Association of NAT2, GSTT1 and GSTM1 gene polymorphism and influence of oxidative stress on prostate cancer risk in Bangladeshi population

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To Whom It May Concern

This is to certify that **Ayatun Nesa,** under our supervision has completed the thesis work titled **"Association of NAT2, GSTT1 and GSTM1 gene polymorphism and influence of oxidative stress on prostate cancer risk in Bangladeshi population"** in the Department of Biochemistry and Molecular Biology, University of Dhaka for the partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry and Molecular Biology, University of Dhaka.

Her work is genuine, and she carried out the work sincerely and devotedly up to our satisfaction.

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Ayatun Nesa

Declaration

I hereby humbly declare that this thesis titled **"Association of NAT2, GSTT1 and GSTM1 gene polymorphism and influence of oxidative stress on prostate cancer risk in Bangladeshi population"** is based on my own work and has been carried out under the direct supervision of Prof. Yearul Kabir, Professor, Department of Biochemistry and Molecular Biology, University of Dhaka, as partial fulfillment of the requirements for the degree of Doctors of Philosophy (PhD).

No part of this thesis work has been submitted for another qualification of the University of Dhaka or any other University or any other institute of learning.

Ayatun Nesa

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List of Acronyms

AJCC	American Joint Committee of Cancer
ASS	
BIRDEM	Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorder
BSMMU	Bangabandhu Sheikh Mujib Medical University
BMI	Body Mass Index
CI	Confidence Interval
DMCH	Dhaka Medical College Hospital
DNA	Deoxy Ribo Nuclease
D/R/E	Digital Rectal Examination
EDTA	Ethylene Di Amine Tetra Acetic Acid
GSH	Glutathione
GST	Glutathione S Transferase
GSTT1	Glutathione S Transferase Theta 1
GSTM1	Glutathione S Transferase Mu 1
MDA	Malondialdehyde
NAT2	N Acetyl Transferase 2
OR	Odds Ratio
PCR	Polymerase Chain Reaction
Pca	Prostate Cancer
PSA	Prostate Specific Antigen

ROS	Reactive Oxygen Species
rmp	Revolutions Per Minute
RFLP	Restriction Fragment Length Polymorphism
SNP	Single Nucleotide Polymorphism
SPSS	Software Statistical Package for Social Science
TNM	Tumor/ Nodes/ Metastasis
USG	Ultra Sonogram
UTI	Urinary Tract Infection

Abstract

Prostate cancer (PCa) is the second leading cause of cancer-related mortality in men. Prostate epithelial cell express phage II metabolizing enzymes, and recent molecular epidemiological studies have analyzed the relationship between N acetyltransferase and Glutathione S transferase gene polymorphism in the etiology of prostate cancer. It has been reported that NAT2 slow acetylators may be at increased risk of prostate cancer, due to their slower inactivation of environmental arylamines carcinogens. A systemic review and meta-analysis of GSTT1 and GSTM1 gene polymorphisms and prostate cancer risk in Asians showed that GSTT1 null genotype and GSTM1 null genotypes were significantly associated with prostate cancer risk. Oxidative stress has long been implicated in cancer development and progression. MDA is the product of lipid peroxidation, and erythrocyte reduced glutathione (GSH) is the major part of the antioxidant defense mechanism. Disturbances in GSH homeostasis are involved in the etiology and progression of many human diseases, including prostate cancer. Zinc is an element with biological functions as catalysts in various enzymatic reactions. Many studies around the world showed serum zinc has an association with prostate cancer. This study was undertaken to evaluate NAT2, GSTT1 and GSTM1 gene polymorphism and the influence of oxidative stress on prostate cancer risk in the Bangladeshi population. This case-control study included 207 histopathologically confirmed cases of prostate cancer that had not undergone any chemotherapy or radiotherapy and 200 age-matched healthy controls with the same geographical areas and ethnicities. After taking informed written consent, preset questioners were filled up, and about 6 ml of venous blood were collected with all ascetic precaution from each study subject. 3 ml blood was collected in an EDTA containing vial and processed for DNA extraction for NAT2, GSTT1, and GSTM1 gene analysis by PCR and erythrocyte reduced glutathione estimation. Serum was separated from the remaining 03 ml blood (contained in clot activator test tube) for biochemical analysis of serum PSA, MDA, GST, and Zinc. All data were plotted in SPSS version 23, and different statistical analyses were done. In this study, the mean age of cases was 67.3±8.3, and among control, it was 62.2±6.8 years. Study of genotype distribution and allele frequency of NAT2 polymorphism in study subjects found that prostate cancer cases had higher frequencies of mutant NAT2*5A (9.7% Vs. 5.5%), NAT2*6A (9.2% Vs. 5.0%) and NAT2*7A (5.3% Vs. 1.5%), in comparison

to control. 19.8% of prostate cancer patients had slow genotypes for NAT2, and 80.2% had fast/rapid acetylator genotype, odds ratio (OR) (95% CI) was 1.90 (1.11-3.32), and results were statistically significant. NAT2 slow acetylator genotypes had a significantly higher risk for the development of moderate to high-grade tumors (Gleason score≥7) (OR=3.91, 95% CI, (2.11-7.15), $p<0.001$). 30% of a prostate cancer patient had null genotypes for GSTT1, and 37.7 % had null genotypes for GSTM1. The frequency of GSTT1 null genotype was slightly higher in prostate cancer cases than control with an OR of 1.45, but it was not statistically significant. On the other hand, the frequency of GSTM1 null genotype was significantly higher, with an OR of 1.71 in prostate cancer cases than control. Significantly higher risks for the development of high-grade tumors were also found for both GSTT1 and GSTM1 null genotypes. The combined genotype study of GSTT1 (null) / GSTM1 (null) showed a highly significant association with prostate cancer risk, and the risk increased 5.75-fold, compared to normal genotype for GSTT1 and GSTM1. However, the risk increased 5.54-fold for NAT2 (rapid) / GSTT1 (null) / GSTM1 (null) combination genotypes and risk increased 9.64-fold for NAT2 (slow) / GSTT1 (null) / GSTM1 (null) combination genotypes. Significantly higher risks for the development of high-grade tumors were also found for both GSTT1 and GSTM1 null genotypes. Oxidative stress marker malondialdehyde was significantly increased in prostate cancer patients than control. On the other hand, antioxidant erythrocyte reduced glutathione, and serum zinc level was significantly reduced in prostate cancer cases than control. In conclusion, significantly higher frequencies of mutant and heterozygote NAT2*6A and NAT2*7A/B genotypes were found in prostate cancer patients of the Bangladeshi population. There was a significant association of NAT2 slow genotypes and GSTM1 null genotypes with prostate cancer in the Bangladeshi population. Individuals with NAT2 slow genotype, GSTT1 null genotype, and GSTM1 null genotype along with positive smoking history/ positive family history of cancer / increased oxidative stress had increased risk for the development of prostate cancer. Presence of combined genotypes GSTT1 (null) / GSTM1 (null) or NAT2 (rapid) / GSTT1 (null) / GSTM1 (null) or NAT2 (slow) / GSTT1 (null) / GSTM1 (null) showed highly significant association with prostate cancer risk.

1. Introduction

Prostate cancer (PCa) is the most widely recognized noncutaneous malignancy among men and is the second leading cause of cancer-related mortality (Jemal et al., 2008; Dianat et al., 2009 and Bray et al., 2018). According to GLOBOCAN 2018 estimates, in the year of 2018, 1,276,106 new prostate cancer cases were enlisted around the world, denoting 7.1% of all cancers in men (Bray et al., 2018). Prostate cancer incidence rates are highly fluctuating across the globe. The age-standardized rate (ASR) was utmost in Oceania (79.1 per 100,000 people) and North America (73.7), trailed by Europe (62.1) (Ferlay et al., 2019). African-American men have the highest incidence of prostate cancer and more prone to be struck with the disease earlier in life in comparison to other racial and ethnic groups (Kheirandish et al., 2011). In Bangladesh, the frequency of prostate cancer is low compared to the developed world, but its prevalence has shown an increasing trend (Salam, 2014). From 2018 to 2040, it is assessed that the mortality will be twofold, with 379,005 deaths worldwide. The highest mortality rate is anticipated to be in Africa (+124.4%), followed by Asia (116.7%), while the least rate will be enrolled in Europe (+58.3%) (Ferlay et al., 2019). Despite the higher incidence rate in developed countries compared to least developed countries (69.5 vs. 14.5), the differences in mortality data were cooperatively modest (10.0 vs. 6.6) (Wong et al., 2016). Considering that limitation in medical care and other facilities in developing countries, it causes high mortality despite the lower incidence (Rawla, 2019).

The etiology of prostate cancer is not well comprehended; however, studies inspecting genetics, diet, lifestyle, and exposure to certain chemicals are increasingly attracting attention (Hein et al., 2000). Susceptibility to most human diseases and disorders is complex and multifactorial, involving genetic and environmental factors. While any two unrelated people share about 99.9% of their DNA sequences, the extra 0.1% is noteworthy. It contains the genetic variants that impact how individuals differ in their risk of disease or their response to drugs and environment exposures (Hung et al., 2004). The biochemical basis for the individual distinction in the vulnerability to carcinogens may be attributed to the genetic polymorphisms of genes related to the metabolic detoxification of environmental carcinogens (Amankwah et al., 2012; Pan et al., 2012). Polymorphic distribution of the enzymes involved in the activation and/ or deactivation of aromatic amines in humans is an important determinant of individual susceptibility to their carcinogenic effects (Hamasaki et al., 2003). Prostate

epithelial cell express phage II metabolizing enzymes, and recent molecular epidemiological studies have reported the relationship between N acetyl transferase and Glutathione S transferase gene polymorphism in the etiology of prostate cancer (Wang et al., 1999; Agundaz et al., 1998; Mittal et al., 2004).

N-acetyltransferases (NATs) are polymorphically expressed enzymes of phase II biotransformation reactions. They are involved in inactivation or detoxification of many carcinogenic compounds and therapeutic drugs (Meisel, 2002; Westwood et al., 2006; Boukouvala and Fakis, 2005). There are two active isoenzymes found in humans, termed as NAT1 and NAT2. Both NAT1 and NAT2 genes are located on the short arm of human chromosome 8. They encode functionally active proteins that share 81% sequence identities. (Meisel, 2002; Pompeo et al., 2002). Though there is correspondence in substrate specificity, NAT2 is predominantly responsible for drug metabolism (Westwood et al., 2006; Boukouvala and Fakis, 2005; Gross et al., 1999). Therefore, individual variations in NAT2 activity may affect the clinical consequence of NAT2-substrates therapy (Bakayev et al., 2004). Most of the NAT2 allelic variants share one or more common missense single nucleotide polymorphism(s) [SNP(s)], that results, low expression, reduced activity, or enzyme instability (Pompeo et al., 2002). Heterocyclic arylamines are the ultimate critical carcinogens that are directly implicated in the tumor initiation process. NAT2 takes part in the bioconversion of those heterocyclic arylamines into electrophilic nitrenium ions (Srivastava et al., 2005b), thus preventing the tumor generation process. There are about 29 NAT2 alleles have been identified, causing the gene polymorphic. Some of these polymorphisms have an impact on enzyme activity. NAT2 activity is expected from the uncovered combination of these NAT2 alleles. The presence of at least one wild type allele results in rapid acetylator, whereas the presence of two mutant alleles is considered a slow acetylator (Inatomi et al., 1999). The NAT2 slow allele is predominant in the Arab population (about 90%), subsequently in Caucasians, including Indians (about 40-60%) and about 5-25% in East Asians (Whoolhouse et al., 1997; Xie et al., 1997). Anitha et al. (2003) found that 74% of South Indians had a Nat2 slow allele in their study. The phenotype of NAT2 studied on the Bangladeshi population showed 79.5% are fast acetylators, and the rest, 20.5% are slow acetylators (Zaid et al., 2004). It has been reported that slow acetylators may be at increased risk of prostate cancer when exposed to environmental arylamine carcinogens due to their slower inactivation (Srivastava et al., 2004). Several studies suggested that NAT2 genotype may play a vital role in identifying the risk of developing prostate cancer. A meta-analysis of NAT1 and NAT2 polymorphisms and prostate cancer by Gong et al. (2011) found a

significant association of NAT2 polymorphism and prostate cancer in Asians, but not in Caucasians. A case-control study in Turkey by Kosova et al. (2010) reported that Nat2* 6A and NAT2 *7A/B gene polymorphisms were significantly associated with prostate cancer in the Turkish population. In another case-control study in India by Srivastava and Mittal (2005b) observed a trend of association of NAT2 (OR=1.452, CI95%, 0.5-1.87, p=0.136) gene in prostate cancer risk. Ethnic differences in NAT2 genotype frequencies may be a factor in cancer incidence.

Glutathione S transferase (s) (GSTs) are a family of phase II metabolizing enzymes involved in the detoxification of various exogenous and endogenous reactive species (Hengstler et al., 1998). The GSTs conjugate glutathione to several potentially carcinogenic compounds, reactive oxygen species, and chemotherapeutic agents, with a variety of substrate specificities (Rebbeck, 1997). In mammals, eight classes of GSTs namely, alpha (α), mu (μ), Pi (π), Theta (θ), sigma (σ), Kappa (κ), Omega (ω) and Zeta (ζ) have been identified based on sequence homology and substrate specificity (Mannervik et al., 1992). Among these, Glutathione S transferase mu 1 (GSTM1) and Glutathione S transferase theta 1 (GSTT1) are extensively studied. The polymorphisms of GSTM1 and GSTT1 gene loci are caused by a gene-deletion, resulting in the absence of enzyme activity in individuals with the GSTM1 and GSTT1 homozygous null genotypes (Sharma et al., 2015). The GSTM1 and GSTT1 null genotypes have widely been studied in various human populations, and their ubiquitous existence is well demonstrated (Garte et al., 2001; Gaspar et al., 2002; Buchard et al., 2007; Saadat, 2007; Fujihara et al., 2009; Piacentini et al., 2011). For example, the prevalence of the GSTM1 null genotype in Caucasians, Asians, and Africans was 47~57%, 42~54%, and 16~36%, respectively, while the prevalence of the GSTT1 null genotype in Caucasians was rather low as 13~26% but common in Asians (35~52%) (Garte et al., 2001). These differences in the frequencies of the GSTM1 and GSTT1 null genotypes among human populations may be related to population-specific disease susceptibilities (Saitou and Ishida, 2015). GSTT1 and GSTM1 polymorphisms have been implicated as risk factors for prostate cancer (Ntais et al., 2005; Mittal et al., 2004). In human GSTT1 and GSTM1 gene families display a genetic variability due to polymorphic deletions in the respective genes (Safarinejad et al., 2011). A systemic review and meta-analysis of GSTT1 and GSTM1 polymorphisms and prostate cancer risk in Asians showed that GSTT1 null genotypes and GSTM1 null genotypes were significantly associated with prostate cancer risk (Hein et al., 2000). However, the epidemiologic studies performed over the past few decades for an association between GSTT1 and GSTM1 polymorphism and prostate cancer

are confusing, predominantly because of heterogeneity in the data and study design or disagreements among the reported investigations.

Oxidative stress is a leading cause of vascular endothelial damage. Ischemia increases the level of reactive oxygen species, growth factors, and cytokines and provokes the development of angiogenesis, resulting in cancer progression (Ohtake et al., 2018). Significant clinical and preclinical evidence was found for increased cellular reactive oxygen species (ROS) and impaired protective mechanisms as drivers of prostate cancer susceptibility (Oh et al., 2016). Glutathione and glutathione-dependent enzymes, such as Glutathione-S-transferases, are involved in cell defense against reactive oxygen species (ROS) (Baltruskeviciene et al., 2016). Besides taking part in the antioxidant defense system, glutathione plays an essential role in many processes on a molecular, cellular, and organism level. Therefore, disruptions in the glutathione system homeostasis are involved in pathogenesis and progression of cancer (Traverso et al., 2013). Some authors have found that erythrocyte reduced glutathione (GSH) levels decreased in prostate cancer patients (Surapaneni et al., 2006; Srivastava and Mittal, 2005a). Glutathione S transferase (GSTs) catalyzes the conjugation of GSH-via a sulfhydryl group-to electrophilic center on a wide variety of substrates, converting the compounds more water-soluble. Previous studies found GSTs activity was altered in prostate cancer patients (Surapaneni et al., 2006). Lipid peroxidation is a free-radical mediated chain of reaction that, once initiated, results in an oxidative deterioration of polyunsaturated lipids, among which most common targets are the biological membrane components (Grotto et al., 2009). Malondialdehyde (MDA) is an end of the peroxidation of polyunsaturated fatty acids and related esters (Merendino et al., 2003). The lack of valid biomarkers has hampered clinical research in the area of lipid peroxidation. Therefore, the most frequently used biomarkers indicating the overall lipid peroxidation level is the plasma concentration of MDA (Nielsen et al., 1997). Highly reactive aldehydes (MDA), products of lipid peroxidation, are capable of modifying both DNA and proteins, resulting in the mutagenic, genotoxic, and cytotoxic events (Merendino et al., 2003). Therefore, many of the literature reported that MDA levels were significantly high in patients with prostate cancer (Grotto et al., 2009). The prostate contains the highest concentration of zinc of all the soft tissues, but zinc concentrations found decreased significantly during prostate cancer. A growing body of experimental evidence supports the notion that high zinc levels are essential for prostate health and may limit prostate cancer development (Cortesi et al., 2009; Wagner et al., 2009).

Several comprehensive studies were done regarding the association of NAT2, GSTT1, and GSTM1 gene polymorphisms and the influence of oxidative stress on prostate cancer, but limited data were available in Bangladesh. Therefore, the present study is aimed to investigate the association of NAT2, GSTT1, and GSTM1 gene polymorphisms, and the influence of oxidative stress in prostate cancer patients in the Bangladeshi population.

Purpose and Objectives of the Study:

Prostate cancer is a common progressive disease of males, and the mortality rate in untreated prostate cancer cases ranges from 75% to 95%. The etiology of prostate cancer is not well understood; however, studies examining genetics variants that contribute to disease risk offer one of the best opportunities to understand the complex causes of disease in humans. Several studies found a relationship between N- acetyltransferase and Glutathione S transferase genes polymorphism and oxidative stress in the etiology of prostate cancer. Antioxidants have a potential role in protecting men from prostate cancer. But no work has been done in Bangladesh on the association of NAT2, GSTT1, and GSTM1 gene polymorphism and the influence of oxidative stress in the risk of prostate cancer development. This study is undertaken to evaluate the association of NAT2, GSTT1, and GSTM1 gene polymorphism and the influence of oxidative stress on prostate cancer risk in the Bangladeshi population.

Specific objectives of the study:

- Find out the phenotypic distribution (fast or slow acetylator phenotype) of the NAT2 gene in prostate cancer patients and healthy control in the Bangladeshi population.
- To identify the genotypic distribution of GSTT1 and GSTM1 gene in prostate cancer patients and healthy control in the Bangladeshi population.
- To rule out the association of NAT2, GSTT1, and GSTM1 gene polymorphism with different pathological grades of prostate cancer.
- To find out the influence of oxidative stress in prostate cancer risk in the Bangladeshi population.
- To explore the relation of oxidative stress with NAT2, GSTT1, and GSTM1 gene polymorphism in prostate cancer patients.

2. Literature Review

2.1 Prostate Gland

The prostate is a small walnut-shaped unpaired accessory structure of the male reproductive system that surrounds the urethra in the pelvic cavity. The prostate contains many small glands. The glandular tissue of the prostate secretes a fluid that contributes 20-30 percent of the total volume of the seminal fluid. It lies immediately inferior to the bladder, above the urogenital diaphragm, posterior to the pubic symphysis, and anterior to the rectum (Fig. 2.1) (Drake et al., 2010). In adult men, a typical prostate is 3 centimeters long and weighs about 20 grams (Aumuller, 1979).

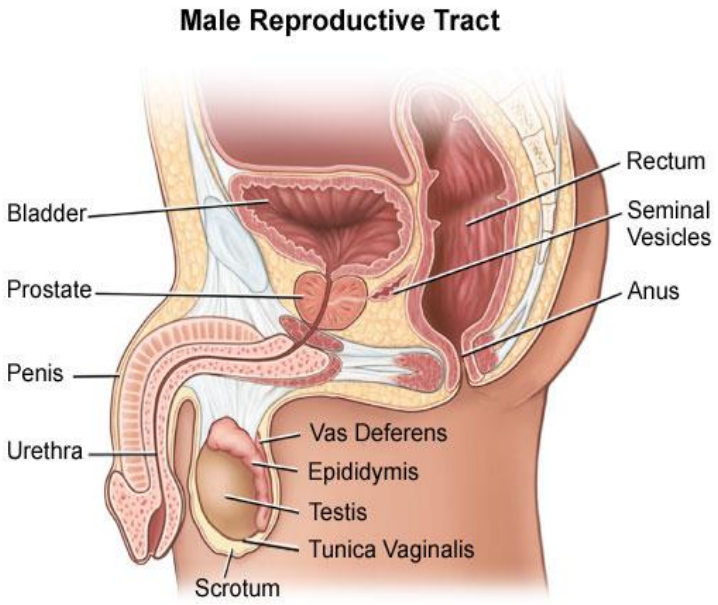


Figure 2.1: Anatomical Location of Prostate Gland

2.2 Common Disorders of Prostate Gland

There are three common disorders of the prostate gland (Xia et al., 2012).

⇒ Inflammatory Prostatitis

⇒ Benign Prostatic Hyperplasia (BPH)

⇒ Carcinoma of Prostate Gland

2.3 Zonal Predisposition of Prostate Disease

Three distinct zones have been identified- the peripheral zone, the central zone, and the transition zone (Fig. 2.2). The zonal anatomy is clinically vital because most carcinomas arise in the peripheral zone, whereas BPH affects the transitional zone (Standring, 2016). Most cancer lesions occur in the peripheral zone (60-70%), fewer happen in the transition zone (10-20%), and almost none arise in the central zone (5-10%) (McNeal et al., 1988).

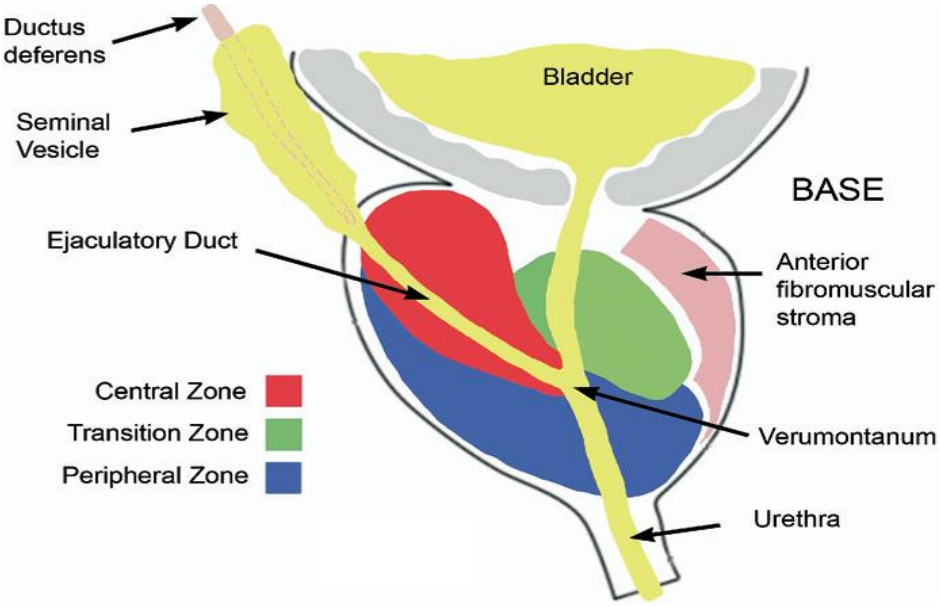


Figure 2.2: Three Distinct Zone of Prostate Gland

2.4 Prostate Cancer

Prostate cancer or carcinoma of the prostate is the development of cancer in the prostate, a gland in the male reproductive system (Mustafa et al., 2016). Generally, prostate cancer is slow-growing cancer, and early detection may allow an effective protocol treatment to achieve a long life (Salam, 2014). The tendency of prostate cancer to develop multifocally in the majority of cases is well documented and presence of two or more separate tumors in a

single prostate range from 60 to 90% (Cheng et al., 2005; Arora et al., 2004; Eichelberger et al., 2004). Because of its location, prostate disease often affects urination, ejaculation, and rarely defecation (Fig. 2.3) (Mustafa et al., 2016).

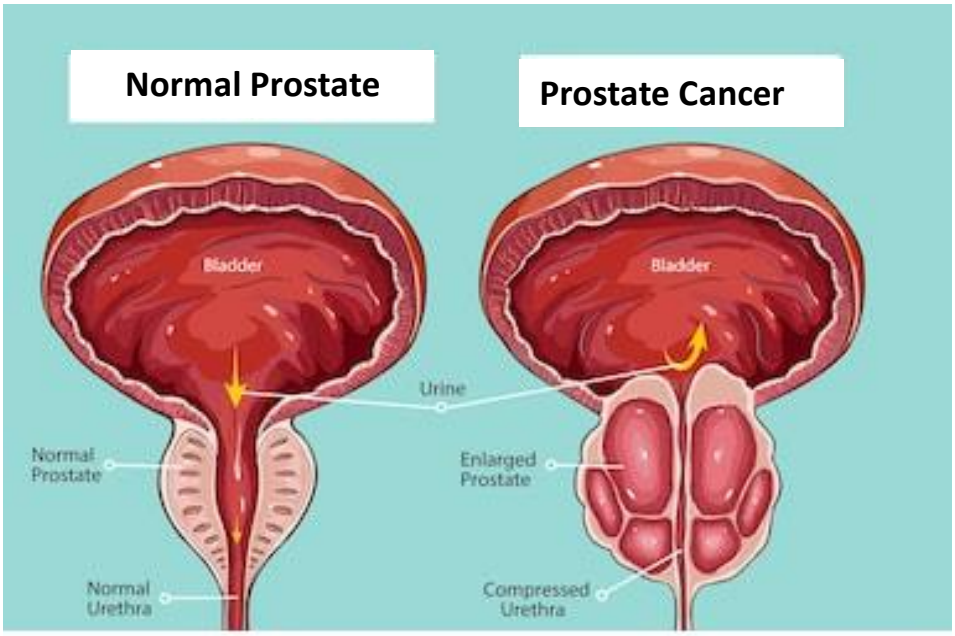


Figure 2.3: Normal Prostate and Prostate Cancer

2.4.1 Epidemiology

Prostate cancer is the second most frequent malignancy (after lung cancer) in men worldwide, counting 1,276,106 new cases and causing 358,989 deaths (3.8% of all deaths caused by cancer in men) in 2018 (Bray et al., 2018; Ferlay et al., 2019). Prostate cancer is the most common non-cutaneous malignant neoplasm in men in Western countries (Jemal et al., 2005). De Marzo et al. (2017) reported that the number of affected men is increasing rapidly in males over the age of 50. Therefore, finding strategies for the prevention of prostate cancer is a crucial medical challenge. In African-American men, the incidence rates are higher when compared to the White men, with 158.3 new cases diagnosed per 100,000 men, and their mortality is approximately twice as white men (Panigrahi et al., 2019). Interestingly, a trend

towards an increase of prostate cancer incidence worldwide with 1,017,712 new cases (+79.7% overall change) up to 2040 is estimated (Table 2.1) (Ferlay et al., 2019). The highest incidence of prostate cancer will be registered in Africa (+120.6%), followed by Latin America and the Caribbean (+101.1%) and Asia (100.9%). On the contrary, the lowest incidence will be registered in Europe (+30.1%). This increase in the incidence rates appears to be related to an increased life expectancy. Increasing incidence rate trends in developing countries is likely due to improved access to medical care as well as improved documentation and reporting of cases. Finally, the fact that incidence rates are increasing in those regions where PSA testing is not routinely used suggests that this phenomenon reflects westernization of the lifestyle, including obesity, physical inactivity, and dietary factors (Baade et al., 2009).

Table 2.1: Estimated Number of Incidents of All Age's Male Prostate Cancer Cases from 2018 to 2040

		2018	**2040**			
		Number	**Number**	**Demographic change**	**Change in risk**	**Overall change**
Africa	Males (APC 0%)	80,971	178,634	97,663 (+120.6%)	97,663 (+120.6%)	97,663 (+120.6%)
Latin America and the Caribbean	Males (APC 0%)	190,385	382,808	192,423 (+101.1%)	192,423 (+101.1%)	192,423 (+101.1%)
North America	Males (APC 0%)	234,278	312,901	78,623 (+33.6%)	78,623 (+33.6%)	78,623 (+33.6%)
Europe	Males (APC 0%)	449,761	585,134	135,373 (+30.1%)	135,373 (+30.1%)	135,373 (+30.1%)
Asia	Males (APC 0%)	297,215	597,180	299,965 (+100.9%)	299,965 (+100.9%)	299,965 (+100.9%)

Globocan 2018 (Ferlay et al., 2019).

2.4.2 Pathophysiology

Prostate cancer is an adenocarcinoma or glandular carcinoma that begins when normal prostate gland cells mutate to cancer cells (Mustafa et al., 2016). 95% of prostate cancer is adenocarcinoma, originating from the glands and ducts in the prostate. The most common sites of prostate cancer in the peripheral zone of the prostate gland. The prostate is an organ in which pre-neoplastic disorders like proliferative inflammatory atrophy (PIA) and prostate intraepithelial neoplasia (PIN) are relatively common (De Marzo et al., 2007). Damage to the tissue by an initiating inflammatory insult leads to PIA, which is a focal regenerative hyper-proliferative response from the epithelium (Packer and Maitland, 2016). Primarily, small clumps of cancer cells remain confined to normal prostate glands, the condition known as carcinoma in situ or prostate intraepithelial neoplasia (PIN). The PIN is not a precursor, but it is closely associated with cancer. Over time, these cancer cells begin to multiply and spread to surrounding tissue (the stroma), forming a tumor (Fig. 2.4) (Mustafa et al., 2016).

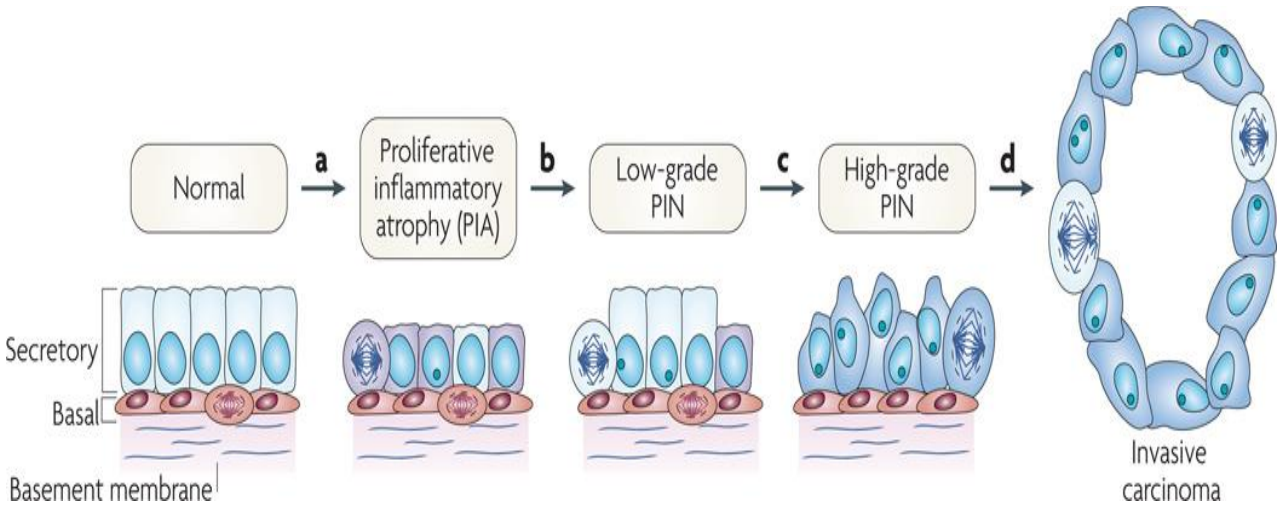


Figure 2.4: Molecular Mechanism of Inflammation-Induced Cancer

2.4.3 Clinical Manifestation

Prostate cancer may be asymptomatic at the early stage and often has an indolent course and may require minimal or even no treatment. However, the most frequent complaint is difficulty with urination, increased frequency, and nocturia, all symptoms that may also arise from prostatic hypertrophy. More advanced stage of the disease may present with urinary retention and back pain, as axis skeleton is the most common site of bony metastatic disease (Fig. 2.5) (Rawla, 2019). The presence of symptoms often presents with the locally advanced or metastasis. Men with the locally advanced or systemic disease usually present with bone pain from skeletal muscle, renal impairment due to ureteric obstruction, bowel symptoms from rectal canal involvement. Spinal metastasis can invade or compress the spinal cord, which may cause lower limb neurological symptoms or autonomic dysfunction like urinary retention or bowel/bladder incontinence (Cross, 2016).

