CYP3A4 AND CYP3A5 GENETIC POLYMORPHISMS AND RISK OF PROSTATE CANCER

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ABSTRACT

Background

Prostate Cancer (PCa) has been the most common cancer in the world for several decades, and by 2008, there were an estimated 899,000 new cases representing 13.7% of the total. Nearly three-quarters i.e. 71.6% cases occur in developed countries whereas 28.4% cases occur in less developed countries. Worldwide, prostate cancer ranks third in cancer incidence and sixth in cancer mortality among men. Incidence rates of prostate cancer varies more than twenty-fold worldwide. The CYP3A4 and CYP3A5 genetic polymorphisms on susceptibility to prostate cancer have received particular interest since these enzymes play a central role in detoxification of major classes of carcinogens, free radicals, xenobiotics and cytotoxic drugs. In the current study we investigated the role of CYP3A4 and CYP3A5 polymorphisms as a genetic modifier of risk for individuals with prostate cancer as susceptible genotypes in Bangladeshi population.

Methods

A case-control study was carried out on 100 prostate cancer patients and 100 controls to investigate two allelic variant of CYP3A4 gene- rs2740574 (CYP3A4*1B) and variant of CYP3A5 gene- rs776746 (CYP3A5*3) using Polymerase Chain Reaction (PCR)-Restriction Fragment Length Polymorphism (RFLP). Risk of prostate cancer was estimated as odds ratio (OR) and 95% confidence interval (CI) using unconditional logistic regression models.

Result

CYP3A5*3 is positively associated with prostate cancer occurrence. For comprehensive examination of the effects of these genes on prostate cancer occurrence, we studied 100 of prostate cancer cases and 100 of controls. An elevated prostate cancer risk was found with heterozygous, mutant and combined heterozygous plus mutant variants of CYP3A4*1B and the results found were not statistically significant (OR = 2.46, 95% CI = 0.62 to 9.81, P = 0.202; OR = 2.11. 95% CI = 0.19 to 23.67, P = 0.545 and OR = 2.37, 95% CI = 0.71 to 7.98, p = 0.162 respectively), whereas a significant association of prostate cancer heterozygous, mutant and combined heterozygous plus mutant variants of CYP3A5*3 was found (OR = 4.36, 95% CI = 1.53 to 12.38, P = 0.003; OR = 3.85. 95% CI = 1.19 to 12.43, P = 0.017 and OR = 4.13, 95% CI = 1.84 to 9.28, p = 0.000 respectively).

Conclusion

Our results indicate that CYP3A5*3 is significantly associated with increased prostate cancer risk. Our findings also suggest that CYP3A4*1B is not significantly associated with prostate cancer susceptibility.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

A	**Adenine (where referring to a nucleotide)**
ADR	**Adverse Drug Reaction**
Ala (A)	**Alanine**
Arg (R)	**Arginine**
Asn (N)	**Asparagine**
Asp (D)	**Aspartic Acid**
BMI	**Body Mass Index**
bp	**Base Pair**
C	**Cytosine (where referring to a nucleotide)**
^oC	**Degrees Celsius**
cDNA	**Complimentary DNA**
CH_3CH_2OH	**Ethanol**
CH_3COONa	**Sodium Acetate**
$C_6H_{16}N_2$	**N, N, N', N'-Tetramethylethylenediamine**
$C_4H_{11}NO_3$	**Tris(hydroxymethyl)aminomethane**
$C_{10}H_{16}N_2O_8$	**Ethylenediaminetetraacetic Acid**
$C_{12}H_{25}OSO_3Na$	**Sodium Dodecyl Sulphate**
C_{max}	**Maximum Serum Concentration**
CMBI	**Centre for Molecular and Biomolecular Informatics**
CNS	**Central Nervous System**
cSNP	**Coding Single Nucleotide Polymorphism**
CYP	**Cytochrome P450**
Cys (C)	**Cysteine**

dbSNP	**Single Nucleotide Polymorphism Database**
ddH_2O	**Double Distilled Water**
dH_2O	**Distilled Water**
DME	**Drug-Metabolizing Enzyme**
DNA	**Deoxyribonucleic Acid**
dNTP	**Deoxynucleotide Triphosphate**
EDTA	**Ethylenediaminetetraacetic Acid ($C_{10}H_{16}N_2O_8$)**
EM	**Extensive Metaboliser**
EtBr	**Ethidium Bromide**
EtOh	**Ethanol (CH_3CH_2OH)**
F	**Forward Primer**
FAD	**Flavin adenine dinucleotide**
FMN	**Flavin mononucleotide**
G	**Guanine (where referring to a nucleotide)**
G6PD	**Glucose-6-Phosphate Dehydrogenase**
Gln (Q)	**Glutamine**
Glu (E)	**Glutamic Acid**
Gly (G)	**Glycine**
HGP	**Human Genome Project**
His (H)	**Histidine**
HPLC	**High performance liquid chromatography**
http	**Hypertext Transfer Protocol**
Ile (I)	**Isoleucine**
IM	**Intermediate Metaboliser**
Inc.	**Incorporated**
indel	**Insertion/Deletion**
ins	**Insertion**

iSNP	**Intergenic Single Nucleotide Polymorphism**
kb	**Kilobase**
KCl	**Potassium Chloride**
kDa	**Kilodalton**
kg	**Kilogram**
K_m	**Michaelis-Menten constant**
L	**Litre**
Leu (L)	**Leucine**
log	**Logarithm**
Lys (K)	**Lysine**
M	**Molar (moles per litre)**
Met (M)	**Methionine**
MDR1	**Multidrug Resistance 1**
mg	**Milligram**
$MgCl_2$	**Magnesium Chloride**
ml	**Millilitre**
min	**Minimum**
max	**Maximum**
mM	**Millimolar**
mm^3	**Cubic Millimetre**
mRNA	**Messenger Ribonucleic Acid**
n	**Sample Size**
N	**Any Nucleotide (where referring to a nucleotide)**
NaCl	**Sodium Chloride**
NAT	**N-acetyltransferase**
NADPH	**Nicotinamide Adenine Dinucleotide Phosphate**

NaOH	**Sodium Hydroxide**
NAT2	**N-acetyltransferase 2**
NCBI	**National Centre for Biotechnological Information**
NEB	**New England Biolabs**
ng	**Nanogram**
PAA	**Polyacrylamide**
PAGE	**Polyacrylamide Gel Electrophoresis**
PCR	**Polymerase Chain Reaction**
PD	**Pharmacodynamics**
PGP	**Phosphoglycoprotein**
p^H	**Potential Hydrogen**
Phe (F)	**Phenylalanine**
PK	**Pharmacokinetics**
PM	**Poor Metaboliser**
Pre-mRNA	**Preliminary Messenger Ribonucleic Acid**
Pro (P)	**Proline**
pSNP	**Perigenic Single Nucleotide Polymorphism**
R	**Reverse Primer**
®	**Registered Trademark**
REase	**Restriction Enzyme/Restriction Endonuclease**
RFLP	**Restriction Fragment Length Polymorphism**
RNA	**Ribonucleic Acid**
RNAse	**Ribonuclease**
rpm	**Revolutions per Minute**
rSNP	**Random Single Nucleotide Polymorphism**
SD	**Standard deviation**

SDS	**Sodium Dodecyl Sulphate ($C_{12}H_{25}OSO_3Na$)**
Ser (S)	**Serine**
SNP	**Single Nucleotide Polymorphism**
SSCP	**Single Strand Conformation Polymorphism**
T	**Thymine (where referring to a nucleotide)**
TBE	**Tris Borate EDTA**
Thr (T)	**Threonine**
TM	**Trademark**
Tris	**Tris(hydroxymethyl)aminomethane**
U	**Unit (enzyme quantity)**
UK	**United Kingdom**
UM	**Ultra-rapid Metaboliser**
UN	**United Nations**
URTI	**Upper Respiratory Tract Infection**
USA	**United States of America**
UTR	**Untranslated Region**
UV	**Ultraviolet**
V	**Volts**
Val (V)	**Valine**
V_{max}	**Maximum Reaction Velocity**
vs	**Versus**
v/v	**Volume per Volume**
WHO	**World Health Organisation**
www	**World Wide Web**
w/v	**Weight per Volume**
$	**Dollar**
>	**Greater Than (except where referring to a**

	nucleotide change)
<	**Less Than**
µ	**Mu (Micro)**
µg/mcg	**Microgram**
mg	**Miligram**
gm	**Gram**
µl	**Microlitre**
L	**litre**
µM	**Micromolar**
%	**Percent**
nm	**Nanometer**

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DECLARATION

Not any portion of this work referred to in this thesis paper has been submitted for another degree or qualification of the University of Dhaka or any other University or any other institute of learning.

DEDICATION

Dedicated to my beloved parents and brothers who always inspire me in every steps of my life

CHAPTER ONE

INTRODUCTION

I

1.1. INTRODUCTION

Prostate Cancer (PCa) is now recognized as one of the most important medical problems facing the male population. In Europe, PCa is the most common solid neoplasm, with an incidence rate of 214 cases per 1000 men, outnumbering lung and colorectal cancer (Boyle P et al., 2004). Furthermore, PCa is currently the second most common cause of cancer death in men (Jemal A *et al*.,2008). In addition, since 1985, there has been a slight increase in most countries in the number of deaths from PCa, even in countries or regions where PCa is not common (Quinn M *et al.,* 2002). Prostate cancer affects elderly men more often than young men. It is therefore a bigger health concern in developed countries with their greater proportion of elderly men. Thus, about 15% of male cancers are PCa in developed countries compared to 4% of male cancers in undeveloped countries (Parkin DM *et al.,* 2001). It is worth mentioning that there are large regional differences in incidence rates of PCa. For example, in Sweden, where there is a long life expectancy and mortality from smoking-related diseases is relatively modest, PCa is the most common malignancy in males, accounting for 37% of all new cases of cancer in 2004 (Persson G *et al*.,2006).

1.2. THE MECHANISM OF PROSTATE GLAND

The prostate gland is a part of the male reproductive system. It develops at puberty and continues to enlarge throughout life. The prostate acts rather like a junction box. It allows the tubes that transport sperm from each testicle and the tubes that drain from the seminal vesicles to meet and then empty their contents into the urethra. The seminal vesicles consist of two pouches that provide nutrients for the sperm and lie immediately behind the prostate. At the point of orgasm, sperm, seminal vesicle fluid and prostatic secretions enter the urethra and mix together, forming semen. This is then ejaculated out through the penis by rhythmic muscular contractions (Stephen Langley *et al.,* 2005).

1.3. THE PROSTATE AND ITS LOCATION

The prostate is a small gland, about the size of a walnut, which lies just below the bladder. The tube draining the bladder, called the urethra, passes through the centre of the gland, to the penis. The valve mechanism, or sphincter, maintains continence and stops urine leaking out of the bladder. It is located below the prostate gland and encircles the urethra (Stephen Langley *et al.,* 2005)

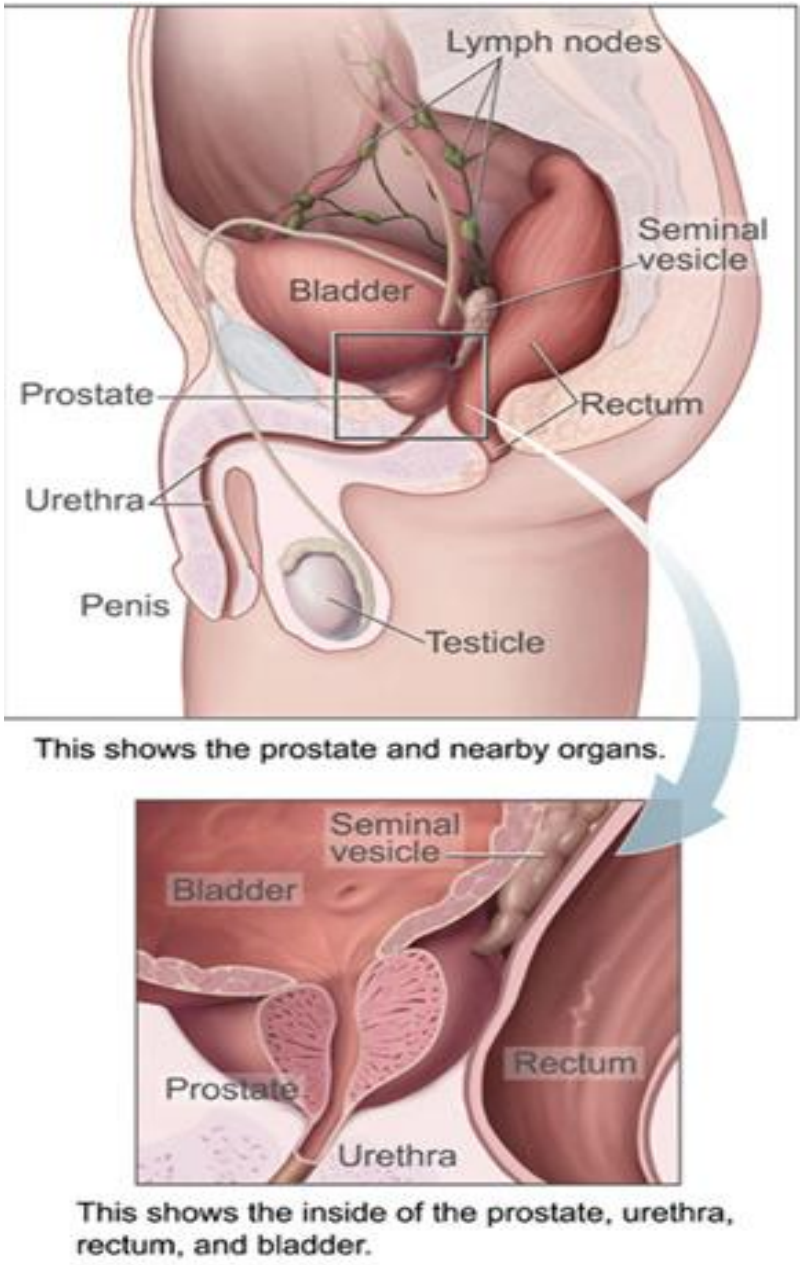

Figure 1.1: The location of prostate gland and nearby organs.

1.4. PROSTATE CANCER

Normally in the prostate, as in the rest of the body, there is a continuous turnover of cells, with new ones replacing old, dying ones. In a cancer, the balance between the new and old cells is lost, with many more new ones being made and older cells living longer, as the process of planned cell death has been disrupted. The malignant growths are known as prostate cancer. They differ from benign enlargements in that the cancerous cells can

spread (metastasise) to other areas in the body. However, sometimes the cancer can be detected before it has spread outside the prostate (Stephen Langley *et al.,* 2005).

1.4.1. SPREAD OF PROSTATE CANCER

Cancer cells can spread by directly growing outwards through the capsule (outer covering of the gland) into the neighbouring parts of the body, such as the seminal vesicles or bladder. They may occasionally spread through the bloodstream and implant and grow in the bones of the spine. Finally, cells can spread through lymph vessels. These vessels are like a second system of veins, except that, instead of blood, they contain a milky fluid that is made up of the cells' waste products. Lymph vessels drain via lymph nodes (special bean-shaped filters), to finally empty back into the blood circulation, and it is these lymph nodes that can also become invaded by cancerous cells (Stephen Langley *et al.,* 2005).

1.5. CONTROLS THE GROWTH OF THE PROSTATE GLAND

The growth of the prostate is controlled by testosterone, the male sex hormone. Most of the testosterone is made by the testicles, although a small amount is also made by the adrenal glands, which lie on top of each kidney. The hormone goes into the bloodstream and finds its way to the prostate. Here, it is changed into dihydro-testosterone (DHT), a more active form which stimulates growth of the gland. The prostate gradually enlarges with ageing, resulting in symptoms such as reduced urine flow and a feeling of incomplete emptying of the bladder, having passed urine. This enlargement is usually benign prostatic hypertrophy (BPH) (non-cancerous) (Stephen Langley *et al.,* 2005).

1.6. RISK FACTORS

The factors that determine the risk of developing clinical PCa are not well known, although a few have been identified. There are three well-established risk factors for PCa: increasing age, ethnic origin, heredity. If one first-line relative has PCa, the risk is at least doubled. If two or more first-line relatives are affected, the risk increases by 5-11-fold (Steinberg GD et al.,1990; Gronberg H *et al.,* 1996). A small subpopulation of individuals with PCa (about 9%) has true hereditary PCa. This is defined as three or more affected relatives, or at least two relatives who have developed early onset disease, i.e. before age 55 (Carter BS *et al.,* 1992). Patients with hereditary PCa usually have an onset 6-7 years prior to spontaneous cases, but do not differ in other ways (Bratt O *et al.,* 2002). The frequency of autopsy-

detected cancers is roughly the same in different parts of the world (Breslow N *et al.,* 1977). This finding is in sharp contrast to the incidence of clinical PCa, which differs widely between different geographical areas, being high in the USA and Northern Europe and low in Southeast Asia (Quinn M *et al.,* 2002). However, if Japanese men move from Japan to Hawaii, their risk of PCa increases. If they move to California their risk increases even more, approaching that of American men (Zaridze DG *et al*., 1984). These findings indicate that exogenous factors affect the risk of progression from so-called latent PCa to clinical PCa. Factors such as food consumption, pattern of sexual behaviour, alcohol consumption, exposure to ultraviolet radiation, chronic inflammation (Nelson WG *et al.,* 2003) and occupational exposure have all been discussed as being aetiologically important. Prostate cancer is an ideal candidate for exogenous preventive measures, such as dietary and pharmacological prevention, due to some specific features: high prevalence, long latency, endocrine dependency, availability of serum markers (PSA), and histological precursor lesions (atypical small acinar proliferation [ASAP] or prostatic intraepithelial neoplasia [PIN]) (Nelson WG *et al.,* 2003). Dietary/nutritional factors that may influence disease development include total energy intake (as reflected by body mass index), dietary fat, cooked meat, micronutrients and vitamins (carotenoids, retinoids, vitamins C, D, and E), fruit and vegetable intake, minerals (calcium, selenium), and phyto-oestrogens (isoflavonoids, flavonoids, lignans), or statins and/or cholesterol intake. Since most studies reported to date are case-control analyses, there remain more questions than evidence-based data available to answer them. Several ongoing large randomised trials are trying to clarify the role of such risk factors and the potential for successful prostate cancer prevention (Schmid H-P *et al.,* 2004). In summary, hereditary factors are important in determining the risk of developing clinical PCa, while exogenous factors may have an important impact on this risk. The key question is whether there is enough evidence to recommend lifestyle changes (lowered intake of animal fat and increased intake of fruit, cereals, and vegetables) in order to decrease the risk (Schulman CC *et al.,* 2000). There is some evidence to support such a recommendation and this information can be given to male relatives of PCa patients who ask about the impact of diet.

1.7. SYMPTOMS OF PROSTATE CANCER

There are often no symptoms associated with early stage prostate cancer. As the disease progresses and the tumour enlarges, it may press on the urethra, which runs through the gland, and obstruct the flow of urine during urination. In this situation, the patient may notice a weak, interrupted stream of urine that requires straining to produce and, on completion, he

may still feel that the bladder is not empty. However, these symptoms are not specific to prostate cancer and are most commonly found in benign (non-cancerous) enlargements of the gland. Blood in the semen may be a sign of prostate cancer, although again this is a common finding and not normally related to malignancy. If the tumour has spread to the bones, it may cause pain. The spine is the most common site for this to occur (Stephen Langley *et al.,* 2005).

1.8. SCREENING AND EARLY DETECTION

Population or mass screening is defined as the examination of asymptomatic men (at risk). It usually takes place as part of a trial or study and is initiated by the screener. In contrast, early detection or opportunistic screening comprises individual case findings, which are initiated by the person being screened (patient) and/or his physician. The primary endpoint of both types of screening has two aspects: 1. Reduction in mortality from PCa. The goal is not to detect more carcinomas, nor is survival the endpoint because survival is strongly influenced by lead-time from diagnosis. 2. The quality of life is important as expressed by quality-of-life adjusted gain in life years (QUALYs). Prostate cancer mortality trends range widely from country to country in the industrialised world (Oliver SE *et al.,* 2001). Decreased mortality rates due to PCa have occurred in the USA, Austria, UK, and France, while in Sweden the 5-year survival rate has increased from 1960 to 1988, probably due to increased diagnostic activity and greater detection of non-lethal tumours (Helgesen F *et al*., 1996). However, this trend has not been confirmed in a similar study from the Netherlands (Post PN *et al.,* 1999). The reduced mortality seen recently in the USA is often attributed to the widely adopted aggressive screening policy, but there is still no absolute proof that prostate-specific antigen (PSA) screening reduces mortality due to PCa (Ilic D *et al.,* 2007). A non-randomised screening project in Tyrol (Austria) may support the hypothesis that screening can be effective in reducing mortality from PCa. An early detection programme and free treatment have been used to explain the 33% decrease in the PCa mortality rate seen in Tyrol compared to the rest of Austria (Bartsch G *et al.,* 2001). In addition, a Canadian study has claimed lower mortality rates in men randomised to active PCa screening (Labrie F *et al.,* 1999), though these results have been challenged (Boer R *et al*., 1999). Positive findings attributed to screening have also been contradicted by a comparative study between the US city of Seattle area (highly screened population) and the US state of Connecticut (seldom screened population) (Lu-Yao G *et al.,* 2002). The study found no difference in the reduction in the rate of PCa mortality, even allowing for the very great diversity in PSA testing and treatment. In 2009, the long awaited results of two prospective, randomised trials were

published. The Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial randomly assigned 76,693 men at 10 US centres to receive either annual screening with PSA and DRE, or standard care as the control. After 7 years' follow-up, the incidence of PCa per 10,000 person-years was 116 (2,820 cancers) in the screening group and 95 (2,322 cancers) in the control group (rate ratio, 1.22) (Andriole GL *et al*., 2009). The incidence of death per 10,000 person-years was 2.0 (50 deaths) in the screened group and 1.7 (44 deaths) in the control group (rate ratio, 1.13). The data at 10 years were 67% complete and consistent with these overall findings. The PLCO project team concluded that PCa related mortality was very low and not significantly different between the two study groups (LE: 1b). The European Randomized Study of Screening for Prostate Cancer (ERSPC) included a total of 162,243 men from seven countries aged between 55 and 69 years. The men were randomly assigned to a group offered PSA screening at an average of once every 4 years or to an unscreened control group. During a median follow-up of 9 years, the cumulative incidence of PCa was 8.2% in the screened group and 4.8% in the control group (Schröder FH *et al.,* 2009). The rate ratio for death from PCa was 0.80 in the screened group compared with the control group. The absolute risk difference was 0.71 deaths per 1,000 men. This means that 1,410 men would need to be screened and 48 additional cases of PCa would need to be treated to prevent one death from PCa. The ERSPC investigators concluded that PSA-based screening reduced the rate of death from PCa by 20%, but was associated with a high risk of over-diagnosis. Both trials have received considerable attention and comments. In the PLCO trial, the rate of compliance in the screening arm was 85% for PSA testing and 86% for DRE. However, the rate of contamination in the control arm was as high as 40% in the first year and increased to 52% in the sixth year for PSA testing and ranged from 41% to 46% for DRE. Furthermore, biopsy compliance was only 40-52% versus 86% in the ERSPC. Thus, the PLCO trial will probably never be able to answer whether or not screening can influence PCa mortality. In an update of the Gothenburg section of the ERSPC trial, which includes 20,000 men, the authors reported a reduction in PCa mortality of 50% after a median follow-up of 14 years. However, this finding was accompanied by a substantial risk of over-diagnosis (Hugosson J et al., 2010). In the complete ERSCP trial, the real benefit will only be evident after 10-15 years of follow-up, especially once the 41% reduction of metastasis in the screening arm has had an impact. A longer follow-upmay reduce the number needed to screen and to treat (Gulati R *et al.,* 2011). Based on the results of these two large, randomised trials, most if not all of the major urological societies conclude that at present widespread mass screening for PCa is not appropriate. Rather, early detection (opportunistic screening) should be offered to the well-informed man. Two key questions remain open: 1. At what age should early detection start?

2. What is the screening interval for PSA and DRE? A baseline PSA determination at age 40 years has been suggested, upon which the subsequent screening interval may then be based (Börgermann C *et al*., 2010). A screening interval of 8 years might be enough in men with initial PSA levels < 1 ng/mL (Roobol MJ *et al.,* 2005). Further, PSA testing in men older than 75 years is not recommended because its early detection would not have any clinical impact (Schaeffer EM *et al.,* 2009).

1.9. DIAGNOSIS

The main diagnostic tools to obtain evidence of PCa include DRE, serum concentration of PSA and transrectal ultrasonography (TRUS). Its definite diagnosis depends on the histopathologic verification of adenocarcinoma in prostate biopsy cores or operative specimens.

1.9.1. DIGITAL RECTAL EXAMINATION (DRE)

Most prostate cancers are located in the peripheral zone of the prostate and may be detected by DRE when the volume is about 0.2 mL or larger. In about 18% of all patients, PCa is detected by a suspect DRE alone, irrespective of the PSA level (Richie JP *et al*., 1993). A suspect DRE in patients with a PSA level of up to 2 ng/mL has a positive predictive value of 5-30% (Carvalhal GF *et al*., 1999). A suspect DRE is a strong indication for prostate biopsy as it is predictive for more aggressive (Gleason score > 7) prostate cancer (Okotie OT *et al.,* 2007; Gosselaar C *et al.,* 2008).

1.9.2. PROSTATE-SPECIFIC ANTIGEN (PSA)

The measurement of PSA level has revolutionised the diagnosis of PCa (Stamey TA *et al*., 1987). PSA is a kallikrein-like serine protease produced almost exclusively by the epithelial cells of the prostate. For practical purposes, it is organspecific but not cancer-specific. Thus, serum levels may be elevated in the presence of benign prostatic hypertrophy (BPH), prostatitis and other non-malignant conditions. The level of PSA as an independent variable is a better predictor of cancer than suspicious findings on DRE or TRUS (Catalona WJ *et al*., 1994). There are many different commercial test kits for measuring PSA, but no commonly agreed international standard exists (Semjonow A *et al*., 1996). The level of PSA is a continuous parameter: the higher the value, the more likely is the existence of PCa. The finding that many men may harbour PCa, despite low levels of serum PSA, has been

underscored by recent results from a US prevention study (Thompson IM *et al.,* 2004). Table **1.1** gives the rate of PCa in relation to serum PSA for 2,950 men in the placebo-arm and with PSA values < 4 ng /mL.

Table 1.1: Risk of PCa in relation to low PSA values

PSA level (ng/mL)	Risk of PCa	Risk of Gleason > 7 PCa
0-0.5	6.6%	0.8%
0.6-1	10.1%	1.0%
1.1-2	17.0%	2.0%
2.1-3	23.9%	4.6%
3.1-4	26.9%	6.7%

The findings in Table 1.1 clearly demonstrate the occurrence of aggressive PCa even at very low PSA levels, precluding an optimal PSA threshold value for detecting non-palpable, but clinically significant, PCa. Use of nomograms may help reducing the number of unnecessary prostate biopsies (Dong F *et al.*, 2008).

1.9.2.1. FREE/TOTAL PSA RATIO (F/T PSA)

The free/total PSA ratio (f/t PSA) is the concept most extensively investigated and most widely used in clinical practice to discriminate BPH from PCa. The ratio is used to stratify the risk of PCa for men who have total PSA levels between 4 and 10 ng/mL and a negative DRE. In a prospective multicentre trial, PCa was found on biopsy in 56% of men with f/t PSA < 0.10, but in only 8% of men with f/t PSA > 0.25 (Catalona WJ et al., 1998). Nevertheless, the concept must be used with caution as several pre-analytical and clinical factors may influence the f/t PSA, e.g. instability of free PSA, variable assay characteristics and very large prostate size (Stephan C et al., 1997). For example, free PSA is unstable at both 4°C and at room temperature. In addition, assay characteristics may vary, and concomitant BPH in large prostates may result in a dilution effect (Stephan C *et al.,* 1997). Furthermore, f/t PSA is of no clinical use in total serum PSA values >10 ng/mL or during follow-up of patients with known PCa.

1.9.2.2. PSA VELOCITY (PSAV), PSA DOUBLING TIME (PSADT)

There are two methods of measuring PSA over time: (1) PSAV, which is defined as an absolute annual increase in serum PSA (ng/mL/year) (Carter HB et al., 1992); and (2) PSADT, which measures the exponential increase of serum PSA over time, reflecting a relative change (Schmid H-P et al., 1993). These two concepts may have a prognostic role in patients with treated PCa (Arlen PM *et al.,* 2008), but they have limited use in the diagnosis of PCa because of background noise (total volume of prostate, BPH), the variations in interval between PSA determinations, and acceleration/deceleration of PSAV and PSADT over time. Prospective studies have shown that these measurements do not provide additional information compared to PSA alone (Heidenreich A *et al*., 2008; Ramirez ML *et al*., 2008; O'Brien MF et al., 2009; Vickers AJ *et al.*, 2009).

1.9.2.3. PCA3 MARKER

An increasingly studied new biomarker is PCA3, detectable in urine sediments obtained after three strokes of prostatic massage during digital rectal examination. The costly Progensa urine test for PCA3 is now commercially available. The amount of the prostate-specific non-coding mRNA marker, PCA3 normalized against PSA mRNA (urine sediment) gives a PCA3 score. The PCA3 score is superior to PSA total, and percent free PSA in detection of PCa in men with elevated PSA as it shows slight but significant increases in the AUC for positive biopsies (Deras IL et al., 2008; Hessels D et al., 2003; Nakanishi H et al., 2008; Hessels D et al., 2010). The PCA3 score may be used together with PSA and other clinical risk factors in a nomogram or other risk stratification tools to make a decision with regard to first or repeat biopsy (Auprich M *et al.,* 2011). The PCA3 score increases with prostate cancer volume, but there is conflicting data about whether the PCA3 score independently predicts the Gleason score and its use as a monitoring tool in active surveillance has not been confirmed (Auprich M *et al.,* 2011). The main current indication of the PCA3 urine test may be to determine whether a man needs a repeat biopsy after an initially negative biopsy outcome, but its cost-effectiveness remains to be shown.

