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Molecular genetic of prostate cancer: association of the candidate genes *CYP17* and *MSR1*

Thesis

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Abbreviations

a, A	adenosine (base)
A	Alanin (amino acid)
ACS	American Cancer Society
AR	Androgen Receptor (gene)
BC	Brain Cancer
bp	base pair
BPH	Benign Prostatic Hyperplasia
c, C	cytosine
CYP17	Cytochrome P450c17α (gene)
DHT	Dehydroxy Testosterone
DNA	Deoxyribonucleic acid
dNTP	desoxyribonucleosidtriphosphate
g, G	guanosine (base)
G	Glycine (amino acid)
H	Histidine (amino acid)
HLOD	Heterogenity lod score
HPC	Hereditary Prostate Cancer
HWE	Hardy Weinberg Equilibrium
K	Lysine
kb	kilobase
L	Leucine
LD	Linkage Disequilibrium
LOH	Loss of Heterozygosity
mRNA	messenger RNA
MSR1	Macrophage Scavenger Receptor 1 (gene)
OR	Odds Ratio
P	Proline (amino acid)
PC	Prostate Cancer
PCAP	Predisposing carcinoma of the prostate

Abbreviations

PCR	Polymerase Chain Reaction
PSA	Prostate Specific Antigen
R	Arginine (amino acid)
RNA	Ribonucleic acid
RT	Room Temperature
S	Serine (amino acid)
SNP	Single Nucleotide Polymorphism
SRD5A2	Steroid-5-alpha-reductase (gene)
t, T	thymidine (base)
UTR	Untranslated Region
X	Stop codon
Y	Tyrosine (amino acid)

1. Introduction

In spite of progress in its diagnosis and treatment, prostate cancer is one of the most frequent lethal cancers in men in many Western industrialized countries. Prostate cancer represents a heterogeneous disease with varying degrees of aggressiveness, patterns of metastasis and response to therapy (31). It arises from a complex etiology that involves both exogenous (diet, environment, etc.) and endogenous (hormonal imbalance, family history) factors.

1.1. Epidemiology of prostate cancer and risk factors

Most prostate cancers start in the glands of the peripheral zone. The earliest precursor detected histologically is prostatic intraepithelial neoplasia (PIN) characterized by thickening of the epithelial layer and loss of distinct basal and secretory layers. Nevertheless prostate carcinoma cells in fact carry markers both of basal cells such as specific cytokeratins and of secretory cells such as the Androgen Receptor (AR) and Prostate Specific Antigen (PSA). Prostate carcinoma is frequently multifocal, varying in the degree of cellular dysplasia, tissue disorganization and genetic alterations. As a practical consequence of this heterogeneity, histological grading (G1–G3) has been largely replaced by Gleason grading which evaluates prostate cancer cells on a scale of 1 to 5, based on their pattern when viewed under a microscope (40).

1.1.1. Incidence of prostate cancer and PSA influence

Due to increases in incidence rates the number of prostate cancer cases is rapidly increasing causing a large and growing public health problem. The highest incidence rates are found in the United States, Canada, Australia, Sweden; European countries have intermediate rates, while Asian countries have the lowest rates (81). For men, prostate cancer is the most common of all cancers (33%; followed by lung and bronchus cancers at 14%), and the second most common cause of death due to

cancer (10% prostate cancer; 31% lung and bronchus cancers) (56). There is an obvious impact of PSA screening on the trend of prostate cancer incidence. In 1986, the Food and Drug Administration approved the prostate-specific antigen (PSA) test for use in monitoring prostate cancer progression. The consequence of PSA screening is a diagnosis of earlier stage disease, with an average lead-time (time by which the PSA advances the diagnosis of prostate cancer) of 4-7 years (50;78).

1.1.2. Age and Ethnicity

Aging is, as a single risk factor, the most significant for the development of prostate cancer. Although PIN can be found in men in their twenties (86), clinically detectable prostate cancer is not generally obvious before the age of 60 or 70. The incidence of prostate cancer and mortality due to prostate cancer are higher in United States and Western Europe than in Asia. In the United States, more than 70% of all prostate cancer cases are diagnosed at >65 years of age (ACS). African Americans have the highest rates of prostate cancer in world (275.3 per 100,000 men) (ACS). The incidence among African Americans is almost 60% higher than among whites (172.9 per 100,000), which in turn, is higher than in Hispanics (127.6 per 100,000) and Asians / Pacific Islanders (107.2 per 100,000).

1.1.3. Diet

High intake of lipids of animal origin appears to be positively correlated with prostate cancer risk (3). It has been estimated that dietary fat intake can account for 10-15 % of the difference of prostate cancer appearance between Caucasians, African-Americans and Asians (104). Beef and dairy products are major sources of dietary branched fatty acids. An enzyme (α-methyl-coenzyme-M-reductase) that plays a key role in the peroxisomal oxidation of these fatty acids is up regulated in prostate cancer but not in the healthy prostate (42). A positive association between plasma concentrations of insulin-like growth factor-I (IGF-1) and prostate cancer risk was observed. This factor is known to regulate the proliferation and differentiation of cancer cells and to prevent them from undergoing apoptosis. Men in the highest quartile of insulin-like growth factor-I (IGF-1) concentrations had a relative prostate cancer risk of 1.7- to 4.3- fold compared with men in the lowest quartile (21). Studies

showing positive correlation between the high BMI (body-mass index) and prostate cancer suggest also a significant role of the diet rich in animal fats as a risk factor for prostate cancer (39). A dietary component, that has been associated with a reduced risk of prostate cancer, is a high plasma levels of the antioxidant lycopene resulting from the increased consumption of tomatoes (35). Other antioxidants like vitamin E and selenium can play the role in reducing the risk of prostate cancer (23;47).

1.1.4. Vitamin D

Vitamin D and vitamin D analogues play an important role in the growth and function of the normal prostate, as well as in prostate carcinogenesis. An active form of human vitamin D 1,25-dihydroxyvitamin (1,25-D) inhibits cell proliferation in normal and malignant prostatic epithelium and plays a role in differentiation (90). The hypothesis that vitamin D may have a protective role against developing of prostate carcinoma was raised by Schwartz *et al*. (87). They showed that the incidence for prostate cancer increases with age and the levels of vitamin D were found to be significantly lower among elderly men. The vitamin D signalling cascade might be altered by genetic changes. A series of common polymorphisms in *VDR* (vitamin D receptor) gene have been identified. The alleles of human *VDR* gene can be distinguished by restriction fragment length polymorphisms (RFLPs) found for *Bsm*I and *Apa*I (intron 8) and *Taq*I (exon 9) (51). The presence (*b*, *a*, *t*) or absence (*B*, *A*, *T*) of a restriction site defines the specific allele. A fourth polymorphism, a poly (A) microsatellite, is located in the 3'-UTR (53). Several studies have evaluated whether the VDR gene polymorphisms could alter the risk of prostate cancer (53;99). Two case-control studies (65;72) reported that serum levels of 1,25-D were significantly higher among individuals who were homozygous for the *BAt* haplotype compared with individuals who were heterozygous or homozygous for the *baT* haplotype. Therefore, the *BAt* haplotype may have a protective effect on developing PCa.

1.1.5. Role of hormones in prostate cancer

Hormones are playing the important role in growth and proliferation of normal prostate cells as well as for the prostate cancer cells; therefore the same hormones can be involved in carcinogenesis. The normal development and maintenance of the prostate depends on androgens. This feature strongly suggests that androgens play a major role in human prostatic carcinogenesis. This is due to the fact that ligand occupied androgen hormone receptors act as transcription factors thereby influencing the rate of cell division and degree of cell differentiation. Prostate cancer growth is dependent on androgens and that has as a consequence that cancers often after androgen ablation therapy, develop the androgen independence nearly in all patients. More than 80% of androgen-independent prostate tumours show high levels of androgen receptor expression. The reasons for the increased androgen receptor levels are gene amplification and/or overexpression, or mutations in the androgen receptor (114).

The male sex hormones testosterone and DHT (dihydrotestosterone) are strongly interrelated in growth and maintenance of normal prostate epithelium as well as in the development of prostate cancer (4). The incidence in prostate cancer between African Americans and Caucasians has been attributed to high serum testosterone levels in African Americans (84). However, higher levels of circulating testosterone in patients with prostate cancer have not been consistently observed (13). Also other hormones, like prolactin and estrogens may have a role in prostate growth and differentiation (13).

Other environmental factors including smoking, alcohol consumption, socioeconomic factors and physical activity have not been shown as prostate cancer risk factors (1;42).

1.1.6. Familial aggregation

One of the strongest risk factors for prostate cancer is a positive family history. Familial prostate cancer is defined by clustering of prostate cancer cases within male members of family. Familial aggregation of prostate cancer was firstly reported by Morganti et *al*. (71). This finding led various case-control and cohort studies to investigate the role of family history as a risk factor for prostate cancer (76;77). Among men with a positive family history for prostate cancer, the risk of developing prostate cancer doubles and risk increases further when multiple first-degree relatives are affected (19;94). The familial clustering of prostate cancer can be caused by inheritance of a susceptibility gene, by exposure to common environmental factors or simply by chance alone because of the high incidence of this malignancy (42). Prostate cancer can be familial, hereditary and sporadic. Hereditary cancers are typically distinguished from sporadic cancers by familial clustering and autosomal-dominant inheritance (not necessarily), multifocality and an early onset. Hereditary prostate cancer has been defined by Carter *et al.* (19) as families that meet at least one of the following three criteria: (1) a cluster of three or more relatives affected with prostate cancer in a nuclear family; (2) the occurrence of prostate cancer in three successive generations in either of the proband's paternal or maternal lineages; or (3) a cluster of two relatives, both affected with prostate cancer at 55 years of age or younger. According to these criteria ~10 % of all prostate cancer cases and up to 40% of those occurring at < 55 years of age may have a hereditary basis (19;20). Prostate cancer involves several genetic loci, but none of them appears to account for a large proportion of susceptibility to the hereditary prostate cancer as a single genetic locus (28;76).

The evidence for the complex genetic basis for prostate cancer is based on a wide range of study designs, including case–control, cohort, twin and family-based studies. Case-control and cohort studies The case–control study is a powerful method to evaluate an association of potential risk factors with prostate cancer assessed for a group of individuals (cases) who developed the disease and another group consisting of unaffected individuals (controls). The odds of the risk factor among cases are compared to the odds of the risk factor among controls and odds ratio is calculated. Nevertheless case–control studies can be biased for several reasons. The information about family history is usually obtained after the case is diagnosed with

prostate cancer. This may have the consequence that cases are more likely to misinterpret prostate problems as cancer or the relatives of cancer patients are more aware of the diagnosis of prostate cancer than the relatives in the control group. An alternative to the case–control study is a cohort study in which men are followed over time and the incidence of the disease is observed. An important advantage of cohort studies is that they are not as prone to recall bias. In addition, these types of studies allow estimation of relative risks, instead of approximating relative risks by ORs.

Twin studies Twin studies can provide information on genetic etiology in contrast to family studies, which cannot distinguish between genetic and non-genetic causes. If the concordance rate of prostate cancer is greater for monozygotic (MZ) than dizygotic (DZ) pairs of twins, then genetic effects are likely to be involved, since MZ twins share 100% of their genes and DZ twins share 50% of their genes.

Family-based studies Family-based analyses provide a model to evaluate whether the observed aggregation of disease in a series of families fits the expected distribution based on a genetic (or purely environmental) model. The genetic model may be based on Mendelian segregation of alleles within families, the population frequency of the putative susceptibility allele, and the penetrance of the underlying genotypes. These analyses are difficult to carry out for complex diseases that are likely to be caused by multiple predisposing genes. However they are required to provide the parametric linkage analyses of cancer (all results are due to relationship within the pedigree).

1.2. Somatic Genetic Alterations in prostate cancer

The modern view of cancer development is that the tumour is arising from cell transformation, loss of contact inhibition and clonal expansion of the cells driven by successive mutations. Molecular studies support the idea that multiple genetic changes are required for tumour progression. At the time of diagnosis, prostate cancer cells exhibit many changes in DNA methylation, chromosomal rearrangements, somatic mutations, gene deletions and gene amplifications. These alterations are accumulating probably over a period of several decades (85).

1.2.1. Alterations in DNA methylation

Some of the genomic alterations in human cancer cells are characterized by abnormal methylation. The patterns of abnormal methylation include hypermethylation, demethylation and redistribution of methylation. Of more biological importance are genomic regions of hypermethylation. The most important site of abnormal methylation resides in regions of high-density C-G dinucleotide sequences, referred to as CpG islands. These CpG islands are generally found in or near the 5'-region of genes, which may contain the promoter and one or more exons of its associated gene. Loss of 5'-methylcytosines, or hypomethylation, has been reported to occur in human PCa, but its significance is not entirely clear (6).

1.2.2. Chromosomal alterations

Early stages of prostate cancer often remain euploid, while numerical and structural chromosomal alterations accumulate at advanced stages. Cytogentic anomalies can be studied via traditional cytogenetic technique, which analyse chromosomal changes relaying on staining metaphase chromosomes of the tumours. Development of the in situ hybridization techniques helped to overcome the main drawback of cytogenetic: the need for the cell culture. The techniques that are belonging to this group are comparative genomic hybridization (CGH) and fluorescence in-situ hybridization (FISH). These techniques have refined the analyses of chromosomal

anomalies through the use of molecular probes. FISH is used to detect, locate and quantify specific nucleotide sequence (DNA or RNA) in chromosome preparations, tissue sections or isolated cells. CGH analyses are designed to reveal the regions that are amplified or lost in the genome, and these analyses confirmed that chromosome deletions are more frequent than chromosome gains in prostate cancer. CGH has a poor preciseness, so it should be combined with FISH to confirm and quantify the observed genetic alternations. Overall, the most frequently altered autosomes in prostate carcinoma include chromosomes 8, 13, 7, 10, 16, 6 and 17. In addition, gains or amplification of X chromosome and loss of Y chromosome are often observed. The two chromosome arms that are mainly altered in the prostate cancer (in CGH analysis) are 8p and 13q. Decreased copy numbers and LOH (loss of heterozygosity) of chromosome 8p are detected in more than a half of the cases (7;14). Likewise, deletions and LOH of chromosome 13q are frequent (52). This leads to the conclusion that inactivation of tumour suppressor genes at 8p and 13q is an early event in the development of prostate cancer. Among the candidate target tumour suppressor genes at 8p are NKX3.1, N33, FEZ1 and PRLTS (12;15;32;55). Concerning the chromosome arm 13q there are a few putative tumour suppressor genes, such as RB1 (retinoblastoma 1) and BRCA2 (24). Gain of the whole long arm of chromosome 8 is the most common aberration and it is associated with aggressive phenotype of the disease (2). Gains of other chromosomes occur with lower frequencies, usually in more advanced tumours.