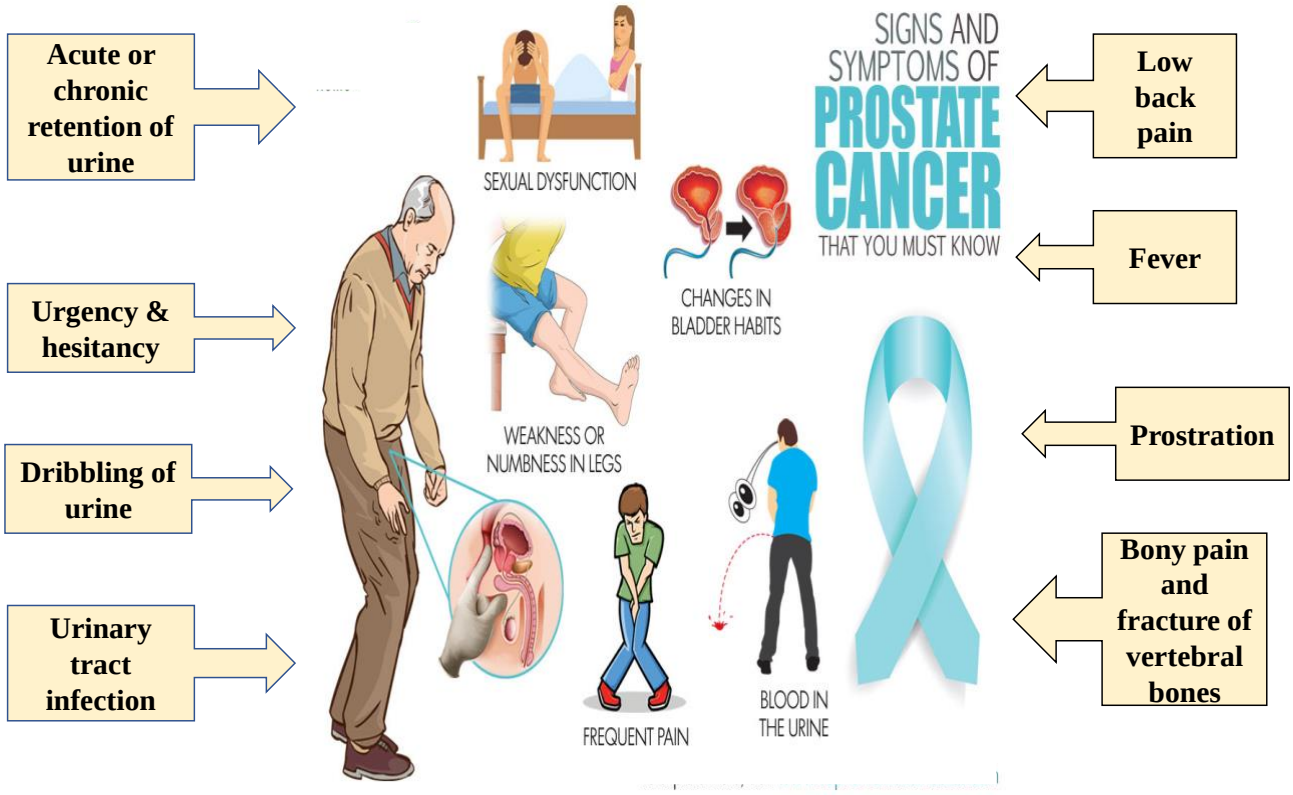


Figure 2.5: Clinical Presentation of Prostate Cancer

2.4.4 Diagnosis

Digital rectal examination: During digital rectal examination (DRE) in prostate cancer patients, there may be increased firmness of the prostatic tissue, nodularity, loss of symmetry, and flexion of the gland due to extension of cancer into the surrounding pelvic wall (Cross, 2016).

Tumor marker: - Prostate specific antigen (PSA): Serum PSA is a serological marker of prostate carcinoma detection, and the efficient use of the combination of DRE, serum PSA and biopsy result is an advantageous protocol for diagnosis of prostate cancer (Standring et al., 2016). The potential value of PSA for the early detection of prostate cancer was described in the early 1990s, both prostate cancer incidence and mortality rates have changed profoundly (Catalona et al., 1991). The American Urological Association (AUA) Foundation changed and updated prostate cancer screening recommendations in May 2013, and the PSA test remains an essential tool in the diagnostic process. Most guidelines mention the traditional cut off of PSA level, 4 ng/ml as an indication for biopsy (Salam, 2014).

Imaging: Magnetic resonance imaging (MRI) is becoming increasingly useful in diagnosing and staging prostate cancer (De Visschere et al., 2010). Computed tomography (CT) and Positron emission tomography (PET) have little or no use in the early detection of prostate cancer. However, they may be helpful in the staging and detection of lymph nodes for distant metastases (De Visschere et al., 2010).

Biopsy: Biopsy is a type of minor surgery. For a prostate biopsy, tiny pieces of tissue are removed from the prostate and looked at under a microscope. This is the only way to confirm a prostate cancer diagnosis. The decision to have a biopsy is based on PSA and DRE results. A family history of prostate cancer, ethnicity, biopsy history, and other health factors are also considered. A prostate biopsy is usually done using an ultrasound probe to look at the prostate and guide the biopsy. If cancer cells are found, the pathologist will assign a “Gleason Score,” which helps to determine the severity/risk of the disease. Among prostatic biopsy Trans Urethral Resection of Prostate (TURP) is commonly performed, however, needle core biopsy,

open biopsy is also done in selected cases (Bonekamp et al., 2011; Barentsz and Richenberg, 2012; Natarajan, 2011).

2.4.5 Staging and Grading

American Joint Committee on Cancer (AJCC) TNM staging

American Joint Committee on Cancer (AJCC) TNM staging system is a standard method for the cancer care team to describe how far cancer has spread. The standard staging system is the four-stage TNM (Tumor/ Nodes/ Metastasis) system (Table 2.2) (Buyyounouski et al., 2017). The TNM staging system is based on the size of the tumor, the number of involved lymph nodes, and the presence of any metastasis. The 2009 TNM classification for prostate cancer is shown in Table 2.3 (Heidenreich et al., 2012).

Table 2.2: Stages of Prostate Cancer

PROSTATE CANCER STAGES	
Stage I	- the cancer is small and only in the prostate
Stage II	- the cancer is larger and may be in both lobes of the prostate but is still confined to the prostate
Stage III	- the cancer has spread beyond the prostate to close by lymph glands or seminal vesicles
Stage IV	- the cancer has spread to other organs such as the bone and is referred to as metastatic cancer. If prostate cancer spreads, or metastasizes, to the bone, you have prostate cancer cells in the bone, not bone cancer

Table 2.3: TNM Classification System of Prostate Cancer

T - Primary tumor		
TX		**Primary tumor cannot be assessed**
T0		**No evidence of primary tumor**
T1		**Clinically in apparent tumor not palpable or visible by imaging**
	T1a	**Tumor incidental histological finding in 5% or less of tissue resected**
	T1b	**Tumor incidental histological finding in more than 5% of tissue resected**
	T1c	**Tumor identified by needle biopsy (e.g., because of elevated PSA level**
T2		**Tumor confined within the prostate[1]**
	T2a	**Tumor involves one half of one lobe or less**
	T2b	**Tumor involves more than half of one lobe, but not both lobes**
	T2c	**Tumor involves both lobes**
T3		**Tumor extends through the prostatic capsule[2]**
	T3a	**Extracapsular extension (unilateral or bilateral) including microscopic bladder neck involvement**
	T3b	**Tumor invades seminal vesicle(s)**
T4		**Tumor is fixed or invades adjacent structures other than seminal vesicles: external sphincter, rectum, levator muscles, and/or pelvic wall**
N - Regional lymph nodes[3]		
NX		**Regional lymph nodes cannot be assessed**
N0		**No regional lymph node metastasis**
N1		**Regional lymph node metastasis**
M -		**Distant metastasis[4]**
MX		**Distant metastasis cannot be assessed**
M0		**No distant metastasis**
M1		**Distant metastasis**
	M1a	**Non-regional lymph node(s)**
	M1b	**Bone(s)**
	M1c	**Other site(s)**

1 Tumor found in one or both lobes by needle biopsy, but not palpable or visible by imaging, is classified as T1c.
2 Invasion into the prostatic apex, or into (but not beyond) the prostate capsule, is not classified as pT3, but as pT2.
3 Metastasis no larger than 0.2 cm can be designated pN1 mi

4 When more than one site of metastasis is present, the most advanced category should be used.

Gleason score

The Gleason score is the grading system used to determine the aggressiveness of prostate cancer. This grading system can be used to choose appropriate treatment options. The Gleason Score ranges from 1-5 and describes how much cancer from a biopsy looks like healthy tissue (lower score) or abnormal tissue (higher score). Most cancers score a grade of 3 or higher. Since prostate tumors are often made up of cancerous cells that have different grades, two grades are assigned for each patient. A primary grade is given to describe the cells that make up the largest area of the tumor, and a secondary grade is given to illustrate the cells of the next largest area. For instance, if the Gleason score is written as 3+4=7, it means most of the tumor is grade 3, and the next most extensive section of the cancer is grade 4, together they make up the total Gleason score. If the cancer is almost entirely made up of cells with the same score, the grade for that area is counted twice to calculate the total Gleason score. Typical Gleason scores range from 6-10. The higher the Gleason score, the more likely that cancer will grow and spread quickly (Penney et al., 2013).

⇒ Scores of 6 or less describe cancer cells that look similar to normal cells and suggest that the cancer is likely to grow slowly.

⇒ A score of 7 suggests and intermediate risk for aggressive cancer. Scoring 7 means that the primary score (largest section of the tumor) scored a 3 or 4. Tumors with a primary score of 3 and a secondary score of 4 have a reasonably good outlook, whereas cancers with a primary Gleason score of 4 and a secondary score of 3, are more likely to grow and spread.

⇒ Scores of 8 or higher describe cancers that are likely to spread more rapidly, and these cancers are often referred to as poorly differentiated or high grade.

2.4.6. Etiology and Risk Factors

The etiology of prostate cancer is not well understood, although several things can increase the risk of developing the condition.

Risk factors of prostate cancer

Age: The risk rises with increasing age, and most cases are diagnosed in men over 50 years of age (Malik et al., 2015).

Ethnic group: Prostate cancer is more common among men of African-Caribbean and African descent than in Asian men. African American men have 1.5 times higher incidence of prostate cancer than white Americans, and the age-adjusted death rates from prostate cancer are 2.4 times higher for African American men than for whites (Brooks et al., 2010).

Family history: Men who have a first degree relative (father or brother) with prostate cancer have twice the risk of developing prostate cancer, and those with two first degree relatives affected have a fivefold higher risk compared with men with no family history (Steinberg et al., 1990).

Obesity: Recent research suggests, there may be a link between obesity and prostate cancer, and a balanced diet and regular exercise may lower the risk of developing prostate cancer. It has been shown that rapid weight gain between the ages of 25 and 40 years doubles the chance of recurrence of prostate cancer disease (Hack et al., 2010).

Diet: Epidemiological studies have revealed a positive association between prostate cancer incidence and mortality with the consumption of meat because in meat, heterocyclic amines found in the highest concentration (Norrish,1999). The mechanism by which meats might stimulate cancer development may be related to the formation of heterocyclic amines, which produced during cooking might be carcinogenic (Sugimura et al., 2004). Research is ongoing into the links between diet and prostate cancer, and there is some evidence that a diet high in calcium is linked to an increased risk of developing prostate cancer.

Infectious agents: Many different pathogenic organisms have been identified to infect and induce an inflammatory response in the prostate. These include sexually transmitted organisms, such as *Neisseria gonorrhoeae, Chlamydia trachomatis, Trichomonas vaginalis and Treponema pallidum*, and non-sexually transmitted bacteria such as *Propioni bacterium acnes* and those known to cause acute and chronic bacterial prostatitis, primarily Gram-

negative organisms such as *Escherichia coli* (Marzo et al., 2007). Viruses can also infect the prostate, and *Human papilloma virus (HPV), Herpes simplex virus type 2 (HSV2), Cytomegalovirus (CMV),* and *Human herpes virus type 8 (HHV8*) have been detected in the prostate (Strickler and Goedert, 2001).

Chemical irritation and urine reflux: Chemical irritation from urine reflux is an etiological agent for the development of chronic inflammation in the prostate and also important both as a route of infection in bacterial prostatitis and as a cause of abacterial prostatitis (Kirby et al., 1982). Although urine contains many chemical compounds that may be toxic to the prostatic epithelium, uric acid itself might be particularly damaging (De Marzo et al., 2007). Crystalline uric acid as a "danger signal" released from dying cells, causes the production of inflammatory cytokines that can increase the influx of other inflammatory cells (Martinon et al., 2006).

Estrogens: Prostate tissue is most probably a target for direct regulation by estrogens and also related to prostatic carcinogenesis. Developing prostate particularly sensitive to an increased level of exogenous/ endogenous estrogens. Perinatal or neonatal exposure of rodents to estrogens leads to the "imprinting" of prostate associated with increased proliferation, inflammation, and dysplastic epithelial change in later life. Prolonged treatment of adult rodents with androgens also leads to epithelial metaplasia, PIN-like lesions, and even adenocarcinoma of the prostate (Harkonen and Makela, 2004).

Genetics: Genetic background may contribute to prostate cancer risk, as suggested by associations with race, family, and specific gene variants. No single gene is responsible for prostate cancer, and many different genes have been implicated. Mutation in BRCA1 and BRCA2, important risk factors for ovarian cancer and breast cancer in women, has also been involved in prostate cancer (Struewing et al., 1997). Other linked genes include the Hereditary Prostate Cancer gene 1(HPC1), the androgen receptor, and the vitamin D receptor (Gallagher and Fleshner, 1998).

2.5 Prostate Cancer in Bangladesh Perspective:

Bangladesh is one of the small developing countries in South Asia. Cancer is predicted to be an increasingly important cause of morbidity and mortality in the next few decades. According to the Bangladesh Bureau of statistics, cancer is the sixth leading cause of death. Word Health Organization (WHO) reported, there were 150,781 new cancer cases in Bangladesh in 2018, and among them, there were 83,715 male and 67,066 female cancer patients (Ferlay et al., 2019). However, the number of cancer patients is increasing day by day, and every year approximately 200,000 people are attacked by cancer, and 150,000 people die of the disease in Bangladesh (Noronha et al., 2012). International Agency for Research on Cancer (IARC) has estimated cancer-related death rates in Bangladesh to 13 % in 2030 (Hussain and Sullivan, 2013). The prostate cancer prevalence in Bangladesh or developing countries is low compared to the developed world. But the prevalence of prostate cancer in Bangladeshi urban population is increasing (Salam, 2014). In Bangladesh, the rate of incidence and mortality are likely unavailable due to a lack of population-based cancer registry system. According to the latest WHO data published in 2017, Prostate Cancer Deaths in Bangladesh reached 738 or 0.09% of total deaths. The age-adjusted Death Rate is 1.50 per 100,000 of population ranks Bangladesh #181 in the world (WHO, 2017). There may be various reasons for such a higher prevalence; the life expectancy of the Bangladeshi population has been increased significantly; more people are health conscious and involved in the health check program (Salam, 2014). Currently, most of the district city general hospitals provide PSA tests. In hospital practice, early detection becomes possible through the PSA test and digital rectal examination (DRE). Increased numbers of early and advanced cases of prostate cancer are detected in clinical practices.

2.6 Molecular Pathology of Prostate Cancer

The molecular pathology of prostate cancer is complex; not only are multiple genes involved in its pathogenesis, but additional environmental factors such as diet and inflammation are also included. The exhaustive research into prostate cancer to date has demonstrated a complex interaction of multiple genes and environmental factors, some of which may be more

important in individuals (Hughes et al., 2005). Although any two unrelated people share about 99.9% of their DNA sequences, the remaining 0.1% is significant because it contains the genetic variants that influence how people differ in their risk of disease or their response to drugs and environment exposures (Hung et al., 2004). Enzymatic activation and detoxification of carcinogens is a major principle in the chemical determinant of individual susceptibility to their carcinogenic effects (Hamasaki et al., 2003). It was reported, prostate epithelial cell express phase II metabolizing enzymes and recent molecular epidemiological studies have analyzed the relationship between N acetyl transferase and Glutathione S transferase gene polymorphism in etiology of prostate cancer (Wang et al., 1999; Agundaz et al., 1998; Mittal et al., 2004). In the presence of polymorphisms of phase II detoxifying enzymes, due to decreased detoxification capacity causes an increased risk of prostate cancer (Fig. 2.6).

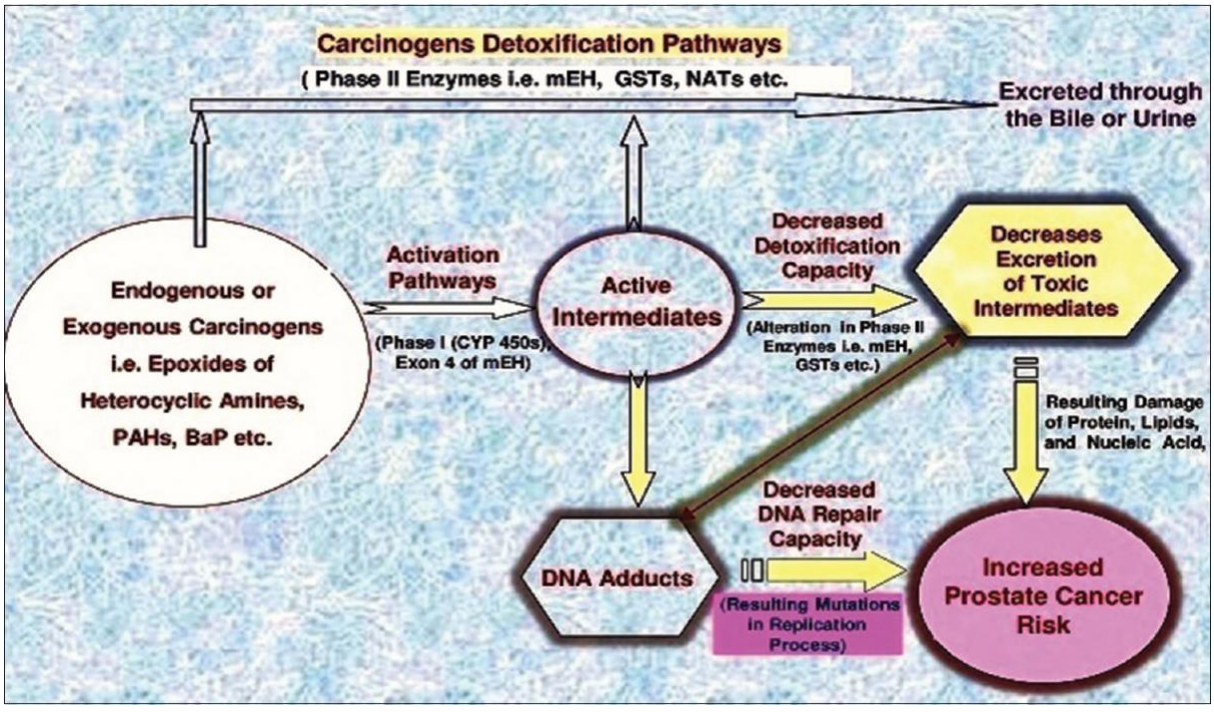


Figure 2.6: Role of Genetic Polymorphism in Phase II Metabolism Enzymes in Prostate Cancer Risk.

2.7 N Acetyl Transferase (NAT) Gene

Arylamine N-acetyl transferases (NATs) are polymorphically expressed enzymes of phase II biotransformation reactions involved inactivation or detoxification of many carcinogenic compounds and therapeutic drugs (Meisel, 2002; Westwood et al., 2006; Boukouvala and Fakis, 2005; Krul and Hageman, 1998). In humans, genetic differences in N acetylation activity have led to a phenotypic classification of individuals as rapid or slow acetylators, with resultant differing rates of metabolism of arylamines. Recent epidemiological studies have demonstrated that the NAT slow acetylator genotype is associated with an increased risk of uroepithelial cancer (Katoh et al., 1999; Inatomi et al., 1999).

2.7.1 NAT Gene Location, Structure and Expression

Human genes for acetyl transferases were cloned by Blum et al. (1990). They used the rabbit cDNA probe from the genomic DNA of an obligate heterozygous rapid acetylator individual identified by a pedigree phenotyping study. The recombinant clones identified three different, but closely related, human NAT genes: NAT1, NAT2, and the pseudogene NATP. Each of the two genes NAT1 and NAT2 has a single, intronless protein-coding exon with an open reading frame of 870 bp that produces a 290 amino acid (33– 34 kDa) protein. Whereas the NAT1 gene produces its entire transcript from a single exon, the NAT2 mRNA is derived from both the protein-coding exon and a second, untranslated exon of 100 bp that is located about 8 kb upstream of the translation start site (Blum et al., 1990; Ebisawa and Deguchi, 1991; Boukouvala and Sim, 2005). The nucleotide homology between NAT1 and NAT2 is 87% in the coding region and are located at 8p22 (Blum et al., 1990; Franke et al., 1994; Hickman et al., 1994), a chromosomal region commonly deleted in human cancers (Bova et al., 1993; Maitra et al., 1999; Lutchman et al., 1999; Brown et al., 1999; Wistuba et al., 1999). There are only 55 amino acid differences, 28 of which are non-conservative changes. The pseudogene NATP has high sequence homology to NAT1 and NAT2 (79%) but contains multiple frameshifts and stop codons. The NAT loci are separated by only 170–360 kb and are in the orientation NAT1 → NATP1 → NAT2, with NAT1 being on the centromeric side of marker D8S261 and NAT2 coinciding with marker D8S21 (Matas et al., 1997).

2.7.2 N-acetyl Transferase Isozymes

N-Acetyl transferase isozymes use acetyl coenzyme A as a cofactor and function as phase II conjugating enzymes. They are capable of N-acetylation, O-acetylation, and N, O- acetylation (Vatsis et al., 1995). Fig. 2.7 displays a two-step, the substituted-enzyme reaction by which NAT isozyme uses acetyl coenzyme A as a cofactor. N-acetylation is a detoxification pathway. O-acetylation and N, O- acetylation occur in alternative metabolic pathways following activation by N-hydroxylation. The isozymes differ in their substrate specificities: Isoniazid and sulfamethazine are NAT2 specific substrates; p-aminobenzoic acid and p-aminosalicylic acid are NAT1 specific substrates. NAT2 is primarily expressed in the liver, whereas NAT1 is expressed mainly at other sites, including the colon (Rodriguez et al., 1993). The NAT2 acetylation polymorphism is significant in clinical pharmacology and toxicology because of its primary role in the activation and/or deactivation of a large and diverse number of aromatic amine and hydrazine drugs used in clinical medicine (Weber, 1985; Weber, 1987; Evans, 1989).

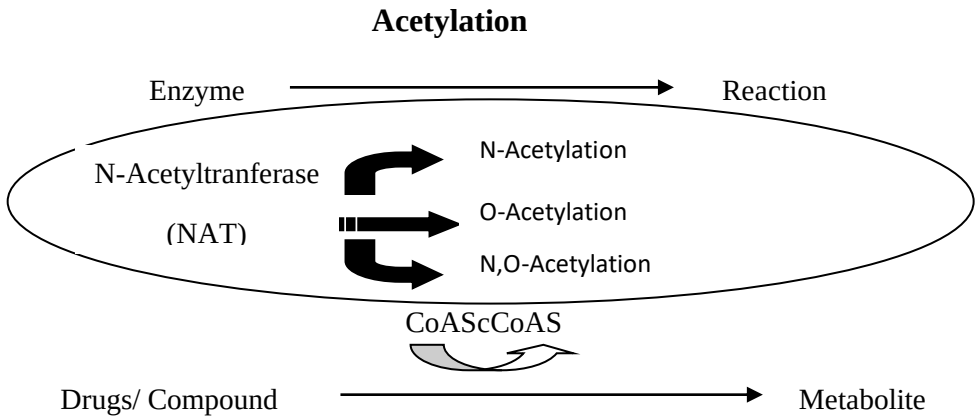


Figure 2.7: The NAT Acetylation Enzymatic Reaction within the Cell Involved in the Metabolism of Certain Drugs and Xenobiotic Compound.

2.7.3 N Acetyl Transferase 2 (NAT2) Enzyme

The N-acetyltransferase-2 (NAT-2) enzyme is involved in human physiological responses to a wide range of xenobiotic compounds, including many clinically useful drugs and a variety of exogenous chemicals present in the diet and environment. Genetic variations of NAT-2 gene,

giving rise to either the ‘slow’ or the ‘fast’ acetylator phenotype, found to influence individual’s variation in responses to environmental toxins, the effectiveness of prescribed medications and different conditions, in particular cancer susceptibility (Ladero, 2008; Agundez, 2008). Beyond its medical relevance, NAT-2 has generated considerable interest in the field of evolutionary genetics, and numerous studies have attempted to decipher the relative roles of population history and natural selection in shaping genetic variation at this locus (Patin et al., 2006; Sabbagh et al., 2008; Luca et al., 2008).

2.7.4 NAT2 Allele Classification and Functional Significance of NAT2 Mutations

The NAT2 alleles described so far may contain up to 4 of 10 reported mutations (Vatsis et al., 1995). The functional significance of most combinations is unknown. However, it is plausible that each combination might result in a different phenotype. There are now 36 alleles registered with the arylamine N-acetyltransferase nomenclature committee. The more common NAT2 alleles or haplotypes are presented in Table 2.4. Some of the mutations change the amino acid sequence of the resultant enzyme, but not all of these have been observed to alter phenotype (e.g., A803G) (Cascorbi et al., 1996). However, some mutations have been consistently found to reduce acetylation activity (e.g., T341C) (Hein et al., 1995). The functional effect on phenotype is due to impairment of the protein translation or stability; messenger RNA levels are not altered (Blum et al., 1991).

The C481T transition does not change the amino acid sequence; however, it has always been found with other mutations. In populations of European origin, C481T most often occurs with T^'C and A803G; when C481T is combined with T341C, enzyme activity is reduced. G191A was discovered because of phenotype/ genotype discordance in African-American subjects (Bell et al., 1993). Site-directed mutagenesis (serial replacement of nucleotides within the gene coding region) showed codon 64 to be highly conserved between species, and it is implicated as the active site for acetyl transfer (Delomenie et al., 1997). Disruption of this region has been demonstrated to abolish enzyme activity in vitro. In European populations, relatively high concordance between acetylator phenotype (for NAT2- specific substrates) and NAT2 gene mutations has been demonstrated (Blum et al., 1991; Smith et al., 1997).

Table 2.4: Human NAT2 Alleles*

Allele	Phenotype	Nucleotide	Amino acid change(s)
NAT2*4	Rapid	None	**None**
NAT2*5A	Slow	T341C,C481T	**IIe114-Thr**
NAT2*5B	Slow	T341C,C481T,A803G	**IIe114- Thr, Lys268-Arg.**
NAT2*5C	Slow	T341C, A803G	**IIe114- Thr, Lys268- Arg.**
NAT2*5D	Slow	T341C	**IIe114- Thr**
NAT2*5E	Slow	T341C, G590A	**IIe114- Thr Lys268- Arg**
NAT2*5F	Slow	T341C,C481T,T759T,A803G	**IIe114- Thr, Lys268- Arg**
NAT2*6A	Slow	C282T, G590A	**Arg197-Gln**
NAT2* 6B	Slow	G590A	**Arg197- Gln**
NAT2*6C	Slow	C282T,G590A,A803G,	**Arg197- Gln, Lys268- Arg**
NAT2*6D	Slow	T111C, C282T, G590A	**Arg197- Gln**
NAT2*7A	Slow	G857A	**Lys286- Glu**
NAT2*7B	Slow	C282T,G857A	**Lys286- Glu**
NAT2*10	Unknown	G499A	**Glu167-Lys**
NAT2*11	Unknown	C481T	**None**
NAT2*12A	Rapid	A803G	**Lys268- Arg**
NAT2*12B	Rapid	C282T,A803G	**Lys268 –Arg**
NAT2*12C	Rapid	C481T,A803G	**Lys268- Arg**
NAT2*13	Rapid	C282T	**None**
NAT2*14A	Slow	G191A	**Arg64-Gln**
NAT2*14B	Slow	G191A, C282T	**Arg64- Gln**
NAT2*14D	Slow	G191A,C282T,G590A	**Arg64- Gln, Arg197- Gln**
NAT2*14E	Slow	G191A, A803G	**Arg64- Gln, Lys268- Arg**
NAT2*14G	Slow	G191A,C282T,A803G	**Arg64 - Gln Lys268- Arg**
NAT2*17	Slow	A434C	**Gln145-Pro**
NAT2*18	Unknown	A845C	**Lys282- Thr**
NAT2*19	Slow	C190T	**Arg64-Trp**

*Adapted from References, Grant et al., 1997 and Hein et al., 2000.

2.7.5. Molecular Genetics of NAT2

NAT2 gene, the focus of this study, is highly polymorphic. This NAT2 polymorphism is widespread in the human population, and individuals can be subdivided into fast, intermediate, and slow acetylator phenotypes (Hein et al., 2000; Weber and Hein, 1985).

A number of single nucleotide polymorphisms (SNPs) have been reported within the 870 bp coding region of the NAT2 gene: 11 missense polymorphisms (C190T, G191A, T341C, G364A, A411T, A434C, G590A, A803G, A845C, G857A, and T859C), four silent polymorphisms (T111C, C282T, C481T, and C759T) and one frameshift polymorphism (859T deletion) (Hein, 2006; Hein, 2002). Among the SNPs mentioned above, seven SNPs (C190T, G191A, T341C, A434C, G590A, A845C, and G857A) lead to a significant decrease in acetylation capacity whereas three SNPs (C282T, C481T, and A803G) do not alter phenotype (Sabbagh and Darul, 2005; Fretland et al., 2001; Hein et al., 2006). Various combinations of SNPs are identified as NAT2 alleles (Vatsis et al., 1995) or haplotypes. Variant NAT2 alleles or haplotypes possessing combinations of SNPs are segregated into clusters possessing a signature SNP either alone or in conjunction with others.

NAT2*4 is considered the ‘wild-type’ allele or haplotype because of the absence of any SNPs and is designated as a fast allele. NAT2*11A, NAT2*12, and NAT2*13 have also been identified as fast alleles. Individuals possessing two fast alleles are assigned as fast acetylators, while those with two variant alleles are slow acetylators, and those with one wild-type and one variant allele are intermediate acetylators. Mutant alleles of NAT2 were first identified by restriction fragment analysis of genomic DNA samples from healthy individuals whose acetylator phenotype had been established by caffeine phenotyping (Blum et al., 1991) or isoniazid (Deguchi et al., 1990) and of DNA from human liver samples with known NAT2 enzyme activity (Blum et al., 1991).

Restriction fragment length polymorphisms (RFLPs) with NAT2 probes generated by Mspl, Kpnl, and Bam HI segregated with acetylator phenotype and defined three mutant alleles of NAT2, initially called M1, M2, and M3 (Blum et al., 1991). To explore the molecular mechanism of slow acetylation, NAT2 genes were cloned from genomic libraries (Blum et al.,

1991) or characterized as cDNAs (Ohsako and Deguchi, 1990) and their sequence determined and compared with the wild-type gene sequence. The M1 allele had mutations at positions 341 and 481, M2 at positions 282 and 590 (Blum et al., 1991), and M3 at position 857 (Ohsako and Deguchi, 1990). Another mutant allele, M4, was also reported, which is exist only in populations of African origin and consisted of a transition of G191A (Bell et al., 1993). It was observed that the three mutant alleles Ml, M2, and M3 account for more than 90% of slow accetylators (Blum et al., 1991).