1.9.3. TRANSRECTAL ULTRASONOGRAPHY (TRUS)

The classic picture of a hypoechoic area in the peripheral zone of the prostate will not always be seen. Grayscale TRUS does not detect areas of PCa with adequate reliability

(Lee F *et al.,* 1989). It is therefore not useful to replace systematic with targeted biopsies of suspect areas. However, additional biopsies of suspect areas may be useful.

1.9.4. PROSTATE BIOPSY

1.9.4.1. BASELINE BIOPSY

The need for prostate biopsies should be determined on the basis of the PSA level and/or a suspicious DRE. The patient's biological age, potential co-morbidities (ASA Index and Charlson Comorbidity Index), and the therapeutic consequences should also be considered (Roobol MJ *et al.,* 2010). Risk stratification is becoming an important tool to reduce unnecessary prostate biopsies (Roobol MJ *et al.,* 2010) The first elevated PSA level should not prompt an immediate biopsy. The PSA level should be verified after a few weeks by the same assay under standardised conditions (i.e. no ejaculation and no manipulations, such as catheterisation, cystoscopy or TUR, and no urinary tract infections) in the same diagnostic laboratory, using the same methods (Eastham JA et al., 2003; Stephan C et al., 2006). It is now considered the standard of care to perform prostate biopsies guided by ultrasound. Although a transrectal approach is used for most prostate biopsies, some urologists prefer to use a perineal approach. The cancer detection rates of perineal prostate biopsies are comparable to those obtained for transrectal biopsies (Hara R *et al.*, 2008; Takenaka A *et al.,* 2008). The ultrasound-guided perineal approach is a useful alternative in special situations, e.g. after rectal amputation.

1.9.4.2. REPEAT BIOPSY

The indications for a repeat biopsy are: (1) rising and/or persistently elevated PSA; (2) suspicious DRE (Epstein JI *et al*., 2006); (3) atypical small acinar proliferation (ASAP); and (4) extensive (multiple biopsy sites) prostatic intraepithelial neoplasia (PIN) (Merrimen JL et al., 2009). High-grade PIN as an isolated finding is no longer considered an indication for repeat biopsy (Moore CK *et al*., 2005). A repeat biopsy should therefore be prompted by other clinical features, such as DRE findings and PSA level. If PIN is extensive (i.e. in multiple biopsy sites), this could be a reason for early repeat biopsy, because the risk of subsequent PCa is slightly increased. If clinical suspicion for PCa persists in spite of negative prostate biopsies, MRI may be used to investigate the possibility of an anterior located PCa, followed by TRUS or MRI guided biopsies of the suspicious area (Lemaitre L *et al.*, 2009).

1.9.4.3. SATURATION BIOPSY

The incidence of PCa detected by saturation repeat biopsy (> 20 cores) is between 30% and 43% and depends on the number of cores sampled during earlier biopsies (Walz J *et al*., 2006). In special situations, saturation biopsy may be performed with the transperineal technique. This will detect an additional 38% of PCa. The high rate of urinary retention (10%) is a drawback (Moran BJ *et al*., 2006).

1.9.4.4. SAMPLING SITES AND NUMBER OF CORES

On baseline biopsies, the sample sites should be as far posterior and lateral as possible in the peripheral gland. Additional cores should be obtained from suspect areas by DRE/TRUS. These should be chosen on an individual basis. Sextant biopsy is no longer considered adequate. At a glandular volume of 30-40 mL, at least eight cores should be sampled. The British Prostate Testing for Cancer and Treatment Study has recommended 10 core biopsies (Donovan J *et al*., 2003). More than 12 cores are not significantly more conclusive (Eichler K *et al*., 2006).

1.9.4.5. DIAGNOSTIC TRANSURETHRAL RESECTION OF THE PROSTATE (TURP)

The use of diagnostic TURP instead of repeat biopsies is a poor tool for cancer detection (Zigeuner R *et al.,* 2003).

1.9.4.6. SEMINAL VESICLE BIOPSY

Indications for seminal vesicle (staging) biopsies are poorly defined. At PSA levels > 15-20 ng/mL, the odds of tumour involvement are 20-25% (Linzer DG *et al*., 1996), but a biopsy is only useful if the outcome will have a decisive impact on treatment, i.e. if the biopsy result rules out radical removal for tumour involvement or radiotherapy with intent to cure.

1.9.4.7. TRANSITION ZONE BIOPSY

Transition zone (TZ) sampling during baseline biopsies provides a very low detection rate and TZ sampling should therefore be confined to repeat biopsies (Pelzer AE *et al.,* 2005).

1.9.4.8. ANTIBIOTICS PRIOR TO BIOPSY

Oral or intravenous antibiotics are state-of-the-art treatment. Optimal dosing and treatment time vary. Quinolones are the drugs of choice, with ciprofloxacin superior to ofloxacin (Aron M *et al*., 2000), but in the last few years increased resistance to quinolones has been reported (Cuevas O *et al*., 2011) associated with a rise in severe infectious complications after biopsy (Loeb S *et al.,* 2011).

1.9.4.9. LOCAL ANAESTHESIA PRIOR TO BIOPSY

Ultrasound-guided peri-prostatic block is state-of-the-art (von Knobloch R *et al*., 2002). It does not make any difference whether the depot is apical or basal. Intrarectal instillation of a local anaesthetic is clearly inferior to peri-prostatic infiltration (Adamakis I *et al*., 2004).

1.9.4.10. FINE-NEEDLE ASPIRATION BIOPSY

Fine-needle aspiration biopsy is no longer state of the art.

1.9.4.11. COMPLICATIONS

Complications include macrohaematuria and haematospermia (Table 1.2) (NCCN Clinical Practice Guidelines in OncologyTM Prostate Cancer Early Detection, 2010). Severe post-procedural infections were initially reported in < 1% of cases, but this rate has increased in the last few years as a consequence of the evolution of antibiotic resistance strains with more post-biopsy hospitalizations for infectious complications while the rate of non-infectious complications has remained stable (Loeb S *et al.,* 2011) . Low-dose aspirin is no longer an absolute contraindication (Giannarini G *et al*., 2007).

Table 1.2: Percentage given per biopsy session, irrespective of the number of cores*

Complications	% of biopsies
Haematospermia	37.4
Haematuria > 1 day	14.5
Rectal bleeding < 2 days	2.2
Prostatitis	1.0
Fever > 38.5°C (101.3°F)	0.8
Epididymitis	0.7
Rectal bleeding > 2 days ± requiring surgical intervention	0.7
Urinary retention	0.2

* Adapted from NCCN Guidelines Prostate Cancer Early Detection. V.s.2010.

1.9.5. PATHOLOGY OF PROSTATE NEEDLE BIOPSIES

1.9.5.1. GROSSING AND PROCESSING

Prostate core biopsies taken from different sites are usually sent to the pathology laboratory in separate vials and should be processed in separate cassettes. Before processing, number of cores per vial and length of each core should be recorded. There is a significant correlation between the length of prostate biopsy tissue on the histological slide and the detection rate of PCa (Iczkowski KA *et al.,* 2002). To achieve optimal flattening and alignment of individual cores, one should embed a maximum of three cores per cassette and use sponges or paper to keep the cores stretched and flat (Van der Kwast TH *et al.,* 2003; Rogatsch H *et al.,* 2000). To optimise the detection of small lesions, blocks should be cut at three levels (Pelzer AE *et al.,* 2005). It is helpful routinely to mount intervening tissue sections in case additional immunostaining is needed.

1.9.5.2. MICROSCOPY AND REPORTING

Diagnosis of prostate cancer is based on histological examination. Ancillary staining techniques (e.g. basal cell staining) and additional (deeper) sections should be considered if a suspect lesion is identified (Novis DA *et al.,* 1999; Iczkowski KA, 2006; Reyes AO *et al.,* 1998). Diagnostic uncertainty in biopsies may often be resolved by intradepartmental consultation or a second opinion from an external institution (Novis DA *et al.,* 1999). Table 1.3 lists recommended concise terminology to report prostate biopsies (Rogatsch H *et al.,* 2000).

Table 1.3: Recommended diagnostic terms to report prostate biopsy findings*

Benign/negative for malignancy. If appropriate, include a description (e.g. atrophy).
Active inflammation, negative for malignancy
Atypical adenomatous hyperplasia/adenosis, no evidence of malignancy
Granulomatous inflammation, negative for malignancy
High-grade PIN, negative for adenocarcinoma
High-grade PIN with atypical glands suspicious for adenocarcinoma
Focus of atypical glands/lesion suspicious for adenocarcinoma/atypical small acinar proliferation suspicious for cancer
Adenocarcinoma

*From Van der Kwast, 2003.

PIN = prostatic intra-epithelial neoplasia.

For each biopsy site, the proportion of biopsies positive for carcinoma and the ISUP 2005 Gleason score should be reported (Epstein JI *et al.,* 2005). A recent study has demonstrated the improved concordance of pattern and change of prognostic groups for the modified Gleason grading (Billis A *et al.,* 2008). According to current international convention, the (modified) Gleason score of cancers detected in prostate biopsy consists of the Gleason grade of the dominant (most extensive) carcinoma component plus the highest grade, irrespective of its extent (no 5% rule). When the carcinoma largely consists of grade 4/5 carcinoma, identification of a small portion (< 5% of the carcinoma) of Gleason grade 2 or 3 glands should be ignored. A diagnosis of Gleason score 4, or lower, should not be given on prostate biopsies (Epstein JI *et al.,* 2005). The presence of intraductal carcinoma and extraprostatic extension should be reported. In addition to a report of the carcinoma features for each biopsy site, an overall Gleason score based on findings in the individual biopsies is commonly provided. The proportion (%) or length (mm) of tumour involvement per biopsy core correlates with tumour volume, extraprostatic extension, and prognosis after prostatectomy (Sebo TJ *et al.,* 2001; Grossklaus DJ *et al.,* 2002; Freedland SJ *et al.,* 2004), and an extent of > 5 mm or > 50% of adenocarcinoma in a single core is used as a cut-off triggering immediate treatment versus active surveillance in patients with Gleason score 6 carcinoma. For these reasons a measure of the extent of cancer involvement (mm or %) should be provided for each core. Length of carcinoma and percentage of carcinoma involvement of the biopsy have equal prognostic impact (Brimo F *et al.,* 2008). The extent of a single, small focus of adenocarcinoma, which is located in only one of the biopsies, should be clearly stated (e.g. < 1 mm or < 1%), as this might be an indication for further diagnostic

workup before selecting therapy as this finding is associated with an increased risk of vanishing cancer (Herkommer K *et al.,* 2004; Postma R *et al.,* 2005; Trpkov K *et al.,* 2006). A prostate biopsy that does not contain glandular prostate tissue should be reported as inadequate for diagnostics, except for staging biopsies.

1.9.6. PATHOHISTOLOGY OF RADICAL PROSTATECTOMY (RP) SPECIMENS

1.9.6.1. PROCESSING OF THE RP SPECIMEN

The histopathological examination of RP specimens aims to provide information about the actual pathological stage, grade, and surgical margin status of the prostate cancer. The weight and dimensions of the specimen are recorded before embedding it for histological processing. It is generally recommended that RP specimens are totally embedded to enable the best assessment of location, multifocality, and heterogeneity of the cancer. However, for cost-effectiveness, partial embedding using a standard method may also be considered, particularly for large prostates (> 60 g). The most acceptable method includes the complete embedding of the posterior (dorsal) part of the prostate in addition to a single mid-anterior left and right section. Compared to total embedding, this method of partial embedding permitted detection of 98% of prostate cancers with a Gleason score > 7 and accurate staging in 96% of cases (Sehdev AE *et al.,* 2001). Upon receipt in the histopathology laboratory, the entire RP specimen is inked in order to appreciate the surgical margin status. The specimen is fixed by immersion in buffered formalin for a few days, preferably prior to incision of the sample, as incision causes distortion of the tissue. Fixation can be enhanced by injecting formalin using 21-gauge syringes, which provides a more homogeneous fixation and sectioning after 24 hours (Ruijter ET *et al.,* 1997). After fixation, the apex is removed and cut with (para)sagittal or radial sections; the shave method is not recommended (Epstein JI *et al.,* 2005). Separate removal and sagittal sectioning of the bladder neck is optional. The remainder of the RP specimen is generally cut in transverse sections at 3-4 mm steps, perpendicularly to the posterior surface. The resultant tissue slices can be embedded and processed either as whole-mounts or after quadrant sectioning. Whole-mount processing provides better topographic visualisation of the carcinoma and faster histopathological examination. However, it is a more time-consuming and more expensive technique that requires specialised equipment and personnel. Although whole-mount sectioning may be necessary for research, its advantages do not outweigh its disadvantages for routine sectioning.

1.9.6.1.1. Recommendations for processing a prostatectomy specimen

Total embedding of a prostatectomy specimen is preferred, either by conventional (quadrant sectioning) or by whole-mount sectioning.
The entire surface of RP specimens should be inked before cutting to evaluate the surgical margin status.
The apex should be separately examined using the cone method with sagittal or radial sectioning.

1.9.6.2. RP SPECIMEN REPORT

The pathology report provides essential information on the prognostic characteristics relevant for making clinical decisions (Table 1.4). As a result of the complex information provided on each RP specimen, the use of synoptic-(like) or checklist reporting is recommended (Table 1.5). Synoptic reporting of surgical specimens results in more transparent and complete pathology reporting (Chan NG *et al*., 2008).

Table 1.4: Information provided by the pathology report

Typing (> 95% of PCa represents conventional (acinar) adenocarcinoma)
Grading according to the Gleason score
(Sub)staging and surgical margin status of the tumour
If appropriate, location and extent of extraprostatic extension, presence of bladder neck invasion, laterality of extraprostatic extension or seminal vesicle invasion, location and extent of positive surgical margins
Additional information may be provided on multifocality, diameter of the dominant tumour and zonal location (transition zone, peripheral zone, anterior horn) of the dominant tumour

Table 1.5: Example checklist - reporting of prostatectomy specimens

Histological type
Type of carcinoma, e.g. conventional acinar, ductal, etc.
Histological grade
Primary (predominant) grade
Secondary grade
Tertiary grade (if applicable)
Total/global Gleason score
Approximate percentage of Gleason grade 4 or 5 (optional)

Tumour quantitation (optional)
Percentage of prostatic gland involved
Tumour size of dominant nodule (if identified), greatest dimension in mm
Pathological staging (pTNM)
Presence of extraprostatic extension (indicate focal or extensive) • If present, specify site(s) • Presence of seminal vesicle invasion
If applicable, regional lymph nodes • Location • Number of lymph nodes retrieved • Number of lymph nodes involved
Surgical margins
Presence of carcinoma at margin • If present, specify sites and extra- or intraprostatic involvement
Other
If identified, presence of angioinvasion
Location (site, zone) of dominant tumour (optional)
Perineural invasion (optional) • If present, specify extra- or intraprostatic location

1.9.6.2.1. GLEASON SCORE

Grading of conventional prostatic adenocarcinoma using the (modified) Gleason score system (Epstein JI *et al.,* 2005) is the single strongest prognostic factor for clinical behaviour and treatment response. The Gleason score is therefore one of the parameters incorporated in nomograms that predict the risk of recurrence after prostatectomy (Partin AW *et al.,* 2001). 1.9.6.2.2 Interpreting the Gleason score The Gleason score is the sum of the most dominant and second most dominant (in terms of volume) Gleason grade. If only one grade is present, the primary grade is doubled. If a grade comprises < 5% of the cancer volume, this grade is not incorporated in the Gleason score (5% rule). Both the primary and the secondary grade are reported in addition to the Gleason score (e.g. Gleason score 7 [4 + 3]). A global Gleason score is given when there are multiple tumours, but a separate tumour focus with a higher Gleason score should also 22 Update February 2012 be mentioned. A tertiary Gleason grade 4 or 5, particularly if exceeding 5% of the prostate cancer volume, is an unfavourable prognostic indicator for biochemical recurrence. The presence of the tertiary

grade and its approximate proportion of the cancer volume should also be reported (Harnden P *et al.,* 2007), in addition to the Gleason score.

1.9.6.2.2. DEFINITION OF EXTRAPROSTATIC EXTENSION

The TNM staging system of the International Union Against Cancer (UICC) is recommended for pathological staging of prostate carcinoma (Epstein JI et al., 2005; Ohori M *et al*., 2004). Pathologic substaging of pT2 prostate cancer is optional, since 1) it does not correlate with clinical T2 substage and 2) it lacks prognostic significance (Van der Kwast TH *et a*l., 2011). Extraprostatic extension is the recommended term for the presence of tumour beyond the confines of the prostate. Extraprostatic extension is defined as carcinoma mixed with periprostatic adipose tissue, or bulging out beyond the contours of the prostate gland, e.g. at the neurovascular bundle or the anterior prostate. Bladder neck invasion is also considered to be an extraprostatic extension. It is useful to report not only the location, but also the extent of extraprostatic extension because extension is related to the risk of recurrence. There are no well-established and internationally accepted definitions of the terms 'focal' and 'non-focal' or 'extensive extraprostatic extension'. Some authors describe focal as 'a few glands' (Epstein JI *et al.,* 1993) or extension < 1 high-power field (Marks M *et al.,* 2007), whereas others measure the depth of extent in mm (Sung MT *et al.,* 2007). Currently, it is considered clinically useful to report the extent of extraprostatic extension (e.g. less or more than 1 high-power field or 1 mm) (Magi-Galluzzi C *et al.,* 2011). At the apex of the prostate gland, tumour mixed with skeletal muscle does not constitute extraprostatic extension. In the bladder neck, microscopic invasion of small fibres of smooth muscle is not equated to (gross) bladder wall invasion, because it does not carry independent prognostic significance for PSA recurrence (Aydin H *et al.,* 2004; Ploussard G *et al.,* 2009) and should be recorded as extraprostatic extension (pT3a). A positive margin at the bladder neck should be reported as an extraprostatic extension (pT3a) with positive margin and not as pT4 disease. Stage pT4 can only be assigned when tumour invades the muscle wall of the bladder as determined by the urologist (Hoedemaeker RF *et al.,* 2000).

1.9.6.3. PROSTATE CANCER VOLUME

The independent prognostic value of the volume of PCa in RP specimens has not been established (Marks M *et al.,* 2007; Stamey TA *et al.,* 2000; Epstein JI *et al.,* 2005; Kikuchi E *et al*., 2004; Van Oort IM *et al*., 2008). Nevertheless, a PCa volume cut-off of 0.5 mL continues to be an important parameter to distinguish insignificant from clinically relevant

cancer (Stamey TA *et al.,* 2000). Continued improvement in radioimaging of the prostate gland has allowed more accurate measurement of cancer volume before surgery. Therefore, it may be recommended to assess the greatest dimension of the dominant tumour nodule, if identified, or to provide a rough estimate of the percentage of cancer tissue in the prostate.

1.9.6.4. SURGICAL MARGIN STATUS

Surgical margin status is an independent risk factor for biochemical recurrence. Margin status is positive if tumour cells are in touch with the ink on the surface of the specimen. Margin status is negative if tumour cells are very close to the inked surface of the margin (Epstein JI *et al*., 2005) or when they are at the surface of the tissue lacking any ink. If the tissue has severe crush artifacts (usually at the apex), it may not be possible to assign a surgical margin status (Evans AJ *et al*., 2008). Surgical margin status is independent of the pathological stage and a positive margin is not evidence of extraprostatic extension (Chuang AY *et al.,* 2008). There is insufficient evidence to prove a relationship between the extent of positive margin and the risk of recurrence (Marks M *et al*., 2007). However, some indication must be given of the multifocality and extent of margin positivity, such as the linear extent in millimetres, or number of blocks with positive margin involvement.

1.9.6.5. OTHER FACTORS

According to the College of American Pathologists consensus statement (Bostwick DG *et al*., 2000), additional potential biomarkers have not been sufficiently studied to demonstrate their additional prognostic value and clinical usefulness outside the standard patient care setting (category III), including perineural invasion, neuroendocrine differentiation, microvessel density, nuclear roundness, chromatin texture, other karyometric factors, proliferation markers, prostate-specific antigen derivatives, and other factors (e.g. oncogenes, tumour suppressor genes, or apoptosis genes).

1.10. CLASSIFICATION OF PROSTATE CANCER

There are different ways of classifying patients with prostate cancer: according to the extension of the tumour (TNM), the histopathological grade (Gleason), the clinical or histopathological stage, or its risk.

1.10.1. TNM CLASSIFICATION [f]

T: PRIMARY TUMOUR [d]

Tx : Unable to assess the primary tumour.

T0 : No evidence of primary tumour.

T1 : Tumour not clinically apparent, not palpable or visible using imaging techniques.

T1a: Tumour detected by chance in an extension less than or equal to 5% of the tissue removed.

T1b: Tumour detected by chance in an extension greater than 5% of the tissue removed.

T1c: Tumour identified by fine needle biopsy (for example, as a consequence of a high PSA).

T2: Tumour confined to the prostate.

T2a: Tumour covers half of a lobe or less.

T2b: Tumour covers more than half of a lobe but not both lobes.

T2c: Tumour covers both lobes.

T3: Tumour extends beyond the prostatic capsule.

T3a: Extracapsular extension unilateral or bilateral

T3b: Tumour invades the seminal vesicle(s).

T4: Tumour is fixed or invades adjacent structures other than the seminal vesicles: bladder neck, external sphincter, rectum, upper anus muscles and/or pelvic wall.

N: REGIONAL LYMPH NODES [e]

Nx: The regional lymph nodes cannot be assessed.

N0: Regional lymph node metastasis is not shown.

N1: Metastasis in regional lymph nodes.

M: DISTANT METASTASIS [e]

Mx: Distant metastasis cannot be assessed.
M0: There is no distant metastasis.
M1: Distant metastasis.
M1a: Non-regional lymph node(s).
M1b: Bone(s).
M1c: Other location(s).

d: prostate adenocarcinoma.

e: The regional lymph nodes are those in the lower pelvis (mainly, the iliopelvic lymph nodes located below the bifurcation of the primitive iliac arteries)[f]

1.10.2. HISTOPATHOLOGICAL GRADING

The grading system proposed by Gleason (Gleason *et al.,* 1974) is recognised internationally, and is based on a pathological examination of prostate tissue obtained by a biopsy. The result is an average index of abnormality for the tissue, for which values between 2 and 10 can be taken (National Institute for Health and Clinical Excellence (NICE), 2008). The classification according to Gleason is as follows (Aus G *et al*., 2007):

Gx: The degree of differentiation cannot be assessed.
G1: Well differentiated (weak anaplasia): Gleason 2-4.
G2: Moderately differentiated G2 (moderate anaplasia): Gleason 5-6.
G3-4: Poorly differentiated/undifferentiated (marked anaplasia): Gleason 7-10.

In 2005, the International Society of Urological Pathology (ISUP) 24 established an international consensus on the diagnosis of a Gleason 2-4, deciding that such a score should be an exception (only in tumours of the transition zone), and will therefore always have to be compared with another expert.

1.10.3. CLASSIFICATION ACCORDING TO THE CLINICAL OR PATHOLOGICAL STAGE

In prostate cancer, the stage at which the patient is found is clinically defined (i e, a stage which is suspected before removing the prostate, taking into account the clinical and

analytical information available at that time, which may be inaccurate or incomplete: cT1 to cT4) or pathologically defined (a stage defined on the basis of information provided by the analysis of a piece surgically extracted by radical prostatectomy: pT1 to pT4). There are different definitions for these phases (Aus G *et al.,* 2007; National Comprehensive Cancer Network (NCCN), 2008; National Institute for Health and Clinical Excellence (NICE), 2008; AATRM, 2004). For example, many studies talk about advanced prostate cancer (Wallen MJ *et al.,* 1999; Segawa N *et al.,* 2001; Miyoshi Y *et al.,* 2003; Culig Z *et al.,*1994; Sadi MV *et al.,*1993; Magi-Galluzzi C *et al.,* 1997) to refer generally to the locally advanced or disseminated form. This guideline uses the following definitions:

1.10.3.1. LOCALISED PROSTATE CANCER

From an anatamopathological point of view, localised prostate cancer is the verified presence of prostate adenocarcinoma without extension to the prostate capsule (pT1-pT2), without lymphatic invasion (N0) and without metastasis (M0). The patient with clinically localised prostate cancer is consistent with the stage cT1-cT2, N0-Nx, M0-Mx.

1.10.3.2. LOCALLY ADVANCED PROSTATE CANCER

From an anatamopathological point of view, locally advanced prostate cancer is the verified presence of prostate adenocarcinoma with extracapsular invasion (pT3a) or invasion to the seminal vesicles (pT3b), but without lymphatic invasion (N0) nor metastasis (M0). The patient with locally advanced prostate cancer at a clinical stage corresponds with thestage cT3, N0-Nx, M0-Mx.

1.10.3.3. PROSTATE CANCER IN PSA RELAPSE

The patient with prostate cancer in PSA relapse is one who, having received primary treatment with intent to cure, has an increased PSA (prostate specific antigen) defined as "biochemical recurrence".

1.10.3.4. DISSEMINATED PROSTATE CANCER

From an anatamopathologicalpoint of view, the patient with disseminated prostate cancer is the verified presence of prostate adenocarcinoma with lymphatic invasion (N1) and/or metastasis (M1) and/or a primary tumour which is fixed or invades adjacent structures other

than the seminal vesicles (pT4). The patient with clinically disseminated prostate cancer spread corresponds to a stage N1, M1 or cT4.

1.10.4. CLASSIFICATION ACCORDING TO RISK

The TNM clinical stage is insufficient to establish the most appropriate treatment for patients with localised prostate cancer. Patients diagnosed with prostate cancer at localised or locally advanced clinical stages can fall into risk or prognosis subgroups on the basis of known risk factors, primarily PSA and Gleason.

This guideline uses the D'Amico classification (D'Amico AV *et al.,* 1998; D'Amico AV *et al.,* 2002).

- Low risk: cT1-cT2a, Gleason < 7 and PSA 10 ng/ml.
- Intermediate risk: cT2b, Gleason = 7 or (PSA > 10 and 20 ng/ml).
- High risk: cT2c or PSA > 20 ng/ml or Gleason > 7.

1.11. TREATMENT OF PROSTATE CANCER

At present, there is no definite evidence as to which is the best treatment for prostate cancer, especially for early stage T1 or T2 tumours, and different Urologists may have differing views. One of the reasons for this is that some patients with early stage disease may live 10 years or more if no treatment at all is used. Therefore, more involved therapies have a hard act to beat. However, in other patients, the disease can be much more serious. Unfortunately, whilst it is possible to generalise, it can be difficult to predict what course the prostate cancer will take in any individual. Also, the side-effects of treatment must be balanced against the overall benefit of therapy. For example, there is little point in undergoing major surgery to take out the prostate if the tumour has spread to areas where it cannot be removed. The treatment of prostate cancer is determined by the stage and the grade of the disease as well as the PSA. There are a number of treatment options for every stage, each with their own advantages and disadvantages. Thus, the therapy needs to be tailored to suit each individual patient. It is possible to cure patients with prostate cancer at an early stage, but even if cure is not a possibility, the disease can normally be kept in check for a number of years (Stephen Langley *et al*., 2005).

1.12. TREATMENT OPTIONS IN PROSTATE CANCER

The different treatment options available to patients diagnosed with prostate cancer are described below. It is important that any patient with such a diagnosis is aware of the different treatments, and they should feel free to discuss these with their Urologist and Oncologist. Some patients feel surprised that they are being offered a choice of different treatments and naturally feel inadequately prepared to make such an important decision. This is a common feeling, which the information in this and the related booklets should help to dispel. One of the prime reasons for including patients in the decision-making process of their treatment is that there is little evidence that either surgery or radiotherapy is more or less likely to cure an individual, or indeed that curative treatment is always necessary, even when possible. Whatever therapy is undertaken, the patient will need regular follow-up examinations, which will involve a PSA blood test and possibly scans or xrays, for a number of years (Stephen Langley *et al.,* 2005).

1.12.1. ACTIVE SURVEILLANCE

If their cancer has been diagnosed accidentally, during an operation to remove prostatic tissue blocking the urinary stream or by a PSA blood test and biopsy, and the patient has no symptoms, a “wait and see” policy may be chosen. This does not mean “do nothing”, but the patient will be regularly monitored by the doctor and if problems develop, appropriate action taken. During this observation period, seeing how quickly the PSA rises can assess the severity of the condition. Frequently, patients opting for such a treatment strategy will be offered a repeat prostate biopsy 2 years after diagnosis, to ensure the grade of the cancer has not worsened. If treatment is ultimately required, curative therapies may still be offered, although often hormone therapy is the treatment of choice. With such a regimen, patients commonly live for a number of years and this option is frequently chosen by patients with low grade cancers and/or who are elderly (Stephen Langley *et al.,* 2005).

1.12.2. PROSTATE SURGERY: RADICAL PROSTATECTOMY

Radical (curative) prostatectomy is an operation to remove the entire prostate and seminal vesicles. This operation can be performed through an incision in the lower abdomen (a radical retropubic prostatectomy) or through an incision made between the anus and scrotum (a radical perineal prostatectomy). In specialist centres, the prostate can also be removed by a keyhole or laparoscopic technique. These are complex, major operations that

usually require a hospital stay of between 1 week for open operations to 2 days with keyhole surgery. Such procedures should not be confused with conventional prostate surgery – transurethral resection of the prostate (TURP) – where only the tissue blocking the urinary flow is removed, leaving part of the gland behind. The advantage of surgery is that it is a one-off procedure and provided the cancer is confined to the prostate, will hopefully cure the disease. It avoids the side-effects of radiotherapy and is thought by some to be the most effective form of treatment for early prostate cancer. However, there are risks associated with radical prostatectomy. It is a major operation and involves a number of weeks of convalescence to make a full recovery. Unfortunately, the prostate lies very close to both the sphincter that controls urinary continence and the nerves that produce penile erections. In the past, removal of the gland often caused damage to these structures, resulting in a significant risk of postoperative urinary incontinence and impotence (inability to achieve an erection). Newer surgical techniques have reduced the recurrence of impotence and severe incontinence is now uncommon. Furthermore, there are a number of new therapies to treat such side-effects, should they occur. Radical prostatectomy, more than any other prostate cancer treatment, is highly dependent on the experience and skill of the surgeon. Few Urologists in the UK are currently trained in the keyhole technique, due to the lack of training opportunities and the lengthy learning curve of the procedure (Stephen Langley *et al*., 2005).