1.2.3. Tumour suppressors and oncogenes

Mutations of tumour suppressor genes (TSGs) are considered generally as recessive, and both copies of these genes must be inactivated before the cell is at risk for transformation. The first mutation of the gene is a somatic event or is inherited in the germline from one of the parents. The second one is likely to appear as inactivation of the normal copy or allele of the gene. Involvement of tumour suppressor genes and consistent loss of specific chromosomal regions suggests their importance in prostate cancer. The major approach used in prostate cancer for searching for tumour suppressor genes is seeking in regions of the genome that are consistently deleted. Among tumour suppressor genes, p53 and PTEN (phosphatase and tensin homolog) are clearly involved in progression of prostate cancer. Losses of

chromosome 17p and 10q occur with moderate frequencies in advanced cancer. The RB1 (retinoblastoma 1) gene is located at 13q14 within one of the most commonly deleted regions in prostate carcinoma. However, it has not been confirmed as a crucial tumour suppressor in prostate carcinoma.

Altered expression of proto-oncogenes contributes to the development and progression of prostate cancer. c-*myc* is a cellular proto-oncogene that encodes a nuclear phosphoprotein. A consistent finding in metastatic tumours is overexpression of the *myc* oncogene, usually associated with an increased gene copy numbers by chromosomal gains or amplification (75). The next extensively studied oncogene is *ras* oncogene. The RAS gene family encodes highly related G proteins and these genes are essential for the transduction of extracellular signals that induce proliferation and differentiation. Point mutations at codons 12, 13, or 61 in the proto-oncogene alter the ability of the *ras* protein to affect signal transduction, leading to unregulated cellular growth. A low frequency of *ras* mutations has been seen in population of American men, while in Japanese prostate cancer samples frequency of *ras* gene mutations was higher (58). This difference may reflect a different etiology causing prostate cancer or affecting its progression between the two populations. Another group of oncogenes that has a role in prostate cancer is the erbB2 (HER2/neu) oncogene. This class of oncogenes encodes a transmembrane tyrosine kinase growth factor receptor with substantial homology to the epidermal growth factor receptor (EGFr). The significance of erbB2 as a prognostic marker in prostate cancer is controversial, because some studies found a higher protein expression or gene amplification in prostate cancer patients compared to non-malignant specimens (115), whereas others didn’t find this correlation (59).

1.2.4. Telomerase and telomere shortening

Telomeres are the repetitive noncoding DNA sequences found at the ends of all eukaryotic chromosomes and act as a protective caps. In humans telomeres consist of six-nucleotide sequence TTAGGG repeated from a few to a thousand times. A reverse transcriptase enzyme, telomerase, synthesizes these sequences.
In normal somatic cells, telomeres shorten with each round of the cell division and, when they reach a critically short length, cells exit from the cell cycle and undergo the replicative senescence. By contrast, immortal cells as well as germline cells adopted mechanisms to bypass the senescence checkpoint. Telomere maintenance in 80–95% of tumour cells is achieved by telomerase. Telomerase activity is typically absent from most normal human cells, while it is expressed in nearly all-human cancer cells as well in the germline cells. It has been observed, that tissues and cell lines of prostate cancer exhibit high levels of telomerase activity, while normal prostate cell lines, BPH and normal prostate tissue do not (62).

1.3. Genes predisposing to hereditary prostate cancer

Genes predisposing to cancer can be divided into two groups: high-penetrance and low-penetrance genes. Mutations in high-penentrance genes are increasing the cancer risk by several fold and tumours with these mutations are often called hereditary cancers, while low-penetrance genes have a moderate effect. Several epidemiological studies demonstrated that men with one first-degree relative and those with two first-degree relatives with prostate cancer have a twofold and fivefold higher risk of developing prostate cancer respectively, compared with men without family history of cancer (19;94). Prostate cancer susceptibility loci identified through linkage analysis, and confirmed in independent studies, are summarized in Table 1.

Table 1. Hereditary prostate cancer loci identified through linkage analyses that are harbouring prostate cancer susceptible genes.

Locus	Name	Ascertainment criteria for the families	Linkage results	First reported
1q24-25	HPC1	Ascertained on the basis of early onset familial PC	Multipoint hLOD score=5.43	Smith et al. (91)
1p36	CAPB	Stratification of pedigrees by number of affected and average age at diagnosis	LOD score=3.22	Gibbs et al. (38)
1q42.2-43	PCAP	Stratification of pedigrees by age of onset	LOD score=3.30	Berthon et al. (10)
8p22-23		Stratification of pedigrees by average age at diagnosis	NPL score=2.64	Xu et al. (110)
17p11	HPC2/ELAC2	A subset of families ascertained using the Utah Population Database	2-point LOD score=4.50	Tavtigian et al. (98)
20q13	HPC20	Stratification of pedigrees by mtm disease transmission, number of affected and average age at diagnosis	3.02	Berry et al. (9)
Xq27-q28	HPCX	A combined study population	Multipoint LOD score=3.85	Xu et al. (108)

mtm= male to male transmition

1.3.1. *HPC1* locus at 1q24-1q25

Smith et *al*. (91) reported the first putative hereditary prostate cancer loci, HPC1. They provided the evidence of linkage for chromosomal region 1q24-25 in 91 North American and Swedish family, each having at least three first-degree affected relatives with prostate cancer. A multipoint LOD score of 5.43 was achieved, with 34% of prostate cancer families linked to this region. One of the candidate genes in the broadly defined HPC1 (hereditary prostate cancer 1) region is *RNASE L* (2'-5'-oligoadenilate-dependent ribonuclease L) (18). The gene encodes a constitutively expressed latent endoribonuclease that mediates the proapoptotic and antiviral activities of the interferon-inducible 2-5A system. Mutation screening of *RNASAE L* preformed by Carpten et al. (18) revealed two interesting mutations: the nonsense mutation Glu265X and the initiation codon mutation Met1Ile. In the follow-up study by Rokman et al. (83) out of 116 index cases with hereditary prostate cancer, the

Glu265X mutation was found in 5 cases. In a mutation screening of a *RNASE L* in Ashkenazi Jews, a novel frame shift mutation 471delAAAG was found, which leads to premature truncation of the protein.

1.3.2. *PCAP* locus at 1q42.2-1q43

A second putative predisposing gene for prostate cancer at 1q42.2-43 was found by Berthon et *al*. (10). They conducted a linkage study using 47 French and German families, having three or more affected with prostate cancer per family. In this set of 9 of 47 families with early-onset prostate cancer (<60 years of age) gave a multipoint lod score of 3.31. The replication of these findings was difficult. Most of the other reporters found no evidence for linkage (8;36;105). For example, Gibbs et al. (36) reported that in 152 hereditary prostate cancer families PCAP may account for a small proportion 4 to 9 %. A likely candidate in this region is PCTA-1 gene (Prostate Cancer Tumour Antigen-1) (95).

1.3.3. *CAPB* locus at 1p36

Epidemiological studies suggested a familial association between prostate and brain cancers, for example Carter et al. (20) reported that families with hereditary PC have a significant excess of BC, and Isaacs et al. (54) showed that such families have a significantly increased relative risk (RR) for tumours of the CNS. Gibbs et al. (38) evaluated 12 families with a history of both prostate cancer and primary brain cancer, and they found a CAPB (Cancer of Prostate and Brain) at 1p36. The overall lod score in these 12 families was 3.22. In the younger age group (mean age at diagnosis < 66) a maximum two-point lod score was 3.65. Gibbs et al. (38) did not observe the significant evidence for chromosome 1p36 linkage in the early- or late-onset families that did not report a family history of primary BC.

1.3.4. *HPCX* locus at Xq27-28

Xu et al. (108) detected significant linkage to chromosome Xq27-28 in a combined study population of 360 prostate cancer families from North America, Sweden and Finland. The maximum LOD score was 3.85, with estimation that HPCX accounts for 16% of the hereditary prostate cancer families. Since the X-linked mode of inheritance represents transmission of susceptibility allele from mother to son, but not from father to son, Xu et al. (108) stratified families according male-to-male (M-M) inheritance of prostate cancer. Following this criterion, 129 families without male-to-male transmission showed the stronger linkage evidence (maximum multipoint lod score 2.46), than 190 families with male-to-male transmission (the maximum lod score was 1.47). The observed lod score is consistent with the hypothesis of X chromosome linkage in this data set.

1.3.5. *HPC20* locus at 20q13

The prostate cancer susceptibility locus at 20q3 was first reported by Berry et al (9), who conducted a genomewide search on 162 North American families with the maximum multipoint lod score for the entire data set of 3.02. The evidence for linkage was strongest in families with an average age of diagnosis ≥ 65 years, no evidence for male-to-male transmission and less then 5 affected men. Several studies confirmed linkage to the HPC20 using independent data sets (11;116). However, three studies were unable to confirm linkage of a HPC20 locus in their data sets (16;48;97).

1.3.6. *HPC2/ELAC2* gene locus at 17p11

Tavtigian et al. (98) in 2001, reported linkage on chromosome 17p based on 33 pedigrees with a multiple lod score of 4.3. Positional cloning and mutation screening lead to the detection of the ELAC2 gene, that harboured mutation that segregate with prostate cancer in two pedigrees. Two common missense variants in the ELAC2 gene that are associated with diagnosis of prostate cancer are: Ser217Leu and Ala541Thr. In contrast, Xu et al. (109) found no evidence for linkage in 159 families, even after investigating subsets of pedigrees according to the age of prostate cancer

diagnosis, number of affected men or race. Rokman et al. (82) screened for mutations of the ELAC2 gene in 66 prostate cancer families, but they didn't find truncated mutation nor an association of missense variants was seen. Also Suarez et al. (96) showed no evidence of linkage to the HPCL2 locus. Overall, it appears that HPC2/ELAC2 has a weak role in prostate cancer.

1.3.7 8p22-23 locus and the MSR1 gene

Frequently loss of heterozygosity in prostate tumours was found at the short arm of chromosome 8, specifically 8p22-23 (25). In 2001, Xu et al. (110) reported suggestive linkage to chromosome 8p22-23 within 159 families with a lod score of 1.84. These findings were confirmed by Wiklund et al. (106). Xu et al. (110) found the strongest evidence for linkage in families with an average age of diagnosis ≥65 years and a larger number of men affected, while Wiklund et al. (106) found stronger evidence in families with younger age of diagnosis and with the fewer than five affected family members. The linkage to chromosome 8p22-23 motivated Xu et al. (112) to perform a mutation screening in hereditary prostate cancer families, where they detected six rare missense mutations and one nonsense mutation in the macrophage scavenger receptor 1 gene (*MSR1*), and these were found to co-segregate with prostate cancer.

1.3.8. Recent genomewide linkage studies and putative HPC loci at 16q, 19q, 11q and other sites

In a genomewide linkage study of 504 brothers with prostate cancer that were from 203 multiplex sibships Suarez et al. (97) found evidence for prostate cancer susceptibility locusies on chromosomes 2q, 12p, 15q, 16p and 16q with the with the strongest linkage on chromosome 16q with Z score=3.15. Subgroup analysis of the late-age-at-onset group gave evidence at of linkage on 4q.

Witte et al. (107) conducted a genomewide linkage analysis of 513 brothers with prostate cancer, using the Gleason score, as a quantitative measure of prostate cancer aggressiveness. Candidate regions were found on chromosomes 5q (P=0.0002), 7q (P=0.0007), and 19q (P=0.0004).

Gibbs et al. (37) performed a genomewide scan of 94 families with hereditary prostate cancer, including 432 affected men. Stratification by age at diagnosis highlighted a

putative susceptibility locus on chromosome 11, among the later-onset families, with a LOD score of 3.02.

1.3.9. Other candidate prostate susceptibility genes

Polymorphisms in a number of genes important in steroid metabolism and signalling have been suggested to be associated with prostate cancer. This includes polymorphisms in androgen receptor (AR) gene, steroide-5α -reductase typeT II (SRD5A2), 17-hydroxilase-cytochrome P450 gene (CYP17).

A candidate prostate cancer gene on the X chromosome is androgen receptor (AR) gene, located at Xq12. The AR gene is polymorphic regarding a variable number of trinucleoted repeats of CAG and GGN in exon 1. The transactivation activity of the product of the AR gene resides in the N'-terminal region of the protein (encoded by exon 1). Short variants may increase the risk for prostate cancer through stimulation of the androgen receptor, whereas the longer AR variants show decreased transactivation activity and decreased binding affinity for androgens. Studies on androgen receptor yielded inconsistent results (49;92).

Elevated levels of DHT (dihydrotestosterone) have been suggested to increase the risk of prostate cancer. The SRD5A2 gene converts testosterone to the more bioactive compound to the DHT. The most common exchange in SRD5A2 gene is an alanin to threonin substitution at codon 49 (Ala49Thr) and increases the catalytic activity of the enzyme, and therefore modifies the risk of prostate carcinoma. Makridakis et al. (68) identified a total of 17 de novo amino-acid substitutions in 13 of 30 microdissected prostate adenocarcinomas. In total, 18 out of 30 (60%) of the examined tumours had de novo somatic substitutions in the prostatic steroid 5alpha-reductase-coding region. The two missense substitutions increased 5alpha-reductase in vitro activity.

1.4. CYP17 (Cytochrome P450c17α) gene

CYP17 belongs to the cytochrome P450 superfamily, a highly diversified set of heme containing proteins. The members of cytochrome P450 superfamily are often called hydroxylases, because hydroxylation is the most frequent reaction that they catalyze. The P450 proteins are performing a wide spectrum of reactions including N-oxidation, peroxidation, deamination, sulfoxidation, etc (103). By convention the cytochrome P450 enzymes with the similar sequences are clustered into families where the first number in the name corresponds to the family group. All enzymes in a family have at least 40% amino acid sequence homology. They are further grouped into subfamilies designated by an alphabet letter. All enzymes in the same subfamily have at least 55% amino acid sequence homology. The last number designates the gene that codes for a specific enzyme (e.g. 11A1, 11A2, 21A2). The class of steroidogenic CYP enzymes is comprised of families 7, 11, 17, 19, 21, 27.

