The Relation between GSTP1 Gene Polymorphism and Prostate Cancer

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Citation: Ghosh S, Dasgupta A (2022) The Relation between GSTP1 Gene Polymorphism and Prostate Cancer. J Cancer Diagn 6: 150.

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regulated in the initial stages of majority of adenocarcinoma including the prostate cancer. e potential GSTP1 gene promoter site remains unmethylated of an "A" base at the 303 position. Another important SNP in the GSTP1 gene was found to be Ile105Val (A to G) that replaced isoleucine by valine at the 105th position of the GSTP1 protein causing signi cant reduction in the detoxifying capability of this GSTP1 isoenzyme.

Interaction of GSTP1 with oxidative stress, drugs, in ammatory mediators and allergic reactions GSTP1 expression is strongly in uenced by oxidative stress as a defence mechanism through the binding of transcription factors Nrf-2 and activator protein (AP) to the antioxidant response. Reverse is also true oxidative stress can inactivate GSTP1 by intermolecular disulphide formation (Oligomerization). Several electrophilic agents induce an irreversible crosslinking of the enzyme. e components that are involved in the modi cation are most reactive cysteines. Both GSTP1 oligomerization and crosslinking a ect its interactions with signaling molecules and stress cascades. GSTP1 displays multiple interactions with drugs, either catalysing their detoxi cation by GSH (S-Glutathionylation) conjugation or being inactivated by them. ese interactions are crucial for cancer therapy [4] and (Figure 1).

Materials & Methods

Subject and specimen collection

Patients diagnosed with adenocarcinoma of the prostate gland on the basis of clinical investigations, histopathology and prostate speci c antigen were selected. At rst, cases were selected provisionally on the basis of clinical investigation. As PSA is speci c for prostate tissue and not for prostate cancer only, there is a considerable overlapping of the PSA values between BHP and CA prostate. So, it is di cult to assign a PSA value with 100% sensitivity and speci city for CA prostate only.

is was a hospital-based case-control study. Case-control studies are o en used to identify factors that may contribute to a medical condition by comparing subjects who have that condition/disease (the "cases") with patients who do not have the condition/disease but are otherwise similar (the "controls"). Here, the control is that human gene of GSTP1 where mutation has not occurred and prostate cancer is not diagnosed. ese will be the basis of comparison to others samples.

Enzymes used in this experiment

Restriction endonuclease: e restriction enzyme used to determine the SNPs (single nucleotide polymorphism) here is BsmA1. It is a unique restriction endonuclease, which is extracted from the organism Bacillus Stearothermophilus. e cleavage site was rst determined from bacteria by the method of Brown et al. Incubation temperature is 55°C.

Recognition site is:

5' GTCTC 3'

3' CAGAG 5'

Cleavage site is:

G T C T C N/N N N N
C A G A G N N N N N/

It is type 1 restriction enzyme.

Isochizomers-Pairs of restriction enzymes speci c to the same recognition sequence.

Isochizomers of BsmA1 are Alw261, BcoDI [5].

Taq polymerase: is is a kind of DNA polymerase that used for PCR which is extracted from the organism *ermus Aquaticus.* is DNA polymerase can tolerate and act in high temperature (upto approx 95°C). It can extend a new DNA strand at temperature of 72°C by template strands in PCR cycle to amplify the gene of interest (Figure 2).

PCR optimization

Perfect thermal cycling conditions.

Speci city depends on the choice of primers and Mg2 + concentration.

Primer Designing

e basic guidelines are:

Self-complementary primers were avoided. Especially complementary more than 3 bp should not be present or else it will form a hairpin loop.