1.12.3. RADIOTHERAPY:

1.12.3.1. EXTERNAL BEAM OR BRACHYTHERAPY

Radiotherapy involves directing high-energy radiation rays at the tumour, aiming to destroy the cancerouscells and leave the healthy ones intact. It may be used in two situations: firstly, to treat early cancers confined to the gland or the surrounding tissues (so-called radical radiotherapy); and, secondly, to treat tumours that have spread to the bone and which are causing pain (palliative radiotherapy). Radiotherapy is a painless procedure, like having an x-ray, although there can be troublesome side-effects associated with the treatment. Radical radiotherapy for a tumour localised to the prostate may be either given by external beam radiotherapy or by brachytherapy (Stephen Langley *et al*., 2005).

1.12.3.2. EXTERNAL BEAM RADIOTHERAPY

Radical (curative) external beam radiotherapy involves beams of radiation passing through the body to be targeted onto the prostate, which is a process similar to x-rays. The treatment

is given on an out-patient basis with the patients attending their local cancer centre for five days a week for 4–7 weeks. At each visit, the patient will receive a small fraction of the radiation dose until the therapy is complete (Stephen Langley *et al*., 2005).

1.12.3.3. BRACHYTHERAPY

Radical (curative) radiotherapy can also be given using radioactive seeds that are approximately half the size of a grain of rice. These seeds, typically 80-100 in number, are inserted directly into the cancerous prostate gland through delivery needles under ultrasound control. The needles are passed through the skin behind the scrotum and in front of the anus to reach the prostate. The procedure is performed under an anaesthetic. It has the advantage of being either a day case or overnight stay procedure, with patients rapidly returning to normal activities. This procedure is relatively new, the first patients being treated in the late 1980s. The results of this technique in curing patients with prostate cancer seem to be as good as for radical prostatectomy or external beam radiotherapy. The advantage of radical radiotherapy is that it can cure early prostate cancer without the need for a major operation. It rarely causes loss of urinary control, and impotence is less common than with surgery. The side-effects of radiotherapy, in general, are normally limited to patients having radical rather than palliative treatment. External beam radiotherapy is lengthier than surgery and often causes tiredness, nausea, and diarrhoea, as well as frequent and painful urination. Although most of these side-effects settle in time, some will occasionally persist. With brachytherapy, the side-effects are usually confined to the urinary system, with patients temporarily experiencing a slow flow and urinary frequency. Some patients may even experience difficulty in passing urine at all after the treatment and require a catheter (tube draining the bladder through the penis) for a short period, normallya week or two, before their urinary symptoms settle. However, incontinence and impotence seem least common with this form of treatment (Stephen Langley *et al*., 2005).

1.12.4. CRYOSURGERY

Cyrosurgery uses extreme cold to destroy the prostate tissue. Using transrectal ultrasound in a fashion similar to brachytherapy, fine cryoneedles are inserted into the prostate gland. Under anaesthetic, argon and helium gases are used to freeze, then thaw, the prostate, causing destruction of the tumour. Temperature can be as low as -140°C. Prostate A warming device and temperature sensors protect vital neighbouring structures, such as the rectum, bladder and sphincter muscles. Patients typically stay in hospital overnight and are

discharged home with a urinary catheter for 2 weeks, to allow the swelling of the prostate to reduce. Cryosurgery is a newer technique being investigated in only a small number of specialist centres in the UK. The impotence rate is higher than with other treatments and incontinence can occasionally occur. Although it has been used to treat men with newly-diagnosed prostate cancers, it is currently primarily reserved for patients with recurrent prostate cancer after treatment by radiotherapy (Stephen Langley *et al*., 2005).

1.12.5. HIGH INTENSITY FOCUSED ULTRASOUND (HIFU)

HIFU treatment involves focusing high intensity sound waves on the prostate, which generate high temperatures over 80°C and cause tissue destruction. These sound waves are generated by a special transrectal ultrasound probe that allows the prostate gland to be visualised and targeted. The aim of the treatment is to obliterate the cancerous tissue, whilst preserving neighbouring organs. The procedure is performed under anaesthetic and takes approximately 3 hours. Some centres routinely perform a telescopic prostate operation to rebore the prostate,TURP,before treating with HIFU. When used with curative intent, the success rate is uncertain and risk of impotence seems high. Whilst the concept of the device is appealing, its real place and value in the treatment of prostate cancer has yet to be established and, at present, the technique is considered experimental (Stephen Langley *et al*., 2005).

1.12.6. HORMONE THERAPY

When the cancer has spread beyond the prostate, going to either the lymph nodes or bones, hormonal therapy may be very effective at shrinking the tumour and reducing the side-effects of the disease. It does not provide a cure, but will often keep the cancer in check for a number of years. Some patients are given a course of hormone therapy before having radical radiotherapy. This is useful if the cancer has spread outside the confines of the prostate gland, but has not yet reached the lymph nodes or bone. As mentioned earlier, the prostate gland and prostate cancer are under the influence of testosterone, the male sex hormone, which drives the tumour to grow and spread. By blocking the body's production of testosterone, or blocking its action, the growth of the tumour may be greatly reduced. There are a number of ways to administer such hormonal therapy (see below). Whatever technique is chosen by the patient, certain side-effects are common, such as hot flushes, a loss of sexual desire, impotence and occasionally breast tenderness, or more occasionally breast enlargement (Stephen Langley *et al*., 2005)

1.12.7. SURGICAL ORCHIDECTOMY

The parts of the testicles that produce testosterone may be surgically removed by a small operation, called an orchidectomy, which can be performed as a day case procedure. This has the advantage of being a one-off treatment, which does not rely on the patient remembering their medication, and tends to cause less breast problems. However, the operation is irreversible and an option that some men find unacceptable. It is not true that men develop a high-pitched voice after such a procedure (Stephen Langley *et al*., 2005).

1.12.8. INJECTION THERAPY

Injection of an agent, known as an luteinising hormone releasing hormone (LHRH) analogue, has a similar effect to removing the testicles, but is reversible and doesn't involve an operation. The injection is given every one or three months by either a doctor or nurse, or the patients can be taught to inject themselves. This new approach to injection therapy has proven very popular with some patients who have been able to master the technique without any difficulty. Because there can be an initial rise in testosterone after the first injection, a two week course of antiandrogen tablets (see below) are normally prescribed to stop this effect. Hot flushes, breast tenderness and impotence are common side-effects with this form of medication (Stephen Langley *et al.,* 2005).

1.12.9. ANTI-ANDROGEN TABLETS

This therapy involves taking tablets to block the action of testosterone. They work by either lowering the level of testosterone in the body, or by blocking its action on the prostate gland. The tablets, which are taken each day, may be used alone to treat prostate cancer, or in combination with an LHRH analogue. All of the available medications have side-effects such as breast tenderness or enlargement, although some have fewer effects on sexual desire and potency (Stephen Langley *et al.,* 2005).

1.12.10. CHEMOTHERAPY

Chemotherapy involves powerful drugs to attack the cancer cells and try to prevent them growing. It is a second line of defence for patients with advanced stage prostate cancer that is no longer controlled by hormonal therapy. There are a number of different agents currently

available, with new drugs having recently been launched which appear effective in controlling the disease in its later stages (Stephen Langley *et al.,* 2005).

1.13. PHARMACOGENETICS

Pharmacogenetics is the study of the role of inheritance in interindividual and interpopulation variations in drug response (Vogel, 1959; Meyer, 2004; Robert *et al.*, 2005). The rationale and ultimate aim of pharmacogenetics is the possibility that knowledge of an individual's genetic make-up could be used to enhance drug therapy by maximising drug efficacy while minimising drug toxicity (Linder *et al.*, 1997; Meyer, 2000; Lesko and Woodcock *et al.*, 2004; Weinshilboum and Wang, 2004). The ultimate goal of pharmacogenetics, therefore, is targeted pharmacological treatment of patients based on their genetic determinants of drug efficacy and toxicity, so that they are more likely to respond favourably with fewer or no unfavourable side effects (Evans and Relling, 1999; Evans and Johnson, 2001).

The results of drug therapy can vary both within a population (Ingelman-Sundberg, 2001; Johnson, 2003) and between different populations or ethnic groups (Meyer and Zanger, 1997; Xie *et al.*, 2001; Meyer, 2004; Westlind-Johnsson *et al.*, 2006). Plasma drug concentrations within two individuals of equal weight on identical drug dosage can vary by more than 600-fold (Eichelbaum *et al.*, 2006). Therefore, the same drug administered in the same dosage to patients with identical disease state may lead to the desired effect in the majority of treated patients, but can prove ineffective in a significant proportion of others and may even produce ADRs (any noxious, unintended or undesirable effects) in some (Evans and Relling, 1999; Nebert, 1999; Meisel *et al.*, 2000; Meyer, 2004). Apart from the detrimental effects of therapeutic failure or inefficacy, the unfavourable effects of ADRs can vary in intensity and severity, ranging from merely bothersome to potentially life-threatening (Lindpaintner, 2003).

Various factors have in the past been held accountable for the observed differences in drug response and include age, concomitant diseases, gender, interactions with other drugs, misdosing, renal and hepatic function, as well as lifestyle variables such as smoking and alcohol consumption (Evans and Relling, 1999; Meisel *et al.*, 2000; Bachmann, 2002; Oscarson, 2003; Schwartz, 2003).

There is, however, an ever-increasing body of evidence that suggests that genetic differences between individuals and even entire populations can be an important and at the same time predominant factor influencing drug response variability (Evans and Relling, 1999; Meyer, 2000; Evans and Relling, 2004; Lesko and Woodcock, 2004). In fact, it is estimated that genetics is responsible for 15% to 95% (depending on the drug or class of drug) of the observed interindividual variability in drug disposition and effects (Evans and McLeod, 2003; Eichelbaum *et al.*, 2006; McGee, 2006a). This increasing awareness of the significant role that genetic polymorphisms play in drug response variability, together with rapid developments in genomic technologies and the completion of the HGP, has given rise to the field of pharmacogenetics.

As the influence of pharmacogenetics on drug discovery and development and drug treatment regimens increases, there will undoubtedly be a move away from the current approach of standardised treatments towards more individualised, 'tailor-made' therapies (Roses, 2000; Liggett, 2004). Despite the fact that this concept of individualised drug treatment seems very helpful and is largely the product of recent advances in human molecular biology, the scientific foundation on which it is based has a relatively long history.

1.13.1 . A BRIEF HISTORY OF PHARMACOGENETICS

Although often viewed as a new discipline, the scientific premise of pharmacogenetics has been recognised since the end of the 19th century. In the late 1800s, Sir Archibald Garrod noted that a subset of psychiatric patients developed porphyria in response to treatment with the hypnotic drug sulphonal (Garrod, 1909; Lindpainter, 2003), and in 1914 proposed that enzymes were somehow implicated in the detoxification of exogenous substances (Garrod, 1914). These observations, along with those by physiological chemists regarding the excretion of drugs in different forms from those in which they were administered, led Sir Archibald Garrod to conclude that the ability to transform drugs into non-toxic conjugates served as a protective mechanism against any poisonous effects and was in fact mediated by enzymes. Furthermore, due to the rediscovery of Mendel's Laws around 1900 and the subsequent flurry of research, Sir Archibald Garrod, along with other researchers, anticipated the connection of enzymes with the genetic material. Sir Archibald Garrod was thus ahead of his contemporaries in recognising that unexpected drug responses could be attributed to the failure of enzymes to detoxify these substances, and that these enzymatic inefficiencies could be genetic in origin (Weber and Cronin, 2000).

Despite the insightful observations of Sir Archibald Garrod, the first experimental identification and study of a pharmacogenetic trait was made during the 1930s and involved not individual response to a drug, but variation in the ability to taste a foreign chemical (Meyer, 2004). It was noted that some individuals expressed an inability to taste ('taste-blindness') phenylthiocarbamide (PTC) (Fox, 1932), which was subsequently found to be inherited in an autosomal-recessive manner and to vary in frequency in populations of different ethnicities (Snyder, 1932). This study of 'taste blindness' was the forerunner of pharmacogenetic studies and, as such, was the first study to document an association between ethnicity and the response to chemical compounds. Further progress within the field slowed, however, until the 1950s when several breakthroughs and the development of new technologies led to further confluence of pharmacology, genetics and biochemistry (Weber and Cronin, 2000).

During the 1950s, researchers realised that certain ADRs were in fact caused by genetically determined variations in enzyme activity (Meyer, 2000; Johnson, 2003). It was discovered that prolonged muscle relaxation and apnoea after suxamethonium treatment (an adjunct to anaesthesia) was due to altered enzyme kinetics of a pseudo cholinesterase and is inherited as an autosomal-recessive trait (Kalow and Staron, 1957). Haemolytic anaemia caused from the antimalarial drug primaquine was resultant of a variant form of the glucose-6-phosphate dehydrogenase (G6PD) enzyme (Carson *et al.*, 1956). Arno Motulsky's seminal paper, concerned with interindividual differences in drug response due to the unique genetic constitution of individuals, was published in the year 1957 (Motulsky, 1957). The term 'pharmacogenetics' was subsequently coined by Vogel in 1959 (Vogel, 1959).

Further progress within pharmacogenetics was made during subsequent decades, characterised by the development of a community of researchers interested in pharmacogenetics and an increasing awareness of gene-drug interactions (Weber and Cronin, 2000; Meyer, 2004). However, with the advent of polymerase chain reaction (PCR) technology in the mid-1980s (Saiki *et al.*, 1985), progress within the field accelerated markedly.

In the late 1980s, the first CYP gene, CYP2D6, was cloned and characterised (Gonzalez *et al.*, 1988), followed by the cloning and characterisation of several other drug metabolism genes, as well as some receptor and transporter genes (Nebert and Vessel, 2004). Developments within the field experienced further advancement with the inception and completion of the HGP (Human Genome Project) , in 1990 and 2003 respectively, and

consequent increased availability of gene sequences (Weber and Cronin, 2000; Lerer, 2004; Robert *et al.*, 2005), the concurrent increase of data on genomic variation (Hoehe *et al.*, 2003), numerous technological advances (Weber and Cronin, 2000; Johnson and Evans, 2002; Meyer, 2004) and the elucidation of entire pathways that may be relevant to drug response (Goldstein *et al.*, 2003). Also assisting progress within pharmacogenetics in more recent years is the increasing interest in pharmacogenetic research by physicians, geneticists, regulatory agencies and, to varying degrees, the pharmaceutical industry-this industry has shown relatively limited interest in pharmacogenetics due to the inherent nature of pharmacogenetics to segment potential drug markets (Breckenridge *et al.*, 2004; Hosford *et al.*, 2004; Meyer, 2004; Weinshilboum and Wang, 2004). Furthermore, there has been considerable investment from, and collaborations and alliances between, numerous biotechnology, genomics and pharmaceutical companies (Webster *et al.*, 2004).

This modern climate of substantial investment, financial and otherwise, and interest in pharmacogenetics is helping improve our understanding of the role that genetic polymorphisms play in drug response.

1.13.2 . GENETIC POLYMORPHISMS

A genetic polymorphism is defined as a deoxyribonucleic acid (DNA) sequence variant which is stable within a population and occurs with a frequency equal to or greater than 1% (Nebert, 1999; Bachmann, 2002; Lash *et al.*, 2003). There is a considerable level of variability between individuals at the genetic level, as manifested by the polymorphisms present within their genome (Sachidanandam *et al.*, 2001; Oscarson, 2003). Over 90% of these polymorphisms are believed to be accounted for by changes in a single nucleotide, namely Single nucleotide polymorphisms (SNPs), with the remainder of the variation caused by insertions and deletions (indels), variable number tandem repeats (VNTRs) and microsatellites (Quirk *et al.*, 2004; Marsh and McLeod, 2006). However, unlike many other previously characterised polymorphisms, such as VNTRs and microsatellites, SNPs are often found within the coding and regulatory regions of genes and thus can have functional consequences for gene expression and gene product functionality (Campbell *et al.*, 2000; Gray *et al.*, 2000).

Initial estimates of 1420000 (Sachidanandam *et al.*, 2001) to 3000000 (Roses, 2002) SNPs within the human genome have since been significantly exceeded. The largest public SNP database, the Single Nucleotide Polymorphism database (dbSNP), currently

has >27000000 submissions with more than 12000000 validated polymorphisms (Build 126, May 2006).(www.ncbi.nlm.nih.gov/projects/SNP/index.html). All of these SNPs can be characterised in terms of their minor allele frequency (rare SNPs <0.01; polymorphic SNPs >0.01; common SNPs >0.05) (Nebert and Vessel, 2004), but are also classifiable into three groups according to their position within the genome (Nebert, 1999).

The vast majorities of SNPs, so-called intergenic or random SNPs (iSNPs/rSNPs), are situated in the non-coding areas between genes (so-called 'junk DNA') and thus have no known function or effect on gene expression and gene product functionality. Perigenic SNPs (pSNPs) occur within or in the immediate vicinity of genes and include SNPs located within introns, non-coding regions of messenger ribonucleic acid (mRNA), upstream regulatory regions from the furthest upstream functional enhancer to the transcription initiation site, as well as silent codon polymorphisms (i.e. synonymous changes) and SNPs within 100 bp downstream of the last exon of a gene. pSNPs are thus similar to iSNPs in that they are non-coding, but differ in that they can still affect gene expression levels or incur functional changes to the gene product (Nebert, 1999). Recent studies have indeed suggested that the presence of sequence variants, such as pSNPs, within intronic regions could affect basic preliminary-mRNA (pre-mRNA) splicing mechanisms and thereby cause altered levels of normal transcripts (Pagani *et al.*, 2003). A pSNP within the 3'-untranslated region (UTR) following the coding sequence may affect the intracellular stability of the mRNA gene transcript (Quirk *et al.*, 2004).

SNPs within the coding regions of a gene which do cause changes in the amino acid sequence of the encoded protein are known as coding SNPs (cSNPs) which, due to greater selective pressures against changes at positions dictating amino acid sequence, are generally less common than iSNPs or synonymous changes in coding sequence (Gray *et al.,* 2000).

A change in the amino acid sequence of a protein can have significant structural consequences, depending on the nature and location of the alteration, which in turn can exert considerable influence on the functionality of the protein, as well as its affinity for its intended substrates.

Despite these myriad effects that single SNPs can have, it should however be noted that single SNP approaches to genotype-phenotype correlations have severe limitations, and that it is in fact patterns of sequence variations that significantly influence the risk for disease and differential drug response (Clark *et al.*, 1998; Nickerson *et al.*, 1998). It has been

demonstrated that gene-based haplotypes (i.e. specific combinations of SNPs throughout the genome) are superior to the use of individual SNPs for predicting association between genomic variation and phenotype (Drysdale *et al.*, 2000; Judson *et al.*, 2000; Johnne *et al.*, 2002). Therefore, when trying to ascertain the genotypic cause of a particular phenotypic trait, it is important to consider not only individual SNPs that may be of interest, but rather combinations of SNPs as well as the different haplotypes.

The determination of these different haplotypes that underlie a specific genotype is vital in the elucidation of the functionality of different forms of a gene (i.e. the form of a gene on each chromosome) (Hoehe *et al.*, 2003). Such determination is, however, complicated by the uncertainty of the phase of heterozygous SNPs - in other words, whether two particular variants reside on the same chromosome ('cis') or on separate chromosomes ('trans'). Therefore, despite having determined the genotype of an individual, there remains uncertainty as to which heterozygous SNP variants came from the same chromosome. Fortunately, however, there are a number of computational techniques that have been developed that can assist in inferring the haplotypes from the genotype data (Halldórsson *et al.*, 2004).

The different types of SNPs are thus multiple, as are their effects. Depending on their location within the genome and their patterns of co-occurrence (i.e. haplotypes), SNPs can alter expression levels of a gene as well as the functionality of the encoded protein product or its affinity for its intended substrates. These effects of SNPs can, as is the case with many other phenotypic characteristics, greatly affect the manner in which a patient responds to drug therapy.

1.13.3. THE EFFECTS OF GENETIC POLYMORPHISMS ON DRUG RESPONSE

Genetic polymorphisms within genes encoding drug targets, drug transporters and drug-metabolizing enzymes (DMEs) can affect the Pharmacokinetic (PK) and Pharmacodynamic (PD) characteristics of drug compounds (Steimer and Potter, 2002; Johnson, 2003). The therapeutic index of a drug (the difference between the minimum effective dose and maximum tolerated dose) and the quantitative role of a drug transporter or DME in the drug's kinetics determine the clinical relevance of such genetic polymorphisms (Meyer, 2000) -e.g. the narrower a drug's therapeutic index, the greater the clinical effects of changes in its PK and PD characteristics. As outlined in Figure 1.1, the clinical effects of

these genetic polymorphisms on the PK and PD of pharmaceutical drugs can lead to variable drug efficacy or risk of toxicity and ADRs.

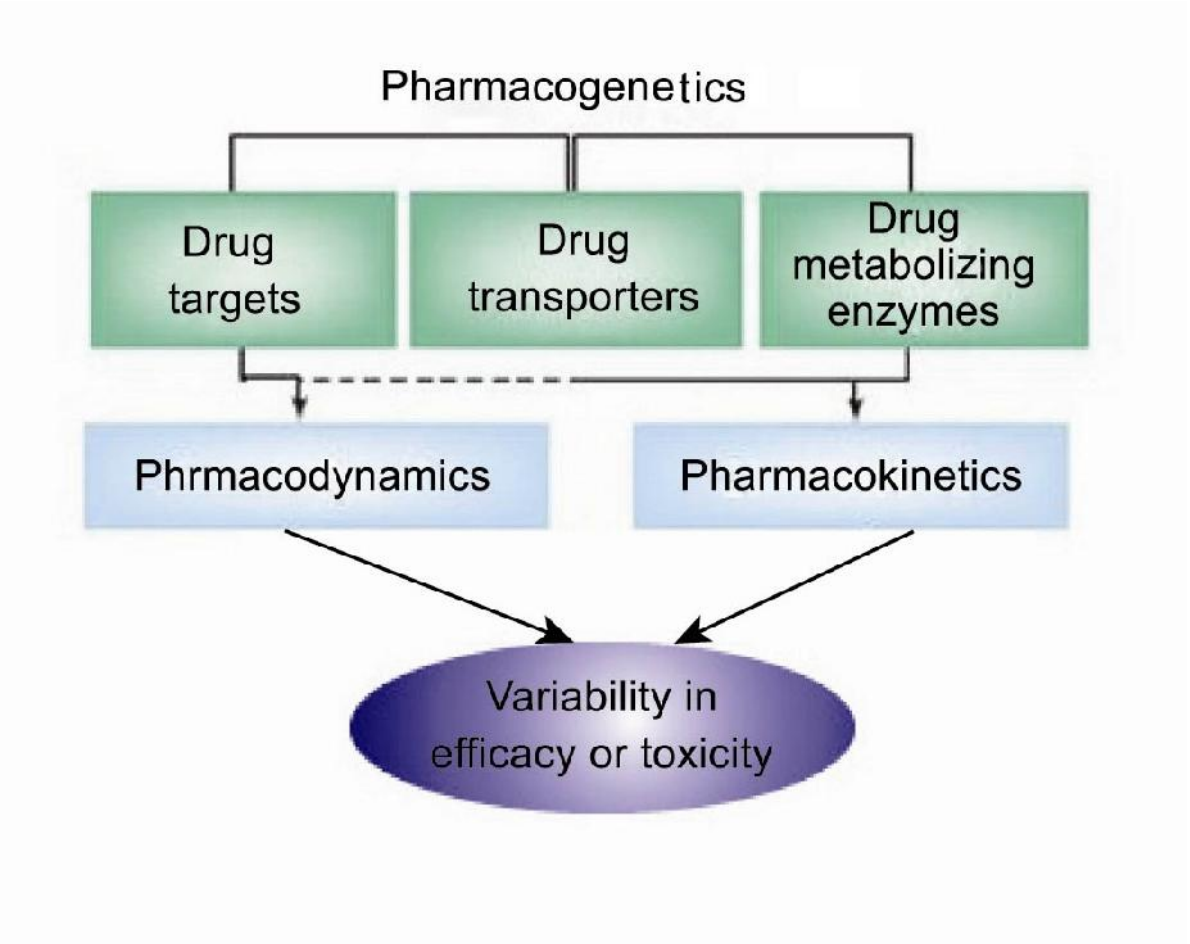

Figure 1.2: Key components in pharmacogenetics (the broken line illustrates that drug transporters are occasionally also the drug target, in addition to affecting drug PK characteristics) (Johnson, 2003).

The effects on the PK nature of a drug include changes in drug disposition (absorption, distribution, metabolism and excretion) (Goldstein *et al*., 2003; Wilkinson, 2005), with a consequent undesirable concentration of the drug and/or drug metabolites at the intended site of action. This non-optimal concentration of the drug and/or drug metabolites at the intended site of action can, in turn, result in either a lack of efficacy or ADRs (Meyer and Zanger, 1997; Lindpainter, 2003). The causes of variations in drug PK include polymorphisms in genes involved in the metabolism and transport of drug compounds, such as the CYP gene super family (Ingelman Sundberg, 2005; Wilkinson, 2005) and MDR1 gene (Ambudkar *et al.*, 1999), respectively. The factors affecting drug PD characteristics include sequence variations in genes affecting how the drug target molecule, or another downstream

member of the drug target molecule's mechanistic pathway, respond to the drug compound (Johnson, 2003). This can result in interindividual differences in drug response, despite the presence of appropriate concentrations of the intended drug compound and/or drug metabolites at the intended site of action (Lindpaintner, 2003).

The effects of genetic polymorphisms on drug response are thus multifaceted, as are the nature of the polymorphisms. As previously mentioned, genetic polymorphisms include sequence variations within the intronic, regulatory and coding regions of genes that influence the PK and PD characteristics of drug compounds. Also of importance, however, are gene duplications that can result in increased enzyme quantity and thus enzyme activity (Johansson *et al.,* 1993; Evans and Johnson, 2001; Ingelman-Sundberg, 2004) with a subsequent lack of therapeutic effect -as can occur with the CYP2D6 gene and treatment with the antidepressant drug nortriptyline (Dalen *et al.*, 1998). The effects of these polymorphisms include alterations in the level of transcription of a gene or functionality and activity of the protein product, thereby altering the PK and/or PD characteristics of a drug and hence the clinical response to it.

The variations in clinical response to drug therapy ascribable to genetically-determined changes in drug PK (due to altered levels of drug metabolism) allow the classification of patients into four clinical groups (Ingelman-Sundberg, 1998; Ingelman-Sundberg *et al.*, 1999; Meyer, 2000; Ingelman-Sundberg, 2004).

These four groups include:

- **Extensive metabolisers (EMs),** who are either homo- or heterozygous for the wild-type or normal-activity enzymes and display a level of drug metabolism observed in the majority of patients;
- **Poor metabolisers (PMs)**, who carry two loss-of-function alleles and therefore have a severely impaired level of drug metabolism;
- **Intermediate metabolisers (IMs),** who carry two decreased-activity alleles, resulting in decreased enzyme activity and subsequent level of drug metabolism (relative to EMs);
- **Ultra-rapid metabolisers (UMs),** who have duplicated or multi-duplicated active copies of a gene and thus exhibit a considerably higher level of drug metabolism relative to EMs.

The two extremes of these four groups, namely PMs and UMs, clearly illustrate the clinical importance and effects of genotype on phenotype in terms of drug metabolism and response, as evident in Table 1.6

Table 1.6: The clinical effects of genotypic influences on phenotype in terms of drug metabolism (Ingelman-Sundberg, 1998; Bean, 2000; Ingelman-Sundber, 2004)

POOR METABOLISERS	ULTRA-RAPID METABOLISERS
Decreased rate of metabolism; increased drug bioavailability	**Increased rate of metabolism; decreased drug bioavailability**
Exaggerated response at standard dosage; side-effects, toxic effects (ADRs)	**Lack of therapeutic effect at standard dosage; explanation for suspected poor adherence**
Active metabolite not formed (in the case of a pro drug); loss of therapeutic efficacy	**Excess of active metabolite formed (in the case of a pro drug); side-effects, toxic effects (ADRs)**

The consequences of either a markedly decreased or increased level of drug metabolism (or drug transport) can thus ultimately manifest in unintended and undesirable side-effects, or ADRs, and variations in levels of therapeutic efficacy.

1.13.4. THE COSTS OF ADRS AND VARIABLE THERAPEUTIC EFFICACY

Unanticipated and undesirable responses to pharmaceutical drugs, both in the forms of ADRs and lack of adequate response, constitute considerable economic, social and healthcare burdens (Haramburu *et al.*, 2000; Schenkel, 2000; Eichelbaum *et al.*, 2006). Most major drugs are effective in only 25 to 60% of patients (Spear *et al.*, 2001) while ADRs are estimated to be the fifth leading cause of death in the USA, responsible for over one and half million hospital admissions and one hundred thousand deaths per year (Lazarou *et al.*, 1998). The monetary costs of ADRs to the USA economy are equally dramatic, with an approximate US$10 billion spent annually on ADR related events, while the costs related to lack of therapeutic efficacy are estimated at a massive US$170 billion (Gurwitz *et al.*, 2006).

1.13.5. CLINICAL APPLICATIONS AND BENEFITS OF PHARMACOGENETICS

There are currently two main approaches in establishing the correct treatment and dosage regimen for the pharmacological management of a condition or disease (Johnson, 2003). The first approach relies on trial-and-error and is used in the treatment of diseases such as depression, diabetes, hypertension and schizophrenia. There are usually numerous first-line therapy drugs that can be used to treat these diseases and the trial-and-error method of establishing which drug, or combination of drugs and at what dosage to use in each patient is time-consuming and can take months to accomplish. The second approach is a more 'one size-fits-all' method, wherein the treatment employed is essentially the same for all patients. Examples of diseases and conditions treated in this manner include most cancers, heart failure and post-transplantation patients. In both approaches, however, a certain proportion of patients will undoubtedly experience a lack of efficacy or ADRs from a given drug.

There are therefore currently two main goals for the clinical application of pharmacogenetics (Johnson and Evans, 2002; Johnson, 2003), namely:

- the ability to predict which patients are at a higher risk of ADRs and which should, therefore, receive a lower dose of a drug or a different drug altogether;
- the ability to predict which patients are most likely to obtain the desired therapeutic effect(s) from a drug.

The subsequent stratification of patient eligibility for a drug or drug dosage level, based on genotypic markers, stemming from these two goals is clearly illustrated in Figure 1.3.