The cytochrome P450c17α enzyme is involved in the biosynthesis of androgens. Steroid hormones have an important role as a determinant of prostate cancer risk. Predisposing variants have been often suggested within genes of the androgen metabolism pathway for several reasons. First, androgens are crucial for the normal development of the prostate gland and in maintaining its functional state in the adult. Second, it has been proven that prostate carcinoma is a highly hormone-dependent tumour (89). Third, it has been reported that increased levels of plasma testosterone are associated with an increased risk for prostate cancer (34). Testosterone is synthesized from cholesterol by a series of enzymatic reactions involving several of the cytochrome P450 enzymes including *CYP17* (Fig. 1).

The cytochrome P450c17α (*CYP17*) encodes the cytochrome P450c17 protein. This enzyme mediates two key reactions in steroid hormone biosynthesis: 17α-hydroxylation of pregnenolone and progesterone, as well as 17,20-lysis of 17α-hydroxypregnenolone and 17α-hydroxyprogesterone. *CYP17* is located on chromosome 10q24.3 and contains 8 exons (79). A single nucleotide polymorphism (SNP) has been described in the 5' untranslated region, 27 bp downstream from the transcription start site and 34 bp upstream from the initiation of translation. It has been assumed that thymidine (T) to cytosine (C) transition creates an additional recognition site (CCACT–CCACC) of the transcription factor Sp–1, and was therefore

expected to alter the expression level of the *CYP17* (30). Two years later, it has been shown by Nedelcheva, Kristensen et *al.* (73) by EMSA that this polymorphism does not create a binding site for Sp–1, but rather that there is an interaction between the *CYP17* polymorphism and some other transcription factor.

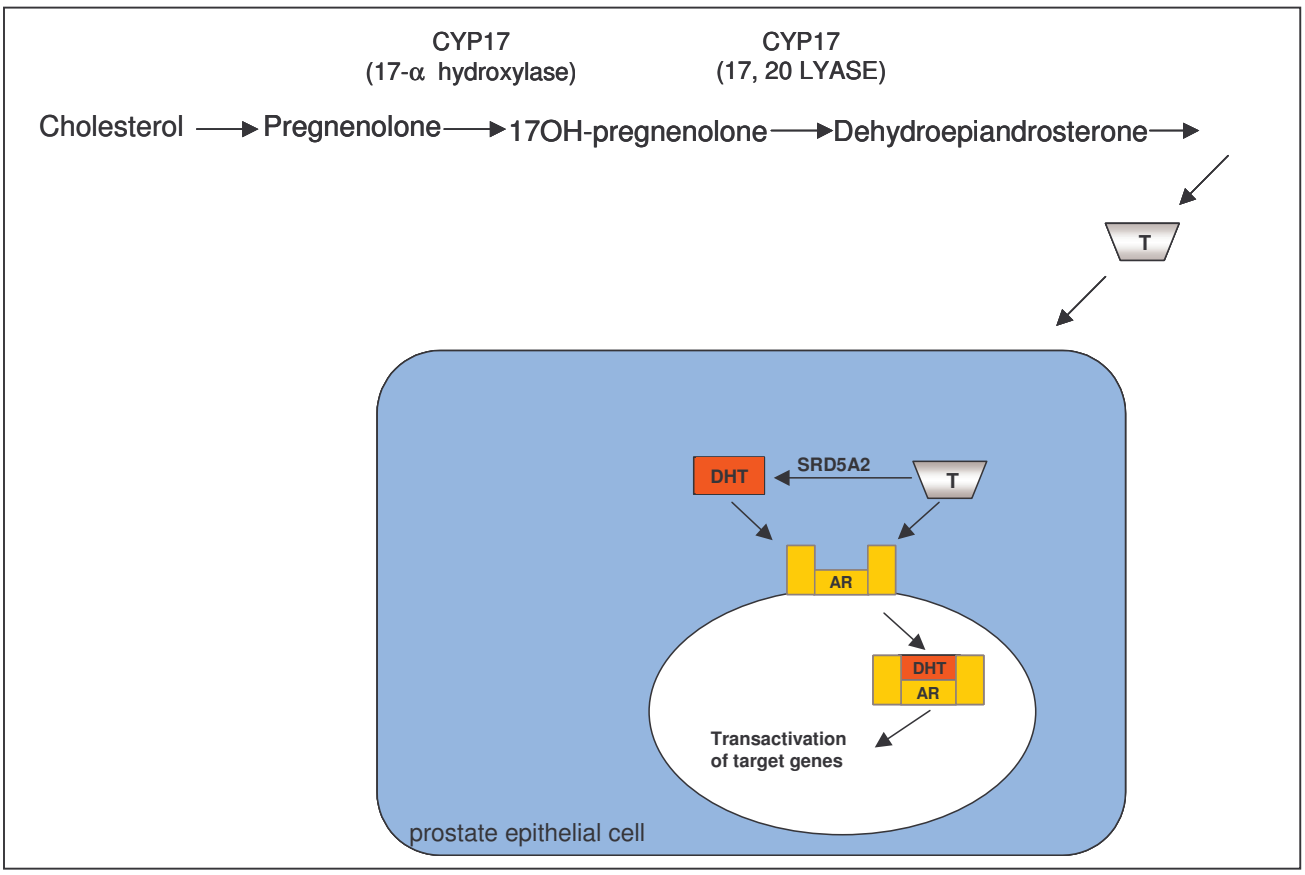


Fig. 1. Diagram of the biosynthesis pathway of T (testosterone) and its conversion to DHT (dihydrotestosterone) (33).

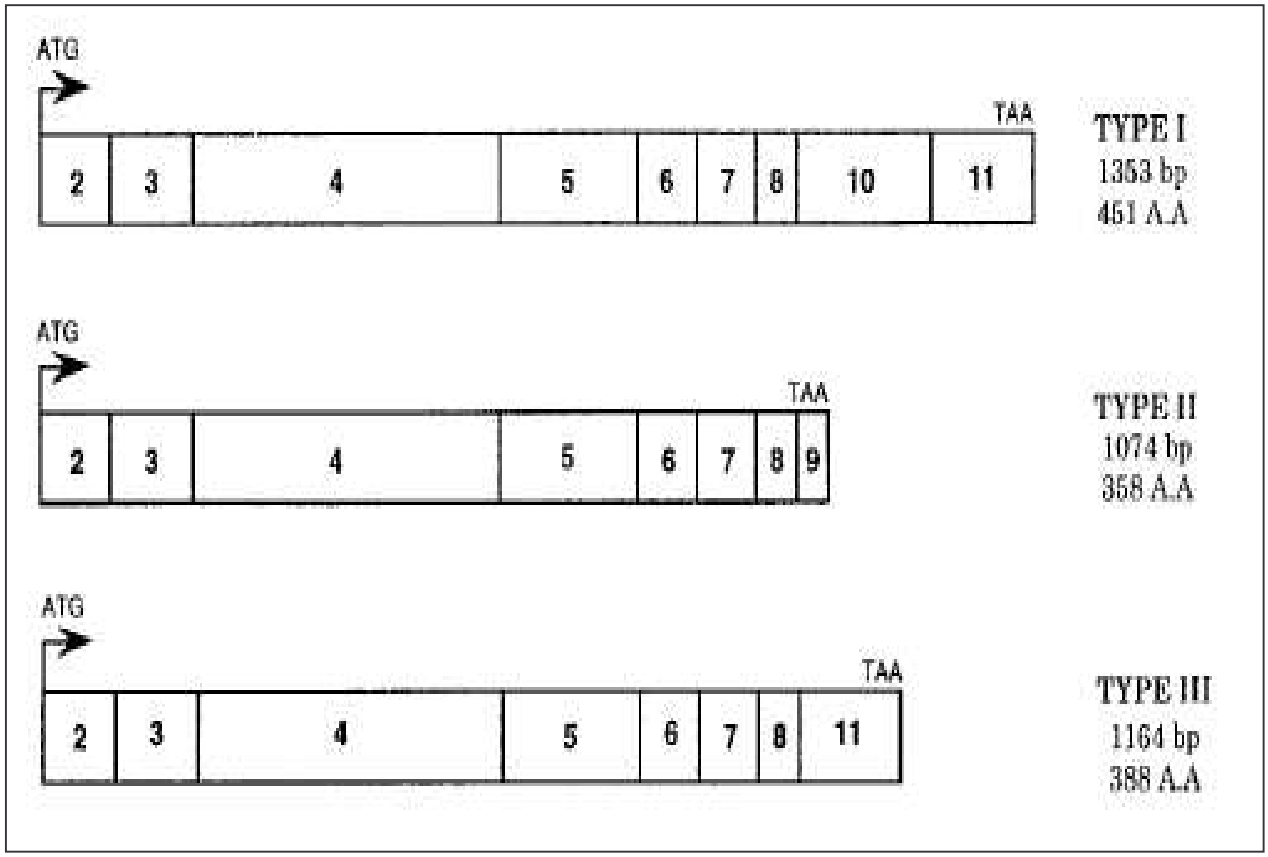
Association studies have been conducted to investigate a possible effect of the *CYP17* polymorphism on the risk for the sporadic prostate cancer. However, the results were inconclusive concerning the question, whether the wild-type allele (referred to as A1 allele) or the altered allele (referred to as A2 allele) can be considered as a risk factor. A recent meta-analysis found no effect of the *CYP17*

polymorphism for the sporadic prostate cancer (74). The question still remains unresolved for familial disease, since only two investigators included prostate cancer families (22;93) . One US American study reported evidence that a familial history of the disease together with the *CYP17* A2 alteration may strongly increase prostate cancer risk (22).

1.5. MSR1 (macrophage scavenger receptor I) gene

The macrophage scavenger receptor I (MSR1) belongs to a group of transmembrane glycoproteins that mediate processing of a wide range of negatively charged macromolecules, ligand internalisation and cell adhesion. Likewise, *MSR1* has been linked to processes such as inflammation, innate and adaptive immunity, as well as to apoptosis (80). The *MSR1* gene is located on chromosome 8p22. The length of the human *MSR1* is approximately 80kb and consists of 11 exons and two types of mRNA. Protein isoformes are generated by alternative splicing from exon 8 to either exon 9 (isoform II) or to exons 10 and 11 (isoform I) (29). The third inactive form (isoform III) (41) is trapped in the enodoplasmatic reticulum, and acts as dominant-negative isoform regulating the activity of the other two active isoforms. The structure of all three isoforms can be seen in Figure 2.

Figure 2. Exon organisation of the *MSR1* isoforms. The numbers in the boxes pertain to the exon numbers (41).



One of the most frequent events in early prostate carcinoma is the loss of the short arm of chromosome 8, which occurs in 80% of prostate tumours, as well in some other carcinomas. Linkage studies of families affected with hereditary prostate cancer (*HPC*) showed that the short arm of chromosome 8, especially 8p22-23, may harbour a prostate cancer susceptibility gene. Xu et al. (110) reported the linkage evidence to locus 8p22-23 in 159 pedigrees affected with the HPC. These results were confirmed by Wiklund et al. (106) in the Swedish HPC families. In a subsequent study, Xu and co-workers (112) identified several missense mutations and one nonsense mutation (Arg293X) by analysing the *MSR1* gene sequence in members of families with the HPC. Association and family-based linkage studies showed statistical evidence that previously identified mutations are associated with prostate cancer. An important role of mutations in the *MSR1* gene to prostate cancer susceptibility was shown in Swedish hereditary and sporadic cases (63). Miller et al. (70) also showed that *MSR1*

mutations are associated with increased prostate cancer susceptibility among African American men. On the contrary, results from Wang et al. (102) do not support *MSR1* as a risk factor for the prostate cancer.

The process of inflammation and proliferative regeneration of prostate epithelium in the presence of increased oxidative stress, probably has a key role in the development of prostate cancer (26). Highly reactive molecules, such as H_20_2 and nitric oxide (NO), are released from inflammatory cells and can interact with DNA in the proliferating epithelium to produce permanent genomic alterations such as point mutations, deletions, and rearrangements. *MSR1* is activated by oxidative stress (69) and it is able to bind to oxidized low density lipoprotein, so it can modify the amount of reactive oxygen intermediates.

1.6. Aims of the study

The first part of the study was focussed on the *CYP17* gene. A thymidine to cytosine transition (designated A2 variant) in the promoter region of the *CYP17* has been used in several studies in order to determine a possible association with the risk of prostate cancer. Till now, no association of a *CYP17* polymorphism with sporadic cases has been shown. The question for familial cases is still unanswered because only two studies dealt with prostate cancer families. In order to further investigate a possible role of *CYP17* in familial disease aggregation we conducted an association study.

The second part of the thesis was focussed on the *MSR1* (macrophage scavenger receptor I) gene. Mutation screening of candidate genes from the 8p region revealed a number of rare mutations in the *MSR1* gene (112). Afterwards the role of *MSR1* in prostate cancer susceptibility was pointed out in African American men and in men of European descent. A genome wide linkage study performed by Maier et al. (66) gave evidence for linkage to 8p22 close to the *MSR1* gene. This led to the screening of the *MSR1* gene in our group (Maier et al, 2005, in press) (67) and several sequence variants were identified, both novel and previously reported.
For the purpose of my thesis the 6 length polymorphisms that span ~ 70kb of the *MSR1* gene were used. The aim of this study was to evaluate if certain alleles or haplotypes made up from six length variants in the *MSR1* gene are associated with prostate cancer.

2. Materials and methods

2.1. Patients and families

2.1.1. Familial prostate cancer cases

The collection and ascertainment of patients with familiar prostate cancer was done mainly on the basis of referrals from urologists at the University of Ulm. The diagnosis of prostate cancer was confirmed by histopathological report or by other adequate medical record. All of the selected families were Caucasian. In one third prostate cancer was diagnosed from their symptoms, while in the two thirds by PSA screening. In order to assess candidate regions that are particularly relevant for the German population, a genome wide linkage search was performed on 139 prostate cancer families from all over the country (66). The DNA samples from this set of the 139 prostate cancer families (with the few more families for the purpose of the CYP17 study) were available for accomplishing the results for this thesis.