The C481T, G590A, and G857A mutations accounted for virtually all of the slow acetylator alleles in Asian and white populations (Lin et al., 1993). The M1 allele is detected by the possession of the C481T substitution. This silent substitution is found in many but not all of the NAT2*5 alleles and is located in NAT2*12C, which is a rapid allele. The M2 allele is detected by the possession of the G590A missense substitution, which is found in NAT2*5, NAT*6, and NAT2*14 allelic clusters. The M3 allele is detected by the possession of the G857A missense substitution, which thus far has been identified only on NAT2*7A and NAT2*7B. The M4 allele, which was initially discovered in African Americans (Ferguson et al., 1996; Bell et al., 1993), is detected by the possession of the G191A missense substitution. Thus far, this substitution has only been identified on the NAT2*14 gene cluster.

Initial studies in liver tissue suggested that the slow acetylator phenotype associated with the presence of specific nucleotide substitutions in the protein-encoding region of the NAT2 gene was due to a marked decrease in NAT2 protein content. In contrast, NAT2 mRNA levels remained unchanged (Grant et al., 1990). Several studies have since investigated the mechanism by which nucleotide substitutions in the NAT2 gene affect acetylation capacity by the use of recombinant expression systems (Dupert et al., 1994; Hein et al., 1994; Hichman et al., 1995; Delomenie et al., 1997; Leff et al., 1999). Hein and coworkers performed a comprehensive study that assessed the acetylation capacity of 16 different NAT2 alleles in a bacterial expression system (Hein et al., 1995). Of the seven specific NAT2 substitutions that they examined, the T341C, G590A, G857A, and G191A substitutions produced recombinant NAT2 allozymes with reduced acetylation capacities. In contrast, the C481T, C282T, and A803G substitutions produced recombinant NAT2 allozymes with acetylation capacities

similar to the wild-type NAT2 4 protein. As a result, NAT2 alleles that contain any of the specific substitutions that produced recombinant NAT2 allozymes with reduced acetylation capacities are associated with a slow acetylator phenotype and include the NAT2*5, NAT2*6, NAT2*7, NAT2*14, and NAT2*17 clusters.

The molecular mechanisms responsible for the production of the slow acetylator phenotypes are not well understood at present. Some base changes appeared to cause a slow acetylation phenotype by producing an unstable protein. NAT2 allozymes encoded by alleles with base substitutions at positions 191, 590, or 857 were found to be significantly more unstable in bacterial expression systems than the wild-type protein (Grant et al., 1997; Delomenie et al., 1997; Hein et al., 1995; Ferguson et al., 1996). This is in contrast to the earlier observations by Grant et al. (1990), who showed that liver NAT2 content was markedly reduced in slow acetylators, suggesting that the artificial environment of bacterial expression systems may not accurately reflect what occurs in mammalian cells with regard to protein degradation.

2.7.6 Geographical and Ethnic Variation of Slow and Fast Acetylator Phenotypes

The ethnic difference in the NAT2 polymorphism has been observed in several studies (Cascorbi et al., 1985; Blum and Ohsako et al., 1990). Various frequencies of slow acetylators have been reported from 6.1%, 16.6-24.1%, 44.3%, and 55.0% in the Japanese, Taiwanese, North Indian, and Caucasian populations, respectively. Taiwan's population is composed of 1.5% aboriginal people, 7.5% mainland Chinese from China after War World II, and 91% Minnan and Hakka from the southeast coast of China since the 17th century (Smith et al., 1997). The frequencies reported in other Southeast Asian populations are 73 percent in Hong Kong (Lin et al., 1993), 72 percent in Malaysia (Zhao et al., 1995), and 58 percent in Singapore (Lee et al., 1998). Studies carried out in other parts of Asia have reported lower frequencies: 32 percent in India (Rothman et al., 1997), 37 percent in the United Arab Emirates (Woolhouse et al., 1997), and 43 percent in Turkey (Aynacioglu et al., 1997).

Researchers often use Chinese as representatives of Asian people and the Minnan /Hakka or so-called Han people as subjects in clinical studies. However, genetic variations in metabolizing enzymes among Taiwan's indigenous tribes and Minnan /Hakka were poorly

investigated. Wu et al. (2008) reported significant differences in the frequency of NAT2 genotype categories according to the 3 SNPs of NAT2 within 7 Taiwanese ethnic groups. The distribution of the frequency of the slow acetylator genotype ranged from 32.0% in the Hakka population to 75.5% in the Taiwan population. A lack of NAT2*5 SNP alleles was found in the Atayal community, while a NAT2*5 allele frequency of 31.6% was observed in the Paiwan group. The frequencies of allele NAT2*6 and NAT2*7 in the investigated ethnic groups ranged from 11.2-37.0% and 17.0-53%, respectively. They also reported a considerable variation in drug-metabolizing enzyme NAT2 genotypes among Taiwan's aboriginal and general population.

Ebeshi et al. (2011) investigated the distribution of arylamine N-acetyltransferase 2 (NAT2) allele frequencies associated with slow acetylation in healthy individuals from the three major Nigerian ethnic groups comprising of Hausa, Ibo, and Yoruba. The allele frequencies of the investigated SNPs indicates that NAT2*4, wild-type (34%; 95% confidence interval (CI): 22-38%) is the most prevalent allele in Hausa, NAT2*6, G>A (29%; 95% CI: 22-37%) is the most common in Ibo while NAT2*5, 481C>T (33%; 95% CI: 21-37%) is the most recorded in Yoruba populations. The most prevalent alleles in the three populations are the wild type, NAT2*4, and the defective alleles, NAT2*5 and NAT2*6. The frequencies of NAT*7, 857G>A, and NAT2 *14, 191G>A are relatively low in these populations except for the 11% recorded for NAT2*14 in the Ibo community. Overall, the NAT2 defective alleles (NAT2*5, *6, *7 and *14) were found to be 66%, 72%, and 71% in the Hausa, Ibo, and Yoruba populations, respectively.

The single nucleotide polymorphism frequencies of the NAT2 gene showed a higher prevalence of the slow acetylator alleles, which was in agreement with previous findings in some African populations (Dandara et al., 2003). The frequencies of the slow alleles in the three major Nigerian ethnic groups are higher than frequencies reported in other African populations such as Tanzanians (49%), South African Vendas (38%) and Zimbabweans (52%) (Dandara et al., 2003) but comparable to the frequency of 56% reported in the Gabonese, a fellow West African ethnic population. The overall prevalence of NAT2*5 (29%) in the Nigerian people was lower than the value of 40% reported for Caucasians (Lin et

al., 1994). The frequency of the NAT2 slow alleles in the Nigerian populations is higher than the frequency of 35-60% reported in the Caucasians (Leffet al., 1999). The high frequencies of the NAT2 slow acetylation alleles are further justified by the reported incidence of 41% slow acetylator phenotype observed in the Nigerian population (Eze and Obidoa, 1978). Despite the tremendous ethnic variability associated with NAT2 as well as its importance in aromatic amines and carcinogen metabolism, its polymorphisms have rarely been studied in Nigerian populations.

Generally, while NAT2*14 has been observed mainly among black populations (Lin et al., 1993), the frequency of NAT2*7 is highest among the Asians compared to other ethnic groups (Hein et al., 2006). Frazier et al. (2001) reported that individuals heterozygous for NAT2*7 had been shown to have a higher risk of developing colorectal cancer, NAT2 genotypes have been associated with prostate cancer and in patients who have bladder cancer (Rovito et al., 2005).

In most European populations, approximately 40 percent of study subjects have genotypes associated with fast or intermediate acetylation. Genotype frequencies within the United States vary by ethnic group: for White subjects, frequencies are similar to those of European populations, and for Asians, they are similar to those of people in Southeast Asia. The lowest frequencies have been reported in two small African studies in which subject selection was not described (Delomenie et al., 1996). Interestingly, higher frequencies have been reported in African Americans (Lin et al., 1993). It was observed that 62% of Berliner Caucasians were classified as slow acetylators (Hildebrand and Seifert, 1989) while in France, it was 53% (Bouchardy et al., 1998). Using isoniazid plasma half-life, 41% of 106 black South African men with tuberculosis were phenotyped as slow acetylators (Bach et al., 1976).

There is considerable variation in the proportion of slow acetylators reported from India in different studies, but on the whole, it is high, at about 60% (Pande et al., 1996). Similar results have been reported from South India. Slow acetylators predominate in countries in the Middle East as well. Numerous studies have been conducted to explain the genetic basis of N-acetylation polymorphism in various ethnic groups (Patin et al., 2006; Rabstein et al., 2006;

Sabbagh et al., 2008). The frequency of fast and slow acetylator NAT2 alleles varies widely across various ethnics groups. Because the NAT2*4 allele does not have any SNPs in its coding region, this allele is considered as a reference allele (fast acetylator). However, the NAT2*4 allele is not the most common allele in many ethnic groups, especially in Caucasians and Africans. NAT2*14 allele, including G191A nucleotide substitution, was found more frequently in African than in Caucasians (Delomenie et al., 1996). Also, NAT2*7 allele including G857 nucleotide substitution is more frequent in South Indians and Koreans than in other populations whereas NAT2*5 allele containing T341C nucleotide substitution is less frequent in Korea than in Europe, North America, India, and Africa (Hein, 2006)

2.7.7 Association of NAT2 Gene Polymorphism with Prostate Cancer Risk

It has been reported that human prostate epithelial cells metabolize potential carcinogens (Lawson and Kolar, 2002). Furthermore, Wang et al. (1999) demonstrated the expression of NAT transcripts in prostate cells. Although the acetylator status of NAT2 in prostate cancers was investigated in several studies (Srivastava and Mittal, 2005b; Costa et al., 2005; Hamasaki et al., 2003), the results were conflicting as to whether the slow or rapid acetylator status did constitute a risk factor. These inconsistencies can be explained by the fact that the NAT2 allele distribution can vary between interethnic populations (Hein et al., 2000), as well as to exposure to environmental factors, which depends on the level of economic development of a country. Another fact is that the NAT2 gene is quite polymorphic, and not all polymorphisms were analyzed at the same time in the studies. Lastly, different groups used various acetylator phenotype detection methods, e.g., the acetylator phenotype can be measured by a caffeine excretion method (Butler et al., 1992). However, this method is influenced by drugs (Hein et al., 2000; Vesell and Gaylor, 1995), liver diseases (Evan, 1989), or foods (Bulovskaya et al., 1978), and could yield false results. Molecular biological tests should preferentially determine the NAT2 acetylator phenotype.

Srivastava et al. (2005b) observed a non-significant association of the fast NAT2 acetylator genotype (OR=1.452, 95% CI: 0.54-1.87, p=0.136) in prostate cancer patients with a North Indian origin. However, when they analyzed the fast NAT2 acetylator genotype in prostate cancer patients that smoked tobacco (OR=3.43, 95% CI: 1.68-7.02, p<0.001), the association

became more evident compared to the controls. These findings agree with previous studies that showed a significant association of prostate cancer for NAT2 fast acetylator genotype in American research (Wang et al., 1999). In contrast, the non-significant association was observed in the Danish population (Wadelius et al., 1999) and the Spanish population (Agundez et al., 1998). On the contrary, there was significant association reported with slow acetylator genotypes of NAT2 in the Japanese population (Hamasaki et al., 2003). In the study, Hamasaki et al. (2003) found that the frequency of cases with a slow acetylator genotype was significantly higher in the prostate cancer group (OR=2.21), associated with a more advanced stage of disease (T3/T4/N1/M1; OR=3.14) and a higher pathological stage of the tumor (OR=4.90). The mutant NAT2*6A genotype was also found in a higher frequency in prostate cancer cases compared with controls in the South European population (Costa et al., 2005).

2.8 Glutathione-S-Transferase (GST) Gene

The glutathione-S-transferases (GST) represent a major group of detoxification enzymes. Glutathione S-transferases (GSTs) are the most important family of phase II isoenzymes known to detoxify a variety of electrophilic compounds, including carcinogens, chemotherapeutic drugs, environmental toxins, and DNA products generated by ROS damage to intracellular molecules. Detoxification via GSTs is achieved by conjugating them with glutathione. GSTs thus play a significant role as cellular antimutagen and in antioxidant defense mechanisms (Hayes et al., 2005).

2.8.1 Classification of GSTs

The superfamily of glutathione transferases (GSTs) is composed of multifunctional proteins widely distributed in nature in both eukaryotes and prokaryotes (Hayes et al., 2005; Allocati et al., 2009; Sheehan et al., 2001). In eukaryotes, GSTs are divided according to their cellular localization into at least three major families of proteins, namely cytosolic GSTs, mitochondrial GSTs and microsomal GSTs (Fig. 2.8) (Hayes et al., 2005; Sheehan et al., 2001; Wu and Dong, 2012). Cytosolic GSTs are readily distributed and, in turn, divided into several major classes based on their chemical, physical and structural properties.

Mitochondrial GSTs are also known as kappa class GSTs and are soluble enzymes that bear structural similarities with cytosolic GSTs. On the contrary, microsomal GSTs, also known as MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism), are integral membrane proteins that are not evolutionary related to the other major classes. Cytosolic GSTs are classified based on sequence similarities and structural properties. Many classes have been recognized so far, some of them with multiple members, which may share sequence identities around 40%. Based on sequence similarity, at least eight members of the cytosolic family have been identified in humans named Mu(M), Kappa (K), Alpha (A), Pi (P), Omega (O), Theta (T), Zeta (Z), and Sigma (S) (Wilce and Parker, 1994; Wu and Dong, 2012). Among candidate genes related to oxidative stress, genes for cytosolic GSTs, particularly GSTM1, GSTT1, were intensively studied in different disease states owing to their potential modulating roles in individual susceptibility to environmentally induced diseases including cancer (Stücker et al., 2002). GSTM1 and GSTT1 genes are polymorphic in humans, and a lack of enzyme activity accompanies the null genotypes. Hence individuals are at risk towards the development of malignancies (Economopoulos et al., 2010; Huang et al., 2015).

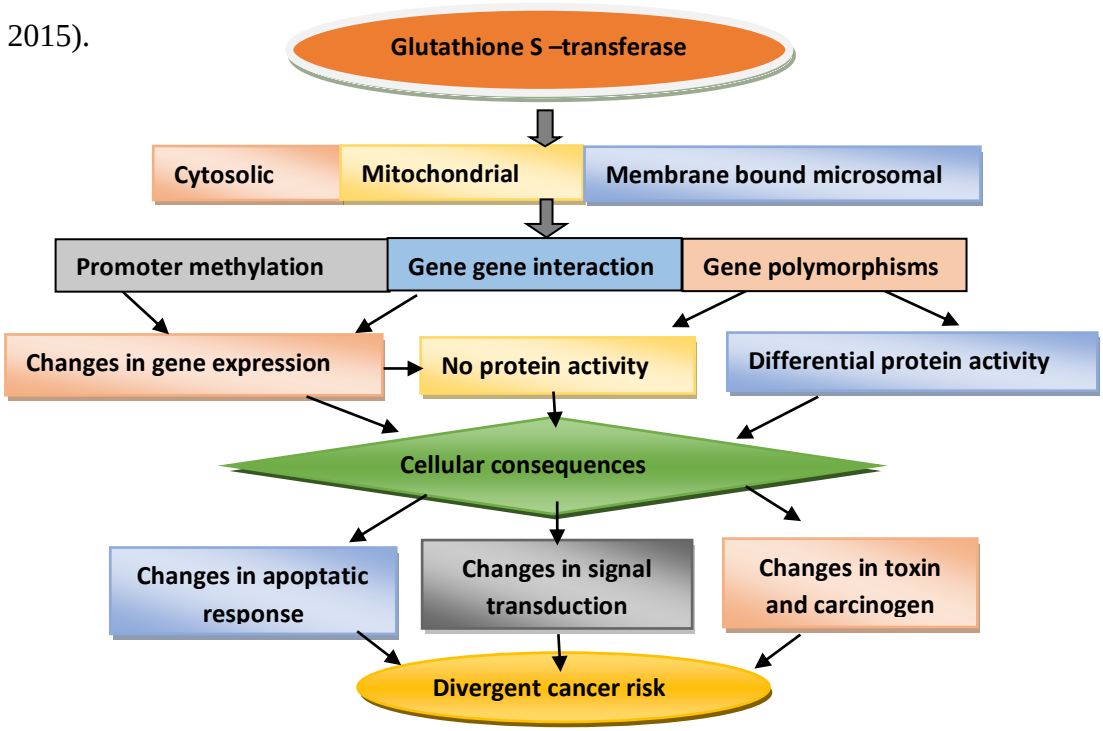


Figure 2.8: Overview of Glutathione-S-Transferase (GST) Classes and the Cellular Consequences of GST Gene Aberrations

2.8.2 Tissue and Organ Localization of GSTs

GSTs are widely expressed in mammalian tissues with a broad substrate specificity (Hayes and Strange, 2000). Many of the cytosolic Phase I and phase II biotransformation GST enzymes are characterized by zone-specific expression in the liver and the pancreas validate their role in detoxification (Oinonen and Lindros, 1995; Standop et al., 2002). The classification and organ localization of Glutathione S Transferases are presented in Table 2.5.

Table 2.5: Classification and Organ Localization of Glutathione S-Transferases

Superfamily	class	Chromosome	protein	Organ
Soluble	Alpha	6p12	GSTA1	testis=liver>>kidney=adrenal>pancreas
			GSTA2	liver=testis=pancreas>kidney>adrenal>brain
			GSTA3	Placenta
			GSTA4	Small intestine=spleen>liver=kidney>brain
Soluble	Kappa	-	GSTK1	liver (mitochondria)
Soluble	Mu	1p13.3	GSTM1	liver>testis>brain>adrenal=kidney>lung
			GSTM2	brain=skeletal muscle=testis>heart>kidney
			GSTM3	testis>>brain=small intestine>skeletal muscle
			GSTM4	brain, heart, skeletal muscle
			GSTM5	brain, heart, lung, testis
Soluble	Pi	11q13	GSTP1	liver, brain>heart=lung=testis>kidney=pancreas
Soluble	Sigma	4q21-22	GSTS1	fetal liver, bone marrow
Soluble	Theta	22q11	GSTT1	kidney=liver>small intestine>brain=prostate
			GSTT2	Liver
Soluble	Zeta	14q24.3	GSTZ1	fetal liver, skeletal muscle
Soluble	Omega	10q23-25	GSTO1	liver=heart=skeletal muscle>pancreas> kidney

MAPEG	Microsomal	12p13.1-13.2	MGST-I	liver=pancreas>prostate>colon=kidney>brain
		9q34.3	MGST-I-Like I	testis>prostate>small intestine=colon
		4q28.31	MGST-II	liver=skeletal muscle=small intestine>testis
		1q23	MGST-III	heart>skeletal muscle=adrenal gland, thyroid
		5q35	LTC_4S	platelets=lung>liver
		13q12	FLAP	lung=spleen=thymus=PBL>>small intestine

Table developed from- Hayes and Strange, 2000.

2.8.3 Role of GSTs in Carcinogen Metabolism

GST represents a large family of enzymes that catalyze the conjugation of a range of alkylating agents, including potential carcinogens and pharmacologically active compounds to the –SH groups of the antioxidant glutathione, thereby neutralizing electrophilic sites and rendering the products more water-soluble compared with the original agents. Glutathione conjugates are metabolized further by cleavage of the glutamate and glycine residues, followed by acetylation of the resultant free amino group of the cysteinyl residue, catalyzed by N‑acetyl transferase, to produce the final product, mercapturic acid which results in detoxification and the elimination of potentially genotoxic compounds by the kidney defense against cytotoxic and potentially carcinogenic chemicals (Eaton and Bammler, 1999).

The level of GST activity depends on genetic differences and may influence individual susceptibility to cancer, particularly in cases of higher exposure to toxic and carcinogenic agents (Strange and Fryer, 1999). GSTs are involved in the biotransformation of several compounds, which are recognized as risk factors for Cancer. Carcinogenic metabolites of environmental pollutants and tobacco smoke (for example, polycyclic aromatic hydrocarbon diol-epoxides) are detoxified by members of GST classes' alpha (GSTA1, GSTA2), mu (GSTM1) and pi (GSTP1). Halogenated solvents, such as dichloromethane, and ethylene oxide, formed endogenously from ethane, which is also present at high levels in cigarette smoke are the substrates for GST class theta enzymes (for example, GSTT1). GSTA1,

GSTA2, GSTM1, and GSTT1 enzymes also show antioxidant activity towards phospholipid hydroperoxides (Coles and Kadlubar, 2005). Thus, GSTs might prevent DNA damage from lipid peroxides formed endogenously, resulting from oxidative stress (Hurst et al., 1998).

2.8.4 Polymorphic Expression of GST and Prostate Cancer Risk

A significant number of genetic polymorphisms among soluble GSTs have been described (Hayes and Strange, 2000). Importantly, variation in GST alleles is prevalent in the population and will presumably make a significant contribution to interindividual differences in carcinogen metabolism. For the most part, polymorphisms in individual GST genes do not confer a markedly increased risk of cancer. Typically, odd ratios associated with any single variant GST allele and the development of particular neoplastic diseases are found to be less than 3.0 (Hayes and Mantle, 1986). However, combinations of variant GST alleles, either with other polymorphic GST or with alleles of other detoxification or antioxidant genes, are likely to have an additive effect in conferring predisposition to the nucleotide number quoted is that found in the cDNA.

GSTs are constitutively found in a wide variety of tissues, with different characteristic patterns of GST isozymes. Certain genes within the GSTM and GSTT subfamilies (GSTM1, GSTT1) are polymorphic in humans, and the levels of individual enzymes expressed can be influenced by induction and genetic polymorphism. Total or partial deletions and (or) single nucleotide polymorphisms in alleles encoding GSTM1, GSTT1 are associated with a reduction of enzymatic activity toward several substrates of different GST isozymes. Individuals with a decreased rate of detoxification, with “high risk” glutathione S transferase genotypes, have a slightly higher level of carcinogen-DNA adducts and more cytogenetic damages.

Polymorphisms in xenobiotic-metabolizing genes may cause alteration in expression, function, and activity and have been suggested to contribute to individual cancer susceptibility as genetic modifiers of cancer risk (Adam et al., 2003). Epidemiological studies indicate that individuals with homozygous deletions of GSTM1 and GSTT1 genes may have impaired ability to eliminate carcinogenic compounds metabolically and may, therefore, be at increased

risk for developing various types of neoplastic diseases, including cancers of the bladder, prostate, colon, skin, lung, and stomach (Strange and Fryer,1998). The frequencies of GSTM1 null alleles display race and ethnic variations, being highest in Europeans (42–60%) and Asians (41–63%) compared with that of Africans (16–36%) However, the frequency of GSTT1 null genotypes is somewhat less in Europeans (13.31%) compared with that of Africans (14–57%) and in Asians (35–48%) (Sharma et al., 2014). Among the primary human classes of this system, GSTM1 and GSTT1 genes exhibit a deletion polymorphism that leads to a lack of active isoforms when in homozygosis, known as the null genotype (Hayes and Strange, 2000). In the case of GSTT1-null, which occurs at frequencies of 11–38% in different populations, 50 kb of genomic sequence containing the entire gene is deleted. While for the GSTM1-null, variable frequencies have a range of 20–70% involving a 15-kb sequence deletion (Pinheiro, 2013).

2.8.5 GSTT1 Activity

Glutathione S-transferase theta (θ) is considered the most ancient of the GSTs (Landi, 2000). The encoded GSTT1 human subunit is about 25,300 Da, and the gene is 8.1 kb long (Coggan et al., 1998; Meyer, 1993). GSTT1 genes are located on chromosome 22q11.23 (Oaklay, 2011). GSTT1 enzymes show lower glutathione binding activity, with increased catalytic efficiency as compared with other GSTs (Jemth and Mannervik, 1997; Bao et al., 1997). However, GSTT1-catalyzed reactions can also increase the toxicity of some essential small dihaloalkanes, such as dichloromethane (Landi, 2000). GSTT1 also catalyzes the detoxification of oxidized lipids and DNA (Bao et al., 1997; Whittington et al., 1999). Halogenated organic compounds, for example, the ethylene dibromide, p-nitrobenzyl chloride, p-nitrophenyl bromide, methyl chloride, and methyl iodide are known substrates for GSTT1 (Meyer et al., 1991; Chamberlain et al., 1998; DeMarini et al., 1997).

2.8.6 GSTM1 Activity

GSTM1 genes are located on chromosome 1p13.3 (Oaklay, 2011). GSTM1 encodes for a class mu GST isoenzyme involved in polycyclic aromatic hydrocarbons (PAHs) detoxification. The substrates of GSTM1 include benzo (a) pyrene, benzo (c) phenanthrene,

benzo (g) chrysene, and other carcinogens (Seidegard et al., 1987; Hu et al., 1998). They can catalyze in-vitro GSH conjugation with several potent carcinogenic epoxides, including aflatoxin B_1-8, 9-epoxide, and electrophilic metabolites of PAHs present in tobacco smoke (Ketterer et al., 1992). Members of mu and alpha GST super families possess selenium-dependent GSH peroxidase activity toward organic hydroperoxides for that cumene hydroperoxide (CuOOH) is used as substrate suggesting that these enzymes may play a role in protection against highly reactive products of oxygen metabolism including those induced by asbestos (Comstock et al., 1994).

2.9 Oxidative Stress and Prostate Cancer

Epidemiological, experimental, and clinical studies suggested that oxidative stress (OS) plays a significant role in explaining prostate cancer development and progression (Lim et al., 2005; Venkataraman et al., 2005; Aydin et al., 2006). Oxidative stress, defined as an imbalance between reactive oxygen species (ROS) production and antioxidant defenses (Sies, 1985; Sies, 1997) has been linked to some prostate cancer risk factors including diet intake (Gronberg, 2003; Jian et al., 2004), recurrent inflammation and aging (Nelson et al., 2002; De Marzo et al., 2007; Kwabi-Addo et al., 2007). Chronic inflammation of prostate epithelium, due to persistent ROS production, has been associated with an increase of oxidative stress and DNA damage leading to neoplastic transformation (Nelson et al., 2002).

At the cellular level, a growing body of evidence indicates that oxidative stress is involved in prostate carcinogenesis (Paschos et al., 2013) Reactive oxygen species can promote carcinogenesis by causing oxidative damage to DNA and macromolecules within cells, altering signal transduction pathways, and developing a malignant phenotype. Furthermore, genetic polymorphisms in antioxidant enzymes have been associated with cancer risk (Klaunig et al., 2010), and prostate cancer risk may be modified by interactions between antioxidant enzyme genotype and dietary antioxidants (Li et al., 2005; Goodman et al., 2006). Men diagnosed with prostate cancer have been shown to have higher oxidative stress, lower antioxidant enzyme activity (Arsova-Sarafinovska et al., 2009). Furthermore, in vitro experiments have implicated the production of reactive oxygen species in the aggressiveness

of prostate cancer (Kumar et al., 2008). Thus, relatively consistent evidence suggests an association between oxidative Fig. 2.9 the stress and prostate cancer. The cellular responses to ROS in prostate cells showing

Oxidative stress can also cause a variegated amount of modifications against DNA structure like base and sugar lesions, DNA- protein crosslinks, strand breaks, and base free sites (Pizzino et al., 2017). The role of oxidative stress has been postulated in many conditions, including atherosclerosis, inflammatory diseases, certain cancers, and the process of aging (Young and Woodside, 2001). Chronic increases in ROS over time are known to induce somatic mutation and neoplastic transformation, and intracellular changes in ROS levels may lead to processes that result in cell proliferation apoptosis and senescence, which are associated with initiation and development of cancer including prostate cancer (Frohlich et al., 2008).

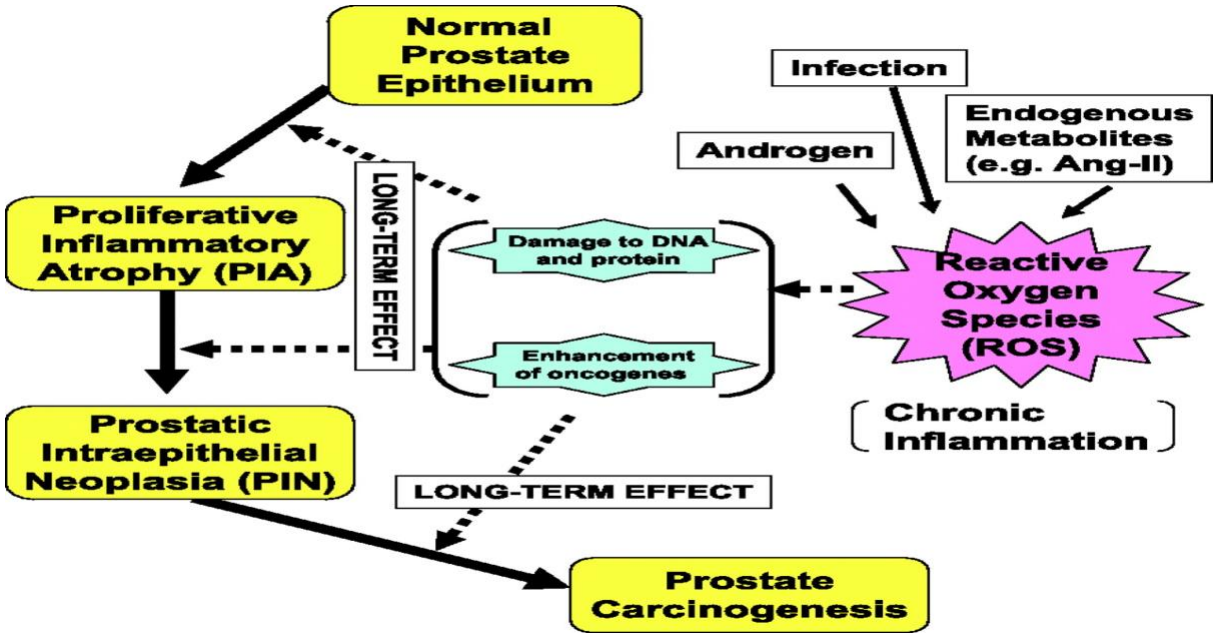


Figure 2.9: ROS Production, and Cellular Response to ROS in Prostate Cells.

2.9.1 Malondialdehyde: A Biomarker of Oxidative Stress

Lipid peroxidation is a process under which oxidants such as free radicals or nonradical species attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs) that involve hydrogen abstraction from carbon, with oxygen insertion resulting in lipid peroxyl radicals and hydroperoxides (Yin et al., 2011). The primary product of lipid peroxidation is lipid hydroperoxides (LOOH). Many different aldehydes, which can be formed as secondary products during lipid peroxidation like malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal (4-HNE). MDA appears to be the most mutagenic product of lipid peroxidation. MDA has been widely used as a convenient biomarker for lipid peroxidation of omega-3 and omega-6 fatty acids. MDA is an end-product generated by decomposition of arachidonic acid and larger PUFAs, through enzymatic or nonenzymatic processes.

MDA can be generated by decomposition of arachidonic acid (AA) and larger PUFAs as a side product by enzymatic processes during the biosynthesis of thromboxane A2(TXA2) and 12-l-hydroxy-5,8,10-heptadecatrienoic acid (HHT), or through nonenzymatic processes by bicyclic endoperoxides produced during lipid peroxidation. Once formed, MDA can be enzymatically metabolized or can react with cellular and tissue proteins, or DNA causes biomolecular damages. The probable biochemical route for MDA metabolism involves its oxidation by mitochondrial aldehyde dehydrogenase, followed by decarboxylation to produce acetaldehyde, which is oxidized by aldehyde dehydrogenase to acetate and further to CO_2 and H_2O (Ayala et al., 2014).