For patients treated with either of the two methods of pharmacological treatment (i.e. trial-and-error and 'one-size-fits-all'), there are numerous benefits of the full, or even partial, realisation of these two goals and consequent stratification of patient populations. These benefits include a shorter time period in which the disease is not properly controlled (e.g. ART for HIV-infection), a decreased risk for negative consequences of the disease not being properly controlled (e.g. suicide in patients suffering from depression), fewer follow-up visits to the physician due to ineffective treatment, the avoidance of the use of ineffective therapies and drug toxicities, and an overall reduction in healthcare costs resulting from all of the above factors (Johnson, 2003; Lindpaintner, 2003).

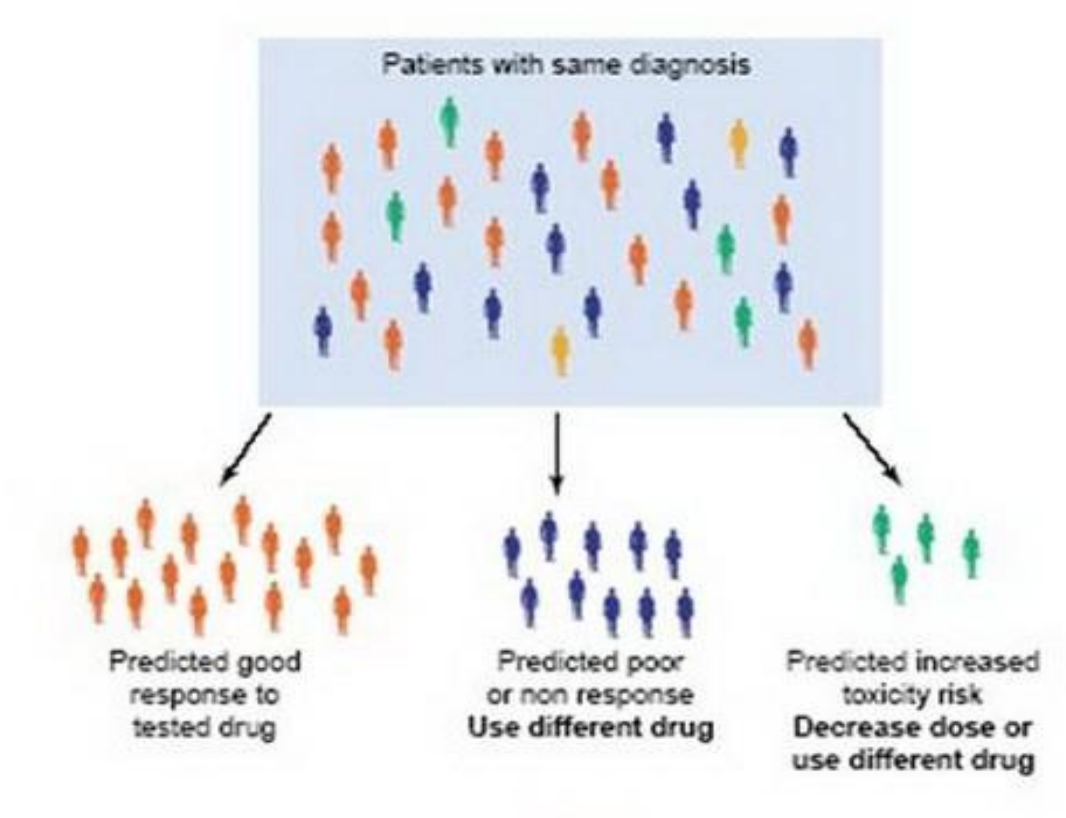

Figure 1.3: Customization of pharmacological treatments through pharmacogenetic testing (Johnson, 2003).

1.14. DRUG METABOLISM

The majority of drugs undergo a variety of chemical reactions in the liver and, to a much lesser extent, in other organs (e.g., intestinal wall, kidney, lungs). Such reactions include oxidation, reduction, hydrolysis, and conjugation (with glucuronic acid, amino acids, acetate, sulphate, and methyl groups) and are directed towards the production of metabolites that are more ionized, more water-soluble, and less capable of penetrating cell membranes and being sequestrated in tissues. The more polar or water-soluble a compound becomes, the more readily it is excreted through the kidney and hepato-biliary system.

This biotransformation is extremely important because most drugs are lipid-soluble weak electrolytes so that they would be readily reabsorbed through the renal tubule or intestine and remain in the body. The rate of metabolism may be influenced by many factors among which the genetic make-up of the individual and drug interactions is the most important. Metabolism of some drugs, the acetylation of isoniazid being the best example, can proceed

at a rapid rate in one subgroup of the population and at a slow rate in another genetically defined subgroup of the population.

A slow rate may be due to the deficiency of a specific enzyme because of some genetic defect and results in an increased sensitivity to drugs. For example, in subjects with acetyl transferase deficiency, the speed of acetylation and inactivation of isoniazid is decreased and consequently the usual doses of the drug will produce toxic effects.

1.14.1. SPECIFIC REACTIONS IN DRUGS METABOLISM

The specific reactions in drugs metabolism are often divided into Phase-I and Phase-II.Phase-I DMEs, many of which are cytochromes P450, sometimes participate in detoxification of reactive substrates. But they are more often involved in the activation of inert protoxicants, promutagens and procarcinogens to electrophilic intermediates that can bind as adducts to proteins or DNA and/or cause oxidative stress (Dalton *et al.*, 1999; Kidd *et al.*, 1999; Nebert, 2000). Phase-II DMEs (e.g. methyltransferases, UDP glucuronosyl transferases, glutathione transferases, sulfo-transferases) are sometimes involved in metabolic activation (Nebert *et al.*, 1996), but they usually conjugate various Phase-I products and other reactive intermediates to form water-soluble derivatives, completing the detoxification cycle. Therefore, it seems likely, that genetic differences affecting the expression of Phase-I and Phase-II DME might be crucial factors in defining susceptibility to toxicity or cancer caused by drugs and other environmental pollutants. Hundreds of genes coding for drug metabolizing enzymes exist in the human genome. Polymorphism in several such genes causing high levels of one enzyme and low levels of another enzyme in a specific pathway involved in the metabolism of a particular environmental pollutant could lead to 30- or more than 40-fold differences between two individuals in response to that foreign chemical (Nebert, 2000).

Table 1.7: Phase-I and phase-II DMEs

Enzyme class	Reaction type	Enzymes
	Phase I DMEs	
Oxidation	Hydroxylation, N- and O- dealkylation, desamination, oxidative dehalogenation	Cytochrome P450-monooxygenase
	N- and S-Oxidation	Cytochrome P450-monooxigenases, flavin monooxigenases
	Dehydratio	Alcohol dehydrogenases
	Dehydration of amines	Monoaminoxidase
Reduction	Dehalogenation of nitro groups	Cytochrome P450-monooxigenases
	Carbonyl reduction	Carbonylreductases
Hydrolysis	Hydrolysis of epoxides	Epoxide hydrolases
	Hydrolysis of esters	Carboxylesterases
	Hydrolysis of peptides	Peptidases
Others	Oxidation of superoxide anions	Superoxide dismutases
	Peroxidation	Glutathione peroxidises
	Phase II DMEs	
Conjugation	Glucuronosylation	UDP-glucuronosyltransferases
	Sulfation	Sulfotransferases
	Acetylation	O- and N-acetyltransferases
	Methylation	O-, N- and S-methyltransferases
	Glutathione S-conjugation	Glutathione S-transferases

Adapted from Elke Störmer, Dissertation, Berlin, 2001.

1.15. CYTOCHROME P450 ENZYMES

The cytochromes P450 are a super family of enzymes which are found in all forms of living organisms. They are responsible for the metabolism of many endogenous compounds, participate in the activation/deactivation of many carcinogens and detoxify many xenobiotics. In particular, in humans they metabolize many drugs and hence are of great interest

to pharmacologists and toxicologists. It is readily identified by a pronounced absorbance band at 450 nm in the soret region of the visible spectrum when the carbon monoxide adduct of the reduced heme protein is formed (Pohl *et al.*, 1984) hence the name P450. Human cytochrome P450 (P450) enzymes catalyze the metabolism of a wide variety of clinically, physiologically, and toxicologically important compounds.

1.15.1. CLASSIFICATION

The classification of the various CYP isozymes employs a three tiered classification based on the conventions of molecular biology: the family (members of the same family display >40% homology in their amino acid sequences), subfamily (55% homology) (Tanaka, 1998).

The highest concentration of P450 enzymes involved in xenobiotic bio-transformation is found in the endoplasmatic reticulum (microsomes) of the liver, but P450 enzymes are expressed in almost all tissues.

The human microsomal P450 enzymes involved in xenobiotic biotransformation belong to three main P450 gene families, namely CYP1, CYP2 and CYP3. Liver microsomes also contain P450 enzymes encoded by the CYP4 gene family, the substrates of which include several fatty acids and eicosanoids but relatively few xenobiotics.

A classification of all existing 57 human P450s based on substrate class is given in (Table 1.8).

Table 1.8: Classification of human P450s based on major substrate class.

CYP Family	Steroids	Xenobiotics	Fatty acids	Eicosanoids	Vitamins	Unknown
1	1A1	1A1	1A1	1A2	1A1	
	1A2	1A2	1B1		1A2	
	1B1	1B1			1B1	
2	2A6	2A6	2A6	2B6	2A6	2A7
	2B6	2A13	2B6	2C8	2B6	2R1
	2C18	2B6	2C19	2C9	2C19	2S1
	2C19	2C8	2D6	2E1	2D6	2U1
	2D6	2C9	2E1	2J2	2E1	2W1
	2E1	2C18	2J2			
	2J2	2C19				
		2D6				
		2E1				
		2F1				
		2J2				
3	3A4	3A4	3A4	3A4	3A4	3A43
	3A5	3A5	3A5		3A5	
	3A7	3A7			3A7	
	3A43					
4	4B1	4B1	4A11	4A11		4A22
		4F12	4B1	4F2		4F11
			4F2	4F3		4F22
			4F3	4F8		4V2
			4F8	4F12		4X1
			4F12			4Z1
5		5A1		5A1		
7	7A1					
	7B1					
8	8A1	8A1		8A1		
	8B1					
11	11A1	11A1			11B1	
	11B1	11B1				
	11B2	11B2				
Others	17	17			19	20
	19	19			24	26C1
	21A2	21A2			26A1	27C1
	27A1	26A1			26B1	
	39A1	51			27A1	
	46A1				27B1	
	51					

Adapted from Guengerich FP (Guengerich, 2004).

1.15.2. CYP3A IN DRUG METABOLISM

Cytochrome P450 3A4 (CYP3A4) is an iso-enzyme involved in Phase I oxidative metabolism of many endogenous and exogenous substances. From a quantitative point of view it is the most important hepatic CYP-enzyme, accounting for approximately 25% of all liver cytochrome P450s. Since CYP3A4 is also present in the small intestine, it has a significant effect on the first-pass metabolism of CYP3A4 substrates.

1.15.3. EXPRESSION AND VARIABILITY OF CYP3A

CYP3A is considered to be the most important drug-metabolizing enzyme subfamily in the human body as it is responsible for the metabolism of 45-60% of currently used drugs (Fig. 1.3), as well as many steroids, environmental chemicals, and carcinogens (Shimada *et al.*, 1994; Li *et al.*, 1995; Thummel *et al.*, 1996; Rebbeck *et al.*, 1998; Evans and Relling, 1999; Guengerich, 1999).

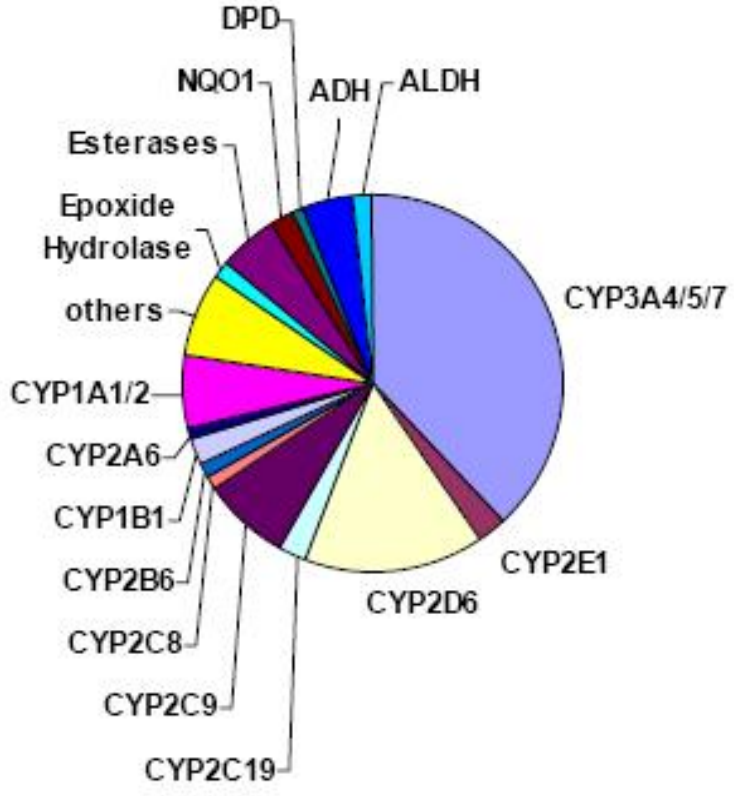

Figure 1.4: Relative contributions of specific enzymes to Phase I drug metabolism.

The percentage of Phase I metabolism of drugs contributed by each enzyme is estimated by the relative size of each section of the chart. ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CYP, cytochrome P450; DPD, dihydropyrimidine dehydrogenase; NQO1, NADPH: quinone oxidoreductase (Evans and Relling, 1999). The members of this enzyme subfamily are the most abundantly expressed CYP enzymes in the liver (30% or more of total CYP content) (Shimada *et al.*, 1994), small intestinal tissue (Kolars *et al.*, 1992; Kolars *et al.*, 1994; Lown *et al.*, 1994; Paine *et al.*, 1997; Koch *et al.*, 2002; Lin *et al.*, 2002) and kidney (Schuetz *et al.*, 1992; Haehner *et al.*, 1996; Koch *et al.*, 2002; Givens *et al.*,2003). The wide substrate spectrum of CYP3A is the reason behind their frequent involvement in drug-drug interactions. Drug interactions may reduce CYP3A metabolic activity through inhibition or may increase it through induction. Such interactions can expand the range of variability of the area under curve (AUC) for CYP3A substrates to about 400-fold (Thummel and Wilkinson, 1998; Levy *et al.*, 2001). However, the marked interindividual differences in CYP3A activity have also been reported to reflect genetic components (Ozdemir *et al.*, 2000). Therefore, there has been a considerable effort to identify CYP3A gene mutations which might affect the expression and function of the CYP3A enzymes.

The human CYP3A locus is comprised of four functional genes (CYP3A4, CYP3A5, CYP3A7 and CYP3A43), but the differentiation between their products has proven difficult, due to the similarities in their protein sequence, in antigenic properties and due to overlapping substrate specificities (Gellner *et al.*, 2001). In consequence, even though the variability in the expression is established for the three most important CYP3A genes (CYP3A4, CYP3A5 and CYP3A7), their respective contributions to the hepatic CYP3A pool and their effects on drug metabolism are still a matter of debate.

Table 1.9: Contribution of each CYP3A enzymes to the total hepatic CYP3A protein pool.

CYP3A Enzyme	Contribution to the total hepatic CYP3A protein pool (%)	References
CYP3A4	40-98%	(Wrighton *et al.*, 1990; Tateishi *et al.*, 1999; Kuehl *et al.*, 2001; Koch *et al.*, 2002; Lin *et al.*, 2002; Westlind-Johnsson *et al.,* 2003).
CYP3A5	2-60%	
CYP3A7	13%, 24%	(Stevens *et al.*, 2003; Sim *et al.*, 2005).
CYP3A43	Not detected	(Domanski *et al.*, 2001; Gellner *et al.*, 2001; Westlind *et al.*, 2001).

1.16. CLINICAL RELEVANCE OF GENETIC POLYMORPHISMS IN DRUG METABOLISM

The genetic polymorphisms in drug metabolism and disposition were typically discovered on the basis of phenotypic differences among individuals in the population (Mahgoub *et al.*, 1977), but the framework for discovery of pharmacogenetic traits is rapidly changing. Adverse drug reactions are common; they are responsible for a number of debilitating side effects and are a significant cause of death following drug therapy (Lazarou *et al.*, 1998). It is now clear that a significant proportion of these adverse drug reactions, as well as therapeutic failures, are caused by genetic polymorphisms, genetically based interindividual differences in drug absorption, disposition, metabolism, or excretion. Most of the commercially available drugs are metabolized by the phase-I cytochrome P450 super family of DMEs. The clinical relevance is best characterised for the genetic polymorphisms in CYP2D6, CYP2C19 and CYP2C9 (Stormer *et al.*, 2000a). CYP2D6 play important roles in the metabolism of beta-blockers, tricyclic antidepressants, antiarrythmic agents, antipsychotic agents and opioids. CYP2C19 is involved in the metabolism of proton-pump inhibitors whereas CYP2C9 metabolizes antidiabetics and anticoagulants.

In the recent advances in molecular sequencing technology, gene polymorphisms [such as single- nucleotide polymorphisms (SNPs), and especially SNPs that occur in gene regulatory or coding regions (cSNPs)] may be the initiating discoveries, followed by biochemical and, ultimately, clinical studies to assess whether these genetic polymorphisms have phenotypic consequences in patients. This latter framework may permit the elucidation of polymorphisms in drug metabolising enzymes that have more subtle, yet clinically important consequences for interindividual variability in drug response. Such polymorphisms may or may not have clear clinical importance for affected medications, depending on the molecular basis of the polymorphism, the expression of other drug-metabolising enzymes in the patient, the presence of concurrent medications or illnesses, and other polygenic clinical features that impact upon drug response. Almost every gene involved in drug metabolism is subject to common genetic polymorphisms that may contribute to interindividual variability in drug response, are given in Table 1.10.

Table 1.10: Examples of clinically relevant genetic polymorphisms influencing drug metabolism and effects.

Gene	Medications	Drug effects linked to polymorphism
CYP2C9 (cytochrome P450 2C9)	Tolbutamide,warfarin, phenytoin, nonsteroidal anti-inflammatoric drugs,	Hypoglycemic effect of oral antidiabetic drugs, anticoagulant effect of warfarin, gastric side effects of nosteroidal anti-inflammatory drugs
CYP2D6 (cytochrome P450 2D6)	Beta-blockers, antidepressants, antipsychotics: codeine, debrisoquin, dextromethorophan, encainide, flecainide, guanoxan, methoxyamphetamine, N-propylajmaline, perhexiline, phenacetin, phenformin, propafenone, sparteine	Cardiac side effects of beta-blockers, Anticholinergic side effects of tricyclic antidepressants, efficacy of antidepressive drugs, tardive dyskinesia from antipsychotics, opioids side effect and efficacy of opioids like codeine and tramadol which are bioactivated by CYP2D6, efficacy and adverse effects (proarrhythmogenic side effects) of antiarrhythmic drugs
DPD (dihydropyrimidine dehydrogenase)	Fluorouracil	Fluorouracil neurotoxicity
TPMT (thiopurine S-methyl-transferase)	6-Mercaptopurine, thioguanine, azathioprine	Thiopurine hematotoxicity and efficacy, risk of secondary cancers
ACE (angiotensin converting enzyme)	Drug targets Enalapril, lisinopril, captopril	Renoprotective effects, cardiac indices, blood pressure immunoglobulin A nephropathy
Potassium channels:		
HERG	Quinidine Cisapride	Drug-induced long QT syndrome Drug-induced torsade de pointes
KvLQT1	Terfenadine, disopyramide, Mefloquine	Drug-induced long QT syndrome
hKCNE2	Clarithromycin	Drug-induced arrhythmia

According to (Evans and Relling, 1999)

1.17. GENETIC VARIABILITY OF DMEs

The frequently studied metabolizing enzymes are the cytochrome P450 (CYP450) isoenzymes, the N-acetyltransferase (NAT) isoenzymes, the UDP glucuro-nosyl transferase, and the methyltransferase. Of these enzymes, the CYP450s are very important because they metabolize drugs into products that are readily excreted into the urine and faeces. In humans, six different forms of CYP P450 (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4) are largely responsible for eliminating drugs.

It is now well recognized that adverse drug reactions may be caused by specific drug metaboliser phenotypes. This is illustrated by the severe and potentially fatal hematopoietic toxicity that occurs when thiopurine methyltransferase-deficient patients are treated with a standard dose of azathioprine or mercaptopurine (Krynetski and Evans, 1998). Another example is the slow acetylator phenotype that has been associated with hydralazine induced lupus erythematosus isoniazid-induced neuropathies, dye-associated bladder cancer, and sulphonamide induced hypersensitivity reactions. In all cases, acetylation of a parent drug or an active metabolite is an inactivating pathway. N-Acetyltransferase is an enzyme that conjugates substrates with a more water- soluble small molecular moiety. Such conjugation reactions are frequently, but not always, detoxifying, in that they often "mask" a more reactive functional group and usually enhance urinary or biliary excretion of substrates.

There are many examples in which the combination of a genetic defect in a conjugation pathway (Fig. 1.5, right) coupled with a wild-type phenotype for an oxidation pathway (Fig.1.5, left), or other chemical modifications, results in a phenotype particularly pre-disposed to adverse effects from a medication or environmental substance.

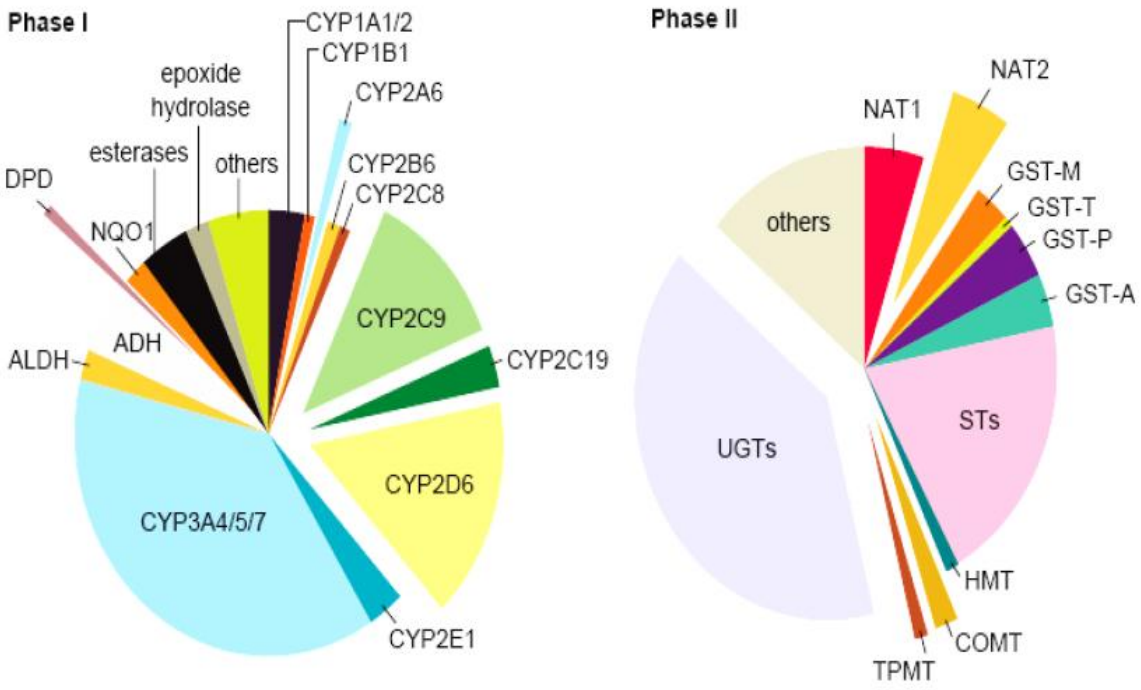

Figure 1.5: Participation of specific human liver cytochrome P450 enzymes (left side) and phase-II-enzymes (right side) in drug metabolism. The sizes of the segments refer to the relative number of drugs metabolized by the respective enzyme, e.g. about 40% of all currently used drugs are metabolized by cytochrome P4503A enzymes (bright blue segment) (Evans and Relling, 1999).

The rate of metabolism by several of the cytochrome CYP450 enzyme subfamilies varies, due to genetically determined polymorphisms in all population studied. Recent research using phenotyping and genotyping techniques has reflected the interest and importance of these pharmacogenetic factors in determining drugs responses (Emilien *et al.*, 2000).

By inhibiting cytochrome P450, one drug can impair the biotransformation of another drug. Such drug-drug interactions can lead to an excessive pharmacological or toxicological response to the second drug. In this regard, inhibition of cytochrome P450 mimics the effects of a genetic deficiency in P450 enzyme expression.

Increased P450 enzyme activity can result from (1) gene duplication leading to overexpression of a P450 enzyme, (2) exposure to environmental factors, such as xenobiotics, that induce the synthesis of cytochrome P450, or (3) stimulation of a pre-existing enzyme by a xenobiotic.

By inducing cytochrome P450 one drug can stimulate the metabolism of a second drug and thereby decrease or increase its therapeutic effect. A dramatic effect of this type of drug interaction is the induction of ethinylestradiol metabolism by phenobarbital and rifampin, which can decrease the contraceptive effect of the former drug and lead to pregnancy (Breckenridge *et al.*, 1980). Allelic variants, which arise from point mutations in the wild-type gene, are another source of interindividual variation in P450 activity. Amino acid substitution can increase or, more commonly, decrease P450 enzyme activity, although the effect may be substrate-dependent. Some of the genetic factors that influence P450 activity identified thus far are summarized (Nagata and Yamazoe, 2002; http://www.imm.ki.se/CYPalleles).

Table 1.11: Human xenobiotics-metabolizing cytochromes P450 (Kalow *et al.*, 1991; Nelson DR *et al.*, 1993; von Moltke *et al.*, 1995; Meyer *et al.*, 1997).

Enzyme	Tissue	Polymorphism[#]	Model substrate(s)
CYP1A1	Many	No	Benzopyrene
CYP1A2	Liver	No	Caffeine, Phenacetin
CYP2A6	Liver	Yes	Coumarin, Diethylnitrosamine
CYP2C9	Liver, intestine	Yes	Tolbutamide, S-warfarin
CYP2C19	Liver	Yes	Mephenytoin, omeprazole
CYP2D6	Liver, intestine, kidney	Yes	Dextromethorphan, sparteine, debrisoquine
CYP2E1	Liver, intestine, leukocytes	No	Ethanol
CYP3A4	Gastrointestinal tract, liver	No	Testosterone, nifedipine, erythromycin

1.17.1. CYP3A4 GENETIC VARIABILITY

Cytochrome P4503A4 (CYP3A4) is an iso-enzyme involved in Phase I oxidative metabolism of many endogenous and exogenous substances. From a quantitative point of view it is the most important hepatic CYP-enzyme, accounting for approximately 25% of all liver cytochrome P450s. Since CYP3A4 is also present in the small intestine, it has a significant effect on the first-pass metabolism of CYP3A4 substrates.

One of the most striking examples is the interaction between CYP3A4 inhibitors and the non-sedating antihistamine, terfenadine. (Honing *et al.*, 1992; Bailey *et al.*, 1998). Terfenadine undergoes extensive pre-systemic elimination by CYP3A4 to terfenadine carboxylate. Terfenadine is a potent blocker of myocyte delayed rectifier potassium current, whereas the metabolite is inactive. This blockade may lead to prolongation of the QTC interval and development of a serious ventricular tachyarrhythmia, torsade de pointes, and may finally lead to death. Inhibition of CYP3A4 activity due to concomitant medication or intake of grapefruit juice lead to increased plasma levels of terfenadine with serious side effects as described above. Approximately 125 deaths linked to terfenadine have been reported, showing the relevance of this interaction. (Bailey *et al.*, 1998).

In Table 1.12 it can be seen that the number of drugs metabolized chiefly by CYP3A4 is large and, including a wide variety of compounds in many different therapeutic classes.

Table 1.12: An overview of typical CYP3A4 substrates (Bertz *et al.*, 1997).

Class	Drugname
Narcotics	Alfentanil Fentanyl Methadone
Anticonvulsants	Carbamazepine Ethosuximide
Antibiotics	Azithromycin Clarithromycin Erythromycin
Antifungals	Fluconazole Ketoconazole Miconazole
Antiparasitis	Quinine
Antivirals and HIV drugs	Indinavir Ritonavir Saquinavir
Cancer Chemotherapy	Etoposide Ifosfamide Tamoxifen
Cardiovascular agents: antiarrhythmics	Amiodarone Disopyramide Lidocaine (lignocaine) Quinidine
Cardiovascular agents: calcium channel blockers	Amplodipine Diltiazem Felodipine Nicardipine Nifedipine Nimodipine Nitrendipine Verapamil
Cardiovascular agents: hypolipidaemics	Fluvastatin Pravastatin
Antihistamines	Loratadine Terfenadine
Gastrointestinal agents	Cisapride
Immunosuppressants	Cyclosporin Tacrolimus
Antidepressants	Nefazodone Sertraline
Sedatives / hypnotics	Alprazolam Midazolam Triazolam Zolpidem
Steroids	Dexamethasone Finasteride Methylprednisolone Prednisone Testosterone

1.18. CYP3A4 POLYMORPHISMS

The four CYP3A genes encoding their respective enzymes are localized in a 231-kb cluster on chromosome band 7q21-q22.1 (Brooks *et al.*, 1988; Spurr *et al.*, 1989; Inoue *et al.*, 1992) and reside in tandem, adjacent to each other in the order: CYP3A43-CYP3A4-CYP3A7-CYP3A5 (Nelson *et al.*, 1996; Domanski *et al.*, 2001; Gellner *et al.*, 2001; Finta and Zaphiropoulos, 2002) in which the CYP3A43 gene is in a head-to-head orientation with its neighbouring gene CYP3A4, and the other three genes lie in head-to-tail orientation. Two pseudo genes (Nelson *et al.*, 2004), CYP3AP1 and CYP3AP2 are present between the intergenic regions of CYP3A7-CYP3A5 and CYP3A4-CYP3A7, respectively (Finta and Zaphiropoulos, 2000).