For the study concerning *CYP17*, we applied selection criteria choosing, the youngest case within a pedigree of multiple affected relatives. We genotyped 82 unrelated familial prostate cancer probands, with a mean age at diagnosis of 60.4 years (range: 47 - 80). The characteristics of the represented families are shown in Table 2.

Table 2. The history of prostate cancer within the pedigrees, represented by 82 unrelated familial prostate cancer probands.

Family characteristic	No. of pedigrees
All	82 (100%)
Families with hereditary prostate cancer[a])	
Yes	26 (32 %)
No	56 (68 %)
No. of affected members	
2	32 (39 %)
3	33 (40 %)
≥4	17 (21 %)

[a]) According to the Hopkins criteria of hereditary prostate cancer (20).

For the analysis of the *MSR1* gene, we used 139 prostate cancer families, that were previously used in the genome wide linkage scan (66). The characteristics of probands are shown in Table 3.

Table 3. Subject characteristics

	Number of pedigrees
All families	139
Hereditary prostate cancer[a]	
Yes	47 (34 %)
No	92 (66 %)
Number of affected	
2	60 (42 %)
3	42 (30 %)
≥ 4	37 (28 %)

[a] - matched the criteria for hereditary prostate cancer according to Carter, et al (20).

Overall, 298 familial prostate cancer cases were available with an average number of 2.2 affected per family for genotyping and 111 unaffected relatives (0.8 per family). The mean age at prostate cancer diagnosis was 63.6 years (47 – 89 years).

2.1.2. Patients with sporadic prostate cancer

Patients who did not report any affected relatives were included as sporadic cases. For the association study concerning the *CYP17* polymorphism we used 92 sporadic cases with a mean age of diagnosis of 63.6 years (range: 43 - 79). The number of sporadic patients used in the *MSR1* study was 324 with the mean age of diagnosis at 63.8 years (range: 42-90).

2.1.3. Control samples

The control group included men who were not diagnosed with prostate cancer before, had a negative family history of the disease and (if available) levels of a serum PSA of ≤ 4 ng/ml. The total number of control samples for *CYP17* study was 89 with the mean age of diagnosis of 56.7 years (range 34 - 79). The control group used in *MSR1* analysis comprised 203 elderly men who were not diagnosed with prostate cancer before with mean age 57.5 (range 32- 88).

2.2. Laboratory material and devices

Chemicals

Agar	Gibco BRL, Neu Isenburg
Agarose	Roth, Karlsruhe
Ammonium chloride	Merck, Darmstadt
Ampicillin	Sigma Aldrich, Munich
Boric acid	AppilChem, Darmstadt
5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal)	Roth, Karlsruhe
Bromphenolblue	Merck, Darmstadt
Dimethylformamide	Fluka, Neu-Ulm
Desoxyribonucleosidetriphosphate	Amersham Bioscience, Freiburg
Ethanol, absolute	Sigma Aldrich, Seelze
EthidiumBromide	Sigma Aldrich, Steinheim
Ethylenediaminetetraacetic acid	Fluka, Neu-Ulm
Formamide	J.T. Backer, Holland
Glycerin, 87%	Merck, Darmstadt
Yeast-extract	Roth, Karlsruhe
Isopropanol	Fluka, Neu-Ulm
Isopropyl-beta-D-thiogalactopyranoside (IPTG)	Roth, Karlsruhe
Potassium chloride	Merck, Darmstadt
Potassium hydroxide	Merck, Darmstadt
Potassiumhydrogencarbonate	Merck, Darmstadt
Sodiumacetate	Merck, Darmstadt
Sodiumchloride	Applichem, Darmstadt
Sodiumdodecylsulphate	Serva, Heidelberg
Tris	Sigma Aldrich, Munich
Ultrapur water	Merck, Darmstadt
Xylene cyanol	Serva, Heidelberg

Buffers and Solutions

Ampicillin stock solutions	25mg/ml in H_2O
EDTA pH 8.0	0.5 M in H_2O pH (NaOH) (autoclaved)
Ethidiumbromide Stock solution (DNA)	10mg/ml EtBr in TE
IPTG Stock solution	100mM in H_2O (sterile filtered)
Loading buffer	50% Glycerin 0.25%(w/v)Bromphenolblue 0.25%(w/v) Xylene cyanol 10mM EDTA, pH 8
LB - Bakteria medium	1% (w/v) Bacto-Tryptone 0.5% (w/v) Yeast Extract 1% (w/v) NaCl pH 7.5 (autoclaved) (+ 50 mg/l Ampicillin)
LB - Freezing medium	50% Glicerin in LB-medium + Ampicillin
LB-Plates	1.5% Agar in LB-medium (autoclaved) + 50mg/l Ampicillin
Lysis buffer	155 mM NH_4Cl 10 mM $KHCO_3$ 0.1 mM EDTA pH 7 (autoclave)
Potassium chloride solution, saturated	6 M NaCl in H_2O (autoclaved)

PCR- buffer (10x)	15 mM $MgCl_2$ 100 mM Tris/HCl pH 8.3 500 mM KCl
Proteinase K using solution	10mg/ml
SDS stock solution	20% SDS (w/v) in H_2O
SE-Buffer	75 mM NaCl 25 mM EDTA NaOH pH 8 (autocalved)
TBE (5x) buffer	89 mM Tris/ HCl 89 mM Boric acid 2 mM EDTA pH 8
TE-buffer (10x)	10 mM Tris/HCl pH 7.5 1 mM EDTA pH 8 (autoclaved)
Tris/HCl	1 M Tris pH 8 (HCl)
X-Gal stock solution	2% in dimethylformamide (sterile filtered)
Bacterial strain	
E.coli TOPO10	Invitrogen, Groningen NL
Vector	
pCR4-TOPO	Invitrogen, Groningen NL

Length markers and Size standards

ϕX174 DNA/*Hae* III Marker	Promega, WI USA
GeneScan™-500 Rox™ Size Standard	Applied Biosystem, Foster City, USA
GeneScan™-120 Liz™ Size Standard	Applied Biosystems Foster City, USA

Oligonucleotides

Different PCR primers	Thermo Hybaid, Neu-Ulm Biomers, Ulm

Enzymes

DNA-Polymerase *Taq*	Amersham Bioscience, Freiburg
Phosphatase SAP (calf intestine)	USB, Cleveland USA
Phosphatase SAP (shrimp alkaline)	USB, Cleveland USA
Proteinase K	Sigma Aldrich, Munich
Eco RI, restriction enzyme	Bio Labs, New England
M*sp* AI, restriction enzyme	Bio Labs, New England

Reaction Kits

BigDye version 3.1 sequencing kit	Applied Biosystems, Foster City, USA
ddNTP SNaP Shot kit	Applied Biosystems, Foster City, USA

Devices

1) ABI prism 3100 Genetic Analyzer	Applied Biosystems Foster City, USA
2) Centrifuge	
Biofuge-pico	Heraeus
Ominfuge 2.ORS	Heraeus
3) Electrophoresis power supply	
CONSORT E452	Belgium
4) Thermoblock	
Thermomixer 5436	Eppendorf AG, Hamburg
5) Thermocycler	
PTC-100™ Thermal controller	MJ Research, Watertown, USA
T-Gradient	Biometra, Göttingen
6) Water bath	
GFL 1086	GFL, Burgwedel
7) Weight	SARTORIUS, Göttingen

Softwars

GeneScan Software	Applied Biosystems Foster City, USA
DNA Sequencing Analysing Software™ version 3.7	Applied Biosystems Foster City, USA
Genotyper® Software version 3.7	Applied Biosystems Foster City, USA

Statistical Programs

Microsoft® Excel 2000	Microsoft, Redmond USA
Statview v 5.0	SAS Institute inc., Madison, USA

FINETTI program	Thomas Wienker, IMSDD Bonn
HARDY program package	Guo SW, Thompson ET (44)
FAMHAP9 program	T. Becker, IMSDD Bonn (5)
FBAT	Laird et al. (60)

2.3. Methods

2.3.1. DNA isolation from peripheral blood

DNA samples of almost all probands in the present study were available when I started. They had been prepared according to the following method, which I used also when I had to prepare a few samples for additional analyses. To lyse the erythrocytes 30 ml of the lyses buffer were added in the 10 ml EDTA tube with patient blood, and the tube was incubated on ice for 15 min. The lymphocytes with nucleus were pelleted by centrifugation at 1000 rpm for 10 min at 4 °C. Afterwards, the supernatant (blood waste) was removed and the 10-20 ml of lyses buffer were added to the pellet. Pellet was resuspended and centrifuged again for 10 min at 4°C (1000 rpm). This step was repeated several times to get rid of erythrocytes as much as possible. After removing the supernatant (blood waste), for the final proteolysis 5 ml of SE-buffer were added and the pellet was thoroughly resuspended. 50 µl of proteinase K and 250 µl 20% SDS were added to the resuspended pellet and the tube was incubated overnight at 55°C in a water bath. In the next step 1,7-1,8 ml of saturated NaCl solution were added to the sample, vortexed 15s and centrifuged at 4000 rpm for 15 min at the RT. The supernatant was transferred into a new tube, and filled up with the two volumes of the RT absolute ethanol. The tube was shaked gently until the DNA was precipitated and then the DNA was captured with the use of glass pipette. The precipitated DNA was then washed in 70% ethanol and dissolved in the sterile TE-buffer.

2.3.2. Amplification of DNA by polymerase chain reaction (PCR)

Utilization of the PCR has been already extensively applied in a big number of research fields, including a diagnosis of genetic disorders. PCR is a rapid, inexpensive and simple mean of producing relatively large copy number of DNA molecules from the small amounts of source DNA material, even when the source DNA is of relatively poor quality. Due to the extreme sensitivity, precautions were taken against contamination of the reaction mixture with the trace amounts of DNA, which could serve as an unwanted template. Positive and negative controls were

included in each run. The PCR consists of 3 steps: first, the double-stranded DNA is denaturated into single strands at 95℃ for 5 min. Second step includes 30 to 35 amplifications cycles and finally the third step includes extension denaturation at 72℃ for 10 min. The amplification cycles of a target sequences consist of denaturation at 95℃ for 30s, then lowering of the temperature to allow annealing of a short oligonucleotides (primers) to complementary sequences in the single-stranded DNA. This annealing phase lasts for 30s to 60s, and at the end extension for 30s to 60s at 72℃.

Detection of the *CYP17* polymorphism via PCR reaction

The genomic DNA available from healthy controls and prostate cancer patients was analyzed for the presence of *CYP17* polymorphism using the PCR. For each sample, 25 µl of the reaction mixture containing 2-4 µl template DNA (final concentration 50 ng/µl), 0.25 µl (20 mM) dNTP's (deoxyribonucleoside triphospates), 0.625 µl of forward and reverse primers specific for *CYP17*, 2.5 µl of 10x concentrated reaction buffer (15 mM MgCl2), 0.3µl of Taq polymerase enzyme (5U/µl), and sterile water to set up.

The oligonucleotides sequences were:

CYP17 forward primer: 5'–GTTCCAAGCCTTGACTCTG–3'
CYP17 reverse primer: 5'–TGAAGACCTGAACCAATCCC–3'
The PCR was performed under the following conditions: 1) initial denaturation at 95℃ for 5 minutes, 2) 30 cycles each consisting of: 30 s at 95℃ (denaturation step), 30 s at 57℃ (annealing step) and 30 s at 72℃ (extension step), 3) final extension step at 72℃ for 10 minutes.

PCR for detection of MSR1 length variants

The genomic DNA was available from prostate cancer patients and healthy controls. The genomic regions containing length polymorphisms of the *MSR1* gene were amplified by PCR reaction (Table 4, the PCR primer sequences). PCR was performed under standard conditions in the total volume of 25 µl (for some PCR reactions extra MgCl2 was added). DNA was first denatured for 3 min at 95℃, following 30 to 35 cycles each consisting of denaturing step (95℃, 30 s), annealing

(30 to 60 s) and extension step (72℃, 30 to 60 s). Final step was extension for 10 min at 72 ℃.

For each length variant one of the primers forward or reverse was labelled with the 6-Fam (6-Carboxyfluorescein) bleu dye or with the 6-HEX (6-carboxy-2", 4,4'', 5'', 7,7''-hexachlorofluorescein) green dye.

Table 4. List of primers used for PCR amplification

Name of the variant	Length of PCR product (bp)[a]	Primer pair
IVS6	296 !!!!	msrepint6h-6Fam msrepint6r
IVS4	225	msrepint4h-6HEX msrepint4r
IVS7(TA)m(CA)n	432	msr1ex7h msr1ex7rn-6Fam
IVS7insTAT	476	msr1ex8h-6Fam msr1ex8r
IVS9(CA)n	124	msrepint10ah-6HEX msrepint10ar
INDEL1	470	msxu1h-6HEX msxu1r

[a]-length of PCR products based on the reference sequence NM_138715

2.2.3. Gel electrophoresis of DNA

The agarose gel electrophoresis is used to check the progression of a restriction enzyme digestion, to determine the yield and the purity of a DNA isolation or PCR reaction, and to size fractionate DNA molecules, which then could be eluted from the gel. Prior to the gel casting, dried agarose was dissolved in 1xTBE buffer by heating and the warm gel solution with added ethidium bromide, was poured into a cast, that is fitted with a well-forming comb (cast was made by wrapping tape around and extending above the edges of plastic mold). The ethidium bromide was included in the gel matrix to enable fluorescent visualization of the DNA fragments under the UV light. For analysis of PCR products, samples were submitted to the electrophoresis using 1.5% and 2% agarose gel. The DNA samples were mixed with gel tracking dye

and loaded into the sample wells in the gel. The size marker ϕX174 DNA/Hae III was co-electrophoresed with a DNA samples. Electrophoresis was usually done at 200 mA for 15-30 min at the room temperature, depending on the desired separation. Subsequently after electrophoresis, the gel was placed at the UV illumination box and a picture of the fluorescent ethidium bromide-stained DNA separation pattern was taken.

2.2.4. Restriction enzyme digestion

Restriction enzyme digestion was performed by incubating the double-stranded DNA molecules with an appropriate amount of restriction enzyme, in its respective buffer as recommended by the supplier, and at the optimal temperature for that specific enzyme. A typical digestion includes one unit of enzyme per microgram of starting DNA (one enzyme unit is usually defined as the amount of enzyme needed to completely digest one microgram of double-stranded DNA in one hour at the appropriate temperature).