2.9.2 Malondialdehyde and Prostate Cancer

MDA levels are considered to be a valuable parameter to evaluate lipid peroxidation and oxidative stress. MDA is a highly reactive aldehyde and has the potential for DNA damage, probably leading to mutagenic, genotoxic, and cytotoxic effects (Olinski et al., 1995). Some investigators documented significantly increased MDA levels in prostate cancer patients when compared with benign prostatic hyperplasia (BPH) and controls (Almushatat et al., 2006; Arsova-Sarafinovska et al., 2009; Surapaneni et al., 2006; Yossepowitch et al., 2007), while

Dogru-Abbasoglu et al. (1999) did not find a significant difference in MDA levels in prostate cancer patients in comparison to benign prostatic hyperplasia (BPH) patients. The application of MDA in prostate cancer diagnosis is growing, and its measurement is now done in combination with prostate-specific antigen (PSA), which is a sensitive and generally accepted marker for prostatic hypertrophy and cancer.

2.9.3 Erythrocyte Reduced Glutathione (GSH) and Its Role as Antioxidant

Erythrocyte reduced glutathione (GSH) is one of the efficient antioxidants due to being endogenously synthesized all through the body (Kerksick and Willoughby, 2005). The majority of GSH is found in the cytosol (about 90%), while mitochondria contain nearly 10% and the endoplasmic reticulum contains a very small percentage (Shelly, 2009)

Biosynthesis

GSH is a tripeptide formed by glutamic acid, cysteine, and glycine. The glutamic acid forms a gamma-peptic bond with cysteine by its gamma-glutamyl group. Two forms of GSH are-

a. The reduced form (GSH) which represents the majority of GSH, reaching millimolar concentration in the intracellular compartment.
b. The oxidized form (GSSG) is estimated to be less than 1% of the total GSH.

The antioxidant function of GSH

In aerobic organisms, there is a certain level of oxidative stress from mitochondrial respiration and forms intermediates like superoxide and hydrogen peroxide. GSH reduces endogenously produced hydrogen peroxide in the presence of GSH peroxidase. In this process, GSH is oxidized to Glutathione disulfide (GSSD), which in turn is reduced back to GSH by GSSD reductase by using NADPH, forming a redox cycle. Catalase can also reduce hydrogen peroxide, but in mitochondria, as there is no catalase, GSH plays a significantly important role of defense against both physiologically and pathologically generated oxidative stress (Fernandez-Checa et al., 1997; Garcia-Ruiz and Fernandez-Checa, 2006).

Under severe oxidative stress, the ability of the cell to reduce GSSG to GSH may be overcome, leading to the accumulation of GSSG. To avoid a shift in the redox equilibrium, GSSG can be actively transported out of the cell or react with a protein sulfhydryl (PSH) to form a mixed disulfide (PSSG) (Shelly, 2009).

2.9.4 Glutathione S- Transferase: An Antioxidant Enzyme

Glutathione S-transferees (GSTs) are a superfamily of multifunctional isoenzymes involved in the cellular detoxification of several endogenous and exogenous compounds. GSTs catalyze the nucleophilic attack of GSH on the electrophilic centers of substrates, including insecticides, toxic compounds, metabolites and organic hydro peroxidases (Kim et al., 2017)

GSTs conjugation activity

GSTs have multiple biological roles against oxidative stress and several toxic molecules, as well as involved in synthesis and modification of leukotrienes and prostaglandins. GSTs protect cellular DNA against oxidative damage that can lead to an increase in DNA mutations or induce DNA damage, promoting carcinogenesis (Allocati et al., 2018). GSTs can conjugate GSH to a wide range of hydrophobic and electrophilic molecules, including many carcinogeneses, therapeutic drugs, and various types of cellular oxidative metabolites to make them less toxic (Kim et al., 2017). Harmful molecules that diffuse across the plasma membrane and inside the cell targeted by the enzymes of Phase I metabolism, where oxidation and reduction of the molecules occurs. In the subsequent Phase II metabolism, the leading role is played by GSTs that catalyze the conjugation of phase I modified xenobiotics to endogenous GSH. The conjugates then actively transported out of the cell by Phase III metabolism (Fig. 2.10) (Allocati et al., 2018)

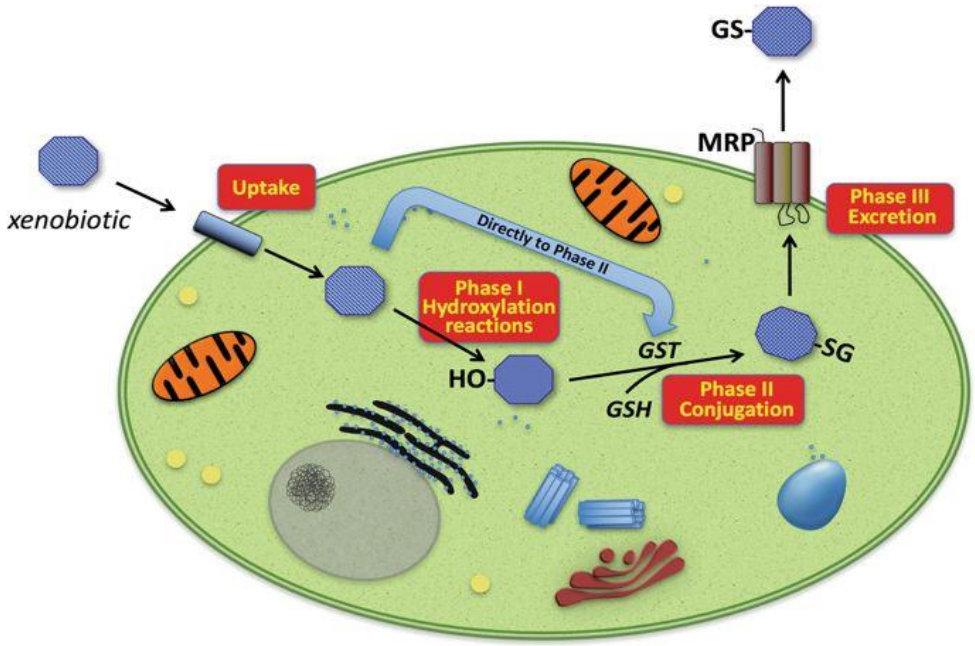


Figure 2.10: Glutathione System (Glutathione and Glutathione S Transferase) in Enzymatic Biotransformation

2.9.5 Glutathione System (Glutathione and Glutathione S Transferase) and Prostate Cancer

Oxidative stress is regulated in both benign and malignant cells via several nonenzymatic and enzymatic antioxidant mechanisms (Paschos et al., 2013). Cancerous cells have relatively higher levels of reactive oxygen species, and compensatory increases in antioxidant enzymes to tolerate increased oxidative stress (Cairns et al., 2011).

Glutathione and glutathione-dependent enzymes, such as GSTs, are involved in cell defense against ROS. The glutathione system participates not only in the antioxidant defense system but also plays a vital role in many processes on a molecular, cellular and organism level; therefore, disturbances in glutathione system homeostasis are involved in pathogenesis and the progression of cancer and liver diseases (Czuczejko et al., 2003; Raza, 2011; Traverso et

al., 2013). GSTs are a family of detoxification enzymes that catalyze the conjugation of reduced glutathione (GSH) to a wide variety of endogenous and exogenous compounds, making them less biologically active, more water-soluble and more quickly eliminated from an organism. Hence, GSTs are essential in controlling toxic products by generating lipid oxidation and oxidative stress.

Glutathione (GSH) is a thiol and tripeptide which is synthesized by glycine, cysteine, and glutamate in the liver and acts as a vital factor in metabolic protective functions, including the reduction of hydroperoxides, the quenching of free radicals, and the detoxification of xenobiotics (Wu et al., 2004) The GSH-dependent antioxidant system consists of GSH and an array of functionally related enzymes, including glutathione S-transferase (GSH-ST), glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Rd). GSH-ST is not only capable of conjugating several potentially toxic electrophilic xenobiotics to the nucleophilic GSH but can also catalyze reactions to reduce peroxides.

Srivastava and Mittal (2005a) reported that the decrease in the levels of GSH in blood haemolysates might be due to the increased utilization of GSH by glutathione S-transferases (GST) in detoxification of endogenous or exogenous exposed carcinogens. Ghalia et al. (2000) also reported that GST activity was higher in malignant tissue as compared to non-malignant tissue. The expression of several enzymes involved in oxidative stress and detoxification is repressed in prostate cancer, in particular, glutathione S-transferase (Singal et al., 2001)

2.10 Zinc and Prostate Cancer

Zinc is essential for prostate function and health. Zinc levels in the prostate gland are higher than in any other soft tissue of the body. Zinc levels in healthy prostate tissue are almost 7 times as high as in prostate cancer tissue (Zaichick et al., 1997). The reduction in antioxidants in prostate cancer patients suggests that micronutrient supplementation could be helpful in the prevention and management of the disease (Duru et al., 2014; Kotrikadze, 2008; Manzanares et al., 2012).

Zn^{+2} has an additional contribution to hormonal function on prostatic tissue (Sapota et al., 2009). Yan et al. (2008) hypothesized that decreased levels of Zn^{+2} are associated with increased DNA damage. Christudoss et al. (2011) and Gomez et al. (2000) have found that Zn^{+2} levels were decreased in prostate cancer patients when compared with control. Both calcium and zinc have been associated with an increased risk of prostate cancer in prospective studies and case-control studies, although their exact role has yet to be determined (Iguchi et al.,1998).

Zinc inhibits human prostatic carcinoma cell growth, possibly due to the induction of cell cycle arrest and apoptosis. Zinc deficiency causes increased oxidative stress that ultimately causes DNA damage, and it also interferes with DNA repair, leading to cell death (Fig. 2.11) (Iguchi et al.,1998).

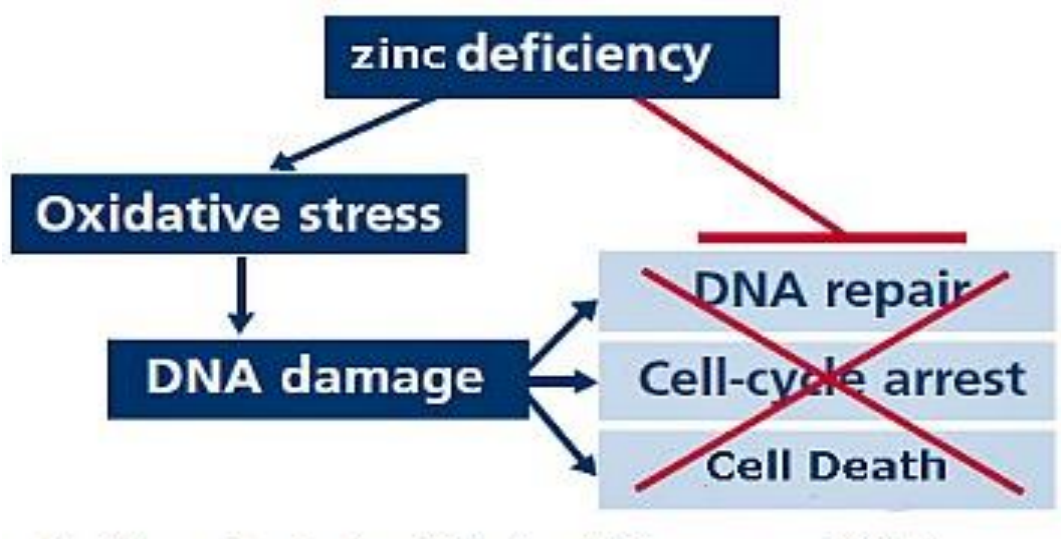


Figure 2.11: Role of Zinc Deficiency in Oxidative Stress and DNA Damage.

There now exists substantial evidence that the loss of a unique capability to retain high levels of zinc is an essential factor in the development and progression of malignant prostate cells (Liang et al., 1999). Zinc was reported to inhibit prostate cancer invasion (Ishii et al., 2001).

Epstein et al. (2011) assessed dietary intake and level of zinc in the blood of prostate cancer patients and reported that those with high dietary zinc intake had a 36% lower risk of dying from prostate cancer than those with low intake.

2.10.1 Physiological and Biological Functions of Zinc

Since Dr. Prasad first reported human zinc deficiency, there has been no doubt as to the role of zinc as an essential micronutrient for human health (Prasad et al., 1963). Tremendous evidence shows that zinc plays a significant role in a variety of biological and physiological functions in humans (Prasad et al., 2009). For example, it is known that zinc participates in the activation of more than 300 enzymes that mediate the regulation of macromolecule biosynthesis in DNA, RNA, and proteins, as well as cell growth and proliferation and other types of metabolism (Prasad et al., 2009). The evidence also shows that zinc is required to stabilize the tertiary structures of more than 300 proteins (for example, zinc finger proteins) and many transcription factors.

2.10.2 zinc as an Antioxidant and Anti-inflammatory Agent

Zinc (Zn) is the second most abundant trace element in the human body. Zn has antioxidant properties and protects tissue from oxidative stress (Miao et al., 2013). By two main mechanisms: (i) protection of protein sulfhydryl groups from free radical attack and (ii) reduction of free radical formation through the antagonism of redox-active transition metals. In biochemical systems, the antioxidant properties of zinc have been demonstrated and, for the most part, appear to be independent of zinc metalloenzyme activity (Saul, 2000). In general, the mechanism of antioxidation can be divided into acute and chronic effects. Chronic effects involve the exposure of an organism to zinc on a long-term basis, resulting in induction in some other substance that is the ultimate antioxidant. On the other hand, chronic zinc deprivation generally results in increased sensitivity to some oxidative stress (last reviewed in Bray and Bettger, 1990). However, the biochemical basis of many of these effects is not clear. The acute effects are generally thought to involve two mechanisms: protection of protein sulfhydryls or reduction in the formation of OH from H2O2 through the antagonism of redox-active transition metals, such as iron and copper (Soul, 2000).

Zinc, as an antioxidant, reduces the formation of free radicals in several ways (Fig. 2.12) (Prasad, 2009). Zinc acts as an inhibitor of NADPH oxidase, inducer of metallothionein (effective scavenger of radicals), and is an integral metal of Cu, Zn-SOD. ROS is known to activate NF-kappa B, which in turn activates growth factors, antiapoptotic molecules resulting in cell proliferation (cancer), inflammatory cytokines, and adhesion molecules (Prasad, 2009). Zinc reduces inflammatory cytokine production by upregulation of a zinc-finger protein, A20, which inhibits NF-kB activation via the TRAF pathway (Prasad, 2008). Thus, zinc functions not only as an antioxidant but also as an anti-inflammatory agent.

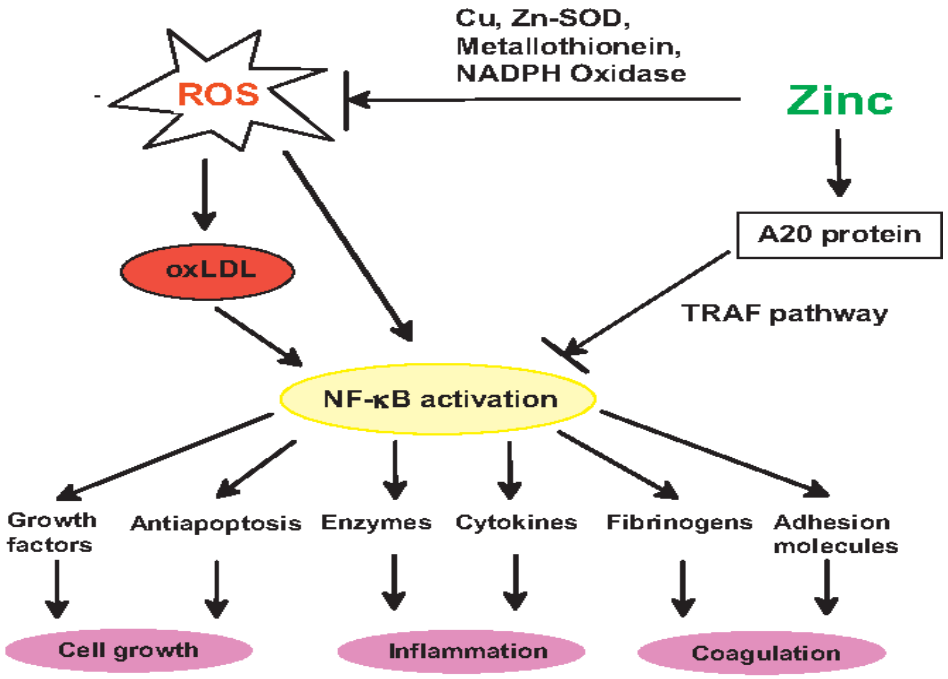


Figure 2.12: Zinc as an Antioxidant and Anti-inflammatory Agent

2.10.3 Zinc and Glutathione System

Several findings relate to glutathione content with zinc status. The decrease in GSH may be related to the observation that in zinc-deficient rats, there is a 7-fold increase in the urinary excretion of glutamic acid (Hsu,1977). Thus, there may be a decreased availability of this precursor of GSH. Also, zinc may have an essential role in the maintenance of reduced

glutathione. Evidence for this is that zinc ions inhibited NADPH oxidase activity in liver microsomes (Chvapil et al., 1976), and this could maintain NADPH levels necessary for GSSG reductase activity and the regeneration of GSH. Finally, blood GSH and zinc have been implicated in the maintenance of erythrocyte membrane integrity. Zinc is an integral component of the membrane (Chvapil et al., 1979) and may play a role in its stabilization, possibly reacting with sulfhydryl groups to form stable mercaptides (Chvapil, 1973). Further, GSH has been implicated as a membrane stabilizer since spontaneous hemolysis was related to glutathione oxidation (Fegler, 1952). For these reasons, the protective effect of zinc may be lost with zinc deficiency and result in a significant decrease in GSH. Zinc is required to maintain adequate levels of glutathione (GSH) in the blood (Mills et al., 1981).

3. Materials and Methods

3.1 Study Design

The study was designed as a case-control study.

(i) Case: Histopathologically confirmed prostate cancer patients (n= 207)

(ii) Control: Healthy subjects without a history of any cancer or chronic diseases (n=200).

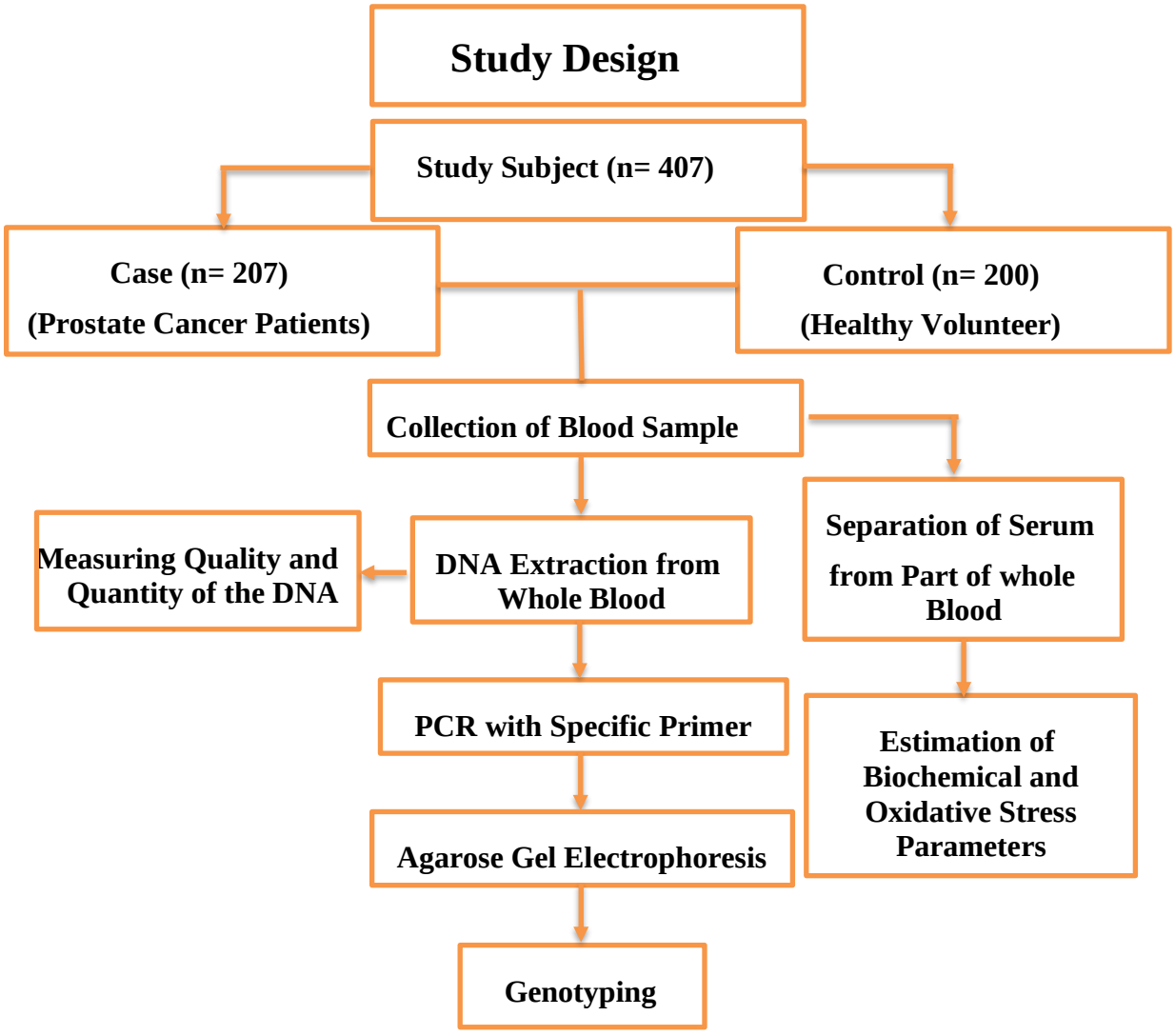


Figure 3.1: Schematic Diagram of Study Design

3.2 Ethical Issue and Consent

The study was approved by the Ethical Review Committee (ERC) of the Department of Biochemistry and Molecular Biology, University of Dhaka **(Appendix III)**. The study was conducted following the declaration of Helsinki and its subsequent revisions (World Medical Association, 2013). All participants were explained by the nature of the study. They were informed about their rights to withdraw from the study at any time. They were also informed that the identity of the individual and data obtained following analysis would not be disclosed. Available data would only be used for research purposes. Participants were included in the study only after signing an "Informed Written Consent Form" (in Bengali) before collecting the blood samples **(Appendix II)**

3.3 Selection of the Study Subject

Prostate cancer patients (n=207) were selected from out and inpatient Department of Urology of BIRDEM General Hospital, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka Medical College Hospital (DMCH) and other tertiary care hospitals of Dhaka.

Controls (n=200) were selected from the outpatient department of Medicine of BIRDEM General Hospital and Bangabandhu Sheikh Mujib Medical University (BSMMU), who had no history of cancer or chronic diseases and came for a routine checkup. The age of the control subjects was matched with the cancer patients.

3.3.1 Eligibility Criteria for Study Subject

3.3.1.1 Inclusion Criteria

Case group:

- Histopathologically confirmed cases of prostate cancer before taking any intervention like chemotherapy/ radiotherapy.

Control group:

- Age-matched healthy control from the same geographic area and ethnicity.

3.3.1.2 Exclusion Criteria

- The subjects had a history of any type of cancer.
- Subjects had any chronic diseases like tuberculosis, chronic kidney diseases, diabetes, etc.
- Subjects had a history of supplementation of zinc or antioxidant.

3.4 Questionnaire

A structured questionnaire was filled up for each study subject, covering information on age, BMI, educational status, residence, occupation, smoking history, and family history of cancer or chronic diseases and other exposure histories. All the clinical features, including digital rectal examination (DRE) findings, ultrasonogram (USG) findings, urine routine examination (R/E) findings, clinical staging, and histopathological grading, were also included in the questionnaire **(Appendix-I).**

3.5 Anthropometric Measurements

3.5.1 Height (m)

Standing height was measured using appropriate scales (Detect-Medic, Detect scales INC, USA) without shoes. The subject was positioned fully erect, with the head in the Frankfurt plane (with the line connecting the outer canthus of the eyes and the external auditory meatus perpendicular to the long axis of the trunk); the back of the head, thoracic spine, buttocks, and heels touched the vertical axis of the anthropometer, and the heels were together. Height was recorded to the nearest 5 millimeters.

3.5.2 Weight (kg)

The balance was placed on a hard flat surface and adjusted to zero before measurement. The subjects were in the center of the platform wearing light clothes without shoes. Weight was recorded to the nearest 0.5 kg.

3.5.3 BMI (kg/m$_2$)

Body mass indexes (BMI) of the subjects were calculated using the following formula.

$$BMI = Weight\ (kg) / [Height\ (m)_2]$$

3.6 Collection and Storage of Blood Sample

About six (6.0) mL of venous blood was drawn from each individual following all aseptic precautions with the help of a trained person, using a disposable syringe. Three (3.0) mL of blood samples were used for serum isolation (collected in a plain vacutainer tube), and three (3.0) mL whole blood were kept for isolation of genomic DNA (collected in EDTA containing tube). The drawn blood was immediately transferred to the tube and kept in an icebox for transportation to the laboratory. Serum was separated from blood containing in the plain tube after centrifugation for10 minutes at 3,000 rpm and collected into the Eppendorf tube by using a micropipette. All samples (serum and whole blood) were stored at -80°C until further use.

3.7 DNA Extraction and Quantification

3.7.1 Extraction of DNA

Genomic DNA was extracted from peripheral blood using the method described by Bailes et al. (2007).

Required Chemicals

EDTA (0.5 M), pH 8.0: 18.61 g of anhydrous EDTA was added to 80.0 mL of distilled water. The pH was adjusted to 8.0 with NaOH. The solution was made up to 100 mL with distilled water. It was autoclaved at 15 p.s.i. for 15 min.

Tris-HC1 (1 M), pH 7.6: 12.11 g of Tris base was dissolved in 80.0 mL of distilled water. The pH was adjusted with concentrated HCl. The solution was cooled at room temperature before the adjustment of the pH. The volume was made up to 100 mL with distilled water and autoclaved at 15 p.s.i. for 15 min.

Red Blood Cell Lysis Buffer: 1.0 mL of Tris (1 M), 10.954 g of sucrose, 0.101 g $MgC1_2$, and finally 1.0 mL of Triton X-100 were taken into 80 mL of distilled water, and the pH was adjusted at 8.0. Then the volume of the solution was made up to 100 mL with distilled water and autoclaved at 15 p.s.i. for 10 min.

Nucleic Acid Lysis Buffer: 1.0 mL of Tris-HC1(1 M, pH 7.6), 0.375 g of anhydrous EDTA (pH 8.0), 1.0 g of SDS, 0.294 g of sodium citrate were taken in a volumetric flask, and the pH was adjusted to 8.0. The volume was made up to 100 mL with distilled water. It was autoclaved for 15 min at 15 p.s.i.

TE Buffer, pH 8.0: 0.5 mL of Tris-HCl (1 M, pH 7.6), 0.2 mL of EDTA (0.5 M, pH 8) were taken in a flask, and the volume was made up to 100 mL with distilled water. The pH was adjusted to 8.0 and autoclaved for 15 min at 15. p.s.i.

Chloroform (Roth), Prechilled to 4 °C

Ethanol (Merck) (100%), Prechilled to -20 °C.

3.7.2 Extraction Procedure

Before starting DNA extraction, the liquid blood sample was shaken gently by rotating the tube.

Steps of DNA extraction:

500 µL of blood was poured into a 1.5 ml Eppendorf tube, and 1000 µL of red cell lysis buffer was added. It was shaking gently (up to homogenizing), then spin for 2 minutes at 7,000 rpm. Then the supernatant was discarded, and the above procedure was repeated two or three more times to remove hemoglobin. The tube was placed on tissue paper for a few seconds downward. Then 400 µL of nucleic lysis buffer, 100 µL of saturated NaCl (5M), and 600 µL of chloroform were added to Eppendorf tube and mixed. Then spin it for 2 minutes at 7000 rpm, and 400 µL of supernatant was transferred to a new 1.5 mL Eppendorf tube. Then 800 µL of cold (–20°C) absolute ethanol was added, and it was shaken gently first then vortex it. DNA appeared as a mucus-like strand in the solution phase. The Eppendorf tube was spin for one minute at 12,000 rpm to precipitate the DNA, then the supernatant was discarded carefully, and the tube was completely dried in room temperature, placing the tube downward on a tissue paper. Finally, 50 µL of TE buffer was added to the Eppendorf tube containing DNA, and after vortex tube was kept at –20°C for later uses.

Steps of DNA extraction are shown in Figure: 3.2.

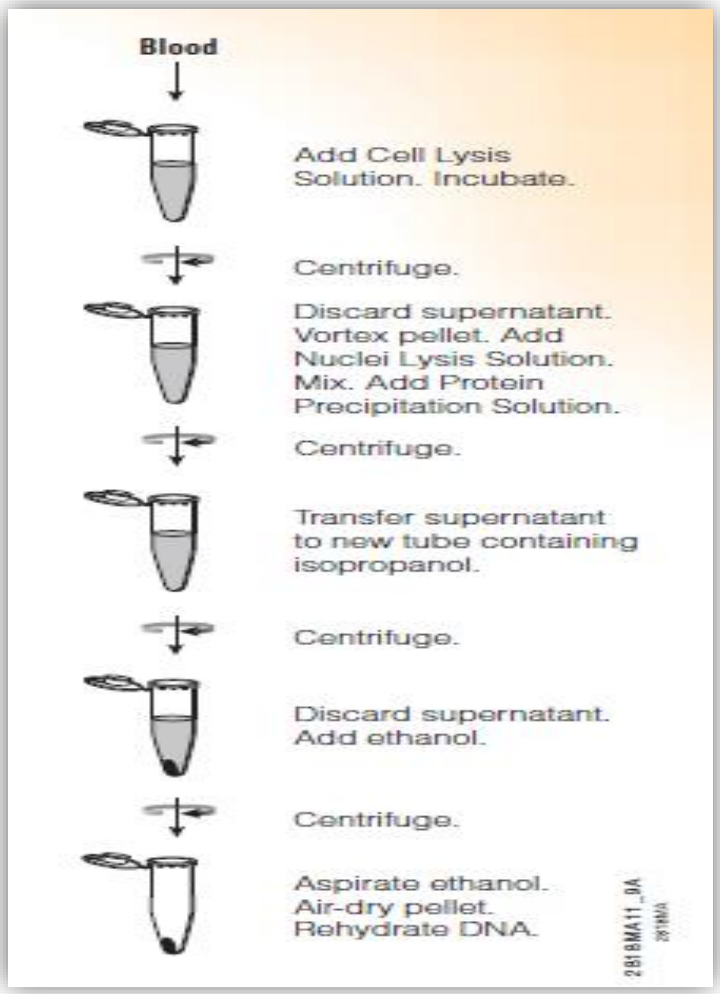


Figure 3.2: Steps of the DNA Extraction Procedure

Routinely, about 1 µL per PCR reaction is used without adverse effects. DNA can be quantified and diluted to a working concentration at this point or simply use 1 µL per PCR reaction.

3.7.3 Quantification of DNA

The quantity of the genomic DNA in the elute was determined by Nano-Drop 1000 spectrophotometer (Nano Drop 1000, US). The absorbance of diluted DNA was measured at 260 nm and 280 nm. The consistency of DNA elutes (5µL) of the samples was also evaluated by agarose gel electrophoresis. A gel containing 0.5% agar was used for this purpose. The electrophoresis was performed at low voltage (40V) for an hour and visualized using a gel picture analyzer.

3.8 PCR Amplification of the Target Genes Segment

The NAT2, GSTT1 and GSTM1 genotypes were determined using the PCR-RFLP method and Multiplex PCR Genotyping.

Reagents

- Go Star Taq Polymerase
- dNTPs
- Primers
- DMSO
- Restriction enzymes
- Agarose
- Ethidium bromide
- 100 bp DNA ladder
- TAE (Tris Acetate EDTA) Buffer

3.9 Identification of NAT2 Gene Polymorphism

3.9.1 PCR of the NAT2 Gene

Polymerase chain reaction (PCR) was carried out in 25 µL reaction volume. PCR product size for the primer set is 815 bp. PCR was carried out using the following primer set:

NAT2	Primer
Forward primer	5′-CTT CTC CTG CAG GTG ACC AT- 3′
Reverse primer	5′-AGG ATG AAT CAC TCT GCT TC -3′

PCR primer was constructed according to the protocol of Inatomi et al. (1999).