The official name of CYP3A4 is cytochrome P450, family 3, subfamily A, polypeptide 4. Identification of single nucleotide polymorphisms (SNPs) in the CYP3A genes has been an active area of research. Currently, 39 CYP3A4 alleles, comprising 65 SNPs have been reported. (Human Cytochrome P450 (CYP) Allele Nomenclature Committee). Available from URL: (http://www.imm.ki.se/CYPallele/cyp3a4.htm). CYP3A4 allelic nomenclature has been shown to Table 1.13

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To date several alleles of CYP3A4 have been reported. By far the most common CYP3A4 genetic variant is the A-392G transition (CYP3A4*1B) located in the 5'-regulatory region (Rebbeck *et al.*, 1998; Westlind *et al.*, 1999). CYP3A4*2 has a change at codon 222, an amino acid substitution serine/proline. Another rare allelic variant in codon 455 designated CYP3A4*3 was found in a single Chinese subject. Three more novel variants of CYP3A4 were found in Chinese subjects (Hsieh *et al.*, 2001). These alleles were designated as CYP3A4*4, CYP3A4*5 and CYP3A4*6. Seven more genetic variants were identified resulted in amino acid substitutions were designated as CYP3A4*7, CYP3A4*8, CYP3A4*9, CYP3A4*10, CYP3A4*11, CYP3A4*12, and CYP3A4*13 (Eiselt *et al.*, 2001). Three new coding-region polymorphisms CYP3A4*17, CYP3A4*18 and CYP3A4*19 are also identified. (Die et al., 2001).

Table 1.13: CYP3A4 allele nomenclature.

Allele	*Protein*	*Nucleotide changes*		*Trivial name*	*Effect*	*Enzyme_activity*		*References*
		cDNA	*Gene*			*In vivo*	*In vitro*	
CYP3A4*1A	CYP3A4.1	None	None	Wild-type		Normal	Normal	Gonzalez *et al.*, 1988
CYP3A4*1B	CYP3A4.1		-392A>G	CYP3A4-V				Rebbeck *et al.*, 1998 Westlind *et al.*, 1999
CYP3A4*1C	CYP3A4.1		-444T>G					Kuehl *et al.*, 2001
CYP3A4*1D	CYP3A4.1		-62C>A					Kuehl *et al.*, 2001
CYP3A4*1E	CYP3A4.1		-369T>A					Hamzeiy *et al.*, 2002
CYP3A4*1F	CYP3A4.1		-747C>G					Hamzeiy *et al.*, 2002
CYP3A4*1G	CYP3A4.1		20230G>A					Fukushima-Uesaka *et al.*, 2004
CYP3A4*1H	CYP3A4.1		20230G>A; 26206C>A					Fukushima-Uesaka *et al.*, 2004
CYP3A4*1J	CYP3A4.1		6077A>G					Fukushima-Uesaka *et al.*, 2004
CYP3A4*1K	CYP3A4.1		-655A>G					Fukushima-Uesaka *et al.*, 2004
CYP3A4*1L	CYP3A4.1		-630A>G					Fukushima-Uesaka *et al.*, 2004
CYP3A4*1M	CYP3A4.1		-156C>A					Fukushima-Uesaka *et al.*, 2004
CYP3A4*1N	CYP3A4.1		14200T>G					Fukushima-Uesaka *et al.*, 2004
CYP3A4*1P	CYP3A4.1		15727G>A					Fukushima-Uesaka *et al.*, 2004
CYP3A4*1Q	CYP3A4.1		15809T>C					Fukushima-Uesaka *et al.*, 2004
CYP3A4*1R	CYP3A4.1		16775A>G					Fukushima-Uesaka *et al.*, 2004
CYP3A4*1T	CYP3A4.1		26013T>C					Fukushima-Uesaka *et al.*, 2004
CYP3A4*2	CYP3A4.2	664T>C	15713T>C		S222P			Sata *et al.*, 2000
CYP3A4*3	CYP3A4.3	1334T>C	23171T>C		M445T			Sata *et al.*, 2000
CYP3A4*4	CYP3A4.4	352A>G	13871A>G		I118V			Hsieh *et al.*, 2001
CYP3A4*5	CYP3A4.5	653C>G	15702C>G		P218R			Hsieh *et al.*, 2001
CYP3A4*6		830_831insA	17661_176622insA		277Frameshift			Hsieh *et al.*, 2001
CYP3A4*7	CYP3A4.7	167G>A	6004G>A		G56D			Eiselt *et al.*, 2001

Allele	*Protein*	*Nucleotide changes* *cDNA*	*Gene*	*Trival name*	*Effect*	*Enzyme* *In vivo*	*Activity* *In vitro*	*References*
CYP3A4*8	CYP3A4.8	389G>A	13908G>A		R130Q		Decreased	Eiselt *et al.*, 2001
CYP3A4*9	CYP3A4.9	508G>A	14292G>A		V170I			Eiselt *et al.*, 2001
CYP3A4*10	CYP3A4.10	520G>C	14304G>C		D174H			Eiselt *et al.*, 2001
CYP3A4*11	CYP3A4.11	1088C>T	21867C>T		T363M		Decreased	Eiselt *et al.*, 2001 Murayama *et al.*, 2002
CYP3A4*12	CYP3A4.12	1117C>T	21896C>T		L373F		Decreased	Eiselt *et al.*, 2001
CYP3A4*13	CYP3A4.13	1247C>T	22026C>T		P416L		Decreased	Eiselt *et al.*, 2001
CYP3A4*14	CYP3A4.14	44T>C	44 T>C		L15P			Lamba *et al.*, 2002
CYP3A4*15A	CYP3A4.15	485G>A	14269G>A		R162Q			Lamba *et al.*, 2002
CYP3A4*15B	CYP3A4.15	485G>A	-845_-844insATGGAGTGA; -392A>G; 14269G>A		R162Q			Hamzeiy *et al.*, 2002
CYP3A4*16A	CYP3A4.16	554C>G	15603C>G		T185S		Decreased	Lamba *et al.*, 2002; Murayama *et al.*, 2002
CYP3A4*16B	CYP3A4.16	554C>G	15603C>G; 20230G>A		T185S		Decreased	Murayama *et al.*, 2002; Fukushima-Uesaka *et al.*, 2004;
CYP3A4*17	CYP3A4.17	566T>C	15615T>C		F189S		Decreased	Dai *et al.*, 2001
CYP3A4*18A	CYP3A4.18	878T>C	20070T>C		L293P	Decreased(m)	Increased (t, c, e)	Dai *et al.*, 2001; Kang *et al.*, 2008
CYP3A4*18B	CYP3A4.18	878T>C	20070T>C; 20230G>A		L293P			Fukushima-Uesaka *et al.*, 2004;
CYP3A4*19	CYP3A4.19	1399C>T	23237C>T; 20230G>A		P467S			Dai *et al.*, 2001
CYP3A4*20		1461_1462insA	25889_25890insA		488Frameshift		None	Westlind-Johnsson *et al.*, 2006

c, chlorpyrifos; e, estrone; m, midazolam; t, testosteron

Adapted from Home Page of the Human Cytochrome P450 (*CYP*) Allele Nomenclature Committee. (http://www.cypalleles.ki.se/cyp3a4.htm).

CYP3A4*1B allelic frequency varies among different ethnic groups: 0% in Asian, 5% in Caucasians and 54% in Africans (Chowbay *et al.*, 2005). CYP3A4*1B demonstrates a frequency of 60% & 4% in Africans and Caucasians respectively and is absent in Chinese & Japanese (Ball *et al.*, 1999; Sata *et al.*, 2000). The other allelic variants occur at much lower frequencies (<1%-2%) or they are selectively prevalent in specific populations (Hamzeiy *et al.*, 2002; Lamba *et al.*, 2002; Floyd *et al.*, 2003). CYP3A4*2 occurred with a frequency of 2.7% in Caucasians and is absent in Africans and Chinese (Sata *et al.*, 2000). Another rare allelic variant in codon 455 designated CYP3A4*3 was found in a single Chinese subject. CYP3A4*4, *5 and *6 occurred with a frequency of 1%, 0.9% and 0.5% respectively in 102 Chinese subjects (Hsieh *et al.*, 2001). CYP3A4*7, *8, *9, *10, *11, *12, and *13 alleles were reported in Caucasians with a frequency of 1.41%, 0.33%, 0.24%, 0.24%, 0.34%, 0.34% and 0.34% respectively (Eiselt *et al.*, 2001). CYP3A4*15, *17, *18, and *19 alleles were reported in Caucasians, Chinese, Africans, and Indo-Pakistanis (Dai *et al.*, 2001).

Chowbay *et al.* reported the absence of CYP3A4*1B, *4, *5 and presence of CYP3A4*6 in Indians residing in Singapore. Another study demonstrates the absence of genetic polymorphism of CYP3A in North Indians on the basis of frequency distribution of urinary 6β-hydroxy-Cortisol/ Cortisol ratio and the absence of variant alleles CYP3A4*2, *4, *5, *6 and *10 (Rais *et al.*, 2006).

Many studies have been carried out on functions of CYP3A4 variants. CYP3A4*1B has been studied to ascertain the effect of the mutation on transcriptional activity and in vivo catalytic activity (Amirimani *et al.*, 1999; Ando *et al.*, 1999). The results of studies with larger numbers of predominantly Caucasian liver samples demonstrate no clear association between CYP3A4*1B variant and CYP3A4 specific content or catalytic activity (Westlind *et al.*, 1999; Lamba *et al.*, 2002).

Compared with wild-type enzyme, there was no significant difference in the rates of CYP3A4*3, CYP3A4*7, CYP3A4*9, CYP3A4*11 and CYP3A4*19 metabolizing the probe substrates testosterone, progesterone, or 7-benzyloxy-4-(trifluoromethyl) coumarin (Sata *et al.*, 2000; Dai *et al.*, 2001; Eiselt *et al.*, 2001). It means that these variants have no pronounced effect on drug metabolism kinetics. The individuals with CYP3A4*8 and CYP3A4*13 genotypes may have lower CYP3A4 protein content, since these variants appear to affect steady-state enzyme levels by altering heme binding and/or protein stability (Eiselt *et al.*, 2001).

For CYP3A4*2, CYP3A4*10, CYP3A4*14, CYP3A4*15 and CYP3A4*16, there was no significant association with midazolam hydroxylation activity (Lamba *et al.*, 2002). Those with CYP3A4*17 genotype exhibited in vitro a significantly lower turnover of testosterone and of the

insecticide chlorpyrifos than those with CYP3A4*1, while those with CYP3A4*18 metabolized both substrates with a higher turnover (Dai *et al.*, 2001). Subjects carrying CYP3A4*4, CYP3A4*5 or CYP3A4*6 exhibited below average 6-beta-hydroxy-cortisol to cortisol ratio, implying reduced catalytic activity for the corresponding protein variants (Hsieh *et al.*, 2001). Indeed, most of the changes in catalytic activity observed for CYP3A4 gene variants are relatively modest. These catalytic findings and consideration of the low allele frequencies for the known structural CYP3A4 variants, implies that they are not the major cause of interindividual differences in CYP3A-mediated drug clearance in the general population.

A summary table of ethnic variation of different CYP3A4 allelic variants has been shown to Table 1.14.

Table 1.14: Ethnic distribution of variant alleles of CYP3A4.

CYP3A4 Genetic variants	Allele frequencies (%) in different ethnic groups							References
	African	Hispanic	Caucasian	Chinese	Japanese	North Indian	Malaysian	
CYP3A4*1B	35-67%	9.3-11%	2-9.6%	0%	0%	1%	ND	Rebbeck *et al.*, 1998; Walker *et al.*,1998; Paris *et al.*, 1999; Sata *et al.*, 2000; Kuehl *et al.*, 2001; Rais *et al.*, 2006.
CYP3A4*2	0%	ND	2.7%	0%	ND	0%	ND	Sata *et al.*, 2000; Rais *et al.*, 2006.
CYP3A4*3	ND	ND	0.47-4%	1.5%	ND	ND	ND	Sata *et al.*, 2000; Rais *et al.*, 2006.
CYP3A4*4	ND	ND	ND	1%	ND	0%	0%	Hsieh *et al.*, 2001; Rais *et al.*, 2006; Ruzilawati *et al.*, 2007.
CYP3A4*5	ND	ND	ND	0.9%	ND	0%	0%	Hsieh *et al.*, 2001; Rais *et al.*, 2006; Ruzilawati *et al.*, 2007.
CYP3A4*6	ND	ND	ND	0.5%	ND	0%	ND	Hsieh *et al.*, 2001; Rais *et al.*, 2006.
CYP3A4*7	ND	ND	1.41%	ND	ND	ND	ND	Eiselt *et al.*, 2001.
CYP3A4*8	ND	ND	0.33%	ND	ND	ND	ND	Eiselt *et al.*, 2001.
CYP3A4*9	ND	ND	0.24%	ND	ND	ND	ND	Eiselt *et al.*, 2001.
CYP3A4*10	ND	ND	2%	ND	ND	ND	ND	Lamba *et al.*, 2002; Rais *et al.*, 2006.

CYP3A4 Genetic variants	**Allele frequencies (%) in different ethnic groups**							**References**
	African	**Hispanic**	**Caucasian**	**Chinese**	**Japanese**	**North Indian**	**Malaysian**	
CYP3A4*11	ND	ND	0.34%	ND	ND	ND	ND	Eiselt *et al.*, 2001.
CYP3A4*12	ND	ND	0.34%	ND	ND	ND	ND	Eiselt *et al.*, 2001.
CYP3A4*13	ND	ND	0.34%	ND	ND	ND	ND	Eiselt *et al.*, 2001.
CYP3A4*14	ND	ND	ND	ND	ND	ND	ND	ND
CYP3A4*15A	24.2%	ND	0%	0%	0%	0%	ND	Dai *et al.*, 2001; Lamba *et al.*, 2002.
CYP3A4*16	ND	5%	ND	ND	5%	ND	ND	Lamba *et al.*, 2002.
CYP3A4*17	0%	0%	0%	2.1%	0%	ND	ND	Dai *et al.*, 2001.
CYP3A4*18	ND	ND	ND	ND	10%	ND	2.09 %	Dai *et al.*, 2001; Ruzilawati *et al.*, 2007.

ND=Not Determined

1.19. CYP3A5 GENETIC VARIABILITY

The genetic basis of the CYP3A5 polymorphism has gradually been elucidated following the publication in 2000 by Paulussen and colleagues that demonstrated the existence of genetic variants in complete concordance with the polymorphic CYP3A5 expression in the liver (Paulussen et al., 2000). To date 10 CYP3A5 alleles, consisting of 22 SNPs, have been identified (Human Cytochrome P450 (CYP) Allele Nomenclature Committee. CYP3A5 allele nomenclature). Of the variants found, CYP3A5*3 (g.6986G) is the only one found in all ethnic groups tested. The frequencies vary from 27% in African-Americans to 95% in Caucasians (Hustert et al., 2001; Kuehl et al., 2001; Fukuen et al., 2002; van Schaik et al., 2002; Hu et al., 2005; Roy et al., 2005). Other variants such as CYP3A5*6 and CYP3A5*7 affecting the CYP3A5 expression are relatively frequent in African subjects (10-22%) but absent in white subjects (Roy et al., 2005). The remaining CYP3A5 genetic variants are rare or occur at much lower allelic frequencies (Lamba et al., 2002; Roy et al., 2005).

In contrast to CY3A4, CYP3A5 expression in human exhibits a bimodal distribution, with the proportion of CYP3A5 “high expressers” and “low expressers” varying depending on the ethnic background. These interethnic differences in the prevalence of the CYP3A5 polymorphism are in part caused by differing allelic frequencies of the major genetic determinant of this trait, the g.6986A>G polymorphism (Hustert et al., 2001; Kuehl et al., 2001). This variant is located in intron 3 of the CYP3A5 gene. The inheritance of an adenine at this position (g.6986A, CYP3A5*1) allows for normal generation of CYP3A5 transcripts, whereas a guanine (g.6986G, CYP3A5*3) generates a cryptic splice with the resulting degradation of CYP3A5 transcripts (Kuehl et al., 2001). In Caucasians the concordance between the presence of g.6986A and increased CYP3A5 expression is high. Among 183 liver samples tested, all 18 livers with increased CYP3A5 protein levels had at least one allele of nifedipine oxidation, the rank order was: CYP3A5*1> CYP3A5*9> CYP3A5*8> CYP3A5*10. Low expression of CYP3A5 maRNA and protein was found to be associated with the most frequent and functionally important CYP3A5*3 genotype, regardless of racial ancestry (Hustert et al., 2001; Kuehl et al., 2001).

1.20. CORRELATION BETWEEN CYP3A4 AND CYP3A5 GENE WITH PROSTATE CANCER

Prostate cancer is the most common nonskin-related malignancyin men in the United States. In 2002 _189,000 men in the United States were diagnosed with prostate cancer, and 30,200 men died from this disease (Jemal, A. *et al.,* 2002). Risk factors include age, ethnicity, family history, and diet (Pienta, K. J. *et al.,* 1993). A strong family history indicative of a highly penetrant gene is believed to account foronly 5–10% of prostate cancers, whereas a larger percentage may be because of common polymorphisms that give rise to a low risk of disease (Coughlin, S. S. *et al.,* 2002; Nwosu, V. *et al.,* 2001). A great deal of interest has focused recently on the role of genes involved in the metabolism, biosynthesis, and regulation of androgens in the occurrence and progression of prostate cancer. The CYP6 family of enzymes functions in a wide variety of metabolic pathways involving both endogenous and exogenous compounds (Gibson, G. G. *et al.,* 2002). Their involvement in the metabolism of steroids, as well as environmental xenobiotics, suggests that some may affect prostate cancer risk (Coughlin, S. S. *et al.,* 2002; Nwosu, V. *et al.,* 2001; Gibson, G. G. *et al.,* 2002). Studies on the activity and expression of CYP3A subfamily members in liver extracts have shown a high degree of polymorphic expression (Gibson, G. G. *et al.,* 2002). TheCYP3A locus consists of four genes, CYP3A4, CYP3A5,CYP3A7, and CYP3A43, all of which reside in a 231-kb region of chromosome 7q21.1 (Gellner, K. *et al.,* 2001).It has been estimated that up to 60% of the variability inCYP3A4 activity may be because of a genetic component (Ozdemir, V. *et al.,* 2000). A SNP in the nifedipine-specific response element in the promoter of the CYP3A4 gene (alternatively termed g.-392A_G, CYP3A4-V, CYP3A4*1B, see website7 for unified nomenclature) has been reported (Rebbeck, T. R. *et al.,* 1998). Case-only studies of Caucasians (Rebbeck, T. R. *et al.,* 1998) and of African-Americans (Paris, P. L. *et al.,* 1999) have detected associations betweenCYP3A4*1B and presentation with biologically aggressive disease. It has been postulated that the presence of theCYP3A4*1B allele decreases the amount of CYP3A4 protein, leading to a reduction of testosterone metabolism and, therefore, more availability of testosterone for conversion to dihydrotestosterone, the most potent androgen affecting the growth and differentiation of prostate cells (Rebbeck, T. R. *et al.,* 1998). However, several invivo studies on the functional effect of CYP3A4*1B have failed to reveal any meaningful link between this polymorphism and activity of the CYP3A4 enzyme (Westlind, A. *et al.,* 1999; Wandel, C. *et al.,* 2000; Ando, Y. *et al.,* 1999; Ball, S. E. *et al.,*1999). CYP3A5 is expressed in a polymorphic manner in 10–29% of adult livers (Wrighton, S. A. *et al.,* 1990; Schuetz, J. D. *et al.,* 1994; Hustert, E. *et al.,* 2001). Several polymorphic variants inCYP3A5 appear to have a functional effect on CYP3A5 activity, including an intronic SNP that affects splicing of the CYP3A5 transcript.

The CYP3A5*1 allele that produces a correctly spliced transcript has a frequency of 0.15 to 0.45 in Caucasians and African-Americans, respectively (Kuehl, P. *et al.,* 2001). The nonfunctional allele (CYP3A5*3, g.6986A_G) occurs in intron 3 of CYP3A5, creating a cryptic splice site leading to the inclusion of a novel exon, and ultimately a premature stop codon (Hustert, E. *et al.,* 2001; Kuehl, P. *et al.,* 2001). Only individuals with at least one CYP3A5*1 allele express CYP3A5 at a high level (Hustert, E. *et al.,* 2001; Kuehl, P. *et al.,* 2001; Lin, Y. S. *et al.,* 2002). CYP3A5 represents at least half of the CYP3A content in the liver and jejunum of most individuals carrying a CYP3A5*1 allele, and CYP3A4 levels in those individuals appear to correlate with CYP3A5 levels (Kuehl, P. *et al.,* 2001; Lin, Y. S. *et al.,* 2002).As no functional significance has been ascribed to theCYP3A4*1B variant allele, an association between CYP3A4*1B and prostate cancer phenotypes may be because of linkage with a functional polymorphism elsewhere in the CYP3A locus.CYP3A5 is an attractive candidate gene for this association because of evidence that it is expressed in normal and tumor prostate tissue (Finnstrom, N. *et al.,* 2001; Wojnowski, L. *et al.,* 2002), whereas CYP3A4 has been reported as expressed in only 0–14% of normal prostate tissues (Finnstrom, N. *et al.,* 2001; Wojnowski, L. *et al.,* 2002; Westlind, A. *et al.,* 2001).The hypothesis that prostate cancer risk may be associated withCYP3A5 genotypes (Kuehl, P. *et al.,* 2001) has been strengthened recently by there port of linkage disequilibrium between the CYP3A4 and CYP3A5 alleles (Wojnowski, L. *et al.,* 2002). To additionally investigate this possibility, we used a family-based case-control study to investigate the association between prostate cancer and the CYP3A4(*1A/*1B) alleles, CYP3A5(*1/*3) alleles, and CYP3A4/CYP3A5 haplotypes.

1.21. RATIONALE OF THE STUDY

Pharmacogenetics and pharmacogenomics examine the genetic factors that contribute to variability in drug response in individual patients. Usually the two terms are used interchangeably, however, pharmacogenetics refers to the field science that focuses on how single genes modulate the effect of a drug, pharmacogenomics refers to the science that focuses on how the genome as a whole affects the action of a drug, referring to the contribution of individual genes, as well as to gene-to-gene interactions.

It is well recognized that different patients respond in different ways to the same medication. These differences are often greater among members of a population than they are within the same person at different times (or between monozygotic twins) (Vesell, 1989). The existence of large population differences with small intra-patient variability is consistent with inheritance as a determinant of drug response; it is estimated that genetics can account for 20 to 95 percent of variability in drug disposition and effects (Kalow *et al.*, 1998).

Unlike other factors influencing drug response, inherited determinants generally remain stable throughout a person's lifetime. More than 1.4 million single-nucleotide polymorphisms were identified in the initial sequencing of the human genome (Sachidanandam *et al.*, 2001), with over 60,000 of them in the coding region of genes. Some of these single-nucleotide polymorphisms have already been associated with substantial changes in the metabolism or effects of medications and some are now being used to predict clinical response (Evans and Relling, 1999; Evans and Johnson, 2001; McLeod and Evans, 2001). Because of pharmacogenetic variations, the pharmacokinetic properties of drug will be different for people of different races. Metabolism and excretion pattern of drugs are different because of involvement of different metabolizing enzymes. Moreover, the formulation itself may be another important factor for variation. Due to these types of variations, the response of a patient towards a drug will be different compared to response produced by patients of other races. These variations may result in severe adverse effects.

Thus, pharmacogenetics helps understand the following: 1) why some individuals do and some do not respond to drugs; 2) why some individuals require higher or lower drug doses to achieve optimal therapeutic response; 3) what is it that determines individual risk of side effects; 4) to what extent is the pharmacologic variability predictable; and 5) how many side effects can thus be prevented, i.e. notify the physician of the patient who is likely to experience toxic therapeutic side effects. A potential role of pharmacogenetics could be the reduction of adverse drug reactions (ADR), a significant cause of morbidity and mortality and excess medical care costs (Johansson *et al.,* 1993*).* Sixty percent of drugs with high frequency of ADR are metabolized by at least one enzyme with a variant allele known to cause poor metabolism. This observation supports the hypothesis that drugs identified in the ADR studies would be more likely to be metabolized by enzymes with genetic variability than drugs not identified in these studies. Interestingly, variant alleles of P450 cytochrome 1A2 (CYP1A2), the enzyme involved in the metabolism of only 5% of all prescribed drugs (Lai *et al.*, 1995), have been found in 75% of the reported adverse drug reactions. On the other hand, P450 cytochrome 2D6 (CYP2D6), the major metabolic pathway for more than 25% of all prescribed drugs, accounted for only 38% of the relevant drugs side-effects. These results suggest that genetic variability in drug metabolizing enzymes is likely to be an important contributor to the incidence of ADR (Lee *et al.*, 2002).

Another potential application for pharmacogenetics is the field of drug discovery (Linder *et al.*, 1997). The traditional approach requires the complete knowledge of the physiological and pathophysiological process and the role of individual factors involved in a given disease. This is typically long, costly and labour intensive. In contrast, genomics may offer powerful

advantages and complementary information. An initial step of functional genomics is filtering through all human genes to identify a much smaller set of genes that may participate in the disease. An obvious approach to target identification is to consider which targets are present or absent in diseased tissue relative to normal tissue. This approach may provide new insights into biological interactions between drug targets and other biological molecules, leading to the identification of new pharmacological targets involved in a given disease. Moreover, application of these technologies will enable effective drugs to be developed and made available more rapidly for clinical use. After the discovery that a specific gene is involved in a disease, chemical 'leads' are identified using chemical libraries or high throughput screening. Thereafter, mutations of genes involved in metabolism, transport, availability and response profiles of active compounds could be identified during phase II clinical trials. These could be used in the selection of patient groups enriched for efficacy in phase III studies. This is likely to make these trials smaller, faster and more efficient. The net result of this innovative approach will be a significant reduction in the waiting-time for new drugs.

1.22. AIMS OF THE STUDY

The aim of this study was to investigate the allelic distribution of CYP3A4 and CYP3A5 genes in Bangladeshi population using a polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method. Genetic variability in drug response occurs as a result of molecular alterations in the enzymes involved in the metabolism of a particular drug in addition to the drug receptors and transport proteins.

Cytochrome P450 3A4 (CYP3A4) is the major cytochrome involved in metabolizing 60% of all drugs used in humans. CYP3A5 also involved in many drug metabolism. A number of allelic variations in CYP3A4 and CYP3A5 gene are known to affect catalytic activity including CYP3A4*1B, CYP3A5*3. Among Asian subjects, a number of allelic variations in CYP3A4 and CYP3A5 gene are known to affect catalytic activity including CYP3A4*1B, CYP3A5*3.

In the present study we characterised CYP3A4*1B, CYP3A5*3 alleles among Bangladeshi population for 100 healthy adult volunteers and 100 prostate cancer patient. This study will help to find different genotypes among this population and which is considered to be very important for dose selection. This will help treatment of patients receiving drugs metabolized by these alleles. This study will be helpful for the adjustment of dosage regimen, reduce the serious adverse reactions to ensure safe, effective and economic treatment.

Bangladesh is a tropical country. Due to its climate variation in different parts of the country, the life style of the people is also different. Ethnic variation and geographical location can also play important roles for polymorphic changes. The study of polymorphism has many uses in medicine, biological research, and other different purposes.

Finally this work is expected to be published in a peer-reviewed international journal

CHAPTER TWO

MATERIALS AND METHODS

II

In a group of hundred adult healthy Bangladeshi volunteers for control group and hundred of prostate cancer patient, the allelic frequency of the CYP3A4*1B and CYP3A5*3 alleles were determined by use of Polymerase Chain Reaction-Restriction Fragment Length Polymorphism assays (PCR-RFLP). This study was carried out in the "**Laboratory of Pharmakinetics and Pharmacogenetics**" in the department of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, University of Dhaka.

2.1. SELECTION OF VOLUNTEERS

2.1.1. VOLUNTEERS

One hundred adult healthy Bangladeshi volunteers and One hundred prostate cancer patients were recruited. Volunteers were informed about the experimental procedures and study aim before giving written informed consents. They were ascertained to be healthy from the medical history, physical examination and routine laboratory tests.

The study protocol was approved by the ethical committees of the respective hospitals and the study was conducted in accordance with the declaration Helsinkis and its subsequent revisions (WMADH, 2008). Ethical permission was taken to approve the protocol and consent form of the clinical investigation from Ahsania Mission Cancer and General Hospital, Dhaka Medical College Hospital and Bangabandhu Sheikh Mujib Medical University (PG Hospital), Dhaka, Bangladesh. Each volunteer (cases and controls) signed an informed consent document before entering the study and was free to withdraw from the study at any time without any obligation.