The T to C transition in the promoter region of the *CYP17* was amplified by polymerase chain reaction (PCR), and the PCR products were than incubated with the restriction enzyme *Msp*A1.

Recognition site for *Msp*A1 enzyme is:

5'…CMG▼CKG...3'

3'...GKC▲GMC…. 5'

The restriction digestion by *Msp*A1 included:

	1x
*Msp*A1 (10.000U/ml)	0.5µl
10 x NEBuffer 4	2µl
10 x BSA	2µl
PCR product	15µl

Reaction was incubated for 3 hours at 37°C, and then restricted fragments were separated on the ethidium bromide-stained 2% agarose gel.

2.2.5. Cloning of the PCR products

Several length variants of the MSR1 gene used for the fragment analysis were the repeats of more than two base pairs. Before starting the fragment analysis, to get the real allele combinations that could be used in the analysis, we performed cloning and sequencing of the two length MSR1 variants. We used the TOPO TA Cloning that is a highly efficient cloning strategy allowing a direct insertion of the *Taq* polymerase-amplified PCR products into the plasmid vector pCR4-TOPO. This plasmid vector contains a single 3´-thymidine (T) overhangs and topoisomerase I covalently bound to the vector. The *Taq* polymerase adds a single deoxyadenosine (A) to the 3´ ends of PCR products that are allowing PCR inserts to ligate efficiently with the vector.

For the TOPO Cloning reaction 0.5-4 µl of PCR product were mixed with 1 µl of vector, 1 µl of salt solution and sterile water to a final volume of 6 µl. Reaction mixture was incubated at the RT for 5-30 min (depending on the size of PCR fragment) and afterwards placed on ice.

Transformation

2µl of the TOPO Cloning reaction were added into a vial of One Shot Chemically Competent *E.coli* and incubated on ice for 5min. The next step was the heat shock of the cells for 30s at 42°C in the water bath and afterwards the vial was immediately transferred on ice. To enable replication of the plasmid in the transformed cells 250µl of SOC medium were added and the mixture was shakend at 37°C for 30-60 min. At the end 10-50 µl of transformation reaction was spreaded on a prewarmed selective agar plate. 50µl of X-Gal and 10µl of IPTG were also added to the agar plates. Agar plates were incubated overnight at 37°C.

Selection of positive clones and DNA amplification

The pCR4-TOPO plasmid contains the LacZ gene that codes the production of the enzyme beta-galactosidase. Normally the beta-galactosidase metabolizes galactose but it can also convert other substrates such as X-Gal (5-bromo-4-chloro-3-indolyl-[beta]-D-galactopyranoside) into a coloured product. X-Gal is a colourless modified galactose sugar; however, when it is metabolized by the beta-galactosidase the products are a bright blue. In order for gene to be actively transcribed from the DNA and then for the enzyme to be produced, an activator called IPTG (isopropyl- [beta]-D-thiogalactopyranoside) has to be added. Within the LacZ gene there is a cloning site where the plasmid can be cutted and a foreign DNA can be added. This produces a plasmid with a foreign DNA located within the LacZ gene. Because of its location within the enzyme the foreign DNA's translated protein product disrupts the

activity and function of the beta-galactosidase enzyme. The disrupted enzyme activity is observed as a white bacterial colony. (If the enzyme is functioning fully each colony is a bright blue colour).

The positive clones (white colonies) were taken with the tip from the agar plate. The tip was firstly resuspended in the master mix for PCR (containing H_2O, PCR buffer, dNTPs, primers and *Taq* polymerase). Afterwards the same tip was resuspended in 1.5ml eppendorf with 30µl LB medium+Amp, and the eppendorf was shaked over night at 37°C and than used for TempliPhi reaction. The PCR was performed to detect the proper clones. After detection of the proper clones via the PCR reaction, the TempliPhi reaction was performed to amplify the DNA.

First step in TempliPhi reaction was mixing 1µl of material to be amplified and 5µl of Sample Buffer and incubating at 95°C for 3min and then cooling to the RT. In the next step 5µl of mix (5µl Reaction Buffer and 0.2µl Enzyme Mix) were added and the reaction was incubated at 30°C for 4-18 hours. Inactivation of the enzyme was done by heating to 65°C for 10 min. After this step the direct sequencing of samples was possible.

The second way for amplifying the sample DNA, instead of TempliPhi, is using the Mini Prep kit (Quiagen).

Mini Prep

1-1.5ml of overnight culture was centrifuged at the 3000rpm for 30s to pellet the cells. The supernatant was removed and the pellet was resuspended completely in 150 µl of solution I by vortexing. The 150 µl of solution II were added and mixed by inverting the tube 10-15 times. Then 300 µl of solution III were added and mixed by inverting the tube until a flocculent precipitate appears. The samples were centrifuged at full speed for 5 min at the RT to pellet the cell debris. The supernatant was transferred to the prepared GFX column (GFX column were placed in vacuum pump) and incubated for 1 min at RT. After incubation the vacuum pump was turned on to allow the solution to be drawn through each column into the collection tray. 400µl of wash buffer were added and the previous step was repeated. After removing the residual wash buffer the GFX column was transferred to a microcentrifuge tube and 50 µl of the TE buffer (or HPLC water) were added directly to the top of membrane in the column. The sample was than incubated at the RT for 1 min and than centrifuged at full speed for 1 min to recover the purified DNA. After amplification, the plasmids

were analysed by restriction digestion with *Eco*RI enzyme to confirm the presence and correct orientation of the insert.

Sequencing of the plasmid

To confirm that our fragments of interest were cloned in the correct orientation and to analyse them we performed sequencing with M13 forward and M13 reverse primers. These primers are located nearby the insert. The sequences of the primers are:

M13 Forward 5´-GTAAAACGACGGCCAG-3´

M13 Reverse 5´-CAGGAAACAGCTATGAC-3´

The sequence reaction was PCR with labelled dNTPs. The reaction components were:

	1x
BDT premix(vers. 3.1)	1µl
5x Buffer	1µl
M13 for (or M13 rev)	0.5µl
Plasmid DNA	2.5µl

All components were mixed together in the PCR tube and submitted to the PCR reaction consisting of 25 cycles. Each cycle had three steps: 1) denaturation at 96°C for 10s, 2) annealing at 55°C for 5s and at the end 3) elongation for 3min at 60°C. After sequencing reaction samples were purified from an unincorporated dye terminators prior to the ABI analysis. Excess of dye terminators can interfere during sequencing, so the cleaning step was necessary.

Firstly, 30µl of Wash Solution were added to the samples, mixed well and transferred to the SEQ plate (for cleaning up). The vacuum pump was turned on for 3-4 min to allow the solution to be drawn through into the collection flask. 30µl of the Wash Solution were added once more to the samples and the step with the vacuum pump was repeated. At the end 30µl of the Injection Solution were added to the samples and the plate was shaken for 10 min. Afterwards, the samples were transferred to the Thermo-Fast Detection plate and submitted to the ABI3100.

2.2.6. Sequencing

To examine whether there were coding or splice site polymorphisms in the *MSR1* gene, amplified PCR products were sequenced in both directions. All exons of the *MSR1* gene were amplified by PCR using the oligonucleotides that anneal to intronic sites 50-100 bp from the intron – exon boundaries. Some of the PCR-amplifications primers were used for sequencing also (Maier et al.). To remove the primers and dNTPs, PCR products were purified using filtration membrane plates (Millipore) prior sequencing. 150µl of sterile water were added pro well in the 96 Millipore filtration plate and the PCR products were added than in the plate. The vacuum pump was turned on for 5 min to allow the solution to be drawn through into the collection flask and when the membrane in the plate was dry the pump was still on for 30s. To the each well 50µl of sterile water were added and plate was shaken for 10 min. Afterwards the samples were transferred into 96-well plate and proceeded with sequencing reaction. Direct sequencing was preformed using the Big Dye Terminator Cycle Sequencing Kit version 3.1., according to the protocol of the manufacturer (Applied Biosystems). The sequencing reaction had consisted of:

	1x
BDT premix(vers. 3.1)	1µl
5x Buffer	1µl
primer	0.5µl
PCR product	2.5µl

The sequencing cycles and cleaning up of the sequencing reaction are described in the previous part (sequencing of the plasmid).

After sequencing on the ABI PRISM 3100 DNA Analyzer, sequences were analysed by the SeqScape v2.0, where the individual sequences from every sample were aligned to a genomic reference sequence (accession number AC023396.4)

2.2.7. SNP genotyping

Sequencing of the MSR1 provided an insight into the different polymorphisms in the *MSR1* gene. Together with the previously reported SNPs additional SNPs were found and have been genotyped using the ddNTP SNaP Shot kit (Applied Biosystems, Foster City, USA). For the purpose of the multi- SNP genotyping, the PCR products

were pooled. Firstly prior to ddNTP primer extension, the PCR products were cleaned by treating with 2.5 U SAP (shrimp alkaline phosphatase) and 1.0 U ExoI (exonuclease I) (USB, Cleveland, USA) per 10 ng PCR product. These two enzymes serve for degradation of not incorporated PCR-primers and dephosphorylation of not incorporated dNTPs. After pooling the PCR products in enzyme-mix (SAP, EXO I and sterile H_2O), reaction mixture was incubated, firstly at 37℃ for 45min and than 15min at 72℃ for enzyme inactivation. The next step was SNaP Shot reaction. 1.5µl of cleaned PCR product was mixed with 3.5µl of master mix (SNaP Shot reagent-including polymerase, fluorescence labelled dNTPs, and SNaP Shot primers). Total volume of the 5µl SNaP Shot reaction was submitted for SNP cycle reaction including 25 cycles (96℃ for 10s, 50℃ for 5s, 60℃ for 30s). After the SNP cycle reaction SNaP Shot products were cleaned with 0.5U of CIP (Calf Intestinal Phosphates) enzyme that catalyses dephosphorylation of not incorporated fluorescence ddNTPs. Reaction included first incubation at 37℃ for 30 min and then 15 min at 72℃ for the enzyme inactivation. Afterwards samples were mixed with formamide and LIZ120 size standard, and submitted to the automatic sequencer ABI3100.

2.2.8. Fragment analysis

The DNA fragment analysis is a useful tool to discriminate individuals from each other on the polymorphic differences in their DNA sequence. These polymorphisms can result in DNA fragments that differ in size from a single to a few nucleotides. Fragment analysis relies on the detection of changes in the length of a specific DNA sequence to indicate the presence or absence of a particular allele. For the purpose of our study we used 6 length variants found in the *MSR1*.

The first step in the fragment analysis was performing the PCR for all six variants. PCR was performed under standard conditions in total volume of 25µl and with the labelled primers (Table 4.). PCR products were than checked by agarose gel electrophoresis for the right fragment size. In the next step 20 µl of master mix (formamide and ROX500 size standard) were placed in the 96 well Thermo-Fast Detection plate. 1µl of PCR from each variant was added in the well and the plate was than denaturated for 2min at 95℃. After cooling down, the plate was submitted to the ABI3100.

2.4. Statistical methods

2.4.1. Hardy-Weinberg equilibrium

One of the most important concepts in the population genetics is the Hardy Weinberg law of equilibrium. The law predicts how gene frequencies will be transmitted from generation to generation under a specific set of assumptions. This set of assumptions includes that an infinitely large, random mating population is free from outside evolutionary forces (i.e. mutation, migration and natural selection). In this case the gene frequencies will not change over time.

Since at any autosomal locus, an individual carries two alleles, for example A and a, and if the relative frequency of the A allele in the population is *p* and *q* is denoted as the relative frequency of the a allele, than the following equation holds true:

$$\mathbf{p^2 + 2pq + q^2 = 1}$$

Where $\mathbf{p^2}$ represents the fraction of the population with the genotype AA, **2pq** represents the fraction of the population with the genotype, Aa, and $\mathbf{q^2}$ represents the fraction of the population with the genotype aa.

This is the case when the Hardy-Weinberg law is used to describe a population with only two alleles for a given gene. The generalized form that describes a population with **n** alleles for a given gene is:

$$(\sum_{i\approx 1}^{n} p_i)^2 = \sum_{i\approx 1}^{n} (\sum_{j\approx 1}^{n} 2\, p_j\, p_i) - \sum_{i\approx 1}^{n} p_i^2$$

(the formula was adopted from http://wiki.cotch.net/index.php/Hardy-Weinberg_law)

Where $\mathbf{p_i}$ is the proportion of the **i**th allele in the population, and each term of the series (after simplification by combining like terms) is the proportion of organisms with the genotype Ai Aj where Ai is the allele with frequency $\mathbf{p_i}$ and Aj is the allele with frequency $\mathbf{p_j}$.

For the purpose of our study Hardy-Weinberg-equilibrium was checked separately in samples and controls, using the exact test implemented in the program FINETTI (Thomas Wienker, IMSDD Bonn) for bi-allelic markers and the programme package HARDY (44) for multi-allelic markers.

2.4.2. Odds ratio and 2x2 contingency tables

Odds ratio is the ratio of the odds of the risk factor in a disease group and in a control group (the ratio of the frequency of presence / absence of the marker in cases to the frequency of presence / absence of the marker in controls). The OR is used in case-control studies and it is a measure of the strength/magnitude of an association. A 2x2 contingency table is the simplest contingency table, required for odds ratio (OR) estimation. A general layout of a contingency table for a disease association study is as follows

		allele i		
		Present	Absent	row totals
outcome	Patients	a	b	a+b
	Controls	c	d	c+d
	column totals	a+c	b+d	N=a+b+c+d

Odds ratio is calculated as ad/bc where a, b, c, d are the entries in a 2x2 contingency table.

$$OR = \frac{a/b}{c/d}$$

which can be simplified to

$$OR = \frac{ad}{bc}$$

The odds ratio is a measure of association in which a value of "1.0" means that there is no relationship between variables. The value of an odds ratio can be less than 1.0 or greater than 1.0. An odds ratio less than 1.0 indicates an inverse or negative association. An odds ratio greater than 1.0 indicates a positive relation.