3.9.2 PCR Condition for NAT2 Gene Amplification

PCR was carried out using GoTaq polymerase. Conditions for the amplification included the initial step of denaturation 95$_o$C for 15 minutes, followed by 35 cycles of denaturation at 95$_o$C

for 45 seconds, annealing at 57₀C for 45 seconds, and elongation at 72₀C for 45 seconds and finally a step of final elongation at 72₀C for 10 minutes. PCR assays were performed in a DNA thermal cycler. A negative control (reagent blank), which contained all components of the reaction mixture without the sample DNA, was included in every PCR procedure (Figure:3.3).

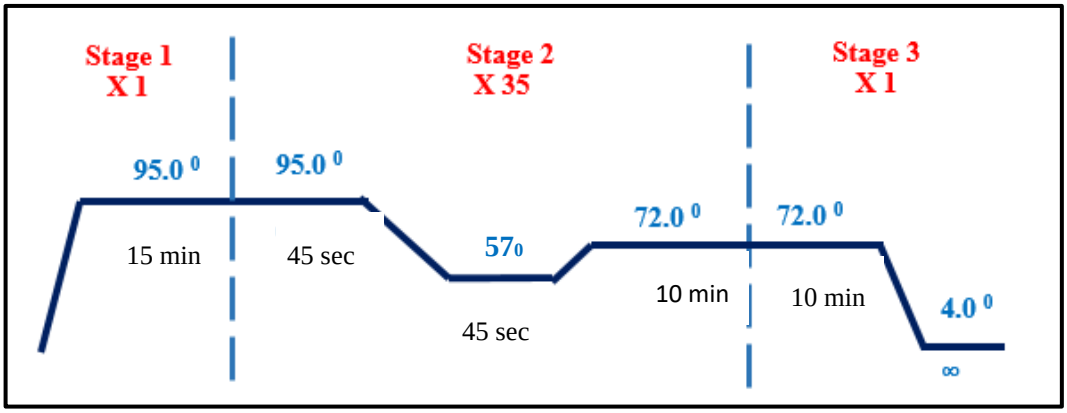


Figure 3.3: PCR Condition for NAT2 Gene Amplification

3.9.3 Composition of Reaction Mix for a PCR

Table 3.1: Composition of Reaction Mix for PCR (25 µL)

Name of the component	Volume (µL)
DNA	5.0
Buffer	2.5
dNTPs	0.2
Forward Primer (F)	1.0
Reverse Primer (R)	1.0
Taq Polymerase	0.175
ddH_2O	15.125
Total	**25.00 µL**

3.9.4 Evaluation of PCR

PCR product was checked for amplification in a 2% agarose gel. The optimum size of the product was ascertained, comparing it with the DNA ladder. The amplified DNA was visualized under UV light and gel image captured and documented (Figure: 3.4).

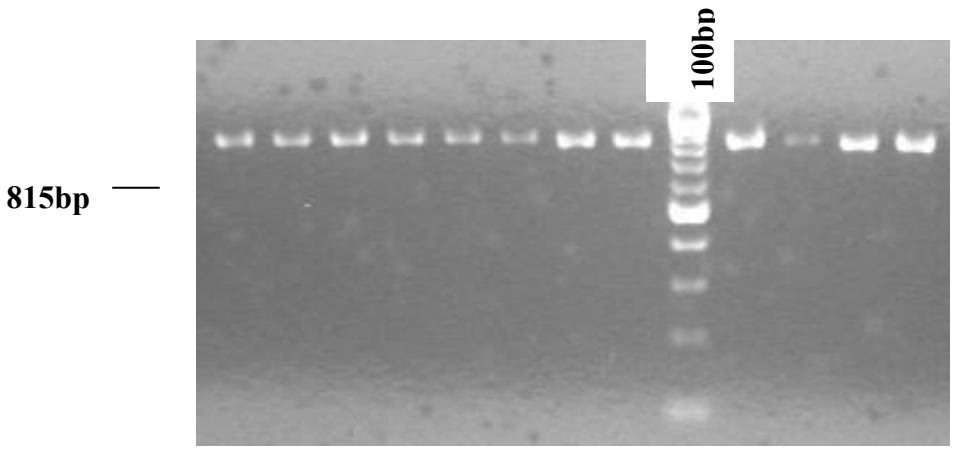


Figure 3.4: ımage of Gel Analysis of NAT2 Gene PCR Products in a 2% Agarose Gel.

3.9.5 RFLP Analysis of NAT2 Gene

NAT2 gene candidate polymorphic markers 481C>T, 590G>A, and 857G>A were analyzed using a site-specific restriction enzyme. Restriction enzyme digestion was performed following standard digestion protocol. Candidate NAT2 variants 481C>T, 590G>A, and 857G>A disrupt restriction enzyme site and were detected by restriction fragment length polymorphism (RFLP) assay (Figure: 3.5).

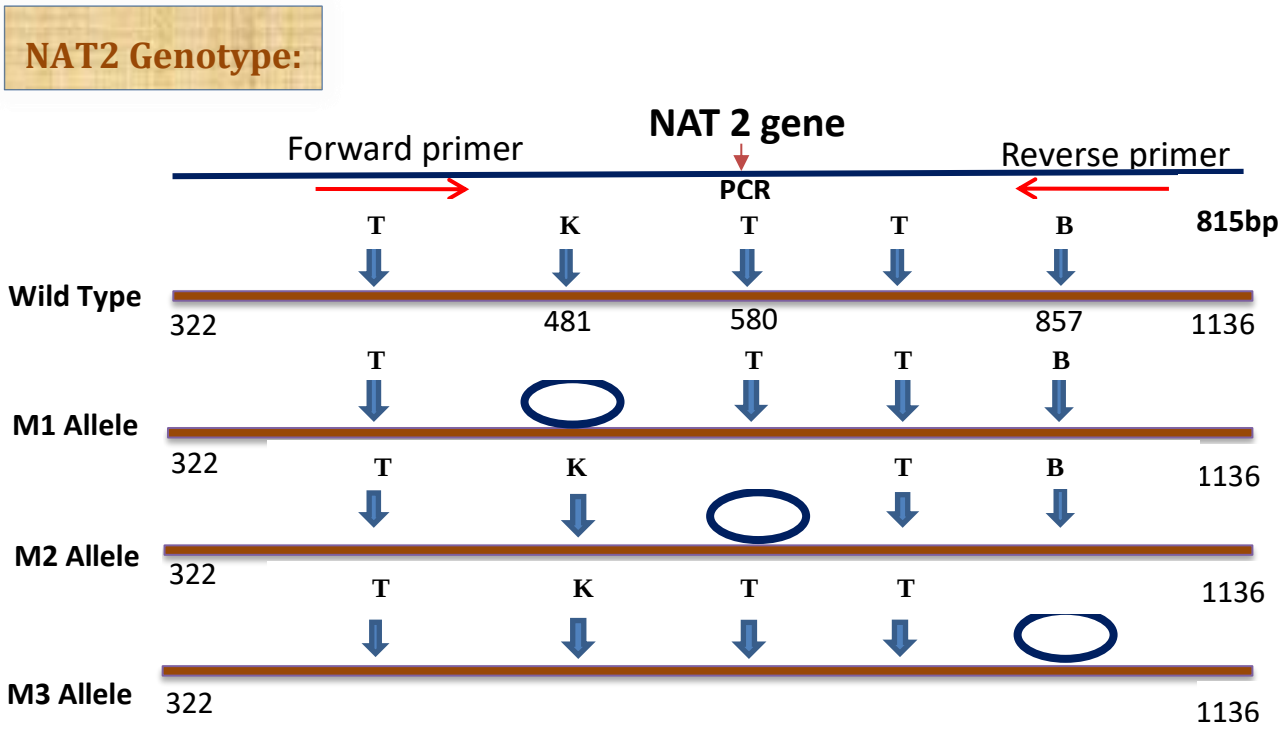


Figure 3.5: NAT2 Genotyping by PCR-RFLP

Kpn I Digestion

NAT2481C>T variant polymorphic marker was analyzed using Kpn I restriction enzyme. The digestion was carried out in a reaction volume of 15 µL. The enzyme digestion protocol was as follows (Table: 3.2):

Table 3.2: Kpn I Restriction Enzyme Digestion Protocol

Name of the component	Volume (µL)
PCR product	5.0
Buffer	1.5
Restriction enzyme (Kpn I)	0.75 (5-10 units)
H_2O	7.75
Total	**15.00 µL**

Kpn I restriction enzyme digestion was carried at 37^{o}C for 2 hours in a water bath. The enzyme digestion product was resolved in 3% agarose gel, and the digested product was visualized using a gel documentation system following ethidium bromide staining.

The 481C>T polymorphism restricts the Kpn I restriction enzyme site (5′…GGTACC…3′). Fragments produced by the Kpn I restriction enzyme digestion of the PCR products were as follows: Homozygous wild genotype 659 bp and 156 bp; heterozygous (Ht) variant genotype 815 bp, 659 bp, and 156 bp; and homozygous (Hz) variant genotype 815 bp (Figure: 3.6).

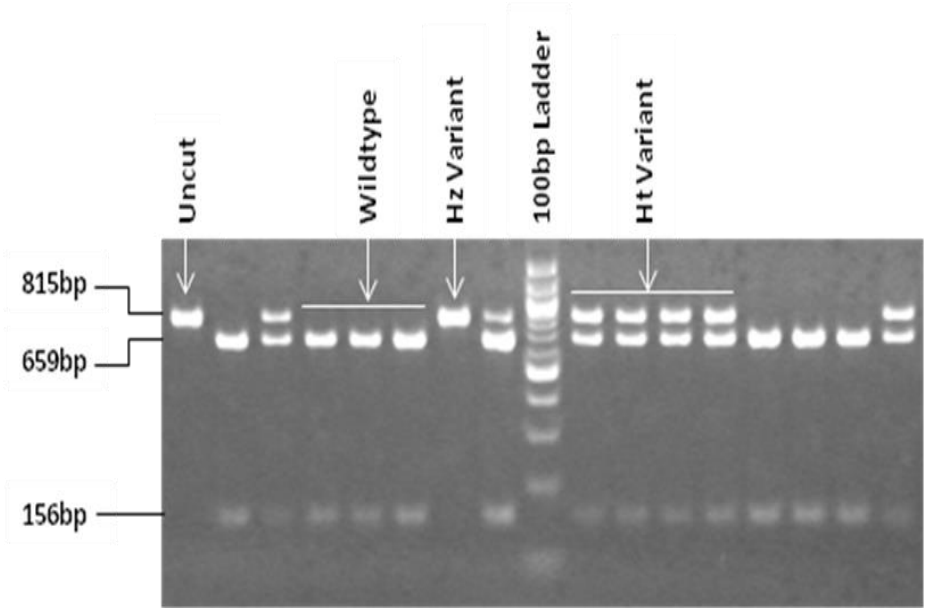


Figure 3.6: Agarose Gel Image of NAT2 Gene 481C>T Candidate Marker Analysis by Kpn I Restriction Enzyme Digestion.

Taq I Digestion

NAT2590G>A variant polymorphic marker was analyzed using the TaqI restriction enzyme. The digestion was carried out in a reaction volume of 15 μL. The enzyme digestion protocol was as follows (Table: 3.3):

Table 3.3: Taq I Restriction Enzyme Digestion Protocol

Name of the component	Volume (μL)
PCR product	5.0
Buffer 3	1.5
Restriction enzyme (Taq I)	0.25 (5-10 units)
BSA	0.15
H_2O	8.10
Total	**15.00 μL**

TaqI restriction enzyme digestion was carried at 65oC for 2 hours in a water bath. The enzyme digestion product was resolved in 3% agarose gel, and the digested product was visualized using a gel documentation system following ethidium bromide staining.

The 590G>A polymorphism restricts the Taq I restriction enzyme site (5′…T↓CGA…3′). The amplified segment of DNA had two internal cutting sites for Taq I common to all NAT2 polymorphic alleles, which generate bands 42 bp and 377 bp in all lanes on digestion with Taq I. Taq I digestion products are 396 bp 226 bp and 170 bp. Fragment 42 bp could not be resolved. For the homozygous wild-type, the product sizes were 226 bp and 170 bp, for the homozygous (Hz) variant the product size was 396 bp, and for the heterozygous (Ht) variant the product size was 396 bp, 226 bp, and 170 bp (Figure: 3.7).

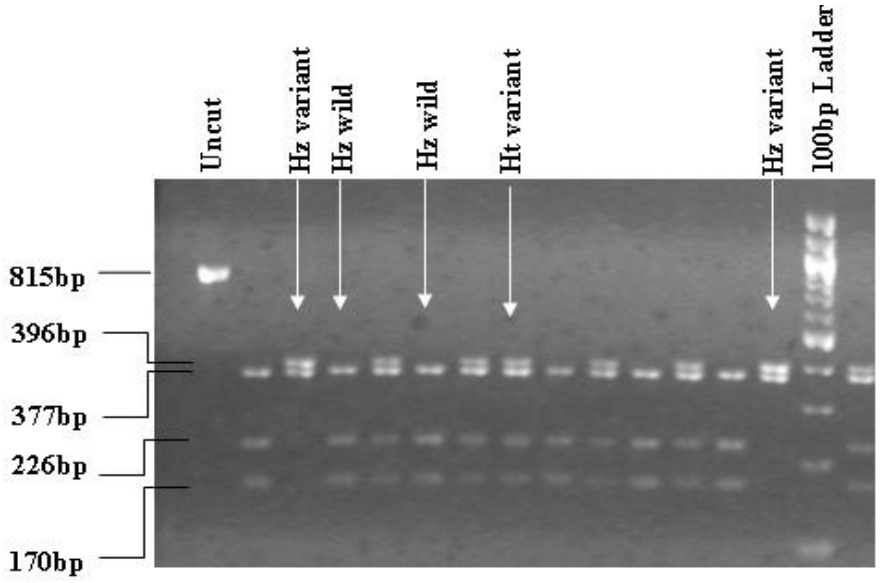


Figure 3.7: Agarose Gel Image of NAT2 Gene 590G>A Candidate by Taq I Restriction Enzyme Digestion.

Bam HI Digestion

NAT2 857G>A variant polymorphic marker was analyzed using the Bam HI restriction enzyme. The digestion was carried out in a reaction volume of 15 μL. The enzyme digestion protocol was as follows (Table: 3.4):

Table 3.4: Bam HI Restriction Enzyme Digestion Protocol

Name of the component	Volume (μL)
PCR product	5.0
Buffer 3	1.5
Restriction enzyme (Bam HI)	0.25 (5-10 units)
BSA	0.15
H_2O	8.10
Total	**15.00 μL**

Bam HI restriction enzyme digestion was carried at 37$_o$C for 2 hours in a water bath. The enzyme digestion product was resolved in 3% agarose gel, and the digested product was visualized using a gel documentation system following ethidium bromide staining.

The 857 G>A polymorphism restricts the Bam HI restriction enzyme site (5′…GGATCC…3′). Fragments produced by the Bam HI restriction enzyme digestion of the PCR product were as follows: Homozygous wild genotype 536 bp and 279 bp; heterozygous variant genotype 815bp, 536 bp, and 279 bp; and homozygous variant genotype 815 bp. (Figure: 3.8)

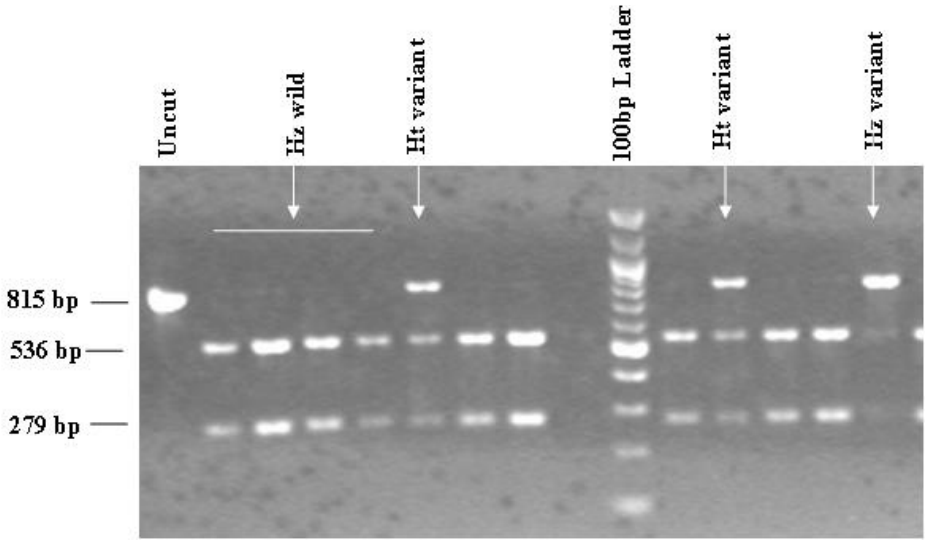


Figure 3.8: Agarose Gel Image of NAT2 gene 857G>A Candidate Marker Analysis by Bam HI Restriction Enzyme Digestion.

3.9.6 Determination of Acetylator Type

The presence of at least two genotype variants (homozygous and heterozygous) of the three candidate markers was taken as slow acetylator type, and others were taken as fast (Borlak et al., 2006).

3.10 Determination of GSTM1 and GSTT1 Gene Polymorphisms

3.10.1 Multiplex PCR for Genotyping

Genotyping of GSTM1 and GSTT1 genes was done using the Multiplex PCR-based method.

Reagents and Chemicals

- Go Taq Polymerase
- dNTPs
- Primers
- DMSO
- Agarose
- Ethidium bromide
- 100 bp DNA ladder

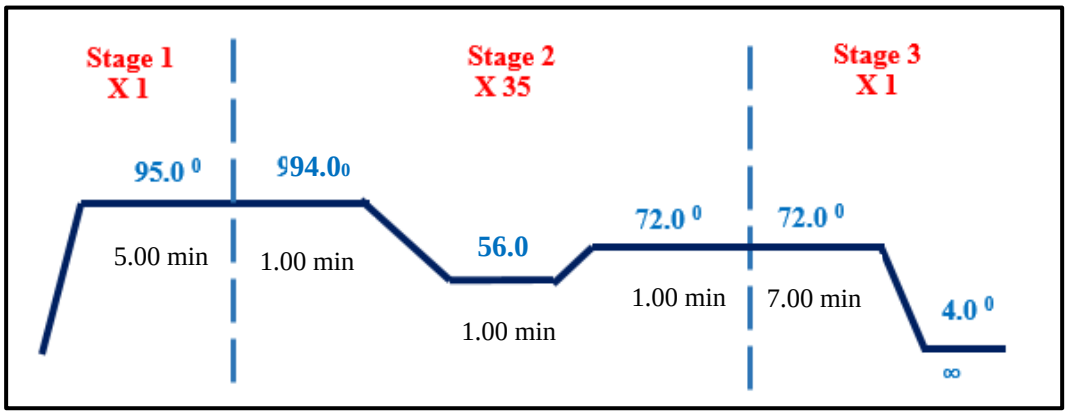
The multiplex PCR-based method was used for the identification of GSTM1 and GSTT1 genotypes. A part of the exon-7 CYP1A1 gene was amplified and used as an internal control to be ensured that a null genotype was due to the absence of GSTM1 and GSTT1 alleles rather than the failure of PCR in this method. Negative controls were also used for all amplifications; the reaction mixtures for these contained all the components except the DNA template.

GSTM1, GSTT1, and exon-7 CYP1A1 fragments were amplified by using the following primers (Yalin et al., 2007).

GSTT1	Primer
Forward primer	5’-TTCCTTACTGGTCCTCACATCTC-3’
Reverse primer	5’-TCCCAGGTCACCGGATCAT-3’
GSTM1	**Primer**
Forward primer	5’-GAACTCCCTGAAAAGCTAAAGC-3’
Reverse primer	5’-GTTGGGCTCAAATATACGGTGG-3’
Exon7-CYP1A1	**Primer**
Forward primer	5’-GAACTGCCACTTCAGCTGTCT-3’
Reverse primer	5’-CAGCTGCATTTGGAAGTGCTC-3’

3.10.2 PCR Condition

PCR was carried out using Go Taq polymerase. Conditions for the amplification included the initial step of denaturation 95₀C for 5 minutes, followed by 35 cycles of denaturation at 94₀C for 1 minute, annealing at 56₀C for 1 minute, and elongation at 72₀C for 1 minute and finally a step of final elongation at 72₀C for 7 minutes. PCR assays were performed in a DNA thermal cycler (Figure: 3.9).



. **Figure 3.9: PCR Condition for GSTT1 and GSTM1 Gene Amplification**

3.10.3 Composition of PCR Product

Table 3.5: Composition of Reaction Mixture for a PCR (15 µL)

Name of the component	Volume (µL)
PCR-H2O	3.2
5X Green Buffer	3.0
Colorless Buffer	0.8
DMSO	3.0
dNTPs	0.4
Forward Primer	0.6
Reverse Primer	0.6
Go Taq Polymerase	0.4
Genomic DNA	3.0
Total	**15.0 µL**

3.10.4 Evaluation of PCR and Genotyping

Finally, the co-amplified products (GSTM1: 215 bp, GSTT1: 466 bp and exon-7 CYP1A1: 312 bp) were analyzed by electrophoresis on 2% agarose gel, and GSTM1 and GSTT1 genotypes were determined. The optional size of the product was ascertained by comparing it with the DNA ladder. The amplified DNA was visualized under UV light, and the gel image captured and documented. The presence of a 215 bp band indicated that this subject wash homozygote or heterozygote for GSTM1, while its absence in the presence of the internal control band indicated the null GSTM1. The presence of a 466 bp band indicated that the subject was homozygote or heterozygote for GSTT1, while its absence in the presence of the internal control band indicated the null GSTT1 (Figure: 3.10).

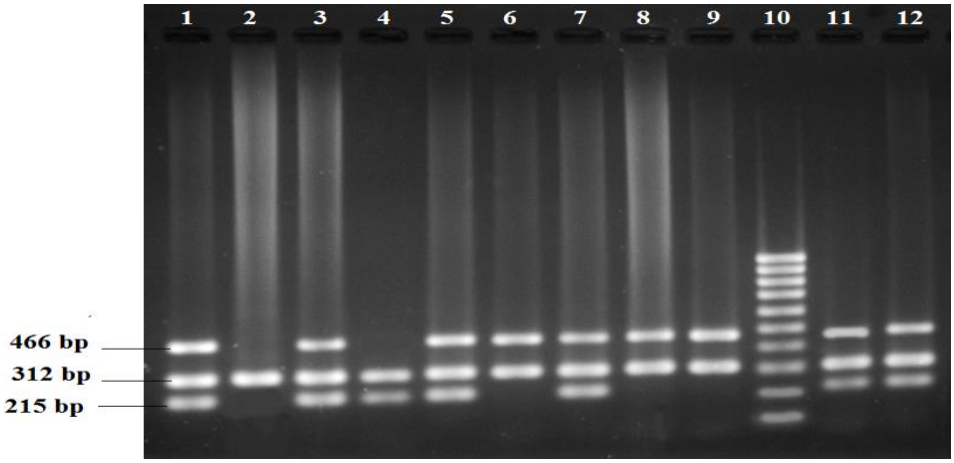


Figure 3.10: Multiplex-PCR Analysis of GSTM1 and GSTT1 Gene Polymorphisms

3.11 Estimation of Biochemical and Oxidative Stress Parameters

All biochemical measurements were carried out at the Clinical Biochemistry Laboratory, BIRDEM General Hospital, and Centre for Advanced Research in Science (CARS). Following investigations was done for each study subjects-

- Serum MDA estimated by the colorimetric method.
- GST activity was estimated from serum by colorimetric method.
- Erythrocyte reduced glutathione (GSH) was estimated from whole blood by the colorimetric method.
- Serum PSA was measured by chemiluminescent immune assay.
- Serum zinc was estimated by AAS Flame.

3.11.1 Estimation of Malondialdehyde (MDA)

Principle

MDA in the catabolite of lipid peroxide can react with thiobarbituric acid (TBA) and produce a red compound, which has a maximum absorption peak at 532 nm. Serum MDA was measured by the methods described by Rice and Anthony (1991).

Reagents

Reagent 1	Clarificant
Reagent 2	Acid reagent
Reagent 3	Chromogenic agent
Reagent 4	Standard (10 mmol/mL)

Preparation of Reagents

Reagent 1 was made warm by keeping it in a 37ºC water bath before use until the liquid turned to transparent before the experiment. Reagent 2 made diluted with double-distilled water at a ratio of 1.2:24 and mixed thoroughly. Reagent 3 (chromogenic agent) was prepared by dissolving the powder with 60 mL of double-distilled water (90ºC- 100 ºC), then 60 mL of glacial acetic acid was added and mixed fully and cooled to room temperature.

Procedure

Four separate clean tubes were taken for the test reactions, one as the blank tube, one as the standard tube, one for sample tube, and one for the control tube. 0.1 mL of absolute ethanol was taken in the blank tube, and 0.1 mL of reagent 4 was taken in the standard tube. On the other hand, 0.1 mL of samples were taken in the sample tube and control tube, respectively. Then 0.1 mL of reagent 1 and 3.0 mL of reagent 2 had been added to all the 4 tubes. After that, 1.0 mL of reagent 3 was added to the blank tube, standard tube, and sample tube, respectively. 1.0 mL 50% glacial acetic acid was added to the control tube and mixed thoroughly. Incubated in 95 ºC water bath for 40 min, then cooled the tubes with running water after incubation. Centrifuged at 3100 g for 10 min, and then the supernatant was collected. The colorimeter was set to zero with double-distilled water and measured the OD value at 532 nm with a 1 cm diameter cuvette.

Calculation of Result

MDA content *(nml/mL*)

$$= \frac{\text{OD sample} - \text{OD control}}{\text{OD standard} - \text{OD Blank}} \times \text{Concentration of Standard (10 nmol/mL)}$$

x Dilution multiple of the sample before the test

3.11.2 Estimation of Glutathione S-Transferase (GSH-ST)

Principle

The Glutathione S-Transferase (GST) Assay Kit utilizes 1-Chloro-2,4-dinitrobenzene (CDNB), which is suitable for the broadest range of GST isozymes. Upon conjugation of the thiol group of glutathione to the CDNB substrate, there is an increase in the absorbance at 340 nm (Habig et al., 1974; Wilce and Parker, 1994).

Reagents

- Dulbecco's Phosphate Buffered Saline
- Sample Buffer
- Substrate (CDNB)
- L-Glutathione reduced
- GST (control), ~0.25 mg/ml

Procedure

The Dulbecco's Phosphate Buffered Saline and the CDNB solution were warmed at 25 °C before starting the assay. A reaction master mix was prepared by adding 10 µL '200 mM L-Glutathione reduced' and 10 µL '100 mM CDNB' with 980 µL 'Dulbecco's Phosphate Buffered Saline.' The solution was freshly prepared for each assay series and used within 60 minutes of preparation. The addition of CDNB to the solution might make the solution slightly cloudy, which was disappeared after mixing the solution completely. 1.0 mL of the freshly prepared substrate solution was transferred to a quartz cuvette and read the Blank absorbance at 340 nm. Then 2-50 µL GST sample or 2 µL of the GST control was added directly to the quartz cuvette containing 1.0 mL substrate solution. After mixing the solution by covering the cuvette with a parafilm and inverting several times, the absorbance readings were taken

according to the kinetics program: reading taken every 30 seconds over a period of 5 minutes after a lag time of 1 minute.

Calculation of Result

The increase in absorbance is directly proportional to the GST activity. The linearity of the reaction determined by plotting the absorbance values against time.

Calculated the change in absorbance ($\Delta A340$)/minute, in the linear range of the plot, for the sample and for the blank using the following equation:

$(\Delta A_{340})/min = A_{340}$(final read) - A_{340}(initial read) / reaction time (min.)

Calculated the GST activity using the following equation:

GST specific activity:

$$\frac{(\Delta A_{340})/min \times V(mL) \times dil}{\varepsilon_{mM} \times V_{enz}\ (mL)} = \mu mol/mL/min$$

Where:

dil = the dilution factor of the original sample
ε mM ($mM^{-1}cm^{-1}$) - the extinction coefficient for CDNB conjugate at 340 nm.
For test in 1 mL cuvette = 9.6 mM^{-1} (path length - 1 cm).

V – the reaction volume: for test in 1 mL cuvette = 1 mL

V_{enz} – the volume of the enzyme sample teste

3.11.3 Estimation of Erythrocyte Reduced Glutathione (GSH)

Principle

The level of erythrocyte reduced glutathione was assayed by the method described by Beutler et al. (1963). The colorimetric substrate that reacts with the free thiol group of GSH to produce the highly colored product.

Reagents

- Glutathione Standard: 250 µM glutathione in a special stabilizing solution
- Detection Reagent; reconstitution with Dry DMSO
- Dry DMSO (Dimethylsulfoxide)
- Assay Buffer Concentration (2X)
- NADPH Concentration (10X): reduced β-nicotinamide adenine dinucleotide 2'phosphate
- Glutathione Reductase Concentrate(10x)
- Oxidized Glutathione Control
- Distilled or deionized water.
- Aqueous 5-sulfosalicylic acid dehydrate

Procedure

Preparation of Erythrocytes: 0.2 mL of whole blood and 1.8 mL of distilled water were mixed into the test tube to prepare a hemolysate. Promptly added 3.0 mL of precipitating solution (glacial meta phosphoric acid 1.67 gm, disodium EDTA 0.20 gm, and 30 gm NaCl dissolved in 100 mL distilled water) and after 5 min, filtered through coarse grade filter paper.

Assay – Two capped cuvettes were marked as 'Assay' and 'Blank.' In Assay cuvette 2.0 mL of filtrate 0.8 mL of Na_2HPO_4 reagent (0.3 mol /L) and 1.0 mL of 5 5' Dithiobis-2 nitrobenzoic acid (DTNB) reagent were taken. In Blank cuvette, 1.2 mL of precipitating solution, and 0.8 mL of distilled water was taken. Then 8.0 mL of Na_2HPO_4 and 1.0 mL of DTNB reagent were added in both the cuvettes. Both the cuvettes were capped and inverted three times. Then absorbance was read at 412 nm within 4 mins.

GSH calibration curve was plotted by assaying different GSH calibrators (50, 10, 5, 4, 2 mg/dL) to determine the GSH concentration of blood samples from the calibrator curve.

Calculation of Result

The GSH concentration was then calculated as micromole per gram of hemoglobin by estimating hemoglobin concentration of whole original blood.

3.11.4 Estimation of Prostate Specific Antigen (PSA)

The chemiluminescence immunoassay (CLIA) kit is used for the quantitative determination of PSA concentration in human serum (Immunoassay KIT, Autobio diagnostic, China)

Principle

The assay system utilizes an anti-PSA monoclonal antibody for solid phase (microtiter wells) immobilization and another anti-PSA monoclonal antibody as an antibody-enzyme (horseradish peroxidase) conjugate reagent. PSA in the reference standards or in the patient's specimens binds to monoclonal antibodies to prostate specific antigen (anti-PSA MAb) on the well, and the anti-PSA second antibody then binds to PSA. Unbound protein and Horseradish Proxidase (HRP) conjugates are removed by washing. Upon the addition of the substrate, the horseradish peroxidase activity bound on the wells is then assayed by a chemiluminescence reaction. The related light unit (RLU) of the reaction is proportional to the concentration of PSA presented in the specimen.

Reagents

- Antibody Coated Microtiter Plate: Microplate coated with monoclonal antibodies to prostate specific antigen (anti-PSA MAb)
- Enzyme Conjugate Reagent: Horseradish Proxidase (HRP) labeled anti-PSA MAb in Stabilizing Buffer
- Reference Standards: 0, 2, 4, 15, 50, and 100 ng/mL PSA in Stabilizing Buffer
- Substrate A
- Substrate B

Preparation of Reagents

All reagents were brought to room temperature (18~25°C) prior to use. Each lyophilized standard was reconstituted with 0.5 ml distilled water, allowed to stand for at least 10 minutes and stored sealed at 2~8°C.