INDIVIDUAL DEMOGRAPHIC DATA FOR CONTROL GROUP:

DNA SERIAL	CODE	AGE	SEX	HEIGHT(cm)	WEIGHT (kg)	BMI(KG/M2)
1	FCD-01	63	M	172.72	65	21.79
2	FCD-02	65	M	165.10	64	23.48
3	FCD-03	68	M	157.48	63	25.40
4	FCD-04	63	M	154.94	70	29.16
5	FCD-05	64	M	160.02	73	28.51
6	FCD-06	65	M	172.72	70	23.46
7	FCD-07	67	M	165.10	78	28.62
8	FCD-08	66	M	175.26	62	20.18
9	FCD-09	69	M	177.80	65	20.56
10	FCD-10	70	M	154.94	60	24.99
11	FCD-11	65	M	162.56	65	24.60
12	FCD-12	69	M	157.48	70	28.23
13	FCD-13	63	M	170.18	58	20.03
14	FCD-14	66	M	160.02	61	23.82
15	FCD-15	63	M	167.64	60	21.35
16	FCD-16	65	M	172.72	62	20.78
17	FCD-17	71	M	162.56	59	22.33
18	FCD-18	63	M	170.18	72	24.86
19	FCD-19	68	M	165.10	60	22.01
20	FCD-20	65	M	175.26	65	21.16
21	FCD-21	64	M	177.80	70	22.14
22	FCD-22	68	M	154.94	67	27.91
23	FCD-23	63	M	162.56	62	23.46
24	FCD-24	64	M	157.48	73	29.44
25	FCD-25	65	M	170.18	70	24.17
26	FCD-26	68	M	162.56	60	22.71
27	FCD-27	66	M	165.10	62	22.75
28	FCD-28	62	M	167.64	71	25.26
29	FCD-29	65	M	165.10	74	27.15
30	FCD-30	63	M	170.18	63	21.75
31	FCD-31	65	M	157.48	58	23.39
32	FCD-32	63	M	154.94	70	29.16
33	FCD-33	71	M	160.02	73	28.51
34	FCD-34	63	M	162.56	70	26.49
35	FCD-35	68	M	165.10	60	22.01
36	FCD-36	63	M	167.64	60	21.35
37	FCD-37	64	M	170.18	58	20.03
38	FCD-38	68	M	160.02	70	27.34
39	FCD-39	62	M	170.18	73	25.21

DNA SERIAL	CODE	AGE	SEX	HEIGHT(cm)	WEIGHT (kg)	BMI(KG/M2)
40	FCD-40	68	M	175.26	65	21.16
41	FCD-41	65	M	162.56	62	23.46
42	FCD-42	64	M	172.72	63	21.12
43	FCD-43	62	M	170.18	73	25.21
44	FCD-44	69	M	167.64	70	24.91
45	FCD-45	72	M	160.02	67	26.17
46	FCD-46	64	M	162.56	62	23.46
47	FCD-47	68	M	152.40	73	31.43
48	FCD-48	63	M	170.18	70	24.17
49	FCD-49	69	M	162.56	60	22.71
50	FCD-50	65	M	165.10	62	22.75
51	FCD-51	64	M	160.02	71	27.73
52	FCD-52	63	M	157.48	74	29.84
53	FCD-53	64	M	170.18	63	21.75
54	FCD-54	68	M	147.32	58	26.72
55	FCD-55	67	M	170.18	70	24.17
56	FCD-56	62	M	157.48	63	25.40
57	FCD-57	65	M	167.64	70	24.91
58	FCD-58	71	M	165.10	73	26.78
59	FCD-59	63	M	175.26	70	22.79
60	FCD-60	66	M	170.18	67	23.13
61	FCD-61	65	M	165.10	65	23.85
62	FCD-62	67	M	162.56	64	24.22
63	FCD-63	64	M	157.48	62	25.00
64	FCD-64	63	M	165.10	59	21.65
65	FCD-65	65	M	167.64	72	25.62
66	FCD-66	69	M	154.94	62	25.83
67	FCD-67	67	M	157.48	58	23.39
68	FCD-68	65	M	162.56	61	23.08
69	FCD-69	68	M	167.64	63	22.42
70	FCD-70	64	M	162.56	62	23.46
71	FCD-71	62	M	165.10	59	21.65
72	FCD-72	72	M	165.10	72	26.41
73	FCD-73	64	M	170.18	66	22.79
74	FCD-74	62	M	160.02	65	25.38
75	FCD-75	68	M	170.18	68	23.48
76	FCD-76	63	M	175.26	62	20.18
77	FCD-77	67	M	162.56	68	25.73
78	FCD-78	71	M	172.72	60	20.11
79	FCD-79	62	M	165.10	69	25.31
80	FCD-80	69	M	167.64	70	24.91
81	FCD-81	70	M	165.10	58	21.28

DNA SERIAL	CODE	AGE	SEX	HEIGHT(cm)	WEIGHT (kg)	BMI(KG/M2)
82	FCD-82	62	M	165.10	74	27.15
83	FCD-83	68	M	157.48	65	26.21
84	FCD-84	64	M	154.94	75	31.24
85	FCD-85	68	M	170.18	63	21.75
86	FCD-86	67	M	165.10	60	22.01
87	FCD-87	66	M	162.56	67	25.35
88	FCD-88	63	M	167.64	65	23.13
89	FCD-89	65	M	172.72	62	20.78
90	FCD-90	66	M	157.48	59	23.79
91	FCD-91	63	M	160.02	65	25.38
92	FCD-92	69	M	175.26	60	19.53
93	FCD-93	63	M	172.72	73	24.47
94	FCD-94	72	M	152.40	75	37.03
95	FCD-95	63	M	157.48	55	22.18
96	FCD-96	68	M	170.18	71	24.52
97	FCD-97	65	M	160.02	59	23.04
98	FCD-98	63	M	162.56	72	27.25
99	FCD-99	68	M	172.72	62	20.78
100	FCD-100	64	M	175.26	58	18.88

INDIVIDUAL DEMOGRAPHIC DATA FOR PATIENT GROUP:

DNA SERIAL	CODE	AGE	SEX	HEIGHT(cm)	WEIGHT (kg)	BMI(KG/M2)
1	FPD-01	68	M	162.56	65	24.60
2	FPD-02	63	M	167.64	62	22.06
3	FPD-03	65	M	165.10	61	22.38
4	FPD-04	63	M	172.72	72	24.13
5	FPD-05	64	M	165.10	58	21.28
6	FPD-06	69	M	170.18	71	24.52
7	FPD-07	63	M	167.64	62	22.06
8	FPD-08	71	M	172.72	61	20.45
9	FPD-09	63	M	175.26	72	23.44
10	FPD-10	65	M	157.48	58	23.39
11	FPD-11	68	M	167.64	71	25.26
12	FPD-12	63	M	170.18	56	19.34
13	FPD-13	68	M	162.56	64	24.22
14	FPD-14	64	M	160.02	54	21.09
15	FPD-15	68	M	160.02	61	23.82
16	FPD-16	65	M	157.48	57	22.98
17	FPD-17	68	M	162.56	58	21.95
18	FPD-18	63	M	170.18	71	24.52
19	FPD-19	65	M	172.72	56	18.77
20	FPD-20	66	M	160.02	58	22.65
21	FPD-21	63	M	160.02	71	27.73
22	FPD-22	64	M	152.40	56	24.11
23	FPD-23	65	M	165.10	64	23.48
24	FPD-24	65	M	172.72	54	18.10
25	FPD-25	68	M	165.10	61	22.38
26	FPD-26	64	M	172.72	72	24.13
27	FPD-27	65	M	167.64	70	24.91
28	FPD-28	72	M	165.10	63	23.11
29	FPD-29	63	M	170.18	70	24.17
30	FPD-30	65	M	170.18	73	25.21
31	FPD-31	64	M	172.72	70	23.46
32	FPD-32	63	M	154.94	60	24.99
33	FPD-33	67	M	165.10	62	22.75
34	FPD-34	71	M	157.48	61	24.60
35	FPD-35	63	M	165.10	62	22.75
36	FPD-36	65	M	167.64	59	20.99
37	FPD-37	68	M	167.64	71	25.26
38	FPD-38	64	M	157.48	62	25.00
39	FPD-39	65	M	175.26	59	19.21

DNA SERIAL	CODE	AGE	SEX	HEIGHT(cm)	WEIGHT (kg)	BMI(KG/M2)
40	FPD-40	68	M	157.48	62	25.00
41	FPD-41	63	M	167.64	62	22.06
42	FPD-42	64	M	167.64	61	21.71
43	FPD-43	65	M	172.72	72	24.13
44	FPD-44	67	M	167.64	58	20.64
45	FPD-45	63	M	160.02	71	27.73
46	FPD-46	64	M	172.72	62	20.78
47	FPD-47	68	M	165.10	61	22.38
48	FPD-48	67	M	165.10	68	24.95
49	FPD-49	63	M	162.56	66	24.98
50	FPD-50	64	M	157.48	65	26.21
51	FPD-51	71	M	170.18	64	22.10
52	FPD-52	63	M	165.10	62	22.75
53	FPD-53	68	M	172.72	68	22.79
54	FPD-54	65	M	167.64	61	21.71
55	FPD-55	63	M	165.10	54	19.81
56	FPD-56	68	M	170.18	55	18.99
57	FPD-57	64	M	170.18	59	20.37
58	FPD-58	68	M	172.72	56	18.77
59	FPD-59	63	M	154.94	60	24.99
60	FPD-60	71	M	167.64	67	23.84
61	FPD-61	63	M	157.48	69	27.82
62	FPD-62	68	M	175.26	63	20.51
63	FPD-63	65	M	157.48	57	22.98
64	FPD-64	63	M	167.64	67	23.84
65	FPD-65	68	M	167.64	59	20.99
66	FPD-66	65	M	157.48	67	27.02
67	FPD-67	64	M	175.26	72	23.44
68	FPD-68	65	M	157.48	64	25.81
69	FPD-9	68	M	167.64	65	23.13
70	FPD-70	64	M	172.72	56	18.77
71	FPD-71	66	M	165.10	55	20.18
72	FPD-72	68	M	170.18	54	18.65
73	FPD-73	63	M	167.64	65	23.13
74	FPD-74	63	M	172.72	74	24.81
75	FPD-75	65	M	175.26	58	18.88
76	FPD-76	71	M	157.48	56	22.58
77	FPD-77	63	M	167.64	65	23.13
78	FPD-78	71	M	170.18	55	18.99
79	FPD-79	63	M	162.56	65	24.60
80	FPD-80	68	M	160.02	54	21.09
81	FPD-81	65	M	162.56	70	26.49

DNA SERIAL	CODE	AGE	SEX	HEIGHT(cm)	WEIGHT (kg)	BMI(KG/M2)
82	FPD-82	63	M	157.48	56	22.58
83	FPD-83	64	M	162.56	57	21.57
84	FPD-84	65	M	157.48	55	22.18
85	FPD-85	66	M	154.94	67	27.91
86	FPD-86	63	M	160.02	65	25.38
87	FPD-87	69	M	167.64	56	19.93
88	FPD-88	70	M	172.72	71	23.80
89	FPD-89	63	M	170.18	56	19.34
90	FPD-90	69	M	162.56	58	21.95
91	FPD-91	63	M	170.18	56	19.34
92	FPD-92	64	M	170.18	66	22.79
93	FPD-93	65	M	165.10	55	20.18
94	FPD-94	63	M	160.02	62	24.21
95	FPD-95	71	M	152.40	60	25.83
96	FPD-96	63	M	170.18	51	17.61
97	FPD-97	68	M	147.32	54	24.88
98	FPD-98	64	M	149.86	61	27.16
99	FPD-99	64	M	152.40	65	27.99
100	FPD-100	68	M	157.48	50	20.16

2.1.2. VOLUNTEER CONSENT FORM

Study number:

Patient or control Identification for this study:

Consent Form

You are being asked to participate in a clinical study. Your decision to take part in this study is strictly voluntary and you are under no obligation to participate. If you decide not to participate or if you choose to withdraw after beginning the study, you will not lose any benefits associated with your medical care. You are encouraged to ask questions before deciding whether you wish to participate as volunteer during this research work. Your identity will be kept confidential throughout. Information will not be associated with participant's name. The research staff will use only a coded number, access will be limited to authorized scientists and any scientific publications, lectures or reports resulting from the study will not identify participant by name.

Title of Research

CYP3A4 AND CYP3A5 GENETIC POLYMORPHISMS AND RISK OF PROSTATE CANCER

Investigator(s)

Principal Investigator:
Department of Clinical Pharmacy and Pharmacology
Faculty of Pharmacy
University of Dhaka

Purpose of the Project

You are being asked to be a volunteer or patients under investigation in a pharmacogenetic study involving the collection of your blood sample from where DNA will be extracted which will be used in the study and excess DNA will be stored for future research. The purpose of this study is to characterize the genotypes of CYP3A4 and CYP3A5 in Bangladeshi

peoples, which will be helpful for the adjustment of dosage regimen, reduce the serious adverse reactions to ensure safe, effective and economic treatment.

Possible Benefits of Participation

Although there may not be any direct benefits to the participant by participating at this stage, family members and future generations may benefit if the researchers succeed. The identification of the genes, and their genetic variants, could in the end lead to the development of methods for prevention, curing or alleviating disease conditions as well as optimizing dose of drugs; In the unlikely event that the research may lead to the development of commercial applications.

Costs

There will be no payment to you for participating in this study. You will not receive any compensation or wages associated with loss of time at your work place.

Risks:

There are no more than minimal medical or psychological risks associated with this study. The participant may feel some pain associated with having blood withdrawn from a vein and may experience discomfort, bruising and/or slight bleeding at the site.

Permission for further studies:

Before the participant's material is used in further projects in the future, the written approval of the, Ahsania Mission Cancer and General Hospital, Dhaka Medical College Hospital and Bangabandhu Sheikh Mujib Medical University (PG Hospital), Dhaka, Bangladesh will be obtained.

VOLUNTEER CONSENT FORM

I, the undersigned, authorize the research student to consider me as a volunteer/patient for his/ her research work. I understand that I can change my mind at any time to withdraw myself as volunteer during this research work.

Volunteer consent to study treatment

Please tick as appropriate

1. Do you have complete idea about the type, ultimate goal and methodology of the research?	Yes	No
2. Are you aware that you don't have to face any physical, mental and social risk for this?	Yes	No
3. There will be no chance of injury in any of your organs; are you aware of this?	Yes	No
4. Have you got any idea about the outcome of this experiment?	Yes	No
5. Have you decided intentionally to participate in this experiment?	Yes	No
6. Do you think this experiment violate your human rights?	Yes	No
7. Are you sure that all the information regarding you will be kept Confidentially?	Yes	No
8. No remuneration will be provided for this experiment, are you aware of this?	Yes	No

After reading the above mentioned points, I am expressing my consent to participate in this experiment as a **volunteer**.

Volunteer signature and Date:_____

Volunteer's Name:_____

Address:_____

Witness:_____

Please return the signed copy to the research student and keep an extra copy for yourself.

_____

Signature of the Researcher

Department of Clinical Pharmacy and Pharmacology

Faculty of Pharmacy

University of Dhaka

DATA COLLECTION FORM

Questionnaires

(CYP3A4 AND CYP3A5 GENETIC POLYMORPHISMS AND RISK OF PROSTATE CANCER)

1. Identification

1.1 I.D. Code: | | | | | | | | | | | | | | | | |

1.2 Name:

1.3 Father's/ Husband's Name:

1.4 Sex:

Male		**Female**	

1.5 Marital Status:

Married		**Unmarried**	

1.6 Date of Birth (dd/mm/yy):

1.7 Age (yr):

1.8 Mailing address:

Vill.: P.O.:

Thana: Dist.:

1.9 Permanent address:

Vill.: P.O.:

Thana: Dist.:

1.10 Telephone No.:

1.11 Religion:

1.12 Nationality:

2. Personal History

2.1 Area of residence:

	Rural	**Urban**	**S-Urban**	**Others**
⁎ Where have you spent your boyhood (1-15 y)?				
⁎ Where have you spent at least ¾th or more of your life time?				

2.2 Education level:

	Illiterate		SSC or equivalent
	Can read only		HSC or equivalent
	Can write a letter		Graduate or higher
	Other		

2.3 Occupation:

	Student		Unemployed
	Professional		Housewife
	Business		Skilled worker
	Technical		Other

2.4 Family expense per month: _____

2.5 Impression about social class:

	Rich		Lower middle
	Upper middle		Poor

2.6 Smoking habit:

	Never		Ex-smoker		Current smoker

2.7 Food Habit (24 hours recall method):

Morning	
Lunch	
Afternoon	
Dinner	

3. Biophysical Characteristics

3.1 Height (cm):		**3.4** Temperature (^{0}F):	
3.2 Weight (kg):		**3.5** BP (Sys/Dias):	
3.3 Pulses/min:			

4. Medical history:

⁜ Previous history of hypersensitivity effect to any of the Macroloid:	Yes	No
⁜ Taking any medication within 30 days prior to experiment:	Yes	No
⁜ Previous history of renal dysfunction:	Yes	No
⁜ Previous history of hepatic dysfunction:	Yes	No
⁜ Any kind of GIT problem:	Yes	No
⁜ Type of prostate cancer : i) Benign prostate cancer	Yes	No
ii) Malignant prostate cancer	Yes	No

5. Medical Test Results:

5.1 Chest X-ray:	
5.2 Tumour Markar:	
- PSA (normal range: 4.0 ng/ml)	
5.3 Kidney Function Test:	
- Serum Creatinine	
- Creatinine Clearance	

Investigated by:

Name: Signature: Date:

2.2. MATERIALS

2.2.1. INSTRUMENTS

Instruments	**Sources**
UNIVERSAL 240V 50i60Hz Refrigerated Bench-Top Centrifuge	Hettich GmbH & Co., Germany
MJ Mini Gradient Thermal Cycler	Bio-Rad Laboratories, USA
Alpha Imager® HP (Gel Doc. System)	Alpha Innotech Corporation, USA
Gel Electrophoresis Machine (Elite)	Wealtech, Germany
UV Probe V.2.1 Spectrophotometer	Shimadzu, USA
p^H Meter (Cyber Scan 500)	Eutech ,Singapore
Micro oven	Rangs, Bangladesh
Micropipette	Bio-Rad Laboratories, USA
Distillation Plant (Distinction D4000)	Bibby Sterlin Ltd., UK
Ultrapure Water System (Arium® 611)	Sartorius, Germany
Microcentrifuge Machine (Mikro 20)	Hettich GmbH & Co., Germany
Freeze (-40^0 C)	Siemens, USA
Freeze (-80^0 C)	DAIREI, Sweden
Vortex Mixer Machine (Rotamixer-9590)	Hook & Tucker Instruments Ltd., UK
Autoclave Machine	Yongfeng Enterprise Co., UK

2.2.2. CONSUMABLE MATERIALS

Materials	**Sources**
Reagent Bottle (250, 500, 1000 ml)	Schott GL-45, Germany
Conical Flasks	Schott GL-45, Germany
Pipettes (Precicolor)	HBG, Germany
Eppendorf Tube (1.5 ml)	Hamburg, Germany
Pipette Tips	ALA, USA
PCR Tubes (0.2/0.5 ml)	Bio-Rad Laboratories, USA
Falcon Tubes (50 ml)	Hamburg, Germany
Polypropylene Tubes (15 ml)	Hamburg, Germany

2.2.3. CHEMICALS AND REAGENTS

2.2.3.1. AGAROSE

Type	DNA Size (kbp)	Gel Strength (gm/cm^2)
HS	0.5-30	>2000 (1.5%)
H	1-200	>2800 (1.5%)
X	0.01-1	>1000 (3%)
1600	0.01-1	>1400 (1.5%)

2.2.3.2. OTHER REAGENTS

Reagents	Sources
Triton-X 100	Sigma Chemical Company, USA
Sodium Lauryl Sulphate	Sigma Chemical Company, USA
Ethanol	Sigma Chemical Company, USA
Chloroform	Sigma Chemical Company, USA
Sodium Perchlorate	Sigma Chemical Company, USA
Glacial Acetic Acid	Sigma Chemical Company, USA
Sodium Chloride	Sigma Chemical Company, USA
Sucrose	Sigma Chemical Company, USA
Magnesium Chloride	Sigma Chemical Company, USA
Tris	Roth, Germany
Tris-HCl	Sigma Chemical Company, USA
EDTA-Na_2	Sigma Chemical Company, USA
Nuclease Free Water	Promega Corporation, USA
Ethidium Bromide	BDH, UK
Boric Acid	Bio Basic Inc., Canada
Acryl Amide/Bisacrylamide 40% solution	Sigma Chemical Company, USA
Ammonium Persulfate (APS)	Sigma Chemical Company, USA
TEMED (N, N, N', N'-tetramethylethylenediamine)	Sigma Chemical Company, USA
Sample Loading Dye,6x	Promega Corporation, USA
Taq DNA Polymerase	NEB, USA
Standard reaction buffer	NEB, USA
$MgCl_2$ Solution	NEB, USA
Deoxynucleotide Solution Mix (dNTP)	NEB, USA
Quick-Load® 50 bp DNA Ladder	NEB, USA
Quick-Load® 2-Log DNA Ladder(0.1-10.0 kb)	NEB, USA
100 bp DNA Ladder	NEB, USA

2.2.4. RESTRICTION ENZYMES (REs)

Genes	RE	Recognition sites	Source
CYP3A4*1B	*Mbo*II	5'....GAAGA(N)$_8$↓....3' 3'....CTTCT(N)$_7$↑....5'	New England BioLabs® Inc., UK.
CYP3A5*3	*Rsa*I	5'....GT↓AC....3' 3'....CA↑TG....5'	New England BioLabs® Inc., UK.

→ **Cutting site**

2.2.5. BUFFERS: (Supplied with REs)

Buffer name	Composition	Applicable for enzymes
1X Buffer C	10 mM Tris-HCl 50 mM NaCl 10 mM $MgCl_2$ (p^H 7.9)	*RsaI*
1X NE Buffer 4	20 mM Tris-acetate 50 mM Potassium acetate 10 mM Magnesium Acetate 1 mM Dithiothreitol (p^H 7.9)	*MboII, RsaI*

2.2.6. SOLUTIONS

NAME	COMPOSITION
TAE Buffer (10x)	0.4 M Tris-Base (Tris (Hydroxymethyl)-amino methane), 11.4 %(v/v)/0.2 M Glacial acetic acid 0.01 M EDTA-Na_2, p^H to 7.6 with Glacial acetic acid or Tris.
TBE Buffer(10x)	0.89 M Tris-Base Boric acid, 20 mM EDTA-Na_2, p^H to 8.0 with Boric acid or Tris.
TE Buffer(1x)	10 mM (Tris-HCl (Tris (Hydroxymethyl) amino methane hydrochloride), 0.001M EDTA-Na_2, p^H to 8.0 with Tris.

2.3. METHODS

2.3.1. GENOMIC DNA ISOLATION

2.3.1.1. VENOUS BLOOD COLLECTION

After explanation and counseling about the study, volunteers were invited to participate in this study. All consent forms were preserved. Approximately 3 ml of whole blood was collected in a sterile appendorf tube containing ethylenediaminetetraacetic acid disodium (EDTA-Na_2) as anticoagulant and preservative. The freezing of blood samples was preferably avoided due to the decreased yield of DNA associated with the freezing process. Blood samples were, therefore, stored at 4ºC until the DNA isolation process could be performed within three to five days of collection. However, if the DNA isolation process was unable to proceed within this time period, then samples were frozen at -80ºC until the process could be carried out.

2.3.1.2. ISOLATION OF GENOMIC DNA FROM WHOLE BLOOD SAMPLES

There are many differing protocols and a large number of commercially available kits used for the extraction of genomic DNA from whole blood. Here Genomic DNA was isolated by using Daly's Method (Daly *et al.*, 1998). This procedure is routinely used in both research and clinical service provision in our laboratory and is cheap and robust.

2.3.1.3. PREPARATION OF DNA ISOLATION REAGENTS

Reagent name	Composition and preparation procedure	Storage condition
Cell Lysis Buffer (1L)	10 mM Tris-HCl, 320 mM Sucrose and 5 mM $MgCl_2$ was added to 850 ml of distilled water. p^H was adjusted to 8.0 by adding NaOH. Then it was autoclaved. 1% Triton X-100 was added to it and the total solution was made up to 1L by adding distilled water.	4°C
Nuclear Lysis Buffer (1L)	400 mM Tris-HCl, 60 mM EDTA-Na_2 and 150 mM Sodium chloride was added to 850 ml of distilled water. p^H was adjusted to 8.0 by adding NaOH. Then it was autoclaved. 1% Sodium lauryl sulphate was added to it and the total solution was made up to 1L by adding distilled water.	Room Temperature
5 M Sodium Perchlorate (100 ml)	61.22 gm of Sodium Perchlorate was dissolved in 100 ml distilled water	4°C
5 mM Tris HCl Buffer (250 ml)	0.197 gm of Tris HCl was added in 150ml of distilled water. p^H was adjusted to 8.0 by adding NaOH. The total solution was made up to 250 ml by adding distilled water. Then it was autoclaved.	4°C

2.3.1.4. GENOMIC DNA ISOLATION PROCEDURE

1. 3 ml blood was taken in a 50 ml Falcon centrifuge tube containing 2 mg of EDTA.
2. 20 ml Lysis Buffer was added to it. Then it was mixed gently for 2 minutes by inversion. It was then centrifuged for 10 minutes at 3000 rpm at 4°C by using UNIVERSAL 240V 50i60Hz Refrigerated Bench-Top Centrifuge Machine (Hettich GmbH & Co., Germany).
3. The supernatant was discarded into a bottle containing enough savlon. The pellet was collected.
4. 2 ml Nuclear Lysis Buffer and 0.5 ml of 5 M Sodium Perchlorate were added to it.
5. Then the tube was mixed in a rotary mixture at room temperature for about 15 min so that pellet was dissolved completely.
6. Then the sample tube was incubated at 65°C for 30 min. (Heidolph Unimax-2010 Incubator, Wolf Laboratories Limited, UK).
7. Then 2.5 ml of chilled Chloroform was added to it.
8. Then it was mixed in a rotary mixture for 10 min at room temperature.
9. Then the tube was centrifuged at 1500 rpm for 5 min. (37°C).
10. The DNA containing phase (uppermost phase) was transferred to a fresh autoclaved 15 ml polypropylene tube using a disposable Pasteur pipette.
11. Two volumes of Ethanol (double that of DNA phase) was added to it.
12. It was then mixed immediately by slow gentle inversion until all cloudiness was disappeared.
13. DNA was seen to come out of the solution as a white ‘cotton-wool’ pellet.
14. The white ‘cotton-wool pellet’ was collected with a disposable microbiology loop.
15. The loop was air dried.
16. The DNA was dissolved in 200 µl TE Buffer contained in a 1.5 ml screw cap tube.
17. Then the tube was kept at 65°C overnight.
18. Then it was taken back and was stored in Freezer.(-20°C)

2.3.2. QUANTIFICATION OF GENOMIC DNA

The quantity and purity of DNA isolated from blood samples were assessed by using a UV Spectrophotometer (UV Prove v2.1) at 260 nm. In order to ensure complete sample homogeneity, which is critical when measuring genomic DNA concentration and purity with this instrument, samples were very gently shaken on a vortex shaker for approximately 30 minutes before measurements were taken. A sample volume of 1.5 to 2 µl was pipetted onto the fibre optic measurement surface. Working solutions of genomic DNA were made up to a standard concentration of 50 ng/µl with Nuclease free water, except in cases where the sample had an initial concentration of less than 50 ng/µl, in which case an undiluted aliquot was taken as a working solution.

For calculation of DNA concentration of samples free of RNA, the following conversion factor is used: 1 OD260 = 50 mg of DNA/ml.

DNA concentration in µg/µl was calculated as follows:

$$\text{DNA Concentration (µg/µl)} = \frac{\text{OD 260} \times 50\ \text{(dilution factor)} \times 50\ \text{µg/ml}}{1000}$$

OD260/OD280 should be=1.7 -1.9. (OD= Optical density).

A value out of this range is not acceptable. It may indicate that the DNA sample is not in solution or that there are contaminants (i.e., protein) in the sample that may inhibit subsequent reactions

All working solutions of genomic DNA were stored at -20ºC until genotype analysis.

The purity and integrity of isolated genomic DNA were also assessed by means of agarose gel electrophoresis. A sample volume of 5 µl (50-70 ng/µl) was resolved on a 1% (w/v) agarose gel or 10% polyacrylamide gel .

2.3.3. GENOTYPING OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) OF CYP3A4*1B, CYP3A5*3

In order to facilitate the accurate genotyping of the patient's/volunteer's DNA samples for the selected SNPs, PCR-RFLP was employed due to its affordability, ease of use and reliability. This method of genotyping entails the restriction enzyme (REase) digestion of polymerase chain reaction (PCR) amplification product. The subsequent digestion or lack of digestion, of PCR amplification product due to the presence or absence of an SNP within the REase recognition site allows for accurate and reliable genotyping and the consequent determination of SNP frequencies within a sample cohort.

The classification of an SNP genotype as 'wild-type' or 'variant' was done according to accepted nomenclature and the relevant reference sequences available from the National Centre for Biotechnological Information (NCBI) Entrez Nucleotides Database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide) last accessed on 12th December, 2013.

2.3.3.1. DNA AMPLIFICATION USING PCR

The relevant genomic target regions, containing the SNPs of interest, were amplified by means of primer-directed PCR using thermostable DNA polymerase, as originally described by (Saiki *et al.,* 1985; Saiki *et al.*, 1988). This primer-directed PCR method facilitates the in vitro amplification of single-copy genomic DNA sequences by a factor of more than ten million with extremely high sequence specificity.

2.3.3.2. PRIMER DESIGN

There are some guidelines for primer design:

• PCR primers should be generally 15-30 nucleotides long.

• Optimal GC content of the primer is 40-60%. Ideally, C and G nucleotides should be distributed uniformly along the primer.

• Should avoid placing more than three G or C nucleotides at the 3'-end to lower the risk of non-specific priming.

• Should avoid primer self-complementarity or complementarity between the primers to prevent hairpin formation and primer dimerization.

• Should check for possible sites of non-desirable complementarity between primers and the template DNA.

• Differences in melting temperatures (Tm) of the two primers should not exceed 5°C.

By considering all the factors, the primers for the study were designed. The sequences of the primers used and their sizes are presented in Table 2.1

Table 2.1: Name of the allele, sequence of the designed primer with their size and melting point

NO	ALLELE	PRIMER SEQUENCE	M.T (°C)	SIZE (bp)
1	CYP3A4*1B **FP**	5'-GGAATGAGGACAGCCATAGAGACAAGGGGA-3'	69.5	30
2	CYP3A4*1B **RP**	5'-CCTTTCAGCTCTGTGTTGCTCTTTGCTG-3'	66.6	28
3	CYP3A5*3 **FP**	5'-CCTGCCTTCAATTTTTCACT-3’	58.0	20
4	CYP3A5*3 **RP**	5'- GGTCCAAACAGGGAAGAGGT-3’	65.0	20

FP=Forward Primer; RP=Reverse Primer; M.T=Melting Temperature

Primers are obtained from 1st BASE, Singapore.

2.3.3.3. PCR PARAMETERS AND CONDITIONS

Primers, GoTaq® DNA polymerase, Standard reaction buffer and dNTPs were used for the PCR amplification of the relevant genomic target regions, containing the SNPs of interest,

Master preparation for eight (8) samples: 20.0 µl of 10X standard reaction buffer, 4.0 µl of dNTPs (2.5 mM), 2.0 µl of each primer (10 µM), 1.0 µl of Taq DNA polymerase (5 U/µl), and 180.0 µl of Nuclease free water. Then transfer 25 µl of master preparation and 1.0 µl of genomic DNA (50-70 ng/µl) into PCR tube. PCR conditions to synthesize various CYP3A4 and CYP3A5 alleles with their respective lengths are given in Table 2.2

Table 2.2: PCR conditions to synthesize various CYP3A4 and CYP3A5 alleles and their respective lengths.