2.4.3. Measures of linkage disequilibrium

Linkage disequilibrium (LD) describes the greater co-occurrence of two genetic markers (on the same chromosome, as a haplotype) in a population than would be expected for independent markers. Usually, LD is generated when the markers are located close together on the same chromosome. The concept of LD was firstly formalized by ***D*** that represents the measure of disequilibrium:

$$D_{ij} = x_{ij} - p_i \cdot q_j$$

where x_{ij} represents the observed frequency of gamete A_iB_j, p_i and q_j are the frequencies of alleles A_i and B_j at loci A and B, respectively. Due to the dependence of ***D*** on allele frequency, numerical value of ***D*** is of little use for measuring the strength of LD and comparing levels of LD. Instead of ***D*** in common use is measure ***D'***.

The absolute value of ***D'*** is:

$$D'_{ij} = \frac{D_{ij}}{D_{max}}$$

where D_{max}= min[p_iq_j, (1– p_i)(1– q_j)] when $D_{ij} < 0$ or D_{max}= min[p_i (1– q_j), (1– p_i) q_j] when $D_{ij} > 0$

Linkage disequilibrium (LD) between markers was quantified using the LD measure D'. Based on genotypes, haplotypes of adjacent variants were constructed and their frequencies were estimated using the program FAMHAP9 (5). Haplotype frequencies were compared using a χ^2 test available in FAMHAP9 program.

Family-based association test was performed for five markers using a software package FBAT (60). FBAT utilizes data from nuclear families, sibships, or combination of the two to test for linkage and linkage disequilibrium between traits and genotypes. FBAT determines an S statistic from the data that is a linear combination of offsprings genotypes and phenotypes. The distribution of the S statistic is generated by treating the offspring genotype data as random and

conditioning on the phenotypes and parental genotypes. When the marker is biallelic, a Z statistic and its corresponding *P* value are calculated. When the marker is multiallelic, a χ^2 test is performed.

These calculations were performed by PD Josef Hoegel.

3. Results

3.1. Role of CYP17 in familial prostate cancer

3.1.1. Detection of *CYP17* polymorphism

The *CYP17* promoter polymorphism was investigated in 82 unrelated familial prostate cancer cases, 92 sporadic and 89 control probands.

The 718 bp DNA fragment including the T to C transition in the promoter region of *CYP17* was amplified by the polymerase chain reaction (PCR) and afterwards analysed by restriction digestion with the *Msp*AI enzyme. The PCR product of the A1 allele is cut into two fragments at a constitutive *Msp*AI site (598 and 120 bp), while in the A2 allele the additional polymorphic *Msp*AI site resulted in three restriction fragments (Fig. 3) (100).

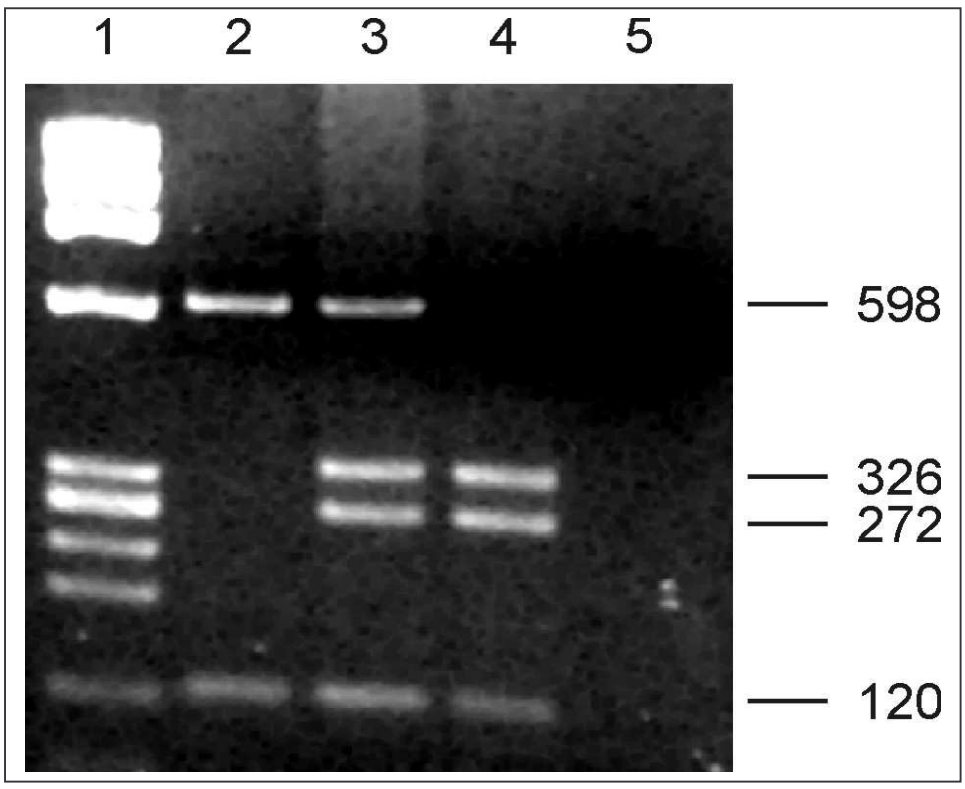


Figure 2: *Msp*AI 1 restriction fragments of *CYP17* alleles, separated by agarose gel electrophoresis (2 % agarose gel in TBE). Fragment length is given as base pairs. Lane 1, DNA molecular weight marker; lane 2, genotype A1/A1 (wild-type); lane 3, genotype A1/A2; lane 4, genotype A2/A2; lane 5, negative control.

3.1.2. Association between the CYP17 polymorphism and prostate cancer

The observed allele frequencies for the familial group were A1: 62% and A2: 38%; for the sporadics A1: 55% and A2: 45%; and A1: 61% and A2: 39% for the controls. For each study group, genotype distributions were verified to be in accordance with the Hardy-Weinberg equilibrium ($p \geq 0.47$) and the observed allele frequencies (A1: 55 – 62%, A2: 38 – 45%) did not differ between the three groups investigated ($p > 0.30$).
A supposed influence of the A2 allele on disease risk was investigated by setting up a dominant and a recessive case control model. For the dominant model, we compared individuals being heterozygous (A1/A2) and homozygous (A2/A2) for the promoter polymorphism against the wild-type genotype (A1/A1). In the recessive model, the homozygous state of A2 representing the risk genotype group was compared to all others (A1/A1 and A1/A2) used as the reference group.

Firstly, we compared familial cases against controls (Table 5A) and sporadic cases versus controls (table 5B) (100).

Table 5. Association of *CYP17* alleles with prostate cancer risk in the groups of familial prostate cancer cases (A) and sporadic disease (B) compared to controls.

A) Families					
Genotype	Controls	Cases	OR	95%CI	*P*-value
Dominant model (A_{12}+A_{22} *vs.* A_{11})					
A_1A_1	29 / 89 (33%)	33 / 82 (40%)	Reference		
A_1A_2+A_2A_2	60 / 89 (67%)	49 / 82 (60%)	**0.72**	0.38 – 1.34	0.30
Recessive model (A_{22} *vs.* A_{12}+A_{11})					
A_1A_1+A_1A_2	79 / 89 (89%)	69 / 82 (84%)	Reference		
A_2A_2	10 / 89 (11%)	13 / 82 (16%)	**1.48**	0.60 – 3.60	0.38

B) Sporadic cases					
Genotype	Controls	Cases	OR	95%CI	*P*-value
Dominant model (A_{12}+A_{22} *vs.* A_{11})					
A_1A_1	29 / 89 (33%)	29 / 92 (32%)	Reference		
A_1A_2+A_2A_2	60 / 89 (67%)	63 / 92 (68%)	**1.05**	0.56 – 1.96	0.90
Recessive model (A_{22} *vs.* A_{12}+A_{11})					
A_1A_1+A_1A_2	79 / 89 (89%)	72 / 92 (78%)	Reference		
A_2A_2	10 / 89 (11%)	20 / 92 (22%)	**2.20**	0.96 – 5.00	0.06

The resulting odds ratios were not statistically significant. In both models (dominant and recessive), comparison between families and controls showed no significant increase of the A2 risk allele among familial cases. A certain trend of the *CYP17* A2-containing genotype has been seen when the comparison was restricted only to sporadic cases *versus* controls, giving an elevated odds ratio of OR = 2.20, but still with an insignificant interval of confidence (CI = 0.96 – 5.00; $p = 0.06$, Table 5B).

In a further step, we examined a possible association between *CYP17* genotypes and the risk of prostate cancer in general. For this purpose, all prostate cancer probands with and without a familial history of the disease were compared with the controls (Table 6).

Table 6. Relationship between the *CYP17* polymorphism and prostate cancer risk in general. All prostate cancer probands, regardless of family history, were compared to controls.

Genotype	Controls	Cases	OR	95%CI	*P*-value
Dominant model (A_{12}+A_{22} *vs.* A_{11})					
A_1A_1	29 / 89 (33%)	62 / 174 (36%)	Reference		
A_1A_2+A_2A_2	60 / 89 (67%)	112 / 174 (64%)	**0.88**	0.50 – 1.50	0.60
Recessive model (A_{22} *vs.* A_{12}+A_{11})					
A_1A_1+A_1A_2	79 / 89 (89%)	141 / 174 (81%)	Reference		
A_2A_2	10 / 89 (11%)	33 / 174 (19%)	**1.85**	0.87 – 3.95	0.11

Under the dominant model, the frequency of the risk genotype (A1/A2 + A2/A2) was equal between all cases and controls with a corresponding odds ratio of 0.88 (95% CI, 0.50 – 1.50). Applying the recessive model, we found a slight but not significantly higher frequency of risk genotype (A2/A2) in the total group of cases as compared to controls with an OR = 1.85 (95% CI, 0.87 – 3.95). The excess of A2/A2 genotypes was mainly due to a high frequency of A2 homozygous carriers in the sporadic prostate cancer sample.

An US American study (93) reported evidence that a familial history of the disease together with the *CYP17* A2 allele may strongly increase prostate cancer risk. We thus estimated the frequency of the *CYP17* A2 allele comparing our familial cases to all other individuals having no affected relatives, i.e. the combined group of sporadic probands and healthy men (Table 7).

Table 7. Association of the *CYP17* A2 risk allele with familial aggregation of prostate cancer. Familial prostate cancer probands were compared to individuals having no affected relatives (sporadic cases and controls).

Genotype	Sporadic cases and controls	Familial cases	OR	95%CI	*P*-value
Dominant model (A_{12}+A_{22} *vs.* A_{11})					
A_1A_1	58 / 181 (32%)	33 / 82 (40%)		Reference	
A_1A_2+A_2A_2	123 / 181 (68%)	49 / 82 (60%)	**0.70**	0.40 – 1.20	0.20
Recessive model (A_{22} *vs.* A_{12}+A_{11})					
A_1A_1+A_1A_2	151 / 181 (83%)	69 / 82 (84%)		Reference	
A_2A_2	30 / 181 (17%)	13 / 82 (16%)	**0.95**	0.50 – 1.93	0.88

The comparison of familial prostate cancer probands with individuals without affected relatives showed no association of the A2 risk allele with prostate cancer neither under the dominant (OR = 0.70; 95% CI, 0.40 – 1.20) nor under the recessive model (OR = 0.95; 95% CI, 0.50 – 1.93) (100).

Stanford et al. (93) gave evidence that men with a family history of prostate cancer who were homozygous for the A2 allele had a significantly increased risk for prostate cancer (OR = 19.2; 95% CI, 2.23 – 157.4) compared to men without a family history who were homozygous for the A1 allele. In our analysis, no evidence for an association was obtained when A2/A2 familial cases were compared to A1/A1 men without affected relatives (OR = 1.10; CI = 0.40 – 2.90; p=0.80).

3.2. MSR1 and risk of prostate cancer

3.2.1. Mutation screening results of MSR1gene

In our group the exons and exon-intron junctions of the *MSR1* gene have been sequenced in 139 family probands (each representing one family) (Maier et al., 2005; in press) (67). The study was carried out in a team and revealed a number of sequence variants Figure 4 and Table 8.

Figure 4. Sequence variants found in the *MSR1* gene (boxes and numbers are corresponding to exons, lines between boxes to introns. Blue boxes indicate the coding region while the 5' and 3'-noncoding region are represented in grey).

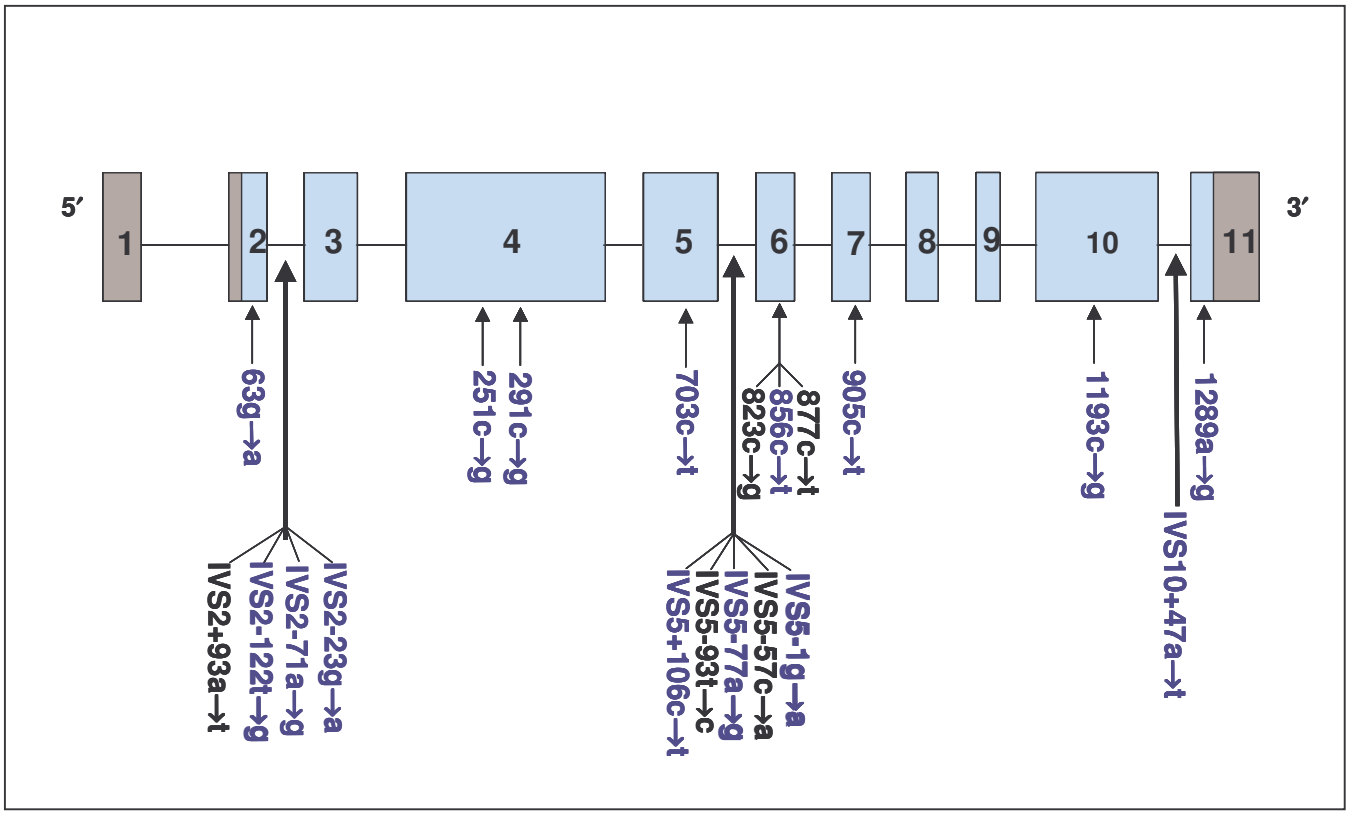


Table 8. Variants identified in *MSR1* coding and non-coding region (from Maier et *al*., 2005; in press) (67)