Primers were chosen that are speci c to the target. Simple sequence repeats or commonly repeated sequences were avoided. If the target has close relatives the primers should be designed in such a way that it anneals to the target site only.
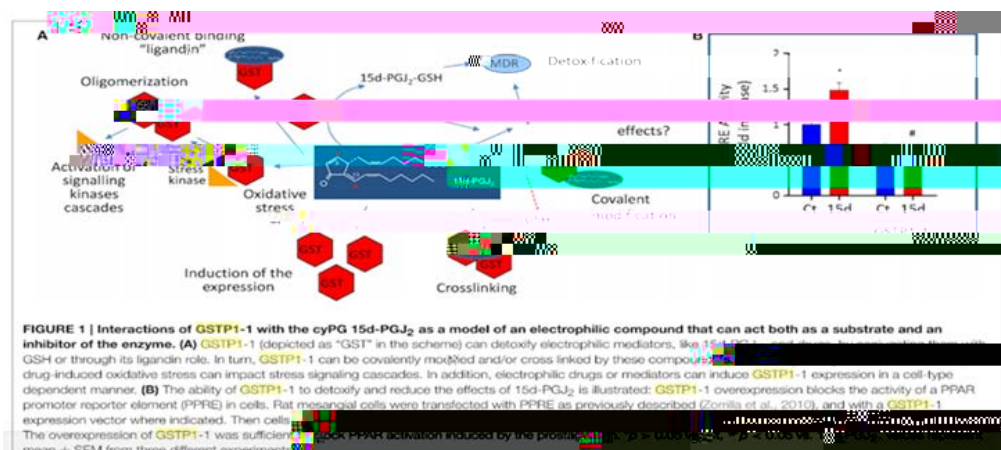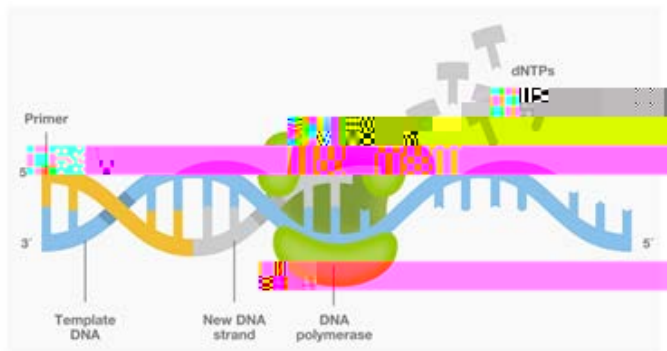

Figure 1: Interaction of GSTP1 with di ferent compounds.

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Figure 2: Taq polymerase mechanism.

Primers between 18 – 25 bp were used that have matched melting temperature (Tm) to each other. A primer greater than 17 bp has a good chance of being unique in the human genome.

It was better to avoid amplifying longer targets, product length less than 500 bases were recommended. Shorter products amplify with higher efficiency.

Complementary between members of primer pairs should be avoided. The 3' complementary is detrimental. Primer dimer will compete for DNA polymerase, primers and dNTPs which will suppress amplification.

Depending on the purpose of the experiment the placement of the priming sites should be taken into consideration. Generally forward and reverse primers bind to sequences in different exons [6].

Positive and Negative control for PCR

Laboratories using PCR should analyze positive and negative quality control samples on a routine basis to demonstrate the adequate performance of PCR-based methods.

Positive controls were used to verify that the method was capable of amplifying the target nucleic acid from the organism of interest. In this, a known sample was given with the same master mix and dNTPs to see if it was properly amplified.

A negative control was used to verify that no contaminating nucleic acid had been introduced into the master mix, distilled water, and dNTPs. In this only master mix, dNTPs and water are given and PCR cycle is performed along with our sample.

A negative control was one expected not to work under the conditions.

A positive control was one expected to work and to provide you with the expected known result.

Methods in the specific order

Genomic DNA extraction: Blood was taken in sufficient amount. Organic (phenol–chloroform) extraction uses sodium dodecyl sulphate (SDS) and protease for cell lysis by the enzymatic digestion of proteins and non-nucleic acid cellular components. A mixture of phenol: chloroform: isoamyl alcohol (25:24:1) was then added to promote the partitioning of lipids and cellular debris into the organic phase and breaking RNA by adding RNase, leaving isolated DNA in the aqueous phase. Following centrifugation, the aqueous phase containing the purified DNA can be transferred to a clean tube for analysis. After

isolation, the DNA was dissolved in slightly alkaline buffer, usually in the TE buffer [7].

Checking the viability of DNA:

Using spectrophotometer –It was used to check the amount of DNA. First the OD was measured at 260 nm for DNA. Then OD was measured at 280 nm for protein. Ratio between the two was taken i.e. OD260/OD280 > 1.8 (Figure 3).

Using gel electrophoresis- It was used to check the integrity of the DNA. If weak DNA is obtained we will get smeared DNA after gel electrophoresis. If DNA is normal we will get bands at specific position. Prepared 0.7% agarose gel.

30 ml TEA buffer+ 0.21 g agarose was taken and mixed properly.

Then microwave for 1 min to dissolve it properly. EtBr was then added to visualize in UV ray. Leave sometimes to cool a bit. Put it in the gel tray, insert the comb and allow solidifying. Put it in the chamber, submerge it with TEA running buffer and insert the DNA samples with the tracking dye (Figure 4).