ALLELE	PCR CONTIONS (35 cycles)	SIZE OF PCR PRODUCTS(bp)
CYP3A4*1B	94°C 1 min	385
	57°C 1 min	
	72°C 1 min	
CYP3A5*3	94°C 1 min	196
	59°C 1 min	
	72°C 1 min	

2.3.3.4. RESTRICTION ENZYME DIGESTION

Preparation of restriction enzyme mixture for ten (10) samples: 1 µl of restriction enzyme (e.g, *Mbo*I *or Rsa*I*),* 10 µl of enzyme buffer, 90 µl of Nuclease free water. After PCR amplification, 10 µl of the PCR products (for CYP3A4*1B or CYP3A5*3) were digested with 10 µl of restriction enzyme mixture *(e.g, Mbo*I *or Rsa*I) obtained from New England Biolabs®, USA. Incubation conditions are listed in Table 2.3. Electrophoreses was done for the digested products using 3% agarose gel or in 10% Polyacrylamide gel.

Table 2.3: The restriction enzymes, digestion condition and length of the expected fragments on digestion to diagnose various CYP3A4 and CYP3A5 alleles

Allele	REs	Digestion conditions	Expected fragments (bp)	References
CYP3A4*1B	*Mbo*II (5000 U/ml)	Incubation at 37° C overnight	**NH** 175, 169, 41 **HE** 210, 175, 169, 41 **MH** 210, 175	Rais et al.,2006
CYP3A5*3	*Rsa*I (5000 U/ml)	Incubation at 37° C overnight	**NH** 94, 102 **HE** 20, 74, 94, 102 **MH** 20, 74, 102	Rais et al.,2006

NH: Normal Homozygote; HE: Heterozygote; MH: Mutant Homozygote

2.3.4 VISUALIZATION OF PCR PRODUCTS AND REase DIGESTION FRAGMENTS

PCR amplification products were visualised by means of agarose gel electrophoresis in order to allow for size estimation and thus confirmation of amplification of the desired genomic target region. REase digestion fragments that were of sufficient size (>100 bp) and size differential between fragments (>30 bp) were also visualized on agarose gel. REase digestion fragments were also visualized on Polyacrylamide gel. EZ Load™ Molecular ruler (100 bp) was used for size estimation of PCR amplification products, which served as confirmation that amplification of the desired genomic target region had occurred, as well as for quantification of PCR product prior to REase digestion reactions. EZ Load™ 50 or 100 bp DNA ladder was also used for size estimation of all REase digestion fragments, allowing for accurate and reliable genotyping of samples. EZ Load™ 50 or 100 bp DNA ladder is thus evident in lane 1 or any other marked position of all agarose and Polyacrylamide gel photos. All agarose and Polyacrylamide gels were visualised under ultraviolet (UV) light and photographed with a Gel Documentation and Analysis System.

2.3.4.1. GEL ELECTROPHORESIS

Electrophoresis is a method of separating substances based on the rate of movement while under the influence of an electric field. Agarose gel electrophoresis of DNA is used to determine the presence and distinguish the type of nucleic acids obtained after extraction and to analyze digestion products. Desired DNA fragments can be physically isolated for various purposes such as sequencing, probe preparation, or for cloning fragments into other vectors. Agarose gels are used for DNA analysis. Typically agarose gels are used for most purposes and polyacrylamide gels are used when small fragments, such as digests of 16S rRNA genes, are being distinguished. Regular agarose gels may range in concentration from 0.6 to 3.0%.

Agarose is a polysaccharide purified from seaweed. An agarose gel is created by suspending dry agarose in a buffer solution, boiling until the solution becomes clear, and then pouring it into a casting tray and allowing it to cool. The result is a flexible gelatine-like slab. During electrophoresis, the gel is submersed in a chamber containing a buffer solution and a positive and negative electrode. The DNA to be analyzed is forced through the pores of the gel by the electrical current.

Under an electrical field, DNA will move to the positive electrode (red) and away from the negative electrode (black). Several factors influence how fast the DNA moves, including; the strength of the electrical field, the concentration of agarose in the gel and most importantly, the size of the DNA molecules. Smaller DNA molecules move through the agarose faster than larger molecules. DNA in the gel will be visualized by the use of Ethidium Bromide, added to the gel. Ethidium bromide binds to DNA and illuminates when exposed to ultraviolet light, causing the DNA to 'glow'.

All PCR products were resolved by electrophoresis in 2% (w/v) agarose gel at 80 volts (V). The REase digestion fragments were also observed in 3% (w/v) agarose gel or 10% Polyacrylamide gel. The REase digestion fragments were resolved at 80 V for agarose gel and 150 V for Polyacrylamide gel, so as to ensure sufficient resolution to allow for accurate genotyping.

2.3.4.2. AGAROSE GEL ELECTROPHORESIS PROCEDURE

All agarose gels were made with and resolved in 1X tris acetate ethylenediaminetetraacetic acid (TAE) buffer, which was made and stored as a 10X stock solution and diluted to the required working concentration as was needed. In order to facilitate the visualization of DNA within the agarose gel under UV light, 1 µg of ethidium bromide (EtBr) per ml agarose solution was added -i.e. 0.01% (v/v) EtBr stock solution (10 mg/ml).

PROCEDURE:

A. Casting a gel

1. An appropriate volume of 1X Tris-acetate-EDTA (TAE) buffer with an appropriate amount of agarose (these values are determined based on the gel dimensions and the desired percentage of agarose) was mixed in a conical flask. The flask was swirled to evenly distribute the agarose.
2. The solution was then heated in the microwave oven for 1 minute. Protective gloves were worn and the flask was removed from the microwave oven (before it boiled over), swirled, and reheated while keeping constant watch to be sure it did not boil over. When it started to boil, boiling was stopped and swirled again repeating the process until all of the agarose went into solution.
3. The flask was allowed to cool. The gel was poured when the temperature of the solution was 55-65° C.

4. The gel apparatus was prepared for casting the gel while the agarose was cooling.
5. Prior to pouring the gel, Ethidium bromide was added to the dissolved agarose and swirled to mix.
7. The gel was poured into the casting tray and the comb was adjusted to keep the wells perpendicular. The gel was allowed to cool and was hardened (20-30 minutes) prior to use.

B. Preparing the gel for electrophoresis

1. A few ml of 1X TAE buffer was added to the well area of the gel and the comb was carefully removed by pulling straight up.
2. The electrophoresis tank was filled with buffer solution (1X TAE) and the gel was placed (In the casting tray) on the tank platform.

C. Preparing samples for loading/running the gel

1. An appropriate volume of loading dye (6X) was added to the sample (1 µl of 6X sample dye for every 5 µl of sample).
2. The sample was loaded using a 1-10 µl micropipette. The marker was also loaded at Lane-1.
3. After the gel had been loaded, the cover was gently placed on the apparatus and the Power leads were hooked up. The power was adjusted to 80 volts (constant voltage). The gel was run until the first dye front (bromophenol blue) had migrated about two-thirds the length of the gel and the second dye front (xylene cyanol) had migrated approximately one-third of the length of the gel.
4. The power was turned off before removing the gel for photographing.
5. The gel was placed on the UV transilluminator to visualize the DNA.

2.3.4.3. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) PROCEDURE

The REase digestion fragments of CYP3A4 and CYP3A5 were also resolved on vertical, non-denaturing 10% (w/v) PAA gel at 150 V. 10% (w/v) PAA gels were made from a mixture containing 16 ml PAA (5% cross-linkage -acrylamide (AA): bisacrylamide (BAA) ratio of 29:1; 30% (w/v)), 5 ml 10X TBE buffer, and 500 µl of 10% (w/v) Ammonium persulphate (APS, $H_8N_2O_8S_2$), 50 µl of N, N, N', N'-tetramethylethylenediamine (TEMED) and 29 ml dH_20 was added subsequently.

PROCEDURE:

1. Water, Acryl amide solution and TBE solution were carefully mixed in a 50 ml Falcon tube. Then APS and TEMED were added and mixed.
2. The gel plate was prepared by washing with 70% alcohol and the two sides were sealed and the bottom spacers were also sealed with Vaseline.
3. The solution was then poured between prepared glass plates.
4. The comb was carefully inserted to form the gel. Polymerization took about 15 minutes.
5. When set, the comb was removed and unpolymerised Acryl amide was washed off.
6. The gel was put into the running tank, with the smaller plate on the inside and 1X TBE buffer was added to the top and bottom reservoirs as required to set up the apparatus.
7. The air bubble that forms between the plates at the bottom of the gel was removed using a syringe.
8. Loading buffer was added to the samples (5µl per 20µl of sample) and the gel was loaded.
9. Electrophoresis was performed in 1X TBE buffer for approximately 3 hours.
10. In order to facilitate the visualisation of DNA within the PAA gel under UV light, gels were soaked for approximately 10 minutes in 1X TBE buffer containing 1 µg of EtBr per ml-i.e. 0.01% (v/v) EtBr stock solution (10 mg/ml). Gels were subsequently destained by soaking in dH_2O for approximately 3 minutes.

2.4. STATISTICAL ANALYSIS

Distributions of demographic variables were compared between cases and controls using χ2- tests and two-sided unpaired t-tests. Genotype and allelic frequencies were reported as percentage. The distribution of genotype frequency was also compared by χ2- test. Unconditional logistic regression was used to estimate crude odds ratio (OR) and their 95% confidence intervals (CIs) using the statistical software package SPSS version 20.0 (SPSS, Inc., Chicago, IL).

CHAPTER THREE

RESULTS AND DISCUSSIONS

III

3.1. RESULT

3.1.1. CASES AND CONTROLS CHARACTERISTICS

The distributions of demographic characteristics and clinical data among study subjects are summarized in Table 3.1. This case-control study consisted of 100 prostate cancer cases and 100 controls. The related factors such as age and smoking history of cases and controls were compared to confirm the observed effects were solely due to the genotype frequency. There were no significant differences in mean age (p = 0.776) and smoking status (p=0.788) between the two groups.

3.1.2. SMOKING STATUS

The observed never smoking rate was 7% in the cases and 8% in controls. Among the smoker 65% and 21% were current smokers & ex-smokers in cases and 69% and 20% were current smokers & ex-smokers in controls, respectively and 7% & 3% were chewing tobacco in cases and controls respectively. There is no significant difference between current smoker, ex-smoker, never smokers & chewing tobacco groups between cases and controls (p=0.788) (Table-3.1).

Table 3.1: Distribution of demographic variables of the prostate cancer patients and controls

Variables	Cases (n=100) (%)	Controls (n=100) (%)	P-value
Age(years)			
Mean age, n(±SD)	66(±2.57)	66(±2.73)	0.776[a]
Range	63-72	62-72	
Smoking status, n (%)			
Current smoker	65	69	
Ex-smoker	21	20	
Chewing tobacco	7	3	
Never smoker	7	8	
Total tobacco user	93	92	0.788[b]
Total tobacco nonuser	7	8	

[a]Unpaired t test, [b]χ^2 test.

3.2. GENOMIC DNA EXTRACTION

From 100 prostate cancer patients and 100 healthy volunteers, genomic DNA was successfully isolated by Daly's method (Daly et al., 1998). The purity of the DNA and their concentrations were measured by SHIMADZU UV-Spectrophotometer at 260 nm. The purity (OD 260/OD 280) of all the DNA samples was found to be in the range between 1.7 to 1.9 and the average concentration was found to be 50 to 70 µgm/ml. Agarose gel electrophoresis was done for all the DNA samples.

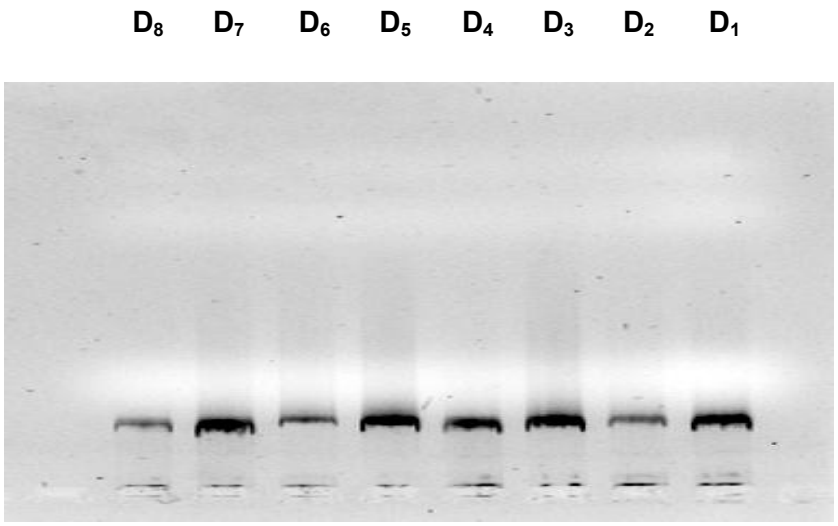

Figure 3.1. Agarose gel electrophoresis (1% w/v agarose) of genomic DNA (DNA No. 1 to 8).

3.3. GENOTYPING OF CYP3A4 GENES

The genotyping of CYP3A4 gene in Bangladeshi population was done by using a polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method.

3.3.1. PCR-RFLP of CYP3A4*1B (rs2740574)

GGAATGAGGACAGCCATAGAGACAAGGGGARGAGAGRGGCGATTTAATAGATTTATGC
CAATGGCTCCACTTGAGTTTCTGATAAGAACCCAGAACCCTTGRACTCCCCAGTAACATT
GAYTGAGTTGTTTRTGATACCTCATAGAATATGAACTCAAAGGAGGTCAGTGAGTGGTG
TGTGTGTGATTCTTTGCCAACTTCCMAGGTGGAGAAGCTTCCAACTGCAGGCAGAGCAC
AGGTGGCCCTGCTACTGGCTGCAGCTCCGCCTGCCTCCTTCTCTAGCATATAAACAATC
CVACAGCCTCACTGAATCACTGCTGGCAGGGCAGGAAAGCTCCATGCACATAGCCCAC
AAAGAGCAACACGAGCTGAAAGG

DARK RED----------> **PRIMER SEQUENCE**

GREEN **----------> OTHER POSSIBLE SNPs**

RED **----------> EXON SEQUENCE**

YELLOW **----------> SNP OF INTEREST**

By using the appropriate pair of primers and other PCR reaction program parameters, the PCR product of CYP3A4*1B was obtained. The PCR product size was 385 bp- and the PCR product was visualized in 2% (w/v) agarose gel.

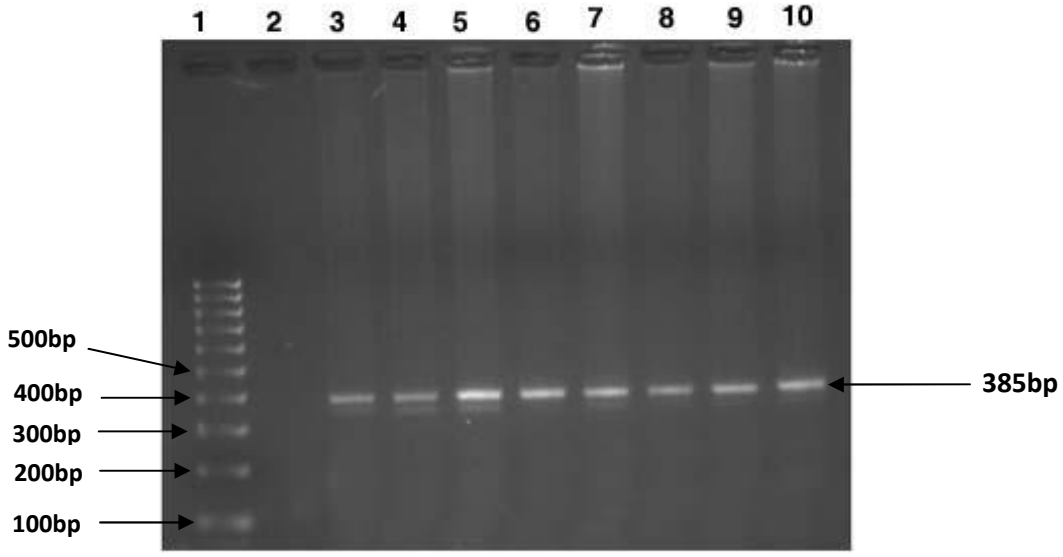

Figure 3.2. PCR product of CYP3A4*1B (385 bp) (Lane 3 to 10) (Lane-1 contains Molecular ruler; Lane-2 contains blank).

Fragmentation Pattern of CYP3A4*1B (rs2740574):

The PCR product was digested with *Mbo*II. The fragments were visualized in agarose gel (3%) or Polyacrylamide gel (10%).

Table 3.2. Name of the restriction enzyme with its sites of digestion in case of CYP3A4*1B

Restriction enzyme	Sites of digestion
*Mbo*II	5'….GAAGA(N)$_8$↓….3' 3'….CTTCT(N)$_7$↑….5'

⟶ **Cutting site**

Table 3.3. Type of nucleotide changes, cutting sites and fragments of the allele in case of CYP3A4*1B (Rais *et al.,* 2006)

SNP	Cutting site	Fragments	Type
When R=A In both chromosome	41, 210	41,169,175	Normal Homozygote
When R=G In one chromosome	41, 210	41,169,175, 210	Heterozygote
When R=G In both chromosome	210	210,175	Mutant Homozygote

When R=A in both chromosomes: (Normal Homozygote)

There are two cutting sites in both chromosomes (41,210). So we will get 3 fragments for each chromosome.

41

GAAATGAGGACAGCCATAGAGACAAGGGGAAGAGAGRGGCGATTTAATAGATTTTAT
GCCAATGGCTCCACTTGAGTTTCTGATAAGAACCCAGAACCCTTGRACTCCCCAGTAA
CATTGAYTGAGTTGTTTRTGATACCTCATAGAATATGAACTCAAAGGAGGTCAGTGAG
TGGTGTGTGTGTGATTCTTTGCCAAC

210

TTCCMAGGTGGAGAAGCCTCTTCCAACTGCAGGCAGAGCACAGGTGGCCCTGCTACT
GG CTGCAGCTCCAGCCCTGCCTCCTTCTCTAGCATATAAACAATCCVACAGCCTCACT
GAATCACTGCTGTGCAGGGCAGGAAAGCTCCATGCACATAGCCCAGMAAAGAGCAA
CACAGAGCTGAAAGG

→ Cuting site

Yellow--- *Mbo*II Recgnition site

GAAATGAGGACAGCCATAGAGACAAGGGGAAGAGAGRGGCGA
(Fragment: 1=41 bp)

ATTTAATAGATTTTATGCCAATGGCTCCACTTGAGTTTCTGATAAGAACCCAGAACCCT
TGRACTCCCCAGTAACATTGAYTGAGTTGTTTRTGATACCTCATAGAATATGAACTCAA
AGGAGGTCAGTGAGTGGTGTGTGTGTGATTCTTTGCCAACTTCCMAGGTG
(Fragment: 2=169 bp)

AGAAGCCTCTTCCAACTGCAGGCAGAGCACAGGTGGCCCTGCTACTGG CTGCAGCTC
CAGCCCTGCCTCCTTCTCTAGCATATAAACAATCCVACAGCCTCACTGAATCACTGCT
GTGCAGGGCAGGAAAGCTCCATGCACATAGCCCAGMAAAGAGCAACACAGAGCTGA
AAGG
(Fragment: 3=175 bp)

When R=G In one chromosome: (Heterozygote)

We will get 2 cutting site (41, 210) for one chromosome, for the polymorphic Chromosome we will get only one cutting site (210).

Not *Mbo*II recognition site

GAAATGAGGACAGCCATAGAGACAAGGGGAGGAGAGRGGCGATTTAATAGATTTTAT
GCCAATGGCTCCACTTGAGTTTCTGATAAGAACCCAGAACCCTTGRACTCCCCAGTAA
CATTGAYTGAGTTGTTTRTGATACCTCATAGAATATGAACTCAAAGGAGGTCAGTGAG
TGGTGTGTGTGTGATTCTTTGCCAAC

210

TTCCMAGGTGGAGAAGCCTCTTCCAACTGCAGGCAGAGCACAGGTGGCCCTGCTACT
GG CTGCAGCTCCAGCCCTGCCTCCTTCTCTAGCATATAAACAATCCVACAGCCTCACT
GAATCACTGCTGTGCAGGGCAGGAAAGCTCCATGCACATAGCCCAGMAAAGAGCAA
CACAGAGCTGAAAGG

→ Cuting site
Yellow--- *Mbo*ll Recgnition site

GAAATGAGGACAGCCATAGAGACAAGGGGAAGAGAGRGGCGATTTAATAGATTTTAT GCCAATGGCTCCACTTGAGTTTCTGATAAGAACCCAGAACCCTTGRACTCCCCAGTAA CATTGAYTGAGTTGTTTRTGATACCTCATAGAATATGAACTCAAAGGAGGTCAGTGAG TGGTGTGTGTGTGATTCTTTGCCAACTTCCMAGGTG
(Fragment: 1= 210 bp)

GAGAAGCCTCTTCCAACTGCAGGCAGAGCACAGGTGGCCCTGCTACTGG CTGCAGC TCCAGCCCTGCCTCCTTCTCTAGCATATAAACAATCCVACAGCCTCACTGAATCACTG CTGTGCAGGGCAGGAAAGCTCCATGCACATAGCCCAGMAAAGAGCAACACAGAGCT GAAAGG
(Fragment: 2= 175 bp)

When R=G In both chromosomes:
We will get 1 cutting site (210) for both the chromosomes. So we will get two fragments for each chromosome.
Fragment-1: 210 bp
Fragment-2: 175 bp

Observed Results of CYP3A4*1B (rs2740574):

Restriction enzyme digestion products were visualized in agarose (3%).

Table 3.4. Name of the allele, PCR product size, restriction enzyme, length of the expected and observed fragments on digestion in case of CYP3A4*1B

Allele Name	PCR Product Size (bp)	RE	Expected Fragments (bp)	Observed Fragments (bp)
CYP3A4*1B	385	*Mbo*II	**NH** 175, 169, 41 **HE** 210, 175, 169, 41 **MH** 210, 175	**175, 169, 41** **210, 175, 169, 41** **210, 175**

NH: Normal Homozygote; **HE:** Heterozygote; **MH:** Mutant Homozygote

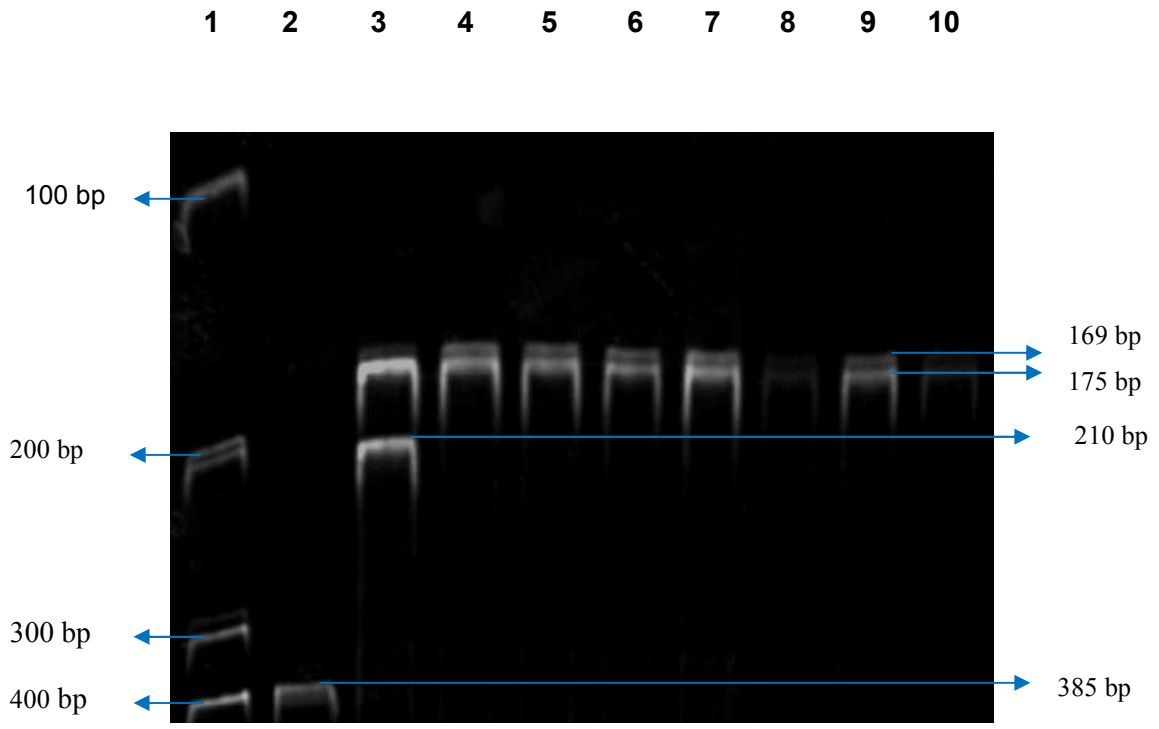

Figure 3.3: Restriction Enzyme (*Mbo*II) digestion fragment of CYP3A4*1B (Lane 3 to 10) (10% Polyacrylamide gel). Lane-1, Molecular ruler; Lane-2, uncut PCR product (385 bp); Lane-3, *1B heterozygote (210, 175, 169, 41 bp); Lane-4 to 10, *1B normal homozygote (175, 169, 41 bp)

3.4. GENOTYPING OF CYP3A5 gene

The genotyping of CYP3A5 gene in Prostate cancer patients and controls was done by using a polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method.

3.4.1. PCR-RFLP OF CYP3A5*3 (rs776746)

CCTGCCTTCAATTTTTCACTGACCTAATATTCTTTTTGATAATGAAGTATTTTAAACATAKA
AAACATTATGGAGAGTGGCA9TAGGAGAKACCCACGTATGTACCACCCAGCTTAACGAA
TGCTCTACTGTCATTTCTAACCATAATCTCTTTAAAGAGCTCTTTTGTCTTTCARTACCTC
TTCCCTGTTTGGACC

Primer sequence Possible SNPs

Exon sequence SNP of interest

By using the appropriate pair of primer and other PCR reaction program parameters the PCR product of CYP3A5*3 was obtained. The PCR product size was 196 bp. The PCR product was visualized in 2% (w/v) agarose gel.

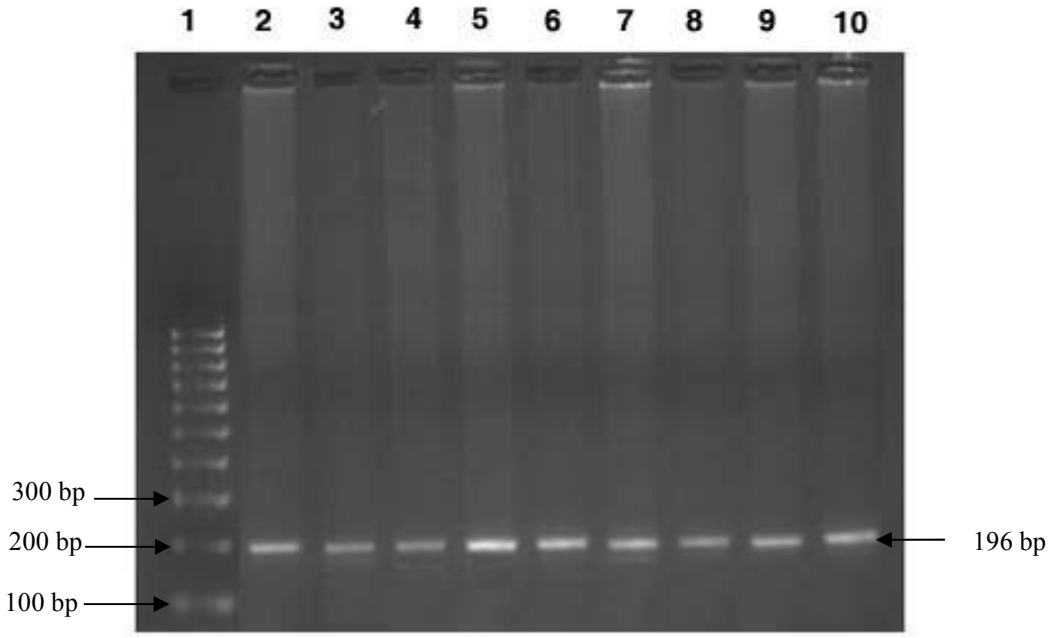

Figure 3.4. PCR product of CYP3A5*3 (196 bp) (Lane 2 to 10)
(Lane-1 contains Molecular ruler)

Fragmentation Pattern of CYP3A5*3 (rs776746):

The PCR product was digested with *Rsa*I. The fragments were visualized in agarose gel (3%) or Polyacrylamide gel (10%).

Table 3.5. Name of the restriction enzyme with its sites of digestion in case of CYP3A5*3

Restriction enzyme	Sites of digestion
*Rsa*I	5'….GTAC….3' 3'….CATG….5'

→**Cutting site**

Table 3.6. Type of nucleotide changes and fragments of the allele in case of CYP3A5*3 (Rais *et al.,* 2006)

SNP	Fragments	Type
When R=A In both chromosome	94, 102	Normal Homozygote
When R=G In one chromosome	20,74,94,102	Heterozygote
When R=G In both chromosome	20,74,102	Mutant Homozygote

When R=A in both of the sister chromosomes: (NORMAL HOMOZYGOTE)

CCTGCCTTCAATTTTTCACTGACCTAATATTCTTTTTGATAATGAAGTATTTTAAACATAKA
AAACATTATGGAGAGTGGCA9TAGGAGAKACCCACGTATGT**=102bp**
ACCACCCAGCTTAACGAATGCTCTACTGTCATTTCTAACCATAATCTCTTTAAAGAGCTC
TTTTGTCTTTCARTACCTCTTCCCTGTTTGGACC=94bp

When R=G in one of the sister chromosomes: (HETEROZYGOTE)

CCTGCCTTCAATTTTTCACTGACCTAATATTCTTTTTGATAATGAAGTATTTTAAACATAKA
AAACATTATGGAGAGTGGCA9TAGGAGAKACCCACGTATGT**=102**

ACCACCCAGCTTAACGAATGCTCTACTGTCATTTCTAACCATAATCTCTTTAAAGAGCTC
TTTTGTCTTTCAGTACCTCTTCCCTGTTTGGACC=94

ACCACCCAGCTTAACGAATGCTCTACTGTCATTTCTAACCATAATCTCTTTAAAGAGCTC
TTTTGTCTTTCAGT=74

ACCTCTTCCCTGTTTGGACC=20

When R=G in both of the sister chromosomes: (MUTANT HOMOZYGOTE)

CCTGCCTTCAATTTTTCACTGACCTAATATTCTTTTTGATAATGAAGTATTTTAAACATAKA
AAACATTATGGAGAGTGGCA9TAGGAGAKACCCACGTAT**GT=102**

ACCACCCAGCTTAACGAATGCTCTACTGTCATTTCTAACCATAATCTCTTTAAAGAGCTC
TTTTGTCTTTCAGT=74
ACCTCTTCCCTGTTTGGACC=20

Observed Results of CYP3A5*3 (rs2740574):

Restriction enzyme digestion products were visualized in agarose (3%) or Polyacrylamide gel (10%).