Nucleeotide variant[a)]	Location	consequence	Allele frequency	Observed in other study population
c.63g→a	exon 2	silent	0.004	novel
IVS2+93a→t	intron 2	–	0.122	USA[b)]
IVS2-122t→g	intron 2	–	0.051	novel
IVS2-71a→g	intron 2	–	0.004	novel
IVS2-23g→a	intron 2	–	0.004	novel
c.251c→g	exon 4	S84X	0.004	novel
c.291c→g	exon 4	silent	0.004	novel
c.703c→t	exon 5	H235Y	0.004	novel
IVS5+106c→t	intron 5	–	0.585	novel
IVS5-93t→c	intron 5	–	0.007	USA[b)]
IVS5-77a→g	intron 5	–	0.004	novel
IVS5-57c→a	intron 5	–	0.044	USA[b,g,d)], Sweden[f)]
IVS5-1g→a	intron 5	unstable RNA	0.004	novel
c.823c→g	exon 6	P275A	0.079	USA[b,c,d)], Finland[e)], Sweden[f)]
c.856c→t	exon 6	P286S	0.004	novel
c.877c→t	exon 6	R293X	0.004	USA[b,c,d)], Finland[e)], Sweden[f)]
c.905c→t	exon 7	P302L	0.004	novel
c.1193c→g	exon 10	A398G	0.004	novel
IVS10+47a→t	intron 10	–	0.004	novel
c.1289a→g	exon 11	K430R	0.004	novel

[a)] variants within the coding region are numbered with respect to the A nucleotide in the start codon of the MSR1 mRNA (cDNA), from reference sequence NM_138715. Nomenclature of variants was according to den Dunnen J.T. and Antonarakis E. (27)

[b)] Wang et *al*. (102)

[c)] Xu et *al*. (112)

[d)] Miller et *al*. (70)

[e)] Seppala et *al*. (88)

[f)] Lindmark et *al*. (63)

[g)] Xu et *al*. (111)

To investigate whether these mutations co-segregate with prostate cancer, the sequence of all available DNA samples from all members (affected and unaffected male relatives) of the 139 families were analyzed. The 371 sporadic probands and 208 unaffected controls were included.

The nonsense mutation R293X reported by Xu et *al*. (112) was present in two of 139 families. In the sporadic and control groups the R293X mutation was more commonly present in the sporadics (7 probands) than in the control group (4 probands).

Additionally, a novel stop mutation in exon 4 (S84X) was identified (Maier et al., 2005, in press) (67). One family (ULM0230) was positive for this mutation. Interestingly, all available members were carriers, two affected and one unaffected proband, who was not yet diagnosed for prostate cancer.

The intronic exchange IVS5-1g→a that alters the splicing site was found in one family (ULM0174) in both brothers affected with prostate cancer.

Neither the S84X variant nor the intronic exchange IVS5-1g→a were identified after screening the sporadic probands and healthy controls.

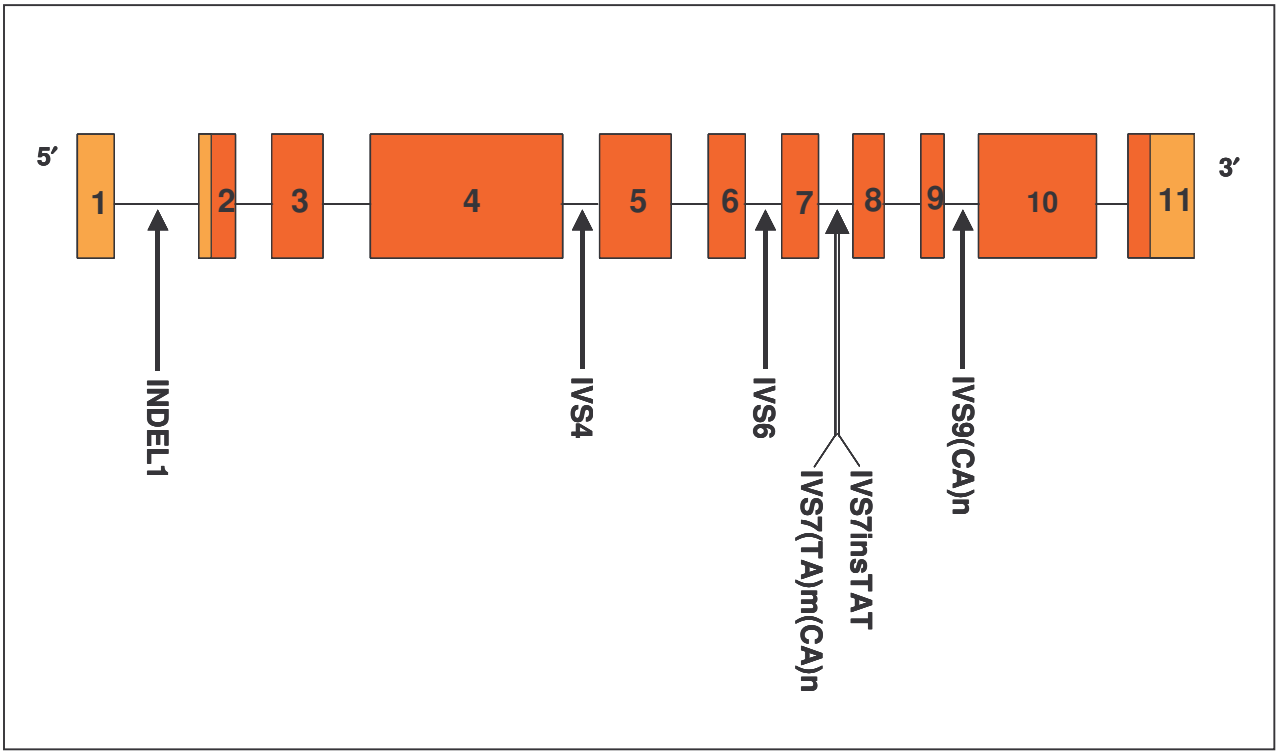
The frequency of subjects carrying the common amino acid variant P275A, reported by Xu et *al*. (112), was 15.1%, 10.6% and 14.7 % among familial cases, sporadic cases and controls, respectively.

In addition to this common sequence variant, five new amino acid substitutions H235Y (exon 5), P286S (exon 6), P302L (exon 7), A398G (exon 10) and K430R (exon 11) were observed (Maier et al., 2005, in press) (67). Concerning the frequency of these five variants in the sporadic and control group, only the P302L variant was found in one sporadic proband, while the others were not present, either in the group of sporadic cases or in controls.

3.2.2. Association analysis between the frequency of the length variants in the *MSR1* gene and prostate cancer

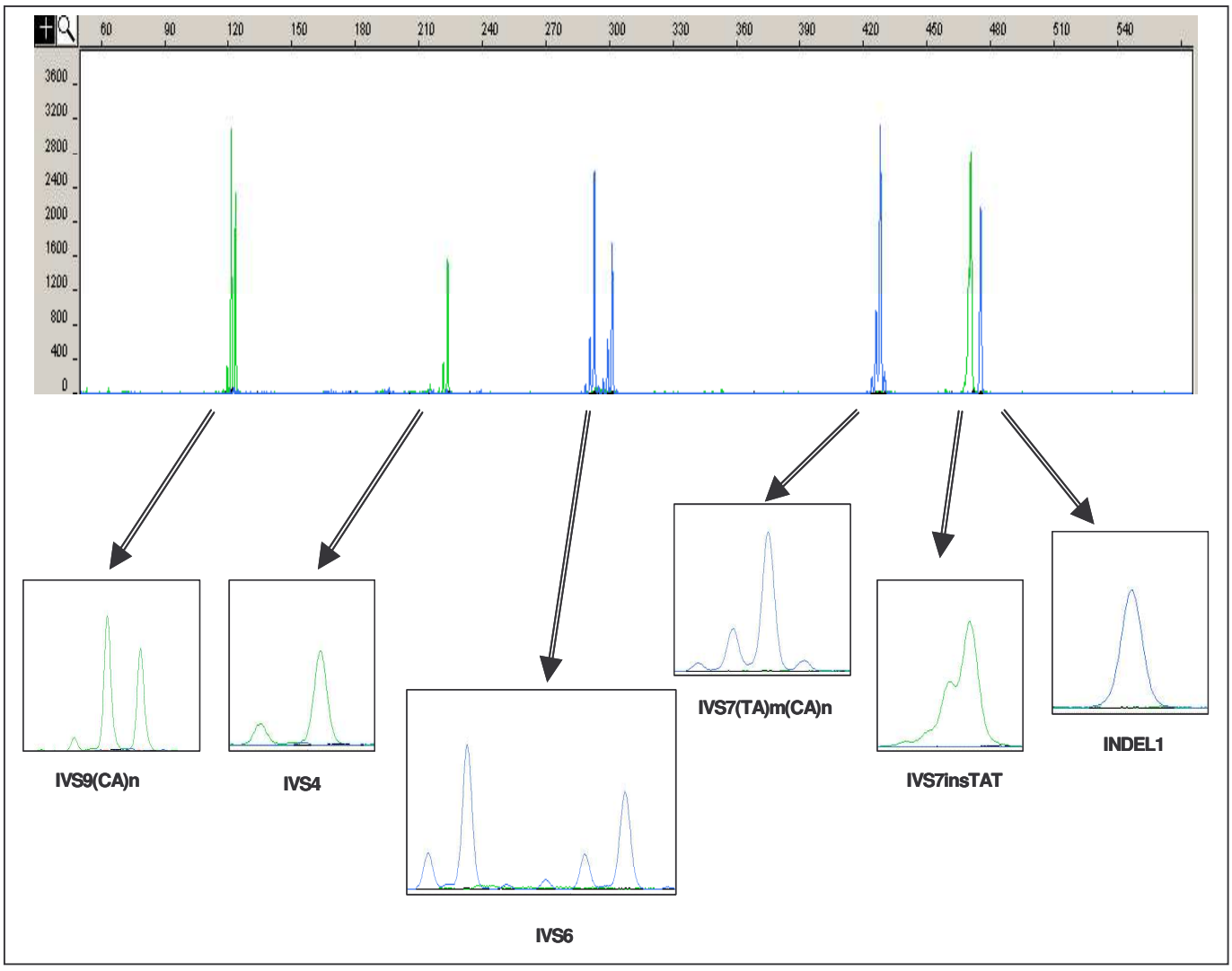
For the purpose of my thesis the 6 length polymorphisms (Figure 5) that span ~ 70kb of the *MSR1* gene were used. Two variants, IVS7(TA)m(CA)n and IVS7insTAT, were observed during sequencing the exons and adjacent intronic sequences, while the other four (IVS4, IVS6, IVS9(CA)n and INDEL1) were obtained from the reference sequence. NM_138715. Three length variants (IVS4, IVS6 and IVS9(CA)n) were two base repeats; the variant IVS7(TA)m(CA)n was four base repeat, while the INDEL1 and IVS7insTAT variants were insertions / deletions of 15bp and 3bp respectively.

Figure 5. Scheme of the six length variants in the *MSR1* gene. Boxes and numbers correspond to exons, lines to introns. The coding part is shown in red, the 5' and 3'-noncoding regions are in orange.



The PCR products of all six variants were pooled together and analysed through fragment analysis. Figure 6 shows an example visualized with the Genotyper Software.

Figure 6. Fragment analyses of six variants pooled together.



After performing the fragment analysis for all three sample groups the allele frequencies were acquired by direct counting (Table 9). All markers were in HWE except the IVS6 marker (was not in HWE in sporadic cases) that was excluded from the further haplotype analysis.

Table 9. Allele frequencies of the markers

Marker	Allele size (bp)	Allele frequency		
		Families	Sporadic cases	Controls
INDEL1	470	0.911	0.906	0.929
	485	0.089	0.094	0.071
IVS4	217	0.003	0.0	0.0
	219	0.009	0.006	0.005
	221	0.011	0.003	0.0
	223	0.838	0.867	0.877
	225	0.137	0.117	0.116
	227	0.003	0.002	0.002
	229	0.0	0.005	0.0
IVS7(TA)m(CA)n	421	0.003	0.008	0.0
	423	0.040	0.043	0.059
	425	0.059	0.051	0.039
	427	0.655	0.662	0.685
	429	0.176	0.156	0.143
	431	0.048	0.048	0.034
	433	0.016	0.020	0.027
	435	0.0	0.009	0.005
	439	0.002	0.002	0.0
	441	0.0	0.002	0.002
	443	0.0	0.0	0.005
IVS7insTAT	474	0.927	0.938	0.941
	477	0.073	0.062	0.059
IVS9(CA)	121	0.955	0.938	0.951
	123	0.016	0.019	0.015
	125	0.024	0.031	0.020
	127	0.0	0.002	0.0
	129	0.004	0.011	0.015

The LD was measured using D' for the pooled sample of sporadic cases and controls and values are given in the Table 10.

Table 10. Values of D' for the adjacent markers for the pooled sporadic and control group.