Procedure

In an antibody-coated microtiter plate, 25 µL of PSA standards, specimens, and controls were dispensed into appropriate wells. Then 100 µl of Enzyme Conjugate Reagent was dispensed

into each well. The mixture was thoroughly mixed for 30 seconds. It was then incubated at 37°C for 60 minutes. The incubation mixture was later removed by flicking plate contents into a waste container. Then rinsed and flicked the microtiter plate 5 times with distilled water. The plate was stroked sharply onto absorbent paper to remove residual water droplets. The volume of the well was about 300 µL. 50 µL of Substrate A and then 50 µL of Substrate B was dispensed into each well and gently mixed for 10 seconds. The microplate was put into the detecting chamber of Luminometer for 5 minutes, then the RLU values of each well were read.

Calculation of Result

In a linear graph paper, RLU (ordinate) value obtained from each reference standard was plotted against the corresponding concentration of PSA in ng/mL (abscissa), and a calibration curve was drawn through the reference standard points by connecting the plotted points with straight lines. Then, the concentration for each control and sample was read by interpolating on the calibration curve. Computer-assisted data reduction and automatic result processing were used. The diluted specimens were corrected by the appropriate dilution factor.

3.11.5 Estimation of Zinc by Atomic Absorption Spectrophotometry (AAS)

The serum zinc level was determined using a flame atomic absorption spectrophotometer (Perkin Elmer, USA, analyst 200).

Principle

The basic principles of atomic absorption spectroscopy are all atoms can absorb light. The wavelength at which light is absorbed is specific for each element. If a sample containing a specific element together with other elements is exposed to light at the characteristic wavelength for a specific element, then only the specific elements atoms will absorb this light. The amount of light absorbed at this wavelength will increase as the number of atoms of the selected element in the light path increases and is proportional to the concentration of absorbing atoms. The relationship between the amount of light absorbed and the concentration of the analyte present in known standards can be used to determine unknown concentrations by measuring the amount of light they absorb. An atomic absorption spectrometer is simply an

instrument in which these basic principles are applied to practical quantitative analysis (Figure: 3.11).

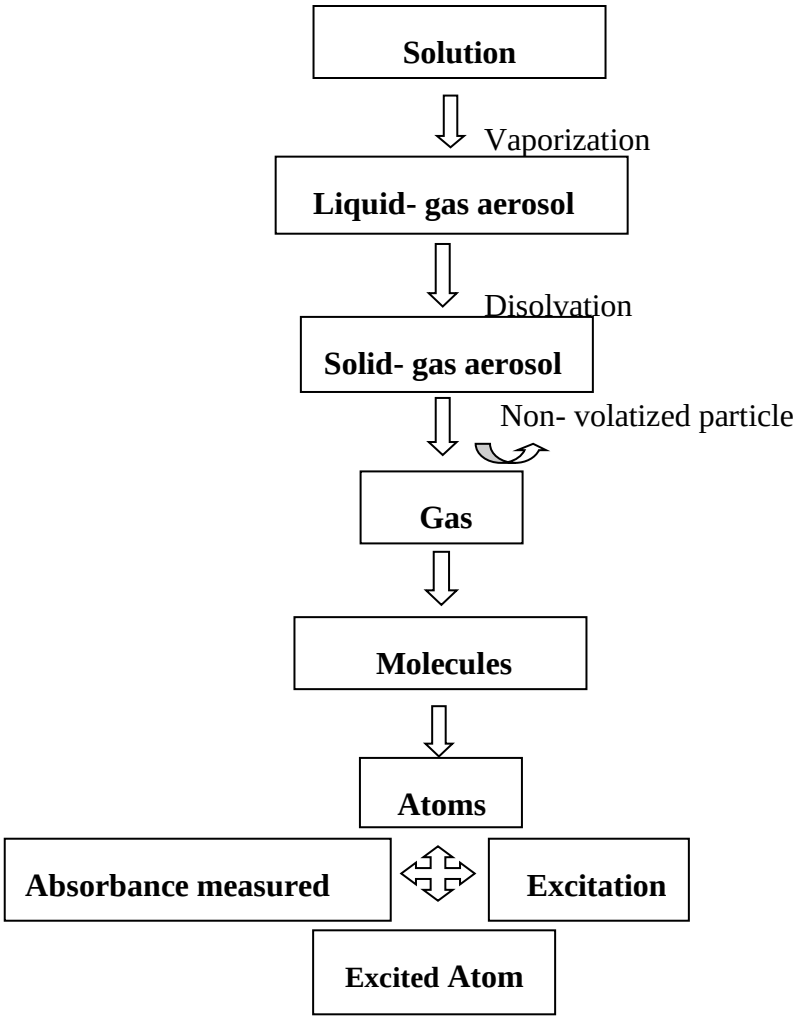


Figure 3.11: Flow Chart of Atomic Absorption Spectroscopy

Procedure

A specific hollow cathode lamp was used to analyzed zinc (wavelength 213.9 nm) in samples by flame AAS. Samples were aspirated through a nebulizer, and the absorbance was measured with a blank as reference. The calibration curve was obtained using standard samples. The instrument has a minimum detection limit of 0.01 mg/L for Zn in the flame method. The sample was diluted 10 folds to keep the results in the analytical range.

Calculation of Result

To calculate how much energy atoms was absorbed, a parallel beam of light was considered, which strikes a cell of N atoms. Let the cell be of unit cross-sectional area, and the light intensity be 10 watts per unit area as it enters the cell. The intensity of light remaining after absorption was given by

$I = I_{0(-KL)}$

Where 'L' is the cell length, and K is the absorption co-efficient energy absorbed per unit area per unit length.

$KL = \log (I_0/I)$

The expression $\log (I_0/I)$ is defined as absorption. Beer-Lambert law, expresses the quantitative relationship more clearly, which is expressed as:

$A = I_0/I = aLc$

Where a= absorptivity constant

c= sample concentration

From the above equation, it was seen that absorbance is directly proportional to concentration. This linearity deviates from ideally where the sample concentration was high. Perhaps this was because of the incomplete atomization in the flame.

3.12 Statistical Analyses

Statistical analyses were performed using the Statistical Package for Social Science (SPSS) version 22.0. The data were expressed as mean ± SD (Standard Deviation). Differences in baseline variables between patients and control subjects were tested using Student's *t* test. A one-way ANOVA test was performed to analyze more than two variables. $p<0.05$ were considered statistically significant. In addition, differences in genotype distribution between subjects with prostate cancer and healthy controls were evaluated using Fisher's exact test. The odds ratios at 95% confidence interval were calculated to estimate the risk. Pearson's correlation was used to find out the correlations between different parameters.

4. Results

This case-control study was conducted with a total of 407 enrolled study subjects, according to the inclusion criteria of the study. Among them, 207 were histopathologically diagnosed cases of prostate cancer, and 200 subjects were age-matched healthy control. After taking written consent from each study subject, a preset questionnaire had been filled up. All the baseline characteristics, along with personal history, family history, clinical history, clinical examination findings, and relevant investigation findings were included in the preset questioner. Genotyping analysis of NAT2, GSTT1, and GSTM1 genes and biochemical analysis of oxidative stress parameters were done from blood, collected from each study subject.

4.1 Baseline Characteristics of the Study Subject

Table 4.1 shows the baseline characteristics of the study subjects. The age of the control was 62.2±6.8 years, and it was 67.3±8.3 years in cases. The study subjects were categorized into four different age group categories (<50, 50-60, 61-70, and >70 years). The majority (51.6%) of the cancer patients belong to 61-70 years age group. BMI among control and cases was 24.1±2.0 and 23.1±2.2, respectively. Statistically, no significant differences were found between the cases and control regarding age and BMI. Among the control, 3% worked in a dye factory, 42% was a farmer, and 55% were from other professions, whereas among the cases, 5.8% worked in a dye factory, 44% was farmer and remaining were from other professions, and statistical analysis showed no significant differences between the two groups. Most of the study subjects of control and cases had completed primary educational level (42.0% and 45.4% respectively), were from middle economic status (49.5% and 45.9% respectively) and were a resident of rural areas (51.5% and 53.6% respectively). No statistically significant differences were found between the cases and control for the educational status, monthly income, and residential status. In the control group, 52.0% were smokers, whereas 60.9% of cases were smokers, but no significant differences between the control and cases were observed regarding smoking status. But the family history of cancer among the study subjects revealed, only 4.5% of the control group had family history cancer,

and on the other hand, 18.4% of cases had a family history of cancer, and it was statistically significant.

Table 4.1: Baseline Characteristics of the Study Subject

Variable	Study Subject (n=407)	
	Control (n=200)	Case (n=207)
Age (year)†	62.2±6.8	67.3±8.3
<50	05 (2.5)	01 (0.5)
51-60	77 (38.5)	43 (20.8)
61-70	94 (47.0)	107 (51.6)
>70	24 (12.0)	56 (27.1)
BMI (kg/m2)†	24.1±2.0	23.1±2.2
Occupation, n (%)		
Dye factory	06 (3.0)	12 (5.8)
Farmer	84 (42.0)	91 (44.0)
Others	110 (55.0)	104 (50.2)
Educational status, n (%)		
Illiterate	13 (6.5)	14 (6.8)
Primary	84 (42.0)	94 (45.4)
Secondary	81 (40.5)	82 (39.6)
Graduation/ above	22 (11.0)	17 (8.2)
Income (monthly), n (%)		
Low (TK<3000)	06 (3.0)	20 (9.7)
Middle (TK3000-6000)	99 (49.5)	95 (45.9)
Upper (TK>6000)	95 (47.5)	92 (44.4)
Residence, n (%)		
Rural	103 (51.5)	111 (53.6)
Urban	97 (48.5)	96 (46.4)
Smoking status, n (%)		
Nonsmoker	96 (48.0)	81 (39.1)
Smoker	104 (52.0)	126 (60.9)
Family history of cancer, n (%)		
No	191 (95.5)	169 (81.6)
Yes	09 (4.5)	38 (18.4)*

Results expressed as number (percentage); †Values are Mean±SD; *p<0.001; BMI: Body mass index, p<0.05 was taken as the level of significance.

4.2 Clinical Characteristics of Prostate Cancer Patient

Table 4.2 presents the clinical characteristics of prostate cancer patients. As shown in Table 4.3, 28.0% of patients had obstructive features with hematuria, and the results of digital rectal examination (D/R/E) indicated that of 63.3% patients had hard masses with an irregular surface and 36.7% had soft masses with a smooth surface in the prostate. Ultrasonography (USG) revealed, 27.5% of patients had a mildly enlarged prostate, 59.9% had moderately enlarged, and 12.6% had a markedly enlarged prostate. Urine routine examination (U/R/E) reported that 64.7 % of patients had features of urinary tract infection (UTI). Clinical staging of the cases found a majority of the cases (61.4%) had localized and organ-confined prostate cancer and histopathological reports showed 54.1% had low grade (Gleason's score <7) tumor and 45.9% had moderate to the high-grade tumor (Gleason's score ≥ 7) (Table 4.2).

Table 4.2: Clinical Characteristics of Prostate Cancer Patient

Clinical Characteristics	Prostate Cancer Patient (n=207)	
	Number (n)	Percentage (%)
Obstructive features with hematuria		
Present	58	28.0
Absent	149	72.0
D/R/E		
Hard mass é irregular surface	131	63.3
Soft mass é smooth surface	76	36.7
Urinary tract infection		
Present	134	64.7
Absent	73	35.3
USG		
Mildly enlarged prostate	57	27.5
Moderately enlarged prostate	124	59.9
Markedly enlarged prostate	26	12.6
Clinical stage		
Localized and organ-confined	127	61.4
Locally advanced or metastatic	80	38.6
Gleason score (Pathological grade)		
< 7	112	54.1
≥ 7	95	45.9

D/R/E= digital rectal examination, USG= ultrasonogram.

4.3 Frequency Distribution according to Age Group

As shown in Figure 4.1, the study subjects were categorized into four different age group categories (<50, 50-60, 61-70, and >70 years). The majority (51.6%) of the cancer patients belong to 61-70 years age group, 27.1% belong to >70 years age group, 20.8% belong to 50-60 years age group, and only 0.5 % of cases belong to <50 years age group. On the other hand, among the control, 47% belong to 61-70 years age group, 38.5% were of 50-60 years age group, 12 % were of >70 years age group, and 2.5 % belong to <50 years age group. Most of the study subjects were belong to 61-70 years age group.

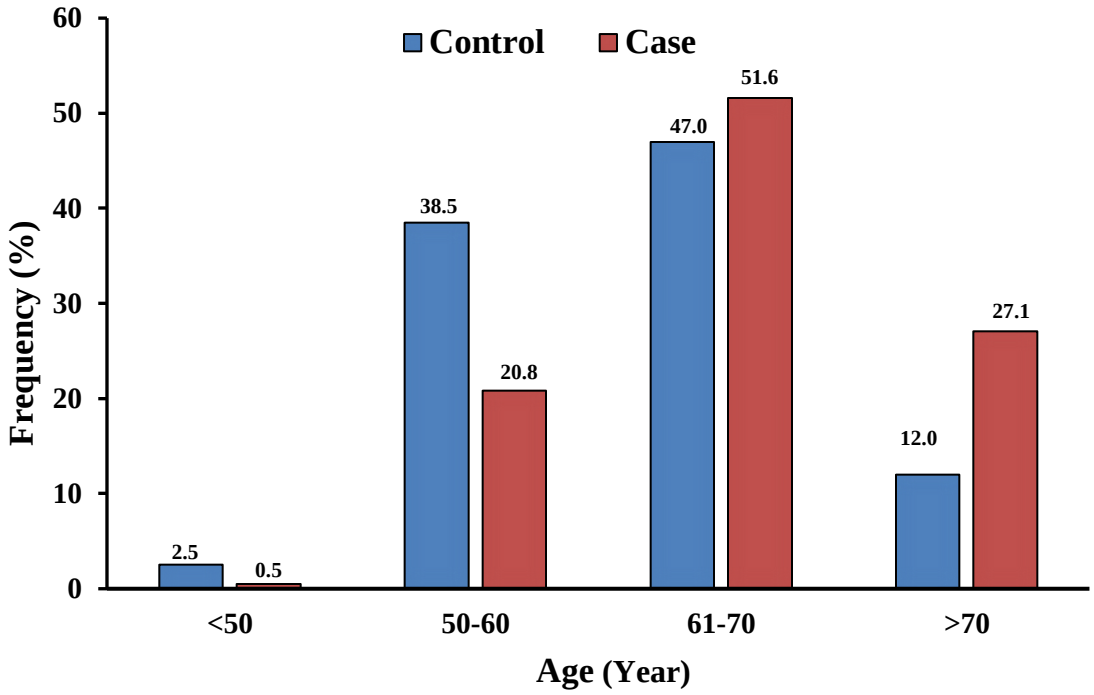


Figure 4.1: Frequency Distribution of Study Subject according to Age Group

4.4 Frequency Distribution of Prostate Cancer Patient according to Serum PSA

Serum PSA (prostatic specific antigen) was estimated in all the study subjects. Serum PSA value >4 ng/mL was found for all prostate cancer patients with a wide range and thus divided into three different categories (<10 ng/ml, 10-50 ng/ml, and >50 ng/mL). It was found that most of the prostate cancer patients (57.0%) had moderately high S.PSA (10-50 ng/mL), 34.3% had very high S.PSA (>50 ng/mL) and only 8.7% had S.PSA <10 ng/mL (Figure 4.2) as all the control had S. PSA <4 ng/mL, so it is not shown in Figure 4.2.

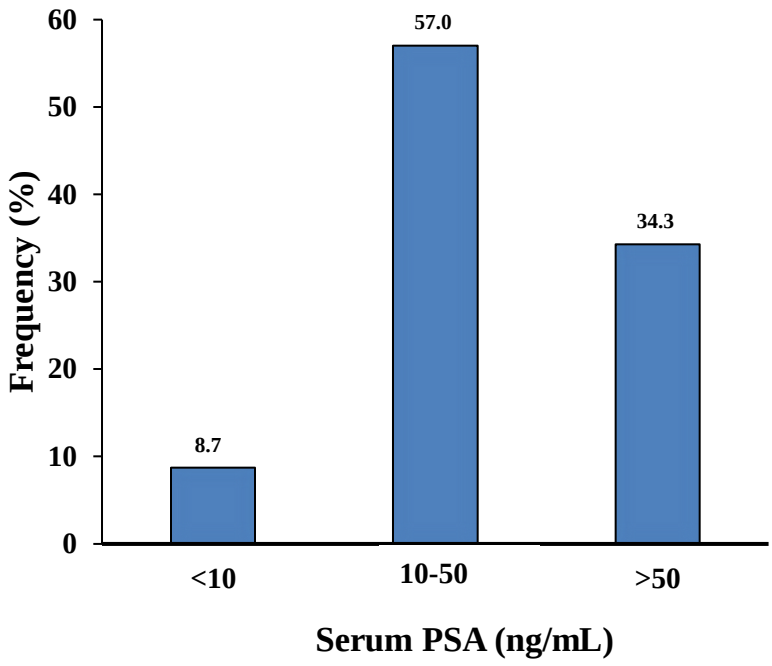


Figure 4.2: Frequency Distribution of PSA (ng/ml) among Prostate Cancer Patient

4.5 Association of Prostate Cancer with Smoking Status and Family History of Cancer

As shown in Table 4.3, there were 52.0% and 60.9% smokers among the control and cases, respectively. Though the odds ratio (OR) showed 1.44 times higher risk in smokers for the development of prostate cancer, compared to the nonsmokers, it was not statistically significant. Among study subjects, 4.5% control and 18.4 % cases had a positive family history of cancer. A significant association was found between the family history of cancer with prostate cancer risk ($p<0.001$) and OR showed that subjects with a positive family history of cancer had 4.77-fold higher risk for the development of prostate cancer compared to the subjects without a family history of cancer (Table 4.3).

Table 4.3: Association of Prostate Cancer with Smoking Status and Family History of Cancer with Estimated Risk

Risk Factor		Study Subject (n=407)		OR (95%CI)	*p* value
		Control (n=200) n (%)	Case (n=207) n (%)		
Smoking status	Nonsmoker	96 (48.0)	81 (39.1)	1.0 (Ref)	>0.05
	Smoker	104 (52.0)	126 (60.9)	1.44 (0.97-2.13)	
Family history of cancer	No	191 (95.5)	169 (81.6)	1.0 (Ref)	<0.001
	Yes	09 (4.5)	38 (18.4)	4.77 (2.24-10.16)	

Results are expressed as number (percentage). $p<0.05$ was taken as the level of significance, OR: odds ratio, CI: confidence interval.

4.6 Biochemical and Oxidative Stress Parameter of the Study Subject

Serum PSA, zinc, malondialdehyde (MDA), Glutathione S transferase (GST), and erythrocyte reduced glutathione (GSH) were estimated in all the study subjects. Mean±SD of S. PSA (ng/mL), zinc (µg/mL), MDA (nmol/mL), erythrocyte reduced glutathione (GSH) (µmol/gm of Hb) and GST (nmol/mL/min) among the control were 3.1±0.5 mg/mL, 0.9±0.3 (µg/mL), 6.7±1.4 (nmol/mL), 2.5±0.8 and 98.6±10.2 (nmol/mL/min) respectively, on the other hand, among the prostate cancer patients, those were 44.5±35.5 (ng/mL), 0.4±0.3 (µg/mL), 13.2±2.2 (nmol/mL), 1.7±0.9 (µmol/gm of Hb) and 101.0±10.9 (nmol/mL/min) respectively (Table 4.4). Significantly increased levels of serum PSA, MDA, and significantly decreased levels of GSH, GST, and Zinc were found between the control and cases.

Table 4.4: Biochemical and Oxidative Stress Parameter of the Study Subject

Variable	Study Subject (n=407)		*p* value
	Control (n=200)	Case (n=207)	
PSA (ng/mL)	3.1± 0.5	44.5±35.5	<0.001
MDA (nmol/mL)	6.7±1.4	13.2±2.2	<0.001
GSH (µmol/gm of Hb)	2.5±0.8	1.7±0.9	<0.001
GST (nmol/mL/min)	98.6±10.2	101.0±10.9	<0.05
Zinc (µg/mL)	0.9±0.3	0.4±0.3	<0.001

Values are presented as Mean ±SD, $p<0.05$ was taken as level of significance, PSA: Prostate specific antigen, MDA: Malondialdehyde, GSH: Erythrocyte reduced glutathione, GST: Glutathione S transferase.

4.7 Correlation among Serum PSA, MDA, Erythrocyte Reduced Glutathione (GSH), GST Activity and Zinc

Table 4.5 depicts the correlation among serum PSA, zinc, MDA, GST activity, and Erythrocyte Reduced Glutathione (GSH) in the study subject. PSA was positively correlated with MDA and GST activity and negatively correlated with serum zinc and Glutathione. Their correlations were statistically significant except with GST activity. Both serum zinc and glutathione had a significant negative correlation with MDA and non-significantly negatively correlated with GST activity, while serum zinc and glutathione were significantly positively correlated.

Table 4.5: Correlation among PSA, MDA, GSH, GST, and Zinc

Variable	PSA	MDA	GSH	GST activity	Zinc
PSA	-	0.539**	-0.233**	0.035	-0.499**
MDA	-	-	-0.400**	0.094	-0.650**
GSH	-	-	-	-0.003	0.333**
GST	-	-	-	-	-0.046
Zinc	-	-	-	-	-

**Correlation is significant at the 0.01 level (2-tailed). PSA: Prostate specific antigen, MDA: Malondialdehyde, GSH: Erythrocyte reduced glutathione, GST: Glutathione S transferase.

4.8 Genotype Distribution and Allele Frequency of the NAT2 Polymorphism

Genotype distribution and allele frequency of NAT2 polymorphisms in the study subject are presented in Table 4.6. As shown in Table 4.6, prostate cancer patients had significantly higher frequencies of homozygous and heterozygous mutant NAT2*6A and NAT2*7A/B in comparison to control. Odd ratio showed that subjects with heterozygous and homozygous mutant NAT2*6A and NAT2*7A/B had a significantly higher risk for the development of prostate cancer compared to NAT2*6A and NAT2*7A/B wild genotypes. It was also observed that NAT2*5A homozygous mutant genotypes had a 2.13 fold higher risk for the development of prostate cancer compared to wild genotype, but it was not statistically significant.

Table 4.6: Genotype Distributions and Allele Frequency of NAT2 Polymorphism

Polymorphism	Genotype	Study Subject (n=407)		OR (95% CI)	*p* value
		Control (n=200) n (%)	Case (n= 207) n (%)		
NAT2*5A (C481T)	CC	103 (51.5)	88 (42.5)	1 (Ref)	
	CT	86 (43.0)	99 (47.8)	1.35 (0.90-2.03)	>0.05
	TT	11 (5.5)	20 (9.7)	2.13 (0.99-4.62)	>0.05
NAT2*6A (G590A)	GG	151(75.5)	120 (58.0)	1 (Ref)	
	GA	39 (19.5)	68 (32.8)	2.19(1.38-3.48)	<0.01
	AA	10 (5.0)	19 (9.2)	2.39 (1.07-5.33)	<0.05
NAT2*7A/B (G857A)	GG	141 (70.5)	119 (57.5)	1 (Ref)	
	GA	56 (28.0)	77 (37.2)	1.63 (1.07-2.48)	<0.05
	AA	03 (1.5)	11 (5.3)	4.34 (1.18- 15.93)	<0.05

Results are expressed as number (percentage). $p<0.05$ was taken as the level of significance, OR: odds ratio, CI: confidence interval.

4.9 Distribution of NAT2 Genotype

The frequency distribution of NAT2 genotype in the study subject is shown in Figure 4.3. In the control group, 88.5% belong to NAT2 rapid genotype, and 11.5% belong to NAT2 slow genotype. In contrast, 80.2% and 19.8% cases (prostate cancer patients) belong to NAT2 rapid and NAT2 slow genotype, respectively. There was a significant difference between control and cases in the frequency of NAT2 slow genotype.

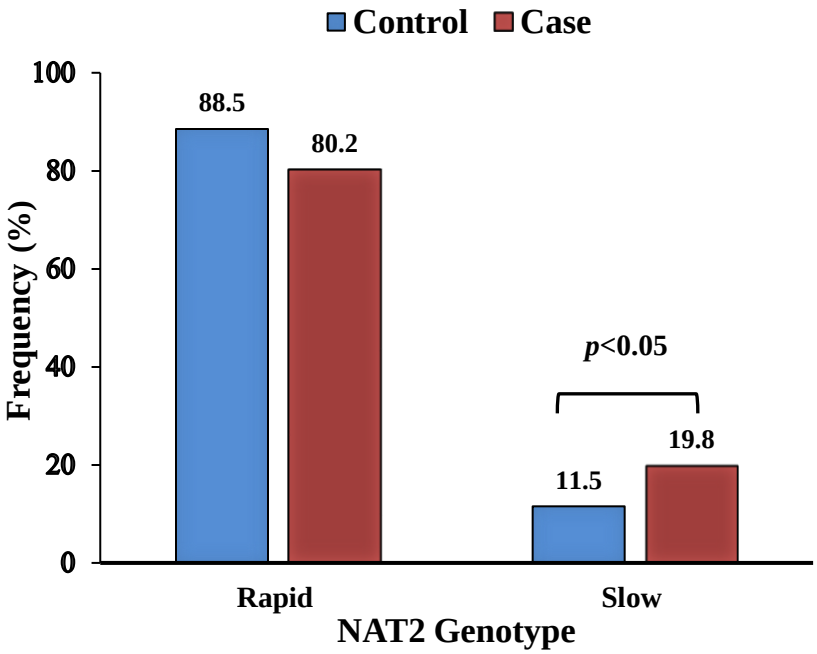


Figure 4.3: Distribution of NAT2 Genotype

4.10 PSA Level in NAT2 Genotype of Prostate Cancer Patient

In prostate cancer patients, the Mean±SD of serum PSA was found 62.1±44.1 ng/mL in NAT2 slow genotypes. In contrast, in NAT2 rapid genotypes, it was found 40.2±31.7 ng/mL. As shown in Figure 4.4, the PSA level was significantly higher ($p<0.001$) in NAT2 slow genotypes compared to NAT2 rapid genotype.

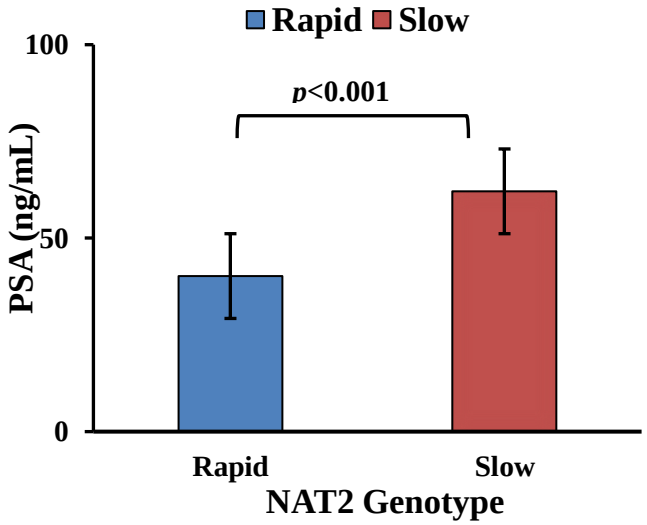


Figure 4.4: PSA level in NAT2 Genotype of Prostate Cancer Patient

4.11 Frequency Distribution of NAT2 Genotype and Risk of Prostate Cancer

Table 4.7 shows the frequency distribution of NAT2 acetylator genotypes in the study subjects with an estimated risk of prostate cancer. A significantly higher frequency of the NAT2 slow acetylator genotype was observed in the cases (prostate cancer patients) when compared with the controls (19.8% Vs. 11.5%, $p<0.05$) and odd ratio shows NAT2 slow genotypes had 1.90-fold higher risk for the development of prostate cancer [OR=1.90 (95% CI: 1.11-3.32)].

Table 4.7: Frequency Distribution of NAT2 Genotype and Risk of Prostate Cancer

Gene	Genotype	Study Subject (n=407)		OR (95% CI)	*p* value
		Control (n=200) n (%)	Case (n=207) n (%)		
NAT2	Rapid	177 (88.5)	166 (80.2)	1.0 (Ref)	
	Slow	23 (11.5)	41 (19.8)	1.90 (1.11-3.32)	<0.05

Rapid genotype: NAT2 wild homozygote or wild/mutant heterozygote, slow genotype: NAT2 mutant homozygote, $p<0.05$ was taken as the level of significance, OR: odds ratio, CI: confidence interval.

4.12 Association of NAT2 Genotype with Category of Age Group

Table 4.8 presents the estimated risk and association of NAT2 genotypes with the category of different age groups of the study subjects. NAT2 slow acetylator was observed to have significant distribution in the age group of 50-60 years in prostate cancer ($p<0.05$, OR=3.13, 95% CI 1.03-9.51), compared to controls.

4.13 Distribution of NAT2 Genotype according to the Smoking Status

Prostate cancer risk in different NAT2 genotypes stratified by smoking habit is shown in Table 4.9. Significant ($p<0.05$) positive association of NAT2 slow genotypes and the smoking habit was observed for prostate cancer development. The study revealed smokers with NAT2 slow genotypes had a 2.38-fold higher risk for the development of prostate cancer in comparison to the smoker with NAT2 rapid genotypes, whereas nonsmokers with NAT2 genotypes did not show any significant risk for the development of prostate cancer.

Table 4.8: Association of NAT2 Genotype with Category of Age Group

Age Strata (year)	NAT2 Genotype	Study Subject (n=407)		OR (95% CI)	*p* value
		Control (n=200) n (%)	Case (n=207) n (%)		
< 50	Rapid	05 (100)	01 (100)		
	Slow	00 (00)	00 (00)		
50 – 60	Rapid	71 (92.2)	34 (79.1)	1 (Ref)	
	Slow	06 (7.8)	09 (20.9)	3.13 (1.03-9.51)	<0.05
61 – 70	Rapid	81 (86.2)	86 (80.4)	1 (Ref)	
	Slow	13 (13.8)	21 (19.6)	1.52 (0.72-3.24)	>0.05
>70	Rapid	20 (83.3)	45 (80.4)	1 (Ref)	
	Slow	04 (16.7)	11 (19.6)	1.22 (0.35-4.31)	>0.05

Results are expressed as number (percentage). $p<0.05$ was taken as the level of significance, OR: odds ratio, CI: confidence interval.

Table 4.9: Distribution of NAT2 Genotype according to the Smoking Status

Smoking Status	Gene	Genotype	Study subject (n=407)		OR (95%CI)	*p* value
			Control(n=200) n (%)	Case (n=207) n (%)		
Smoker		Rapid	90 (86.5)	92 (73.0)	1.0 (Ref)	
	NAT2	Slow	14 (13.5)	34 (27.0)	2.38 (1.20-4.76)	<0.05
Non-smoker		Rapid	87 (90.6)	74 (91.4)	1.0 (Ref)	
		Slow	09 (9.4)	07 (8.6)	0.91 (0.35-2.46)	>0.05

Results are expressed as number (percentage). $p<0.05$ was taken as the level of significance, OR: odds ratio, CI: confidence interval.

4.14 Distribution of NAT2 Genotype according to the Family History of Cancer

Table 4.10 presents the prostate cancer risk in different NAT2 genotypes stratified by the family history of cancer. A significant ($p<0.05$) association of NAT2 slow genotypes with the family history of cancer was observed in prostate cancer patients (OR= 7.06, 95% CI: 1.27-39.18). On the other hand, none of the variants of NAT2 genotype were found significantly associated with prostate cancer risk in subjects having no family history of cancer.

Table 4.10: Distribution of NAT2 Genotype according to the Family History of Cancer

Family History of Cancer	Gene	Genotype	Study subject (n=407)		OR (95% CI)	*p* value
			Control (n=200) n (%)	Case (n=207) n (%)		
Yes	NAT2	Rapid	06 (66.7)	09 (23.7)	1.0 (Ref)	
		Slow	03 (33.3)	29 (76.3)	7.06 (1.27-39.18)	<0.05
No		Rapid	171 (89.5)	157 (92.9)	1.0 (Ref)	
		Slow	20 (10.5)	12 (7.1)	0.60 (0.28-1.29)	>0.05

Results are expressed as number (percentage). $p<0.05$ was taken as the level of significance, OR: odds ratio, CI: confidence interval.

