
Between groups	10.44	2	5.22	4.04	.019
Within groups	286.48	222	1.29		
Total	296.93	224			

*P value significant at P < 0.05 for 95% confidence interval.

Table 3B: Post hoc ANOVA with Bonferroni's correction showing the distribution of ISUP gradings between the heterozygote Ile/Val allele and the homozygote wild and mutant alleles.

Bonferroni

Genotype	Group means	Mean difference (I-J)	Standard error	P value*
Ile/Ile and Ile/Val (AA and AG)	3.73; 4.06	-0.33333	0.15	0.11
Val/Val and Ile/Val (GG and AG)	4.40; 4.06	0.34000	0.27	0.66
Ile/Ile and Val/Val (AA and GG)	3.73; 4.40	-0.67333	0.27	0.04*

*P value significant at $P < 0.05$ for 95% confidence interval.

Table 4: Logistic regression analysis showing the effect of G allele on ISUP grading in prostate cancer patients.

Step 1[a]	Intercept	Slope	Exp(slope)	Regression coefficient R2	P value
Allelic variation (A or G)	-2.11	0.31	1.37	0.50	0.0068*

[a]: Dependent variable ISUP grading.
*P value significant at $P < 0.05$.