Marker 1	Marker 2	D'
INDEL1	IVS4	0.850318
INDEL1	IVS7(TA)m(CA)n	0.578649
INDEL1	IVS7insTAT	0.236516
INDEL1	IVS9(CA)n	0.102103
IVS4	IVS7(TA)m(CA)n	0.423514
IVS4	IVS7insTAT	0.072533
IVS4	IVS9(CA)n	0.104424
IVS7(TA)m(CA)n	IVS7insTAT	0.876916
IVS7(TA)m(CA)n	IVS9(CA)n	0.162838
IVS7insTAT	IVS9(CA)n	0.056128

Comparison of allele frequency distribution as well as haplotype frequency distribution between controls and sporadic cases, using appropriate χ^2 test, did not yield significant test results at the 0.05-level of significance.

Concerning the families the first analysis was performed with the FBAT package program. Only in one marker, INDEL1, some allelic transmission imbalance was seen; allele 1 was transmitted slightly more frequently than expected under the hypothesis of no association (p= 0.016). However, these results were gained from only 14 informative families.

Due to this lack of information in the family-based approach, we performed a case-control-like analysis as for the sporadic cases and controls. For the purpose of this comparison we took from each family one index person (the first person diagnosed

with the prostate cancer in the corresponding family). This yields a set of unrelated patients, which can be compared with controls, as described. The allele and haplotype frequencies of the five length variants (markers) were not significantly different between these familial cases and controls on the basis of the χ^2 test implemented in FAMHAP9 (Table 11).

Table 11. Allele frequencies of the markers

Marker	Allele size (bp)	Allele frequency	
		Family-index-person	Controls
INDEL1	470	0.917	0.929
	485	0.083	0.071
IVS4	217	0.0	0.0
	219	0.007	0.005
	221	0.007	0.0
	223	0.849	0.877
	225	0.133	0.116
	227	0.0	0.0
	229	0.0	0.0
IVS7(TA)m(CA)n	421	0.007	0.0
	423	0.043	0.059
	425	0.043	0.039
	427	0.676	0.685
	429	0.165	0.143
	431	0.047	0.034
	433	0.018	0.027
	435	0.0	0.0
	439	0.0	0.0
	441	0.0	0.0
	443	0.0	0.0
IVS7insTAT	474	0.924	0.941
	477	0.076	0.059
IVS9(CA)	121	0.960	0.951
	123	0.014	0.015
	125	0.022	0.020
	127	0.0	0.0
	129	0.004	0.015

4. Discussion

4.1. Polymorphism in CYP17 and prostate cancer risk

The growth and differentiation of the prostate gland is under androgen control. Accordingly, polymorphisms in genes involved in androgen biosynthesis, transport, and metabolism and the activation of androgen-responsive genes in prostate cells may be markers of prostate cancer susceptibility. The *CYP17* gene is a likely candidate for prostate cancer because it is directly involved in the production of testosterone. The first report of a positive association (17) between the *A2* allele of *CYP17* and hyperandrogenic diseases, polycystic ovarian syndrome, and male pattern baldness, led to the selection of *CYP17* as a candidate gene for study in relation to hormonal-related cancers. Consequently, the A2 allele has been examined in numerous case control studies as a candidate for prostate cancer and was suggested as a low penetrance modifier. These approaches, which did not take into consideration a familial disease history, led to inconsistent results on the association between the polymorphism in *CYP17* and the development of prostate cancer. Two studies observed an elevated risk in men homozygous for the frequent A1 allele (45;101), while six investigations noticed a borderline significance for A2 allele (A2/A2 or A1/A2 genotypes) associated with prostate cancer (43;46;57;64;93;113) and two studies (22;61) did not detect any effect of the A2 allele at all. This inconclusive situation has been recently clarified by a meta-analysis combining ten single studies, which dealt predominantly with sporadic prostate cancer probands (74). The authors found no correlation between *CYP17* and disease risk when the study was restricted to Caucasian populations.

In our study, we identified an unequal distribution of *CYP17* genotypes among sporadic cases and controls (100). The comparison under the dominant model gave a small value of odds ratio (OR=1.05, Table 5B) that did not differ significantly from 1.0; the value under the null hypothesis. Under the recessive model probands homozygous for A2 risk allele were compared to all other probands (being heterozygous for A1/A2 or homozygous for A1 allele) and the corresponding odds

ratio was elevated to OR = 2.20, but its confidence interval (CI = 0.96 – 5.00) still covers the value 1 meaning an insignificant result (p = 0.06, Table 5B).
Although a certain trend can be seen, where the A2 allele increases susceptibility to prostate cancer, the overall results are consistent with the conclusion that *CYP17* has no influence on prostate cancer risk in general. However, the power of our sample could have been limited by two factors. First, our sample size might have been too small to detect moderately small effects of the disease. Second, the disease-free status is not histologically confirmed, and thus a residual prevalence of prostate cancer among controls could bias the results towards null hypothesis. Due to potential undetected prevalence of prostate cancer among a few controls the statistical test might fail to show significance.

To investigate the involvement of this polymorphism in familial prostate cancer we compared familial cases with controls. We started from the assumption that if the A2 allele confers a risk, this may be due to the presence of one or two A2 allele in the genotype depending on the mode of action. To be able to discriminate between a recessive and a dominant mode of action we designed a dominant and a recessive model by combination of the corresponding genotypes in the analysis. After applying both models the resulting odds ratios were 0.72 (CI = 0.38 – 1.34) for dominant and 1.48 (CI = 0.60 – 3.60) for recessive (table 5A). Thus, we did not observe a statistically increased risk for familial prostate cancer in subjects with the A2 variant of the 5' promoter polymorphism in the *CYP17* gene.
Comparing all prostate cancer cases (with and without family history) led to similarly insignificant results (100). The odds ratio under the recessive model was slightly higher than under the dominant model, due to a high frequency of A2 homozygous carriers in the sporadic prostate cancer sample. An explanation for the lack of significance in the results may-be due to the fact that this sequence variant increases the risk only slightly (low penetrance). Accordingly, this variant does not completely segregate with prostate cancer in our family sample.

To our knowledge, only two studies (22;93) have examined a putative role of the *CYP17* polymorphism in familial aggregation of prostate cancer.

Recently, US American investigators (22) applied a family-based association test using the software package FBAT to pedigrees with at least three first-degree relatives affected by prostate cancer. These thoroughly selected families come close to the definition of hereditary prostate cancer. The results of this study did not support a role for *CYP17* as a high-risk factor for prostate cancer. Stanford et al (93) performed a large population-based study in which they included familial prostate cancer cases. The authors observed a strong association with the proposed risk genotype A2/A2 and familial disease history. The odds ratio for being homozygous for the A2 allele associated with having a family history of prostate cancer was 26.1 (95% CI, 3.41 −199.6) relative to men without a family history of disease. In our study, we asked whether the reported association could be verified in a European population. Our results show no evidence that the *CYP17* genotype might predispose for a familial aggregation of prostate cancer either under the dominant or under the recessive model (Table 7). This result may be due to our small sample size, which, therefore, limits the power to detect moderate effects of the potential risk genotype. However, with respect to the obtained confidence interval (0.6 to 3.6) our results are not compatible with a disease impact of the strength reported by the previous American study (93).

Several reasons are under discussion to explain the divergent outcomes of association studies. The most plausible interpretation of a positive test result, that is compatible with the null hypothesis of no disease effect, simply is chance. On the other hand, if there are true disease effects which are not detected by small individual studies, a meta-analysis might provide a significant test result by pooling data. Such an approach has already been applied to the role of *CYP17* in sporadic prostate risk, and may also be helpful to explore a putative influence on familial aggregation of the disease. There have been arguments (57;64) that divergent outcomes would indicate true disease effects, especially if single studies represented different populations. The impact of a risk gene under study might be confounded by environmental factors and the genetic backgrounds specific for ethnicity. Furthermore there is an assumption that a gene-gene interaction between the *CYP17* and another gene that influences development of prostate cancer, may account for these results. Further analysis investigating the SNPs in genes involved in androgen biosynthesis and metabolism, may give more insight into predisposition for prostate cancer. Finally a reason may be that the 5' promoter polymorphism is not by itself causal, but might instead be in linkage disequilibrium with a disease mutation within the *CYP17* gene and thus causes divergent results.

4.2. Association between MSR1 sequence variants and prostate cancer

The *MSR1* gene represents a strong candidate for hereditary prostate cancer. This led Xu et *al.*(112) to suggest that rare mutations tend to impose a high risk, while common *MSR1* sequence variants tend to have low risk for prostate cancer. Additionally, Xu et *al*. (111;112) and Wiklund et *al*. (106) reported suggestive linkage to chromosome 8p22-23 with the *HLOD* of 1.84 and 1.08, respectively. In contrast, results gained by Wang (102), Seppala (88) and Lindmark (63) did not support *MSR1* as a risk factor for prostate cancer.

The *MSR1* mutations reported in our German study group (Maier et al., 2005, in press) (67) showed that most of the variants were rare and only found in one family per mutation, while the missense variant P275A was more common. In an earlier mutation analysis of *MSR1* in the same samples of probands a nonsense variant S84X and a splice site mutation IVS5−1g→a were additionally detected to the known R293X variant. The frequency of the nonsense variant R293X was 1.9% in prostate cancer cases and 2.0% in controls. The other two variants S84X and IVS5−1g→a were found only in one family per variant. The exchange R293X leads to a loss of most of the extracellular ligand-binding domain and of the conserved extracellular scavenger receptor cystein-rich domain (89). The S84X nonsense variant in exon 4 is in the spacer domain, which connects the membrane spanning domain and the fibrous coiled coil domain, and is situated in the first cluster of two potential N-linked glycosylation sites. Thus, this polymorphism may play an important role in proper folding and trimerization of the *MSR1* protein. The third variant IVS5−1g→a leads to an unstable transcript.

Beside these nonsense variants, the common P275A exchange and five new missense variants (H235Y, P286S, P302L, A398G and K430R) were identified (Maier et al., 2005, in press) (67). The P275A variant was found in all three groups (familial, sporadic cases and controls) with similar frequency. Concerning the new missense variants, four were present only in the single family and not in the sporadic group or controls, while only the P302L has been seen in one sporadic proband,

In summary, when screening the *MSR1* gene for mutations several sequence variants were identified, both novel and previously reported (Maier et al., 2005, in press) (67). Although these results do not support *MSR1* as a strong candidate for

hereditary prostate cancer all conspicuous variants were found in early onset prostate cancer families. In order to assess the potential disease risk of this newly identified rare variants a larger sample size would be necessary. Further functional analyses using combinations of these variants could provide insight into the function of each variant.

To evaluate if certain alleles or haplotypes made up from six length variants in the *MSR1* gene are associated with prostate cancer we performed genotyping of familial probands, sporadic cases and controls for these variants.

The IVS6 marker was not in HWE in the sporadic group. The observed frequencies of homozygotes and heterozygotes of this nine-allelic marker deviated from what was expected under HWE (p=0.0024). This led to exclusion of this marker from the further analysis. After comparison allele frequencies as well as haplotype frequencies between sporadic cases and controls, using appropriate χ^2 tests, we did not observe any significant difference. The same results were achieved when a comparison was performed between familial cases and controls. Some allelic transmission imbalance was seen in one marker, INDEL1 when analysing the families with family-based association approach. Allele 1 of the INDEL1 marker was transmitted slightly more frequently than expected under the hypothesis of no association (p= 0.016). Nevertheless, the latter results were obtained from only 14 informative families. All these findings suggest that the *MSR1* gene is unlikely to be a high-risk gene for prostate cancer.

5. Summary

Familial history is one of the strongest risk factor for prostate cancer. The search for genes associated with inherited forms of prostate cancer is very difficult. Nevertheless, the investigation of prostate cancer families has yielded several candidate genes that co-segregate with prostate carcinoma.

One of the prostate cancer candidate genes is the *CYP17* gene. A thymidine (T) to cytosine (C) transition (designated A2 variant) in the promoter region of the *CYP17* gene has been used in several studies in order to determine a possible association with the prostate cancer risk. A recent meta-analysis found no effect of the *CYP17* polymorphism for the sporadic prostate cancer (74). The question still remained unresolved for familial cases, since only two investigators included prostate cancer families (22;93). In order to evaluate the role of the *CYP17* A2 allele in familial aggregation of prostate cancer we performed an association study. A putative influence of the A2 allele on disease risk was investigated by designing a dominant and a recessive model. In our study we realized a slight difference of *CYP17* genotypes between sporadic cases and controls. However, this unequal distribution was not significant. Although a certain trend can be seen, that the A2 allele increases susceptibility to prostate cancer, our results are consistent with the conclusion, that *CYP17* has no effect on prostate cancer risk in general. To investigate the involvement of this polymorphism in familial prostate cancer we performed comparison of the familial cases with controls. Our results showed no evidence that the CYP17 genotype might predispose for a familial aggregation of prostate cancer neither under the dominant nor under the recessive model. Our results do not suggest a role of *CYP17* as a high-risk susceptibility gene for familial prostate cancer nor as a modifier for the disease risk.

Rare germline mutations of the macrophage scavenger receptor 1 (*MSR1*) gene were reported to be associated with prostate cancer risk in families with hereditary prostate cancer (HPC) and in probands with non-HPC (112). A genome wide linkage study performed by Maier et al. (66) gave evidence for linkage to 8p22 close to the *MSR1* gene. This linkage results led us to evaluate the role of *MSR1* as a candidate

gene for prostate cancer. The *MSR1* gene was screened in our group (Maier et al, 2005, in press) (67) and several sequence variants were identified, both novel and previously reported. Most of the variants were rare and only found in one family per mutation.

For the purpose of the study I used 6 length polymorphisms (Figure 5) that span ~ 70kb of the *MSR1* gene. One of the markers (IVS6 marker) was not in the HWE, so it was excluded from the further analysis. The results gained from analysing the five length polymorphisms did not lead to significant result concerning the allele and haplotype frequency distribution between the cases and controls. Some allelic transmission imbalance was seen in the INDEL1 marker when families were analysed with family-based association approach. Allele 1 of the INDEL1 marker was transmitted slightly more frequently than expected under the hypothesis of no association ($p= 0.016$). Nevertheless, these results were obtained from only 14 informative families. Taken together our results do not support *MSR1* as a high-risk gene for prostate cancer.

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