4.15 Distribution of NAT2 Genotype according to the Tumor Grade (Gleason score) and Clinical Stage (TNM classification) of Prostate Cancer

NAT2 slow acetylator genotypes had a significantly higher risk for the development of moderate to high-grade tumors (Gleason score ≥7) (OR=3.91, 95% CI: 2.11-7.15, p<0.05). This study also found that NAT2 slow acetylator genotypes had significantly higher frequency and risk in the development of locally advanced or metastatic prostate cancer (OR=3.71, 95% CI: 1.96-7.15, p<0.05), in compared to control group (Table 4.11).

4.16 Association of Oxidative Stress with NAT2 Genotype

Table 4.12 shows the Mean±SD value of different oxidative stress-related parameters among NAT2 genotypes. The Mean±SD value of malondialdehyde (MDA), erythrocyte reduced glutathione (GSH), glutathione s transferase (GST) and zinc in NAT2 rapid genotypes were 9.8±3.7 (nmol/mL), 2.2±0.9 (µmol/g of Hb), 99.5±10.5 (nmol/mL/min) and 0.7±0.4 (µg/mL) respectively. On the other hand, the Mean±SD value of MDA, GSH, GST and zinc in NAT2 slow genotypes were 11.0±3.8 (nmol/mL), 2.0±0.9 (µmol/g of Hb), 101.8±11.4 (nmol/mL/min) and 0.5±0.3 (µg/mL) respectively. Significantly reduced zinc (p<0.01) were found in NAT2 slow genotypes in comparison to rapid genotype, but no significant differences were found for MDA, GSH, and GST.

Table 4.11: Distribution of NAT2 Genotype according to the Tumor Grade (Gleason score) and Clinical Stage (TNM classification)

Tumor Grade and Stage	NAT2 genotype		OR (95% CI)	*p* value
	Rapid n (%)	Slow n (%)		
Tumor grade				
Control (n=200)	177 (88.5)	23 (11.5)	1 (Ref)	
Case (n=207)				
Gleason score <7	103 (62.0)	09 (22.0)	0.67 (0.29-1.44)	>0.05
Gleason score ≥7	63 (38.0)	32 (78.0)	3.91 (2.11-7.15)	<0.05
Tumor stage				
Control(n=200)	177 (88.5)	23 (11.5)	1 (Ref)	
Case (n=207)				
T1a-c/T2a-b N0 M0 (organ confined group)	112 (67.5)	15 (36.6)	1.03 (0.55-2.01)	>0.05
T3a-b/T4/N1/M1 (locally advanced or metastatic)	54 (32.5)	26 (63.4)	3.71 (1.96-7.15)	<0.05

TNM classification, T1a-c: Clinically inapparent tumor not palpable or visible by imaging; T2a-b: Tumor confined within the prostate; T3a-b: Tumor extends through the prostatic capsule; T4: Tumor is fixed or invades adjacent structures; N0: nodal involvement absent; N1: nodal involvement present; M0: metastasis absent; M1: metastasis present; $p<0.05$ was taken as the level of significance, OR: odds ratio, CI: confidence interval.

Table 4.12: Association of Oxidative Stress with NAT2 Genotype

Variable	NAT2 Genotype		*p* value
	Rapid	Slow	
MDA (nmol/mL)	9.8±3.7	11.0± 3.8	>0.05
GSH (µmol/g of Hb)	2.2±0.9	2.0±.09	>0.05
GST (nmol/mL/min)	99.5±10.5	101.8±11.4	>0.05
Zinc (µg/mL)	0.7±0.4	0.5±0.3	<0.01

Values are presented as Mean±SD, SD: standard deviation, MDA: Malondialdehyde, GSH: Erythrocyte reduced glutathione, GST: Glutathione S transferase, $p<0.05$ was taken as the level of significance.

4.17 Distribution of GSTT1 and GSTM1 Genotype

Distribution of GSTT1 and GSTM1 genotypes in the study subjects are presented in Figure 4.5. The frequency of GSTT1 null genotype was 30.0% in prostate cancer patients and 22.5% among the controls. On the other hand, the frequency of GSTM1 null genotype was 37.7% in prostate cancer patients and 26.0% among the control, which was significantly higher than that of the control (Figure 4.5).

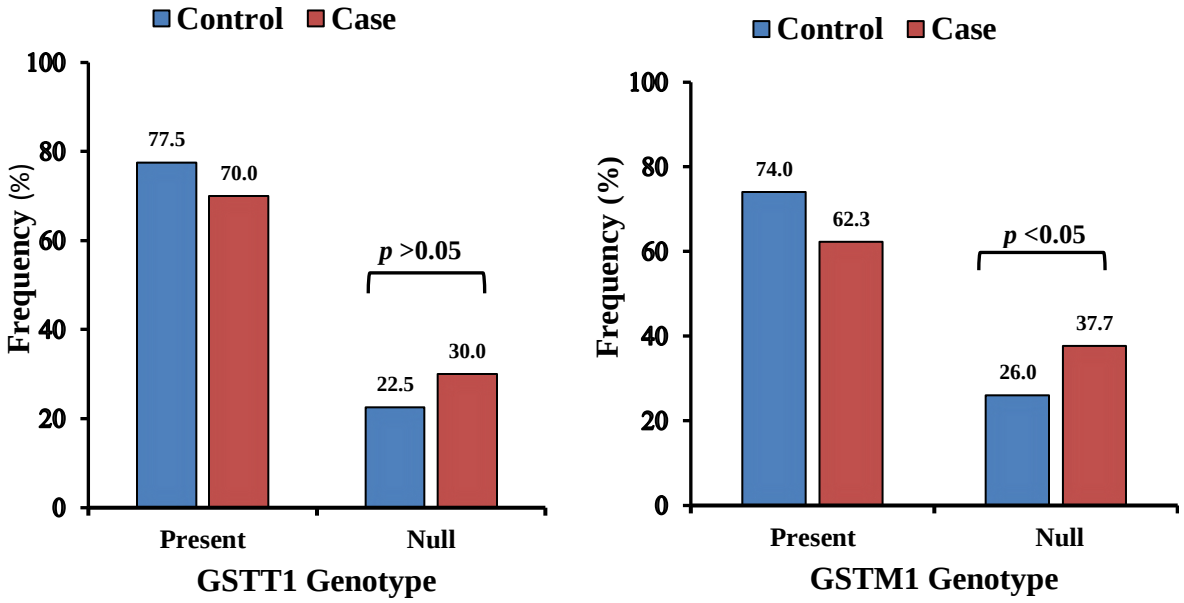


Figure 4.5: Distribution of GSTT1 and GSTM1 Genotype

4.18 PSA Level in GSTT1 and GSTM1 Genotype of Prostate Cancer Patient

Figure 4.6 showing the Mean ± SD of serum PSA in GSTT1 and GSTM1 genotypes of prostate cancer patients. The Mean±SD of serum PSA in prostate cancer patients with GSTT1 present genotypes was 44.5±34.1 ng/mL, and in GSTT1 null genotypes, it was 44.6±38.8 ng/mL. No significant differences were observed. Similarly, the Mean±SD of serum PSA in prostate cancer patients with GSTM1 present genotypes and null genotypes were found 42.2±31.3 ng/mL and 48.4±41.5 ng/mL respectively, and no significant differences were observed (Figure 4.6).

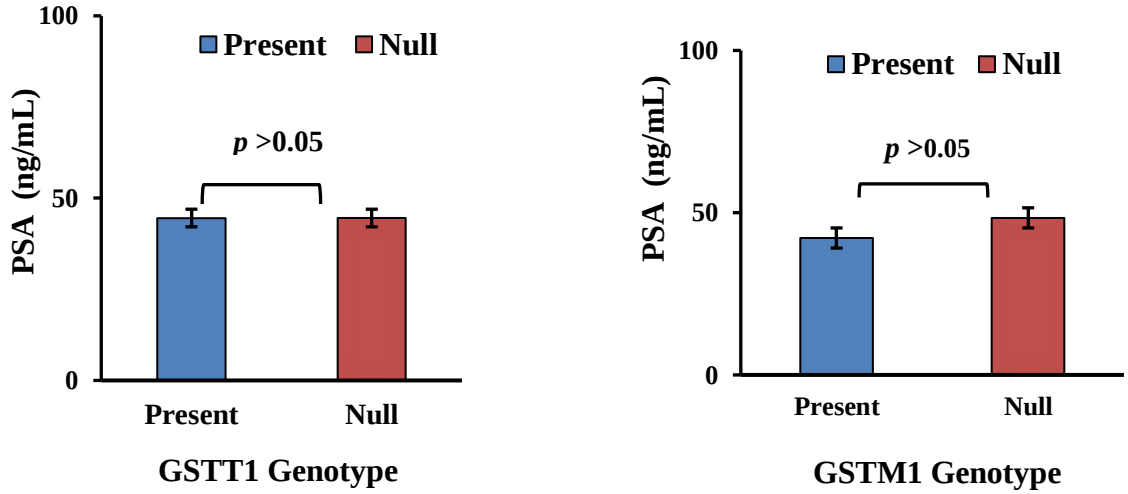


Figure 4.6: PSA Level in GSTT1 and GSTM1 Genotype of Prostate Cancer Patient

4.19 Frequency Distribution of GSTT1 and GSTM1 Genotype and Risk of Prostate Cancer

The frequency distribution of GSTT1 and GSTM1 genotypes with their estimated risk is presented in Table 4.13. The frequency of GSTT1 null genotype was slightly higher in prostate cancer patients than control with an odds ratio (OR) of 1.45 (95% CI, 0.93-2.28), which was not statistically significant. On the other hand, the frequency of GSTM1 null genotype was significantly higher in prostate cancer patients compared to the control with an OR, 1.71 (95% CI, 1.12-2.61).

4.20 Association of GSTT1 and GSTM1 Genotype with Category of Age Group

Association of GSTT1 and GSTM1 genotypes in different age groups of study subjects with estimated risk for prostate cancer is presented in Table 4.14. Study subjects were grouped into four age group categories (<50, 50-60, 61-70 and >70 years) and found that GSTT1 null genotypes in the age group of 50-60 years had a significantly higher risk for development of prostate cancer than GSTT1 present genotypes with an odds ratio of 3.70 (1.65-8.27). On the other hand, no significant association was observed for the different age groups with GSTM1 genotypes.

Table 4.13: Frequency Distribution of GSTT1 and GSTM1 Genotype and Risk of Prostate Cancer

Gene	Genotype	Study Subject (n=407)		OR (95% CI)	*p* value
		Control (n=200) n (%)	Case (n=207) n (%)		
GSTT1	Present	155 (77.5)	145 (70.0)	1.0 (Ref)	
	Null	45 (22.5)	62 (30.0)	1.45 (0.93-2.28)	>0.05
GSTM1	Present	148 (74.0)	129 (62.3)	1.0 (Ref)	
	Null	52 (26.0)	78 (37.7)	1.71 (1.12-2.61)	<0.05

GSTT1: Glutathione S Transferase Theta 1; GSTM1: Glutathione S Transferase Mu1, present: both allele present, null: deletion mutation present, $p<0.05$ was taken as the level of significance, OR: odds ratio, CI: confidence interval.

Table 4.14: Association of GSTT1 and GSTM1 Genotype with Category of Age Group

Age Strata (year)	Genotype	Study Subject (n=407)		OR (95% CI)	*p* value
		Control (n=200) n (%)	Case (n=207) n (%)		
	GSTT1				
< 50	Present	05 (100)	01 (100)		
	null	00 (00)	00 (00)		
50 – 60	Present	60 (77.9)	21 (48.8)	1 (Ref)	
	Null	17 (22.1)	22 (51.2)	3.70 (1.65-8.27)	<0.05
61 – 70	Present	70 (74.5)	80 (74.8)	1 (Ref)	
	Null	24 (25.5)	27 (25.2)	0.98 (0.52-1.86)	>0.05
>70	Present	20 (83.3)	43 (76.8)	1 (Ref)	
	Null	04 (16.6)	13 (23.2)	1.51 (0.44-5.22)	>0.05
	GSTM1				
< 50	Present	03 (60.0)	01 (100)		
	Null	02 (40.0)	00 (00)		
50 – 60	Present	64 (83.1)	32 (74.4)	1 (Ref)	
	Null	13 (16.9)	11 (25.6)	1.69 (0.68-4.20)	>0.05
61 – 70	Present	65 (69.1)	64 (59.8)	1 (Ref)	
	Null	29 (30.9)	43 (40.2)	1.51 (0.84-2.70)	>0.05
>70	Present	16 (66.7)	32 (57.1)	1 (Ref)	
	Null	08 (33.3)	24 (42.9)	1.50 (0.55-4.08)	>0.05

Results are expressed as number (percentage). $p<0.05$ was taken as the level of significance; OR: odds ratio, CI: confidence interval.

4.21 Distribution of GSTT1 and GSTM1 Genotype according to the Smoking Status

Prostate cancer risk in different genotypes of GSTT1 and GSTM1 stratified by smoking habit is presented in Table 4.15. No significant association between GSTT1 null genotype and the smoking habit was observed for prostate cancer patients [OR=1.58 (0.85-2.97)]. On the other hand, smokers with GSTM1 null genotypes showed a significant association with prostate cancer risk compared to GSTM1 present genotypes [OR= 2.14 (1.23-3.75)].

Table 4.15: Distribution of GSTT1 and GSTM1 Genotype according to the Smoking Status.

Smoking Status	Gene	Genotype	Study Subject (n=407)		OR (95% CI)	*p* value
			Control (n=200) n (%)	Case (n=207) n (%)		
Smoker	GSTT1	Present	83 (79.8)	90 (71.4)	1.0 (Ref)	
		Null	21 (20.2)	36 (28.6)	1.58 (0.85-2.97)	>0.05
Non-smoker		Present	72 (75.0)	55 (68.0)	1.0 (Ref)	
		Null	24 (25.0)	26 (32.0)	1.42 (0.72-2.66)	>0.05
Smoker	GSTM1	Present	77 (74.0)	72 (57.1)	1.0 (Ref)	
		Null	27 (26.0)	54 (42.9)	2.14 (1.23-3.75)	<0.01
Non-smoker		Present	71(74.0)	57 (70.4)	1.0 (Ref)	
		Null	25 (26.0)	24 (29.6)	1.20 (0.64-2.26)	>0.05

Results are expressed as number (percentage). $p<0.05$ was taken as the level of significance, OR: odds ratio, CI: confidence interval.

4.22 Distribution of GSTT1 and GSTM1 Genotype according to the Family History of Cancer

Table 4.16 shows the distribution of GSTT1 and GSTM1 genotypes in the study subjects stratified by the family history of cancer. No significant association was observed between positive family history of cancer and GSTT1 and GSTM1 genotypes in cancer patients. Although it was not statistically significant, the OR were higher in GSTT1 and GSTM1 null genotypes [2.02 (95% CI: 0.30-13.89)] and [2.70 (95% CI: 0.47-15.39)] respectively for patients having a positive family history of cancer.

Table 4.16: Distribution of GSTT1 and GSTM1 Genotype according to the Family History of Cancer

Family History of Cancer	Gene	Genotype	Study Subject (n=407)		OR (95% CI)	*p* value
			Control (n=200) n (%)	Case (n=207) n (%)		
Yes	GSTT1	Present	06 (66.7)	27 (71.1)	1.0 (Ref)	>0.05
		Null	03 (33.3)	11 (28.9)	2.02 (0.30-13.89)	
No		Present	149 (78.0)	118 (69.8)	1.0 (Ref)	>0.05
		Null	42 (22.0)	51 (30.2)	1.52 (0.94-2.45)	
Yes	GSTM1	Present	06 (66.7)	16 (42.1)	1.0 (Ref)	
		Null	03 (33.3)	22 (57.9)	2.70 (0.47-15.39)	>0.05
No		Present	142 (74.3)	113 (66.9)	1.0 (Ref)	
		Null	49 (25.7)	56 (33.1)	1.44 (0.91-2.29)	>0.05

Results are expressed as number (percentage). $p<0.05$ was taken as the level of significance, OR: odds ratio, CI: confidence interval.

4.23 Distribution of GSTM1 and GSTM1 Genotype according to the Clinical Stage (TNM Classification) of Prostate Cancer

GSTM1, GSTT1 genotype distribution according to different stages of prostate cancer is shown in Table 4.17 and found that GSTM1 null genotype had a significantly higher risk for the development of locally advanced or metastatic prostate cancer [OR, 2.99 (95% CI: 1.76-5.14), p<0.001] in comparison to healthy control. Similarly, GSTT1 null genotypes also had a significantly higher risk for the development of locally advanced or metastatic prostate cancer [OR=2.82, (95% CI: 1.61-4.92), p<0.001] than healthy control.

Table 4.17: Distribution of GSTT1 and GSTM1 Genotype according to Clinical Stage (TNM Classification)

Tumor stage	Genotype		OR (95% CI)	*p* value
	Present n (%)	Null n (%)		
	GSTT1			
Control (n=200)	155 (77.5)	45 (22.5)	1 (Ref)	
Case (n=207)				
T1a-c/T2a-b N0 M0 (n=127) (organ confined group)	101 (69.7)	26 (41.9)	0.89 (0.52-1.55)	>0.05
T3a-b/T4/N1/M1 (n=80) (locally advanced or metastatic**)**	44 (30.3)	36 (58.1)	2.82 (1.61-4.92)	<0.05
	GSTM1			
Control (n=200)	148 (74.0)	52 (26.0)	1 (Ref)	
Case (n=207)				
T1a-c/T2a-b N0 M0 (n=127) (organ confined group)	90 (69.8)	37 (47.4)	1.17 (0.71-1.94)	>0.05
T3a-b/T4/N1/M1(n=80) (locally advanced or metastatic)	39 (30.2)	41(52.6)	2.99 (1.76-5.14)	<0.05

TNM classification, T1a-c: Clinically inapparent tumor not palpable or visible by imaging; T2a-b: Tumor confined within the prostate; T3a-b: Tumor extends through the prostatic capsule; T4: Tumor is fixed or invades adjacent structures; N0: nodal involvement absent; N1: nodal involvement present; M0: metastasis absent; M1: metastasis present; OR: odds ratio, CI: confidence interval.

4.24 Distribution of GSTT1 and GSTM1 Genotype according to the Pathological Grade (Gleason score) of Prostate Cancer

Table 4.18 shows the distribution of GSTT1 and GSTM1 genotype according to the histopathological grade of prostate cancer. As shown in Table 4.18, the GSTT1 null genotypes had a significantly higher risk for high-grade tumors [OR= 2.73, (95% CI: 1.59-4.52), p<0.001] compared to the control group. Similarly, GSTM1 null genotypes also had a significantly higher risk for high-grade tumors [OR=2.91, (95% CI: 1.75-4.89) p<0.001].

Table 4.18: Distribution of GSTT1 and GSTM1 Genotype according to the Pathological Grade (Gleason score)

Tumor Grade	Genotype		OR (95% CI)	*p* value
	Present n (%)	Null n (%)		
	GSTT1			
Control (n=200)	55 (77.5)	45 (22.5)	1 (Ref)	
Case (n=207)				
Gleason score <7	92 (63.4)	20 (32.3)	0.75 (0.42-1.35)	>0.05
Gleason score ≥7	53 (36.6)	42 (67.7)	2.73 (1.59-4.52)	<0.05
	GSTM1			
Control (n=200)	148 (74.0)	52 (26.0)	1 (Ref)	
Case (n=207)				
Gleasons score <7	82 (63.6)	30 (38.5)	1.04 (0.62-1.75)	>0.05
Gleasons score ≥7	47 (36.4)	48 (61.5)	2.91 (1.75-4.89)	<0.05

GSTT1: Glutathione S Transferase Theta 1, GSTM1: Glutathione S Transferase Mu1, positive: both allele present, null: deletion mutation present, $p<0.05$ was taken as the level of significance, OR: odds ratio, CI: confidence interval.

4.25 Association of Oxidative Stress with GSTT1 and GSTM1 Genotype

Table 4.19 shows oxidative stress-related parameters among GSTT1 and GSTM1 genotypes. The Mean±SD of MDA, erythrocyte reduced Glutathione (GSH), GST and zinc level in GSTT1 present genotypes were 9.8±3.8 (nmol/mL), 2.1±0.9 (µmol/gm of Hb), 101.1±10.1 (nmol/ mL/ min) and 0.7±0.4 (µg/mL) respectively. In GSTT1 null genotypes, the Mean±SD of MDA, erythrocyte reduced glutathione, GST and zinc level were 10.5±3.6 (nmol/mL), 2.1±1.0 (µmol/gm of Hb), 96.3±11.4 (nmol/mL/min) and 0.6±0.4 (µg/mL) respectively. There were no significant differences in MDA, GSH, and Zinc levels in between GSTT1 present genotypes and null genotypes, except serum GST activity. GST activity was found significantly lower in GSTT1 null genotypes than present genotypes ($p<0.01$). On the other hand, the Mean±SD of MDA reduced Glutathione, GST and zinc level in GSTM1 present genotypes were 9.6±3.7 (nmol/mL), 2.2±0.9 (µmol/gm of Hb), 101.2±10.0 (nmol/mL/min) and 0.7±0.4 (µg/mL) respectively. Whereas in GSTM1 null genotypes, the Mean±SD of MDA, reduced Glutathione, GST, and zinc level were 10.8±3.9 (nmol/mL), 2.0±.09 (µmol/gm of Hb), 97.0±11.5

(nmol/mL/min) and 0.6±0.4 (µg/mL) respectively. There were no significant differences in MDA, GSH, and GST activity, and Zinc levels in between GSTT1 present genotypes, and null genotypes were observed.

Table 4.19: Association of Oxidative Stress and Antioxidant Parameter with GSTT1 and GSTM1 Genotype

Variable	GSTT1 Genotype			GSTM1 Genotype		
	Present	Null	*p* value	Present	Null	*p* value
MDA (nmol/mL)	9.8±3.8	10.5±3.6	>0.05	9.6± 3.7	10.8±3.9	>0.05
GSH (µmol/gm of Hb)	2.1±0.9	2.1±1.0	>0.05	2.2±0.9	2.0±0.9	>0.05
GST (nmol/mL/min)	101.1±10.1	96.3±11.4	<0.01	101.2±10.0	97.0±11.5	>0.05
Zinc (µg/mL)	0.7±0.4	0.6±0.4	>0.05	0.7±0.4	0.6±0.4	>0.05

Values are expressed in Mean± SD, GSTT1: Glutathione S Transferase Theta 1, GSTM1: Glutathione S Transferase Mu1, present: both allele present, null: deletion mutation present, MDA: malondialdehyde, GSH: Erythrocyte reduced glutathione, GST: Glutathione S Transferase. $p<0.05$ was taken as the level of significance.

4.26 Distribution of MDA in Combined Genotype of GSTT1 and GSTM1

Figure 4.7 shows the Mean±SD of MDA in the serum of different GSTT1 and GSTM1 combined genotypes of prostate cancer patients and control. The MDA level in all combined genotypes found higher than that of control, and significant differences were observed in all combined genotypes except GSTT1(-)/GSTM1(+) combined genotype.

4.27 Distribution of Erythrocyte Reduced Glutathione (GSH) in Combined Genotype of GSTT1 and GSTM1

Figure 4.8 shows the Mean±SD erythrocyte reduced glutathione in the different GSTT1 and GSTM1 combined genotypes of prostate cancer and controls. The erythrocyte reduced glutathione in all combined genotypes found lower than that of controls, but no significant differences were revealed.

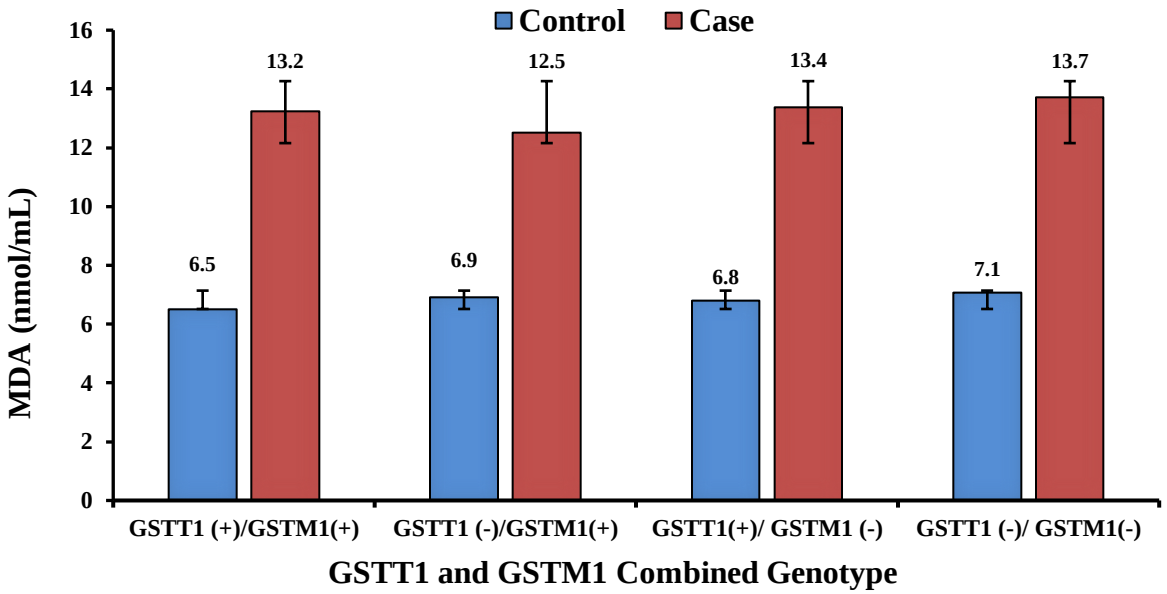


Figure 4.7: Distribution of MDA in Combined Genotype of GSTT1 and GSTM1 in the Study Subject

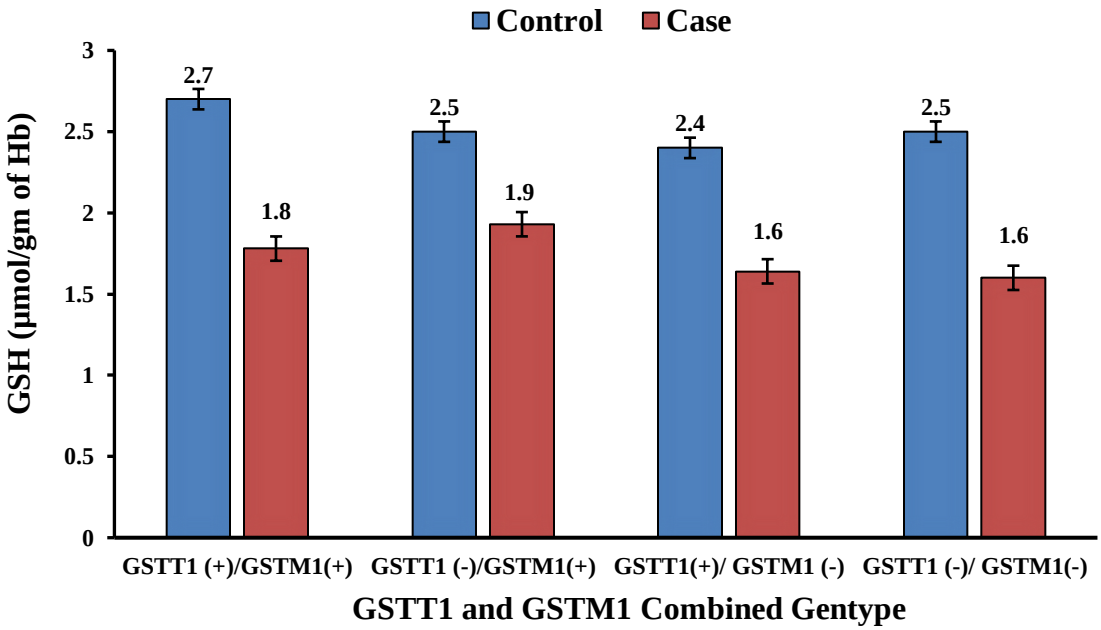


Figure 4.8: Distribution of Erythrocyte Reduced Glutathione (GSH) in Combined Genotype of GSTT1 and GSTM1 in the Study Subject

4.28 Distribution of GST Activity in Combined Genotype of GSTT1 and GSTM1

Figure 4.9 shows the Mean±SD GST activity of different GSTT1 and GSTM1 combined genotypes of prostate cancer and controls. The GST activity of all combined genotypes found higher than that of control except GSTT1(-) /GSTM1(-) combined genotype. Statistically significant differences were observed for GSTT1(-)/GSTM1(+) and GSTT1(-) /GSTM1(-) combined genotypes but no significant differences were observed for GSTT1(+) /GSTM1(+) and GSTT1(+) /GSTM1(-) combined genotypes.

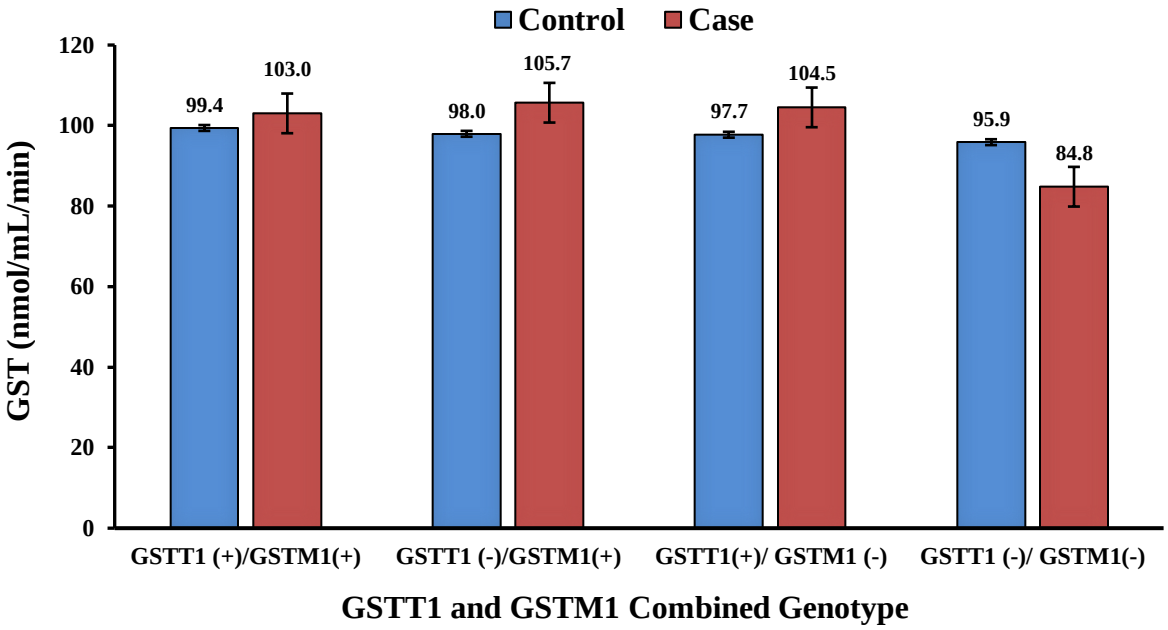


Figure 4.9: Distribution of GST Activity in Combined Genotype of GSTT1 and GSTM1 in the Study Subject

4.29 Distribution of Zinc in Combined Genotype of GSTT1 and GSTM1

Figure 4.10 shows the Mean±SD serum zinc in the different GSTT1 and GSTM1 combined genotypes of prostate cancer and controls. The serum zinc in all combined genotypes found lower than that of controls, but no significant differences were observed.