PCR amplification of GSTP1: For PCR amplification four things are required-

i. Primer (Forward & reverse)

ii. Master mix (MgCl2 , buffer, dNTPs, Taq polymerase)

iii. DNA Template

iv. Deionised & nuclease free double distilled water.

Primers were designed accordingly from NCBI primer BLAST. The forward and reverse primers selected were 5ʹ-GTCTCTCATCCTTCCACGCA-3ʹ and
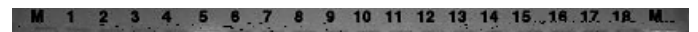

Figure3: DNA bands.

Figure4: Smeared DNA.

5 -CTGCACCCTGACCCAAGAA-3 respectively.As per the protocol each PCR tube should be of 25µl   lled. 12.5µl Master mix, 2µl primers, 1µl DNA template, 9.5µl distilled water are added in each PCR tube.
   e PCR should be programmed as follows-

3) and 32nd (lane 5) samples were uncut and sharp band occurred at 365 bp position. Sample number 34th (lane 4) & 36th (lane 6) was cut into 3 bands at 365 bp, 225 bp, 140 bp positions measured by 100 bp ladder (Figure 7).

Case II

i.   Some prostate adenocarcinoma positive DNA samples were taken and those sample numbers are 51, 52, 54, 55, 57, 58, 59, 61, 62, 64, 66, 82, 83 & 84. Then we check the amount of DNA of those samples by spectrophotometer and check the integrity of DNA of those samples through electrophoresis by 0.7% agarose gel. We saw by gel doc system that 57th sample give a very week band and 64th, 66th & 84th samples give bright and strong band. So we took 57,64,66,84 number samples for PCR.

ii.   Then create a master mix as per above protocol by taking amount for one extra sample means for 5 samples. In each PCR tube, 24 μl master mix and 1.2 μl selected DNA samples were distributed. These 4 samples were set to run in gradient PCR for 30 cycles with same programming.

iii.   After PCR completed, loading dye was added in each of 10μl sample. Centrifuge all samples by 6000 rpm for 1 min. Then samples were run to electrophoresis by 2% agarose gel by putting samples in wells (lane 1- ladder, lane 2- 57th, lane 3- 64th, lane 4- 66th, lane 5- 84th).

A er running we saw in gel doc system that 57th & 84th sample not ampli ed and bands not occurred. 64 & 66 samples able to occur clear band at 365 bp measured by ladder.

iv.   Then we took the previous DNA samples 35th & 37th which were uncut a er restriction digestion. So we ready more 3 PCR tubes with these 2 samples as 35th, 37th, & 37th duplicate (37D). Performed in gradient PCR for 30 cycles with same programming. A er PCR completed, loading dye was added in each of 10μl sample. Centrifuge all samples by 6000 rpm for 1 min. Then samples were run to electrophoresis by 2% agarose gel by putting samples in wells along with 64th & 66th samples.

A er running we saw in gel doc system that every sample gave a clear band at 365 bp position (Figure 8). But as 35th & 37th sample was performed upto digestion previously so that we didn't take 35, 37, 37D samples now for digestion. We took 64th & 66th samples for digestion.

v.   For restriction digestion, the amount of BsmA1, distilled water, 10x bu ers was taken for total 2 samples. is mixture was equally distributed in rest of each samples as per above protocol and made the total volume of 25 μl each. en incubate it for approx. 2 hrs. A er incubation, those digested 2 samples were allow to run in electrophoresis by 2.5% agarose gel a er adding tracking dye into the samples (lane 1- ladder, lane 2- 64th, lane 3- 66th).

A er running we saw in gel doc system that both samples remain uncut and gave a clear band at 365 bp position (Figure 9).

Case III (control)

i.   Some prostate adenocarcinoma negative DNA samples (un-mutated DNA) were taken and samples are 2, 4, 10, 16, 24, DNA control (C). We check the integrity of DNA of those samples through electrophoresis by 0.7% agarose gel. We saw by gel doc system that sample number 2 (lane 2) & 24 (lane 6) gave very weak band and sample number 4, 10, 16, C (lane 3,4,5,7 respectively) gave very strong band (Figure 10). So we took 4, 10, 16, C samples for PCR.

ii.   Then create a master mix as per above protocol by taking amount for one extra sample means for 5 samples. In each PCR tube, 24 μl master mix and 1.2 μl selected DNA samples were distributed.

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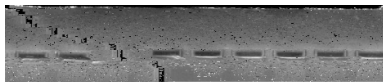**Figure10:** Lane 2, 6- gave weak band. Lane 3, 4, 5, 7- gave strong band.