Table 3.7. Name of the allele, PCR product size, restriction enzyme, length of the expected and observed fragments on digestion in case of CYP3A5*3

Allele Name	PCR Product Size (bp)	RE	Expected Fragments (bp)	Observed Fragments (bp)
CYP3A5*3	196	*Rsa*I	**NH** 94, 102 **HE** 20, 74, 94, 102 **MH** 20,74,102	**94, 102** **20, 74, 94, 102** **20, 74, 102**

NH: Normal Homozygote; **HE:** Heterozygote; **MH:** Mutant Homozygote

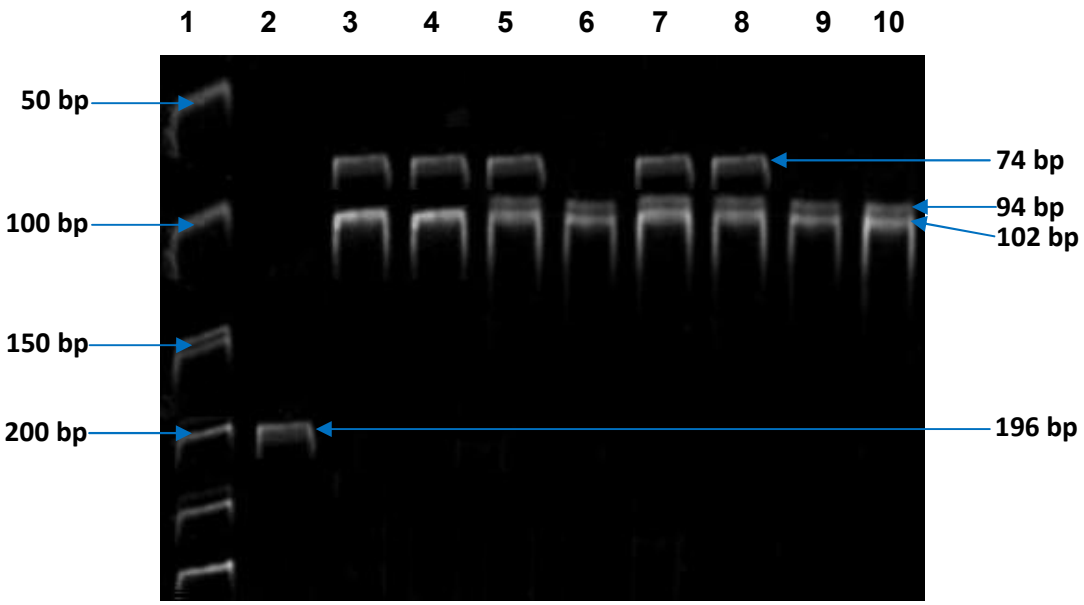

Figure 3.5: Restriction Enzyme (*Rsa*I) digestion fragment of CYP3A5*3 (Lane 3 to 10) (10% Polyacrylamide gel). Lane-1, Molecular ruler; Lane-2, uncut PCR product (196 bp); Lane-6, 9 & 10, normal homozygote (94, 102 bp); Lane-3 & 4, mutant homozygote (20, 74, 102); Lane-5, 7 & 8, heterozygote (20, 74, 94, 102 bp)

3.5. INDIVIDUAL RESULTS: CONTROL GROUP

SERIAL NO	CODE	Type of Allele	
		CYP3A4*1B	CYP3A5*3
1	FCD1	NH	NH
2	FCD2	NH	NH
3	FCD3	NH	NH
4	FCD4	NH	NH
5	FCD5	NH	NH
6	FCD6	NH	NH
7	FCD7	NH	NH
8	FCD8	NH	NH
9	FCD9	NH	NH
10	FCD10	NH	NH
11	FCD11	NH	NH
12	FCD12	NH	NH
13	FCD13	NH	MH
14	FCD14	NH	NH
15	FCD15	NH	NH
16	FCD16	NH	NH
17	FCD17	NH	MH
18	FCD18	NH	NH
19	FCD19	NH	NH
20	FCD20	NH	NH
21	FCD21	NH	NH
22	FCD22	NH	NH
23	FCD23	NH	NH
24	FCD24	NH	NH
25	FCD25	NH	NH
26	FCD26	NH	NH
27	FCD27	NH	MH
28	FCD28	NH	MH
29	FCD29	NH	NH
30	FCD30	NH	NH
31	FCD31	NH	NH
32	FCD32	NH	NH
33	FCD33	NH	NH
34	FCD34	NH	HE
35	FCD35	NH	NH
36	FCD36	NH	HE
37	FCD37	NH	NH
38	FCD38	NH	NH
39	FCD39	NH	NH
40	FCD40	NH	NH

SERIAL NO	CODE	Type of Allele	
		CYP3A4*1B	CYP3A5*3
41	FCD41	NH	HE
42	FCD42	NH	NH
43	FCD43	NH	NH
44	FCD44	NH	HE
45	FCD45	NH	HE
46	FCD46	NH	NH
47	FCD47	NH	NH
48	FCD48	NH	NH
49	FCD49	NH	NH
50	FCD50	NH	NH
51	FCD51	NH	NH
52	FCD52	NH	NH
53	FCD53	NH	NH
54	FCD54	NH	NH
55	FCD55	NH	NH
56	FCD56	NH	NH
57	FCD57	NH	NH
58	FCD58	NH	NH
59	FCD59	MH	NH
60	FCD60	NH	NH
61	FCD61	NH	NH
62	FCD62	HE	NH
63	FCD63	NH	NH
64	FCD64	NH	NH
65	FCD65	NH	NH
66	FCD66	NH	NH
67	FCD67	NH	NH
68	FCD68	NH	NH
69	FCD69	NH	NH
70	FCD70	NH	NH
71	FCD71	NH	NH
72	FCD72	NH	NH
73	FCD73	NH	NH
74	FCD74	NH	NH
75	FCD75	NH	NH
76	FCD76	NH	NH
77	FCD77	HE	NH
78	FCD78	HE	NH
79	FCD79	NH	NH
80	FCD80	NH	NH
81	FCD81	NH	NH

SERIAL NO	CODE	Type of Allele	
		CYP3A4*1B	CYP3A5*3
82	FCD82	NH	NH
83	FCD83	NH	NH
84	FCD84	NH	NH
85	FCD85	NH	NH
86	FCD86	NH	NH
87	FCD87	NH	NH
88	FCD88	NH	NH
89	FCD89	NH	NH
90	FCD90	NH	NH
91	FCD91	NH	NH
92	FCD92	NH	NH
93	FCD93	NH	NH
94	FCD94	NH	NH
95	FCD95	NH	NH
96	FCD96	NH	NH
97	FCD97	NH	NH
98	FCD98	NH	NH
99	FCD99	NH	NH
100	FCD100	NH	NH

(**NH=**NORMAL HOMOZYGOTE)
(**MH=**MUTANT HOMOZYGOTE)
(**HE=**HETEROZYGOTE)

3.6. INDIVIDUAL RESULTS: PATIENT GROUP

SERIAL NO	CODE	Type of Allele	
		CYP3A4*1B	CYP3A5*3
1	FPD1	NH	HE
2	FPD2	NH	NH
3	FPD3	HE	NH
4	FPD4	NH	NH
5	FPD5	HE	NH
6	FPD6	NH	NH
7	FPD7	NH	HE
8	FPD8	NH	NH
9	FPD9	NH	HE
10	FPD10	NH	NH
11	FPD11	NH	HE
12	FPD12	NH	NH
13	FPD13	NH	NH
14	FPD14	NH	NH
15	FPD15	NH	NH
16	FPD16	NH	NH
17	FPD17	NH	NH
18	FPD18	NH	NH
19	FPD19	NH	NH
20	FPD20	NH	NH
21	FPD21	NH	NH
22	FPD22	HE	MH
23	FPD23	NH	HE
24	FPD24	NH	MH
25	FPD25	NH	MH
26	FPD26	NH	MH
27	FPD27	MH	NH
28	FPD28	NH	NH
29	FPD29	NH	NH
30	FPD30	NH	NH
31	FPD31	NH	HE
32	FPD32	NH	NH
33	FPD33	NH	HE
34	FPD34	NH	NH
35	FPD35	NH	NH
36	FPD36	NH	NH
37	FPD37	NH	MH
38	FPD38	NH	HE
39	FPD39	NH	MH
40	FPD40	NH	MH

SERIAL NO	CODE	Type of Allele	
		CYP3A4*1B	CYP3A5*3
41	FPD41	NH	NH
42	FPD42	NH	NH
43	FPD43	NH	MH
44	FPD44	NH	MH
45	FPD45	HE	HE
46	FPD46	HE	HE
47	FPD47	NH	NH
48	FPD48	NH	NH
49	FPD49	NH	NH
50	FPD50	NH	NH
51	FPD51	NH	NH
52	FPD52	NH	NH
53	FPD53	NH	NH
54	FPD54	NH	NH
55	FPD55	MH	NH
56	FPD56	NH	NH
57	FPD57	NH	NH
58	FPD58	NH	NH
59	FPD59	NH	NH
60	FPD60	NH	NH
61	FPD61	NH	HE
62	FPD62	HE	NH
63	FPD63	NH	NH
64	FPD64	NH	NH
65	FPD65	NH	NH
66	FPD66	NH	MH
67	FPD67	NH	NH
68	FPD68	NH	NH
69	FPD69	NH	NH
70	FPD70	NH	NH
71	FPD71	NH	NH
72	FPD72	NH	HE
73	FPD73	NH	NH
74	FPD74	NH	NH
75	FPD75	NH	NH
76	FPD76	NH	NH
77	FPD77	NH	NH
78	FPD78	HE	HE
79	FPD79	NH	NH
80	FPD80	NH	NH
81	FPD81	NH	NH

SERIAL NO	CODE	Type of Allele	
		CYP3A4*1B	CYP3A5*3
82	FPD82	NH	NH
83	FPD83	NH	NH
84	FPD84	NH	NH
85	FPD85	NH	HE
86	FPD86	NH	NH
87	FPD87	NH	NH
88	FPD88	NH	NH
89	FPD89	NH	NH
90	FPD90	NH	MH
91	FPD91	NH	HE
92	FPD92	NH	NH
93	FPD93	NH	HE
94	FPD94	NH	NH
95	FPD95	NH	NH
96	FPD96	NH	HE
97	FPD97	HE	MH
98	FPD98	NH	NH
99	FPD99	NH	NH
100	FPD100	NH	NH

(NH=NORMAL HOMOZYGOTE)
(MH=MUTANT HOMOZYGOTE)
(HE=HETEROZYGOTE)

3.7. SUMMARY OF GENOTYPING RESULTS

Table 3.8: Identification of each allele present in each DNA sample for control group

Allele	RE	PCR product size (bp)	Expected fragments (bp)	Observed fragments for all samples	Conclusion about samples
CYP 3A4*1B	*Mbo*II	385	**NH** 210, 169, 41 **HE** 210,175,169,41 **MH** 175, 210	210, 169, 41 210,175,169,41 175, 210	NH=96 HE=03 MH=01
CYP 3A5*3	*Rsa*I	196	**NH** 94, 102 **HE** 20,74,94,102 **MH** 20,74,102	94, 102 20,74,94,102 20,74,102	NH=91 HE=05 MH=04

RE=Restriction enzyme; **NH:** Normal Homozygote; **HE:** Heterozygote; **MH:** Mutant Homozygote

Table 3.9: Identification of each allele present in each DNA sample for patient group

Allele	RE	PCR product size (bp)	Expected fragments (bp)	Observed fragments for all samples	Conclusion about samples
CYP 3A4*1B	*Mbo*II	385	**NH** 210, 169, 41 **HE** 210,175,169,41 **MH** 175, 210	210, 169, 41 210,175,169,41 175, 210	NH=91 HE=07 MH=02
CYP 3A5*3	*Rsa*I	196	**NH** 94, 102 **HE** 20,74,94,102 **MH** 20,74,102	94, 102 20,74,94,102 20,74,102	NH=71 HE=17 MH=12

RE=Restriction enzyme; **NH:** Normal Homozygote; **HE:** Heterozygote; **MH:** Mutant Homozygote

3.8. OBSERVED GENOTYPING RESULT OF CYP3A4 FOR PROSTATE CANCER CASES AND CONTROL

Table 3.10: CYP3A4 genotype and allelic frequencies among prostate cancer cases and controls and their association with prostate cancer

Genotype	Cases	Controls	OR	95%CI	P
NH (*1A/*1A)	**91**	**96**	1	--	--
HE (*1A/*1B)	**7**	**3**	2.46	0.62 to 9.81	0.202
MH (*1B/*1B)	**2**	**1**	2.11	0.19 to 23.67	0.545
HE+MH (*1A/*1B+*1B/*1B)	**9**	**4**	2.37	0.71 to 7.98	0.162

Allele	Cases	Controls	OR	95% CI	P
CYP3A4*1A	**189**	**195**	1	--	--
CYP3A4*1B	**11**	**5**	2.27	0.77 to 6.66	0.135

NH = Normal Homozygote, HE= Heterozygote, MH = Mutant Homozygote, OR= Odds ratio, CI= Confidence interval

Compared with the NH genotype of CYP3A4*1B (Table 3.10) heterozygous (HE), mutant homozygote (MH) and combined heterozygous plus mutant variants (HE+MH) has increased risk of prostate cancer (OR = 2.46, 95% CI = 0.62 to 9.81, p = 0.202; OR = 2.11, 95% CI = 0.19 to 23.67, p = 0.545 and OR = 2.37, 95% CI = 0.71 to 7.98, p = 0.162 respectively). The distribution of the CYP3A4 genotypes were not significantly different (p= 0.356) between the cases and controls [NH(*1A/*1A), HE(*1A/*1B) and MH(*1B/*1B)] 91%, 7% and 2% vs 96%, 3% and 1% respectively.

Heterozygote, HE(*1A/*1B) and Mutant Homozygote, MH(*1B/*1B) genotypes has increased risk of prostate cancer 2.46 and 2.11 times respectively compared with NH (*1A/*1A) genotype, whereas HE+MH(*1A/*1B+*1B/*1B) combined genotype has 2.37 times more risk of prostate cancer compared with NH genotype. The obtained results are not statistically significant (p>0.05).

3.9. OBSERVED GENOTYPING RESULT OF CYP3A5 FOR PROSTATE CANCER CASES AND CONTROL

Table 3.11: CYP3A5 genotype and allelic frequencies among prostate cancer cases and controls and their association with prostate cancer

Genotype	Cases	Controls	OR	95%CI	P
NH (*1/*1)	71	91	1	--	--
HE (*1/*3)	17	5	4.36	1.53-12.38	0.003
MH (*3/*3)	12	4	3.85	1.19-12.43	0.017
HE+MH (*1/*3+*3/*3)	29	9	4.13	1.84-9.28	0.000

Allele	Cases	Controls	OR	95%CI	P
CYP3A5*1	159	187	1	--	--
CYP3A5*3	41	13	3.71	1.92-7.17	0.001

NH = Normal Homozygote, HE= Heterozygote, MH = Mutant Homozygote, OR= Odds ratio, CI= Confidence interval

Compared with the NH genotype of CYP3A5*3 (Table 3.11) heterozygous (HE), mutant homozygote (MH) and combined heterozygous plus mutant variants (HE+MH) is significantly associated with the risk of prostate cancer (OR = 4.36, 95% CI = 1.53 to 12.38, p = 0.003; OR = 3.85, 95% CI = 1.19 to 12.43, p = 0.017 and OR = 4.13. 95% CI = 1.84 to 9.28, p = 0.000 respectively).The distribution of the CYP3A5*3 genotypes were significantly different (p= 0.001) between the cases and controls [NH(*1/*1), HE(*1/*3) and MH(*3/*3)] 71%, 17% and 12% vs 91%, 5 % and 4% respectively.

Heterozygote, HE (*1/*3) and Mutant Homozygote, MH (*3/*3) genotypes has increased risk of prostate cancer 4.36 and 3.85 times respectively compared with NH (*1/*1) genotype, whereas HE+MH (*1/*3+*3/*3) combined genotype have 4.13 times more risk of prostate cancer compared with wild type genotype and that is statistically significant (p< 0.05).

3.10. Comparison of our study results with different ethnic groups

A summary of different CYP3A4*1B and CYP3A5*3 alleles in different ethnic population including present study has been shown in table 3.12 and 3.13

Table 3.12: Ethnic distribution of variant alleles of CYP3A4 (Comparison with our study results).

CYP3A4 Genetic variants	**Allele frequencies (%) in different ethnic groups**						
Allele	**African**	**Caucasian**	**Chinese**	**Japanese**	**Malaysian**	**North Indian**	**Bangladeshi (Our study)**
*1B	60%	4%	0%	0%	ND	0%	5.5%

ND: Not Detected

Table 3.13: Ethnic distribution of variant alleles of CYP3A5 (Comparison with our study results)

CYP3A5 Genetic variants	**Allele frequencies (%) in different ethnic groups**							
Allele	**Canadian**	**Caucasians**	**Hispanic**	**Zimbabweans**	**Koreans**	**Japanese**	**Chinese**	**Bangladeshi (Our study)**
CYP3A5*3	93%	70%	75%	77.6%	70%	71-85%	65-73%	**20.5%**

3.11. Discussions

In the present study we characterised CYP3A4*1B and CYP3A5*3 alleles among Bangladeshi population in 100 healthy adult (male) volunteers as control and 100 prostate cancer patient as cases. All of the results were compared with the results of control groups. The study was done by using PCR-RFLP assay method for the detection of genetic polymorphisms. From the distributions of demographic characteristics and clinical data among study subjects, the related factors such as age and smoking history of cases and controls were compared. There were no significant differences in mean age (p = 0.776) and smoking status (p=0.788) between the two groups (Table 3.1). The observed never smoking rate was 7% in the cases and 8% in controls. Among the smoker 65% and 21% were current smokers & ex-smokers in cases and 69% and 20% were current smokers & ex-smokers in controls, respectively and 7% & 3% were chewing tobacco in cases and controls respectively.

It has been found that different patients respond in different ways to the same medication. These differences are often greater among members of a population than they are within the same person at different times (or between monozygotic twins) (Vesell, 1989). CYP3A4 is involved in the metabolism of >60% of all drugs used in human (Thummel *et al.,* 1996). It is found in the human livers and intestines (Shimada *et al.,* 1994) and plays important roles in the metabolism of a wide variety of drugs such as antidiabetics, antiarrhythmics, antihistamines and synthetic estrogens (Watkins, 1994).

CYP3A4*1B is an A-392G transition in the 5′-promoter region (Shimada *et al.,* 1994). CYP3A4*1B demonstrates a frequency of 60% and 4% in Africans and Caucasians, respectively, but has not been found in Chinese and Japanese. (Rebbeck *et al.,* 1998; Walker *et al.,*1998; Paris *et al.,* 1999; Sata *et al.,* 2000; Hsieh *et al.,*2001; Kuehl *et al.,* 2001).

Our result revealed that, CYP3A4*1B, heterozygous (HE), mutant homozygote (MH) and combined heterozygous plus mutant variants (HE+MH) has increased risk but not significantly associated (p >0.05) with prostate cancer compared with normal homozygote (NH) genotype (OR = 2.46, 95% CI = 0.62 to 9.81, p = 0.202; OR = 2.11, 95% CI = 0.19 to 23.67, p = 0.545 and OR = 2.37, 95% CI = 0.71 to 7.98, p = 0.162 respectively). The distribution of the CYP3A4 genotypes were not significantly different (p= 0.356) between the cases and controls [NH(*1A/*1A), HE(*1A/*1B) and MH(*1B/*1B)] 91%, 7% and 2% vs 96%, 3 % and 1% respectively.

Significant associations ($p < 0.05$) between the variant and the occurrence and severity of prostate cancer has been suggested in some previous reports (Paris PL *et al.,* 1999; Tayeb MT *et al.*, 2002; Rebbeck TR *et al.*, 1998). Plummer *et al* (Plummer SJ *et al.,* 2003) observed an inverse association between CYP3A4*1B and prostate cancer risk. CYP3A4*1B variant was positively associated with prostate cancer among Caucasians with more aggressive disease [odds ratio (OR), 1.91; 95% confidence interval (CI), 1.02–3.57; P =0.04], and inversely associated with risk among Caucasians with less aggressive disease (OR, 0.08; 95% CI, 0.01– 0.49; P = 0.006) and men with an age of diagnosis <63 (OR, 0.51; 95% CI, 0.26 –1.00; P = 0.05) (Plummer SJ *et al.,* 2003). CYP3A4*1B was associated inversely with the probability of having prostate cancer in Caucasians (age-adjusted odds ratio=0.54, 95% confidence interval, 0.32– 0.94) (Charnita Zeigler-Johnson *et al*., 2004). CYP3A4 is involved in the metabolic deactivation (hydroxylation) of testosterone (Waxman DJ *et al.,* 1998; Domanski TL et al., 2001). Unfortunately, we had limited ability to obtain statistical significance because of small sample size. However, the function of CYP3A4*1B has been controversial.

A number of authors have studied the relationship of CYP3A4 expression or function of CYP3A4*1B with prostate cancer (Westlind A *et al.,* 1999; Amirimani B *et al.,* 1999; Ando Y *et al.,* 1999; Spurdle AB *et al.,* 2002; Floyd MD *et al.,* 2003; Amirimani B *et al.,* 2003; Hamzeiy H *et al.,* 2003).

Most of the authors reported a small magnitude of association of CYP3A4 and CYP3A5 with prostate cancer prognosis. However, almost all studies have reported consistent elevations in expression associated with CYP3A4*1B in the range of 20–200% increase over the consensus CYP3A4*1A. Although it is possible that this magnitude of effects will not confer clinically meaningful effects on drug disposition, it is not clear whether this phenotypic perturbation is sufficient to alter metabolism of exposures (e.g., steroid hormones) that may confer disease risk over the lifetime of an individual. For example, a 20% greater metabolism of testosterone by CYP3A4*1B over the course of a man's lifetime may be sufficient to increase prostate cancer risk and therefore explain epidemiologic associations between CYP3A4*1B and prostate cancer. Waxman DJ *et al* concluded that, the metabolic effect of CYP3A4*1B to increase CYP3A4 expression is consistent with the epidemiologic association reported here, CYP3A4 converts testosterone to 2β-, 6β-, or 15β-hydroxytestosterone and therefore shunts testosterone away from the more biologically active form of dihydrotestosterone (Waxman DJ *et al.,* 1998).

Genetic variants that are associated with increased CYP3A4 activity, such as CYP3A4*1B, would be expected to decrease prostate cancer risk if the effect of the polymorphism is to decrease bioavailability of dihydrotestosterone.

Genotype of CYP3A5*3, heterozygous (HE), mutant homozygote (MH) and combined heterozygous plus mutant variants (HE+MH) is significantly associated (p< 0.05) with the risk of prostate cancer (OR = 4.36, 95% CI = 1.53 to 12.38, p = 0.003; OR = 3.85, 95% CI = 1.19 to 12.43, p = 0.017 and OR = 4.13. 95% CI = 1.84 to 9.28, p = 0.000 respectively).The distribution of the CYP3A5*3 genotypes were significantly different (p= 0.001) between the cases and controls [NH(*1/*1), HE(*1/*3) and MH(*3/*3)] 71%, 17% and 12% vs 91%, 5 % and 4% respectively.

Charnita Zeigler-Johnson *et al* (Charnita Zeigler-Johnson *et al.,* 2004) found that no association of CYP3A5 genotypes with prostate cancer or disease severity. S Leskela *et al* (S Leskela *et al.,* 2007) found that CYP3A5 mRNA in non-tumoral prostate tissue was 10% of the average amount of liver samples, whereas the expression of the other CYP3A genes was much lower. CYP3A5 protein was detected in non-tumoral prostate microsomes by western blot, and immunohistochemistry (IHC) localized CYP3A5 exclusively in the basolateral prostate cells express high levels of CYP3A5 which dramatically decrease in tumoral tissue. This finding supports an endogenous function of CYP3A5 related to the metabolism of intra-prostatic androgens and cell growth, and that polymorphisms affecting CYP3A5 activity may result in altered prostate cancer risk and aggressiveness (S Leskela *et al.,* 2007). The high CYP3A5 prostatic expression suggests that CYP3A5 may play a relevant function in the prostate and, since the prostate is not a tissue relevant for drug metabolism, this function must be related to the metabolism of prostatic endogenous CYP3A5 substrates, such as androgens (Ohmori *et al*., 1998, Miller *et al.,* 2004). In other tissues CYP3A5 has also been shown to play an important endogenous function, and CYP3A5*3 has been shown to influence the systolic blood and pulse pressure, presumably by altering CYP3A5-mediated glucocorticoid metabolism (Kreutz *et al*., 2005).

KU *et al* observed that CYP3A5 and CYP3A4 play a significant role in the metabolism of phosphodiesterase type 5 inhibitors (PDE5Is). The genetic polymorphism of CYP3A5 may contribute to interindividual variability in the disposition of PDE5Is, especially vardenafil (K U *et al.,* 2008).

Among Asian subjects, a number of allelic variations in CYP3A5 gene are known to affect catalytic activity including CYP3A5*3, CYP3A5*6. The clinical implications for CYP3A5 polymorphic variants are more robust and unequivocal than those for CYP3A4, CYP3A7 and CYP3A43. CYP3A5 activity displays a bimodal distribution and high interindividual variation (Haehner, Gorski *et al.* 1996; Kuehl, Zhang *et al.* 2001; Koch, Weil *et al*. 2002).

The most frequent SNP in the CYP3A5 gene is CYP3A5*3, a A6986G transition within intron 3 (Kuehl, Zhang *et al.* 2001). This mutation results in a cryptic splice site leading to transcripts with premature stop codons at the junction between exons 3 and 4. The resulting mRNAs are rapidly degraded via a nonsense-mediated decay mechanism (Busi and Cresteil 2005). Finally, there are some other CYP3A5 SNPs variants existing at low frequencies and thus less likely to play a major role in the CYP3A5 variable expression and activity. The effect of polymorphism on CYP3As expression and activity is generally moderate, with the exception of CYP3A5.

CYP3A5*3 (g.6986G) is the only one found in all ethnic groups tested. The frequencies vary from 27% in African-Americans to 95% in Caucasians (Hustert *et al.,* 2001; Kuehl *et al.,* 2001; Fukuen *et al.,* 2002; van Schaik *et al.,* 2002; Hu *et al.,* 2005; Roy *et al.,* 2005). The role of CYP3A5 in the metabolism of hormones or other putative prostate carcinogens is not as well understood as that for CYP3A4 (Kuehl P *et al.,* 2001).

It has been reported that up to a 2 to 3 fold enhancement of CYP3A5 activity can be achieved in any given ethnic population (Schuetz *et al.,* 1994). This observation has led to the suggestion that the genetic variation in CYP3A5 expression is a major determinant of CYP3A-dependent drug metabolism in humans (Chou *et al*., 2001). Although some previous studies have attempted to correlate the metabolic capabilities of different patients with genotype, a clear relationship between the levels of CYP3A5 expression and/or activity and genetic markers remains to be established (Wrighton *et al.,* 1990; Jounaidi *et al*., 1996; Kuehl *et al.,* 2001; Westlind *et al*., 2001). Ultimately, the metabolic capacities of CYP3A5 are intertwined into a complex procedure determined by the genetic makeup of individual as well as external factors, such as xenobiotics, influencing gene expression (Gibson *et al*., 2002).

However, the genetic polymorphism of CYP3A5 alone cannot explain the inter-individual differences reported in CYP3A-mediated metabolism. Additional studies will have to be undertaken examining a large number of parameters, including genotype, mRNA expression, and drug-drug interactions, as well as external influences, to more fully understand the factors that determine the metabolic capabilities of the CYP3A5 family enzymes.

In conclusion, our results confirm that CYP3A4*1B gene has increased risk on prostate cancer and CYP3A5*3 gene is significantly associated with prostate cancer occurrence, and further elucidate the relationships of multiple genotypes at the CYP3A locus with prostate cancer occurrence. Combined with information about the function of these genes, there is growing evidence that one or more of the genes in the CYP3A locus are involved in prostate cancer occurrence.

CHAPTER FOUR

CONCLUSION AND FUTURE RESEARCH

IV

Pharmacogenetics is the study of the role of inheritance in interindividual and interpopulation variations in drug response. The rationale and ultimate aim of pharmacogenetics is the possibility that knowledge of an individual's genetic make-up could be used to enhance drug therapy by maximising drug efficacy while minimising drug toxicity.

The present study is the investigation of establishing CYP3A4 and CYP3A5 genotypes in Bangladeshi prostate cancer cases (n=100) comparison with healthy population as control (n=100). It can be hypothesized that CYP3A4 and CYP3A5 are well conserved among the Bangladeshi populations because it is an important enzyme metabolizing >60% of current drugs used but yet no mutations were detected for CYP3A4*1B and CYP3A5*3. The present study confirmed that CYP3A4*1B has increased risk with prostate cancer whereas CYP3A5*3 is significantly associated with prostate cancer.

The study will continue for another 100 prostate cancer cases. Genotyping of CYP3A4*1B, CYP3A5*3 alleles in all the cases will also be done. This study is under investigation. Phenotype study will be done in future by measuring urinary ratio of 6β-hydroxy-cortisol /cortisol, with the phenotype result we can correlate between phenotype and genotype of CYP3A4 and CYP3A5 in Bangladeshi prostate cancer cases.

CHAPTER FIVE

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V

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