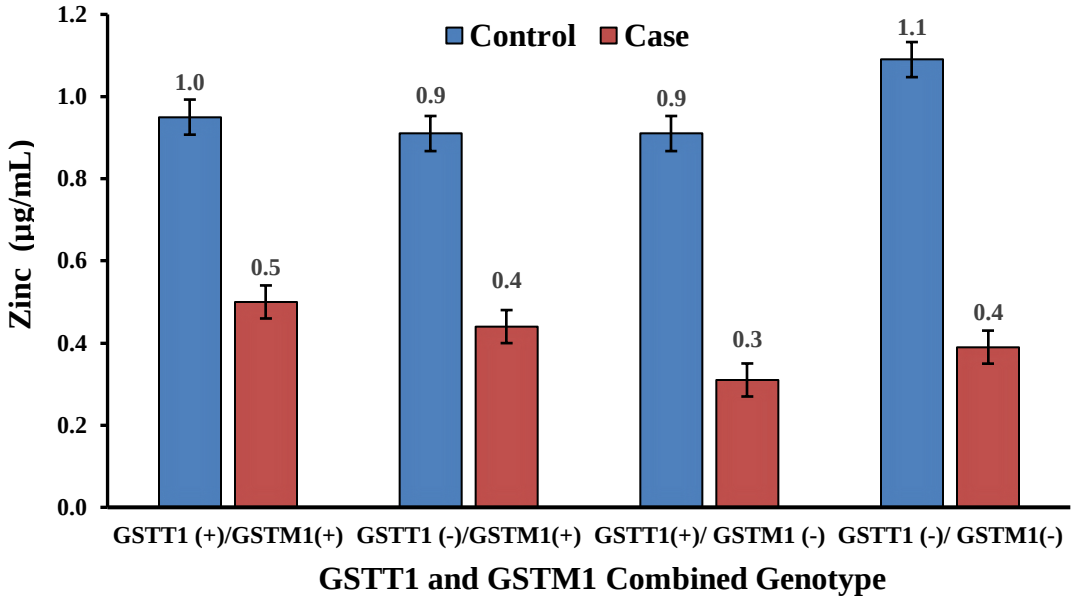


Figure 4.10: Distribution of Zinc in Combined Genotype of GSTT1 and GSTM1 in the Study Subject

4.30 Distribution of NAT2, GSTT1 and GSTM1 Combined Genotype (Double and Triple) in the Study Subject and Associated Risk

Distribution of combined genotypes (double and triple) of NAT2, GSTT1 and GSTM1 in the study subjects and their associated risk for cancer is presented in Table 4.20. As shown in Table 4.20, significant association were present in combined genotypes of NAT2 (slow)/ GSTT1(Present), NAT2 (slow) / GSTM1(null), NAT2 (rapid) / GSTM1 (null), GSTT1 (null) / GSTM1 (null), NAT2 (rapid) / GSTT1 (null) / GSTM1 (null) and NAT2 (slow)/ GSTT1 (null) / GSTM1 (null) with prostate cancer. The combined genotype GSTT1 (null) /GSTM1 (null) showed highly significant association with prostate cancer risk and the risk was 5.75-fold higher in compared to normal genotype for GSTT1 and GSTM1. Further, the risk increased to higher level (5.54-fold) for NAT2 (rapid) / GSTT1 (null) / GSTM1 (null) combined genotype and risk increased to much higher level (9.64-fold) for NAT2 (slow)/ GSTT1 (null) / GSTM1 (null) combined genotypes compared to normal genotype for NAT2, GSTT1 and GSTM1.

Table 4.20: Distribution of NAT2, GSTT1 and GSTM1 Combined Genotype (Double and Triple) in the Study Subject and Associated Risk

Combined Genotype	OR (95% CI)	*p* value
NAT2 / GSTT1		
Rapid / Present	1 (Ref)	---
Slow / Present	1.83 (0.91-2.40)	<0.05
Slow / Null	2.83 (0.97-8.28)	>0.05
Rapid / Null	1.90 (1.00-3.60)	>0.05
NAT2 /GSTM1		
Rapid/ Present	1(Ref)	---
Slow/ Null	3.00 (1.33-6.78)	<0.05
Slow/ Present	1.79 (0.85- 3.79)	>0.05
Rapid/ Null	1.63 (1.02-2.63)	>0.05
GSTT1 / GSTM1		
Present / Present	1(Ref)	---
Present / Null	0.88 (0.51-1.52)	>0.05
Null / Present	1.16 (0.71-1.90)	>0.05
Null / Null	5.75 (2.30-14.36)	<0.001
NAT2 /GSTT1 / GSTM1		
Rapid / Present / Present	1 (Ref)	---
Rapid / Present / Null	1.08 (0.62 - 1.86)	>0.05
Rapid / Null / Present	0.93 (0.52 - 1.66)	>0.05
Rapid / Null / Null	5.54 (2.02 - 15.22)	<0.01
Slow / Present / Present	2.01 (0.84 - 4.82)	>0.05
Slow / Present / Null	2.11 (0.84 - 5.27)	>0.05
Slow / Null / Present	1.21 (0.29 - 4.97)	>0.05
Slow / Null / Null	9.64 (1.18 – 78.6)	<0.05

NAT2: N-acetyltransferase 2, Rapid genotypes: NAT2 wild type, homozygote wild/mutant heterozygote, slow genotypes: NAT2 mutant type homozygote, GSTT1: Glutathione S Transferase Theta 1, GSTM1: Glutathione S Transferase Mu1, present: both allele present, null: deletion mutation; OR: odds ratio, CI: confidence interval.

5. Discussion

This case-control study was undertaken to evaluate the association of NAT2, GSTT1 and GSTM1 gene polymorphisms, and the influence of oxidative stress on prostate cancer risk in the Bangladeshi population. The additive effect of smoking, family history of cancer, oxidative stress on different NAT2, GSTT1, and GSTM1 genotypes of prostate cancer was also being studied. This study performed on 207 cases of prostate cancer and 200 age-matched healthy control. All the study subjects (prostate cancer cases and healthy control) were male, belonged to the same ethnic background with the same age group, and all shared a common geographic origin. The results of basic demographic data showed no significant differences in age, BMI, occupation, educational status, residence, monthly income, and smoking status between prostate cancer cases and control except the family history of cancer.

In this study, the age of cases (prostate cancer patients) was 67.3±8.3 year, and among control, it was 62.2±6.8 year (Table 4.1), which is similar to that of the age group (62.3±9.9 year and 63.3±8.0 year in prostate cancer patients and control respectively) reported by Taroiguchi et al. (2009). Similar to this study, Kosova et al. (2010) stated, no significant age difference between prostate cancer patients and control. This study found, most of the prostate cancer patients were of 61-70 years of age, which is consistent with the previous research of Plaskon et al. (2003). Age plays a vital role in the development of prostate cancer, and its incidence increases with increased age (Malik et al., 2015).

In this study, there were 60.9% and 52.0% smokers among the cases and control, respectively. Though the odds ratio (OR) showed a smoker had a 1.4 times higher risk for the development of prostate cancer, it was not statistically significant ($p > 0.05$) (Table 4.3). Similarly, Taroiguchi et al. (2009) did not found any significant association of smoking with prostate cancer risk. On the contrary, Shahabi et al. (2014) reported in their study that current smokers had a statistically significant increased risk of advanced prostate cancer. The increased risk is probably due to the presence of polycystic aromatic hydrocarbon, nitrosamines, and aromatic amines in tobacco smoke (Shaker et al., 2009).

Regarding the family history of cancer, this study found,18.4 % cases and 4.5% control had a positive family history of cancer, and there was a significant association of family history of cancer with the risk of prostate cancer ($p<0.001$). Odds ratio (OR) showed that subjects with a positive family history of cancer had 4.8 times higher risk for the development of prostate cancer (Table 4.3). Similarly, Jr Rovito et al. (2005) also reported that the family history of prostate cancer in the first-degree blood relatives (father and brother) was a significant risk ($p=0.01$) factor for prostate cancer.

Study of clinical characteristics revealed, 38.6% of prostate cancer cases of our study subjects had a locally advanced or metastatic tumor, and on histopathological grading found, 45.9 % had a high-grade tumor (Gleason score ≥7) (Table 4.2). The finding was in accordance with the previous studies (Berber et al., 2013; Kosova et al., 2010; Shahabi et al., 2014). The diagnosis of cancer at the late stage of the disease might be the reason.

Following the previous studies by Sivonova et al. (2009) and Kaya et al. (2017), the present study also found, men with prostate cancer had significantly high ($p<0.001$) serum PSA compared to healthy men (Table 4.4). On the categorization of S. PSA level into three different categories, <10 ng/ml, 10-50 ng/ml, and >50 ng/ml, we found that most of the prostate cancer cases had moderately high PSA value (10-50 ng/ml) (Fig 4.2). A study done by Gretzer and Partin (2002) described that PSA usually found in low concentrations in serum. Still, elevations during disease processes are believed to be a product of the disruption of the typical cellular architecture of the prostate gland.

Study of different oxidative stress-related parameters (MDA, reduced Glutathione, GST, and zinc) revealed significant differences in the level of MDA, reduced Glutathione, GST, and Zinc between the prostate cancer patients and control (Table 4.4). Increased level of lipid peroxidation is associated with carcinogenesis. The malondialdehyde (MDA), which is a marker of lipid peroxidation, was found significantly raised in prostate cancer patients ($p<0.001$) in comparison to the control group (Table 4.4) in our study. Our finding is in agreement with Aydin et al. (2006), Oh et al. (2016), and Kaya et al. (2016). The rise in MDA could be due to increase generation of Reactive Oxygen Species (ROS), due to the excessive

oxidative damage generated in cancer patients. Erythrocyte reduced glutathione (GSH) is an endogenous antioxidant, and its primary function is to protect cells against ROS during the metabolism of the living organism. GSH level was significantly low in prostate cancer patients in this study ($p<0.001$) compared to the control group (Table 4.4). Similarly, Srivastava and Mittal (2005a) reported significantly decreased GSH in prostate cancer patients than in the control group. Reduced GSH levels in cancer patients were also reported by Bhuvanamurthy et al. (1996) and Faber et al. (1995). In a study by Surapaneni and Ramana (2006) suggested that the lower GSH levels may be due to the increased turnover of GSH for preventing oxidative damage in cancer patients.

GST enzyme is potentially crucial in regulating susceptibility to cancer due to its ability to metabolize reactive metabolites of carcinogens. Srivastava and Mittal (2005a) reported in their study that GST activity was significantly higher in malignant tissue as compared to non-malignant tissue. Similarly, in the present study, the GST level was found significantly higher ($p<0.05$) in prostate cancer patients than control (Table 4.4). But Malik et al. (2015) did not found any significant association between GST and prostate cancer. Similar results also reported in South Indian, American, and African populations (Caceres et al., 2005). Zinc is one of the essential minerals for human health. It acts as a co-factor for several vital enzymes that contribute to the proper functioning of the antioxidant defense system (Marreiro et al., 2017). There was a significantly decreased ($p<0.001$) serum zinc level in prostate cancer cases in comparison to the control group in our study (Table 4.4). Several previous studies also reported that the level of plasma and tissue zinc were substantially lowered in the cancerous prostate than in healthy prostate (Christudoss et al., 2011; Malm et al., 2000; Gomez et al., 2007). It has been attributed to the loss of zinc from catabolic tissue and increased urinary excretion of zinc.

The correlational study of oxidative stress parameters and serum PSA found a significant positive correlation ($p<0.01$) of serum PSA with MDA in our study (Table 4.5). Similar findings also reported by Merendino et al. (2003). On the other hand, a significant negative correlation of serum PSA with GSH and zinc was observed in our study (Table 4.5). A significant negative correlation of MDA with GSH was also found in this study (Table 4.5),

which is in agreement with the findings of Srivastava and Mittal (2005a), indicates the generation of more free radicals that may result in the destruction of protein structure or formation of DNA adduct.

Variations in the frequency of NAT2 genotype/phenotype among the different populations and ethnic groups had been reported in several studies carried out in various regions around the world. Study of genotype distribution and allele frequency of NAT2 gene polymorphism in this study found that prostate cancer cases had significantly higher rates of mutant and heterozygote NAT2*6A and NAT2*7A, in comparison to control group (Table 4.6). The odds ratio also showed a higher risk for the development of prostate cancer in the presence of mutant and heterozygote NAT2 genotypes. Our findings are compatible with the results reported by Kosova et al. (2010), and they concluded that NAT2*6A and NAT2*7A/B polymorphisms were significantly associated with prostate cancer risk in the Turkish population.

In our study, 19.8% of prostate cancer patients had slow genotypes for NAT2, and 80.2% had NAT2 rapid acetylator genotypes. On the other hand, only 11.5 % of controls had slow genotypes for NAT2, and 88.5 % had rapid acetylator genotypes, odds ratio (OR) showed NAT2 slow genotypes had 1.9-folds significantly higher risk for development of prostate cancer in comparison to rapid genotypes (Table 4.7). Similarly, a significantly higher frequency of the NAT2 slow acetylator was observed in a study on the Turkish population (Kosova et al., 2010) and the Japanese people (Hamasaki et al., 2003) when compared with the control group. On the other hand, Kidd et al. (2011) and Wadelius et al. (1999), analyzed the NAT2 acetylator genotype frequency in prostate cancer patients and controls in men of African descent and Swedish and Danish populations, respectively and reported the absence of association between NAT2 slow acetylator genotype and the development of prostate cancer. The differences in results, observed among the different epidemiological studies, could reflect exposure or tissue specificity effects. Moreover, these inconsistent findings in several studies might be due to some confounding factors such as ethnicity, geographical location, inadequate sample size, and gene-gene and gene-environmental interactions.

Serum PSA was found significantly higher ($p<0.001$) in NAT2 slow genotypes compared to NAT2 rapid genotype in this study (Fig 4.4). On the contrary, no association was found between NAT2 genotype and PSA in the study done by Srivastava and Mittal (2005b). The present study also observed that the NAT2 slow genotypes have significant distribution in prostate cancer of 50-60 years age group ($p<0.05$, OR=3.13, 95% CI 1.03-9.51), compared to the control (Table 4.8).

The combination of the NAT2 slow acetylator genotype and tobacco smoking had previously been implicated as a risk factor for the development of uroepithelial cancer (Katoh et al., 1999; Inatomi et al., 1999). In this study, the combined effect of smoking and NAT2 genotypes on prostate cancer development found that smokers with NAT2 slow genotypes had a significantly higher risk (odds ratio, 2.38), compared to the smoker with rapid genotypes (Table 4.9). The results of our study suggested that, when smoking and NAT2 gene polymorphism are considered together, a modestly increased risk is associated with the slow acetylation. Hamasaki et al. (2003) also reported, significantly increased risk of prostate cancer development, among smokers with NAT2 slow genotypes (OR=3.78, $p=0.004$).

The distribution of NAT2 genotypes, stratified by a family history of cancer in the study subjects, revealed that a significantly higher frequency of NAT2 slow genotypes was present among the subjects with a positive family history of cancer (Table 4.10). This study also found that subjects with NAT2 slow genotypes with a positive family history of cancer had 7.06-folds higher risk for the development of prostate cancer (Table 4.10). Therefore, it revealed that a family history of cancer might be a risk factor for the patient having these gene variants.

This study found NAT2 slow acetylator genotypes had a significantly higher risk for the development of moderate to high-grade tumors (Gleason score ≥ 7) (OR=3.91, $p<0.05$) (Table 4.11). This study also found that NAT2 slow acetylator genotypes had significantly higher frequency and risk in the development of locally advanced or metastatic prostate cancer (OR=3.71, $p<0.05$) in comparison to the control group (Table 4.11). Similarly, Hamasaki et al. (2003) also reported that the occurrences of the NAT2 slow acetylator genotype were

significantly associated with a more advanced stage of disease (T3/T4/N1/M1; OR=3.14) and a higher pathological grade of the tumor (OR= 4.90). Study of oxidative stress parameters (MDA, reduced Glutathione, GST, and zinc) in NAT2 genotypes revealed significantly reduced zinc ($p<0.01$) in NAT2 slow genotypes in comparison to rapid genotypes, but no significant differences were found for MDA, GSH and GST (Table 4.12).

Deletion of GSTT1 and GSTM1 genes (null genotypes) leads to failure to the expression of the GST proteins, which may cause reduced detoxification of potential carcinogens and lead to a higher risk of cancer (Spurdle et al., 2001). In our study, 30% of prostate cancer patients had null genotypes for GSTT1, and 70% had positive genotypes. On the other hand, only 22.5 % of control had null genotypes for GSTT1, and 77.5 % had positive genotypes. Though the odds ratio showed, GSTT1 null genotypes had 1.45-folds higher risk for the development of prostate cancer compared to positive genotypes, but statistically, no significant associations were observed (Table 4.13). Berber et al. (2013) also did not found any significant association of GSTT1 gene polymorphism with prostate cancer risk. However, Srivastava et al. (2005) reported a significant association of GSTT1 genotypes for the risk of prostate cancer in Indian populations.

Study of GSTM1 genotypes in our study found, 37.7% of prostate cancer patients had null genotypes for GSTM1, and 62.3% had present genotypes (Fig 4.5). On the other hand, only 26.0% of controls had null genotypes for GSTM1, and 74.0% had present genotypes (Fig 4.5). Odd Ratio showed GSTM1 null genotypes had a significantly higher risk for the development of prostate cancer (Table 4.13), which is in agreement with the study of Srivastava et al. (2005). On the contrary, Berber et al. (2013) did not found any significant association of GSTT1 gene polymorphism with prostate cancer risk. The discrepancy of the present study results with other populations in the assessment of specific GSTT1 and GSTM1 genotype profiles may be due to differences in ethnicity as well as the degree of exposure to environmental procarcinogens.

Study of GSTT1 and GSTM1 genotypes in different age groups of study subjects revealed, GSTT1 null genotypes in the age group of 50-60 years had a significantly higher risk for the

development of prostate cancer, but no significant association was observed for GSTM1 genotypes (Table 4.14). Mittal et al. (2004) reported that there was significant variation in GSTT1 and GSTM1 null genotypes between control and prostate cancer patients for at 50-60 years age group. A similar finding was also reported by Chen et al. (2001). So, it revealed that GSTT1 null genotypes were prominent in 50-60 years of age and corresponds to cancer susceptibility. Our study also observed that both GSTT1 and GSTM1 null genotypes had a significantly higher risk for the development of locally advanced/ metastatic cancer with a high Gleason score (>7) (Table 4.17 and Table 4.18).

The results of our study suggested that, when smoking and genetic polymorphisms are considered together, a modestly increased risk of prostate cancer is associated with the null genotypes of GSTT1 and GSTM1. However, for GSTT1 genotypes, it was not statistically significant (Table 4.15). On the other hand, in GSTM1 null genotypes, it was found that prostate cancer risk was significantly increased with an odds ratio of 2.14 (Table 4.15). But Berber et al. (2013) reported that prostate cancer risk did not differ with the smoking habit in GSTM1 null individuals. Still, they also said that prostate cancer risk was significantly increased in GSTT1 null genotypes with smokers., which are disagreement with our findings. Whereas, no significant association of family history of cancer with GSTT1 and GSTM1 genotypes were observed in our study (Table 4.16).

Study of different oxidative stress-related parameters (MDA, reduced Glutathione, GST, and zinc) among GSTT1 genotypes found, no significant differences except GST activity, GST activity found significantly decreased in GSTT1 null genotypes than present genotypes ($p<0.01$) (Table 4.19). On the other hand, the study of different oxidative stress-related parameters among GSTM1 genotypes revealed no significant association. Similarly, Reszka et al. (2007) did not observe any significant differences of erythrocyte GST activity and serum zinc level in between different genotypes of GSTT1 and GSTM1. Study of MDA level in different GSTT1 and GSTM1 combined genotypes of prostate cancer revealed that the MDA level was higher in both null [GSTT1(-)/GSTM1(-)] genotypes than both positive [GSTT1(+)/GSTM1(+)] genotypes (Fig 4.7). Similar results were also reported by Kumar et al. (2011). Erythrocyte reduced GSH in GSTT1 and GSTM1 combined genotypes also found

lower in all combined genotypes than that of control in this study, but none of them was statistically significant (Fig 4.8). Whereas, GST activity in combined GSTT1 and GSTM1 genotypes revealed, GST activity was higher than that of control in all combined genotypes, except in GSTT1(-)/GSTM1(-) combined genotypes (Fig 4.9). A study on Bangladeshi tannery workers found that GST activity was higher in individuals having GSTT1(+) /GSTM1(+) and GSTT1(-) /GSTM1(-) but lower in individuals having either GSTT1 or GSTM1 null genotypes (+/-, -/+) (Akther et al., 2016). The role of GST genotypes in regulating GST activity to cope up the oxidative stress, the expression of other isoenzymes of GST might be the reason.

Considering the combined genotype distribution of GSTT1 and GSTM1 in the study subjects, it was found that the risk of prostate cancer increased by 5.75-folds higher in the presence of combined GSTT1 (null) and GSTM1 (null) genotypes, compared to protective combined GSTT1 and GSTM1 genotypes. (Table 4.20). Similarly, a study in North India by Srivastava et al. (2005) also found that the presence of combined null genotype distribution of GSTT1 and GSTM1 had significantly higher risk with an odds ratio of 3.73, for the development of prostate cancer. The presence of combined NAT2 slow genotypes and GSTM1 null genotypes also had a significantly higher risk for the development of prostate cancer (Table 4.20). Besides this, our study also explored the combined genotype distribution of three genes (NAT2, GSTT1, and GSTM1) with their associated risk in the development of prostate cancer. It was found that the risk of prostate cancer development increased 5.54-folds for NAT2 (rapid)/ GSTT1 (null)/ GSTM1 (null) combination genotypes and risk increased to 9.64-folds in the presence of NAT2 (slow)/ GSTT1 (null)/ GSTM1 (null) combination genotypes, compared to protective combined NAT2, GSTT1 and GSTM1 genotypes (Table 4.20). The analysis indicates that the higher frequency of polymorphic mixed variants is significantly associated with the risk of prostate cancer.

This case-control study suggested a prospective association of genetic polymorphisms and oxidative stress with increased susceptibility to prostate cancer. This study found significantly higher frequencies of mutant and heterozygote NAT2*6A and NAT2*7A/B genotypes in prostate cancer patients of the Bangladeshi population. A significant association of NAT2

slow genotypes and GSTM1 null genotypes with the risk of prostate cancer was observed in the Bangladeshi people. This study revealed significantly increased oxidative stress in prostate cancer patients compared to control. Unfortunately, studies on genetic polymorphisms and prostate cancer are scanty, especially among the Bangladeshi population. Since prostate cancer exhibits distinct ethnic variation, the present research concentrates on exploring and identifying susceptibility markers in the Bangladeshis. The study is the first of its kind in Bangladesh and has proposed susceptibility markers and genotype profiles attributed to an increased risk of prostate cancer.

There are several limitations to this study. Our study had a relatively small sample size, and a narrow geographical area consisted of only the Bangladeshi male race. Our data did not reflect the subjects' dietary habits and lifestyles, which may affect the prostate. The exact roles of oxidative DNA damage in the pathogenesis of disease were not completely defined. Although it is unlikely, due to study design and genetic background of the subjects, population admixture is still a possibility, which cannot be ruled out, for the observed positive findings in this study. Larger and more rigorous analytical studies will be required to confirm our findings and determine the real impact of genetic susceptibility in prostate cancer in Bangladesh.

In conclusion, while this study has some restrictions, it is the first study that established the association of NAT2, GSTT1 and GSTM1 gene polymorphisms, and oxidative stress on prostate cancer risk in the Bangladeshi population. Although the results were significant, this was a preliminary evaluation, and studies with a larger sample are needed to confirm these promising results. Additionally, these findings may contribute to developing more rational therapeutic approaches.

As a future perspective, the followings can be done:

1. Polymorphisms in other genes, which are thought to be involved in bladder cancer pathogenesis and have essential roles in the cellular pathways, can be studied. The combined effect of their interaction with the studied genes and with each other on an individual’s

prostate cancer risk can be determined. Extension of the present study with more samples and SNP profiling of other genes will enable the identification of additional susceptible SNPs in the Bangladeshi people. Construction of an SNP profile of susceptibility will allow the development of an SNP array to screen for genetically predisposed individuals. Further, the SNPs' functional characterization will enable an understanding of the exact molecular mechanism of cancer susceptibility and progression.

2. Gene expression studies should be performed, and the expression profile of latent and metastatic cancer will enable characterization of differential gene expression

3. The analysis of a large number of DNA variations (polymorphisms and mutations) on a genome-wide scale can be carried out by utilizing microarray technology.

6. References

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Appendix-I

Questionnaire

Association of NAT2, GSTT1 and GSTM1 gene polymorphism and influence of oxidative stress on prostate cancer risk in Bangladeshi population

Patient ID: **Age:**

Address:

Contact No:

Chief Complain: Dysuria / Increased urinary frequency / Urinary incontinence / Hematuria/ Abdominal mass / any other symptom (----------)

Past History: Any cancer: Yes No (if yes: ----------)

Other chronic disease: Yes No (if yes :----------)

Family History Any cancer: Yes No (if yes :----------)

History of smoking: Yes No

Educational status: Illiterate/ Primary/ Secondary/ Graduation or above

Occupation: Works at dye factory / Farmer / Others

Monthly income: Low (TK<3000) / Middle (TK 3000-6000) / Upper (TK>6000)

Residence: Urban / Rural

D/R/E: Normal / Mild / Moderate / Hugely Enlarged Prostate

USG: Normal / Enlarged Prostate (volume---------- cc)

Histopathological Diagnosis: ---------- (Gleasons score----------)

Urine R/E: Pus cell:-------- /HPF

Epi cell:---------/HPF

RBC: ----------/HPF

Anthropometric measurements:

Variables	**At present**
Weights (kg)	
Height (m_2)	
BMI (kg/m_2)	

Biochemical and oxidative stress parameters:

Variables	**At present**
Serum PSA (ng/ml)	
Erythrocyte reduced glutathione (µmol/gm of Hb)	
Malondialdehyde (nmol/ml)	
Gluthathione s-transferase (nmol/min/ml)	
S. Zinc ((µg/ml)	

Genotype study:

- NAT2 Genotype……
- GSTT1 Genotype……..
- GSTM1 Genotype……..

Date: **Signature of the investigator**

Appendix-II

অব্যহতি ক্রমে সম্মতিপত্র

আমি ডাঃ **আয়াতুন নেছা,** আমার পি.এইচ. ডি গবেষনার জন্য আপনার রোগ সম্মন্ধে বিস্তারিত তথ্য নিব। এই সম্মতিপত্রের উদ্দেশ্য হল আপনাকে প্রয়োজনীয় তথ্য প্রদান করা, যে তথ্যগুলো আপনাকে সিদ্ধান্ত নিতে সাহায্য করবে, আপনি এই গবেষণায় অংশগ্রহণ করবেন কিনা।

উদ্দেশ্যঃ

বাংলাদেশে প্রসটেট ক্যান্সার এর রুগীর সংখা দিনে দিনে বৃদ্ধি পাচ্ছে।বিভিন্ন দেশের গবেষনায় পাওয়া যায়, কিছু জীন এর পলিমরফীজম (যেমন, ন্যাট-২, জ্রি এস টি টি-১ ও জ্রি এস টি এম-১) এবং অক্সিডেটীভ স্ট্রেস এর প্রধান কারন গুলর অন্যতম। এই গবেষনা্র মাধ্যমে বাংলাদেশের প্রসটেট ক্যান্সার রুগীদের রক্ত পরীক্ষা করে উক্ত কারন গুলর ভূমিকা নির্ণয় করা হবে।

পদ্ধতিঃ

এই গবেষণার স্বার্থে অভিজ্ঞ নার্সের মাধ্যমে আপনার কাছ থেকে ৬ মিলি লিটার রক্ত সংগ্রহ করব। এতে আপনার সামান্য ব্যাথা অনুভূত হতে পারে।

গবেষণার ঝুঁকিঃ

এই গবেষণায় অংশগ্রহণ করার কারণে আপনার শারীরিক কোন ঝুকি থাকবে না। আপনার নিয়মিত রক্ত পরিক্ষার তথ্য দিয়ে গবেষণাটি সম্পন্ন করা যাবে।

গবেষণায় অংশগ্রহণের সুবিধাদিঃ

এই গবেষণায় আমরা প্রসটেট ক্যান্সার এ ন্যাট-২, জ্রি এস টি টি-১ ও জ্রি এস টি এম-১ জীন এর পলিমরফীজম এবং অক্সিডেটীভ স্ট্রেস এর ভূমিকা নির্ণয় করব। এর ফলে যথাযথ কারন নির্ণয় এবং প্রয়জনীয় ব্যবস্থা গ্রহনের মাধ্যমে ভবিস্যতে প্রসটেট ক্যান্সার ঝুকি ও মৃত্যুর হার কমানো সম্ভব হবে।

বিকল্পঃ

আপনার এই গবেষণায় অংশগ্রহণ করা কিংবা না করার ব্যাপারে অথবা যেকোন সময় গবেষণা থেকে সরে যওয়ার ব্যাপারে আপনি নিজেই সিদ্ধান্ত নিতে পারেন।

খরচঃ

এই গবেষণায় অংশগ্রহণের জন্য আপনার কোন খরচ করতে হবে না বা আপনাকে কোন টাকা পয়সা দেয়া হবে না।

গোপনীয়তাঃ

গবেষণা চলাকালীন ও পরবর্তিতে সকল তথ্য কঠোর ভাবে গোপন রাখা হবে। আপনার ব্যক্তিগত বিষয়াদি অন্য কোন তথ্য বিশ্লেষণে, প্রতিবেদন তৈরিতে এই গবেষণার পরিক্ষক ব্যতিত কারো কাছে প্রকাশ করা হবে না। ফলে আপনার কোন তথ্য অন্য কেউ জানতে পারবে না।

স্বেচ্ছামূলক অংশগ্রহণঃ

এই গবেষণায় আপনার অংশগ্রহণ সম্পূর্ণ স্বেচ্ছামূলক। আপনি এই গবেষণায় অংশগ্রহণে অস্বীকৃতি জানাতে পারেন অথবা গবেষণা চলাকালীন যে কোন সময় তাকে সরিয়ে নিতে পারেন। তাতে আপনার চিকিৎসার কোন তারতম্য হবে না। এই ফরমে স্বাক্ষর করলে আপনার অধিকার খর্ব হবে না।

সম্মতির স্বীকারোক্তিঃ

আমি গবেষণায় নিয়োজিত চিকিৎসকের সাথে এই গবেষণা নিয়ে আলোচনায় সন্তুষ্টি প্রকাশ করছি। আমি এটা বুঝেছি যে গবেষণায় আমার অংশগ্রহণ স্বেচ্ছামূলক এবং আমি যে কোন সময় কোন বাধ্যবাধকতা ছাড়াই গবেষণা থেকে বিরত রাখতে পারি। আমি উপরোক্ত শর্তগুলো পড়েছি এবং স্বেচ্ছায় আমি এই গবেষণায় অংশগ্রহণ করাতে সম্মতি জ্ঞাপন করছি।

সাক্ষাৎ গ্রহণকারীর স্বাক্ষর ও তারিখ

অংশগ্রহণকারীর স্বাক্ষর / বৃদ্ধাঙ্গুলির ছাপ

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ঢাকা বিশ্ববিদ্যালয়
ঢাকা - ১০০০
বাংলাদেশ

OFFICE OF ETHICAL REVIEW COMMITTEE
Department of Biochemistry and Molecular Biology
University of Dhaka, Bangladesh

November 19, 2017

BMBDU-ERC/EC/04/2018

Ms. Ayatun Nesa
Ph.D Student
Department of Biochemistry and Molecular Biology
University of Dhaka

Subject: Institutional Ethical Review Committee (ERC) Clearance

Dear Ms. Ayatun Nesa,

With reference to your application on the above mentioned subject, this is to inform you that your Ph.D research titled "**Association of NAT2, GSTT1 and GSTM1 gene polymorphism and influence of oxidative stress on prostate cancer risk in Bangladeshi population**" has been reviewed and approved by the Departmental Ethical Review Committee in its 5th meeting held on 18th November, 2017.

You are requested to follow the Institutional Ethical Review Committee guidelines. Please note that failure to comply with the conditions of approval may result in withdrawal of ethical clearance approval of the proposal.

Expiry date and Expected submission date of thesis: June, 2020.

Prof. Dr. Yearul Kabir
Chairman
Departmental Ethical Review Committee