Figure 11: Lane 2, 3, 4, 5- amplified & give band at 365 bp but lane 5 give very weak band Lane 1- 100 bp ladder.

ese 4 samples were set to run in gradient PCR for 30 cycles with same programming.

iii.   A er PCR completed, loading dye was added in each of 10μl sample. Centrifuge all samples by 6000 rpm for 1 min.   en samples were run to electrophoresis by 2% agarose gel by putting samples in wells (lane 1- ladder, lane 2- 4th, lane 3- 10th, lane 4- 16th, lane 5- C).

A er running we saw in gel doc system that all samples were ampli ed and 4th, 10th, 16th samples gave clear sharp band and sample C gave very li ed and 4tyere set to run in gradient PCR for 30 cycles with same
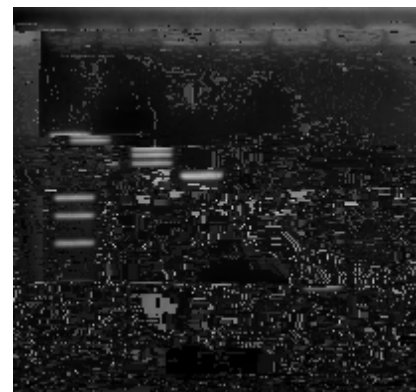

Figure 13: PCR product not amplifed during 1st time case II Lane 1- 100 bp ladder.

16 remains uncut and give a clear band at 365 bp position and sample C also uncut and give a so much mild band at 365 bp position. But the sample number 10 didn't give any band and get smeared may cause of over digestion (Figure 12).

Note that:- In case II we repeated the PCR stage for 2nd time because in 1st time the PCR products were not ampli ed and not come any bands while viewing through gel doc system causes any kind of manual errors (Figure 13).

Statistical analysis

e results are represented on pie chart (Figure 14) -

Comparison of the distribution of di erent GSTP1 alleles between the case and control groups is performed by chi square test and odds ratio analysis.

Chi Square test:

Case (n=7) & Control (n=4)

Due to very small sample size chi square test cannot be performed (Table 1).

Odds Ratio:

Now,

O.R. = 0.5, Range = 0.056 to 4.474 at 95% con dence interval (Table 2).

Discussion

By this project we used to found that how many types of genotypes or alleles may occur by SNPs which are the main cause of prostate adenocarcinoma.

We saw that AG and GG alleles of GSTP1 gene polymorphisms are considered as risk for prostate cancer. In case I digestion we obtain AG genotype of polymorphism; in case II digestion we don't obtain any polymorphic genotype; but in case III of control sample digestion we unfortunately obtain GG genotype of polymorphism that instead of control sample, it also very near to the case which are positive in prostate adenocarcinoma.

Chi-square test cannot be performed due to low data TP1 gse. It is also true that the chi-square test is performed only if at least 80% of the cells have an expected frequency of 5 or greater, and no cell has an expected frequency smaller than 1.0.

A signi cantly higher association of prostate cancer with the G allele in GSTP1 gene by the odds ratio of 0.5 with a 95% con dence interval of 0.056 to 4.474. is means that the chances of having prostate cancer with G allele are 0.5 times more than with association to an allele. However, a major drawback of this result is due to extremely small TP1 gs size.

GSTP1 gene polymorphisms may be considered as factors increasing the susceptibility to and risk of Hepatocellular carcinoma (HCC). HCC is one of the most frequent malignant neoplasms in the world. Genetic polymorphism has been reported to be a factor increasing the risk of HCC [8].

Expression of GSTP1 is regulated mainly at the transcriptional level. It has been suggested that replacement of isoleucine with the gses 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 [9].

GSTP1 polymorphism is signi cantly associated with risk of prostate cancer. GSTP1 polymorphism should be considered as a prognostic indicator for prostate cancer patients [10].

We have observed the polymorphism in the GSTP1 gene. Polymorphisms are the low-penetrable mutations that make us all To subtlety di erent. It is estimated that up to 85% of such theoretical SNPs cannot be con rmed in population statistic studies but in this project, we are already aware of the population statistics from the literature available. GSTP1 polymorphisms are important in mediating clinical phenotypes for cancers including prostate cancer. We have undergone to prove the relation between GSTP1 polymorphism and prostate cancer and we have learned the processes involved, to perform the experiment.

Conclusion

From our study, we conclude that prostate cancers have been found to be associated more with the G alleles or substitution of valine from isoleucine in the GSTP1 gene. It is also true that GSTP1 polymorphism of A to G is signi cantly associated with the risk of prostate cancer. us, it should be considered as an important biomarker for detecting prostate cancer.

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