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PROGINS-polymorphism in the human progesterone receptor gene: a potential genetic risk factor for prostate cancer?

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Abbreviations used:

BP        base pair
BPH       benign prostatic hyperplasia
CAT       chloramphenicol acetyltransferase
DHT       dihydrotestosterone
DNA       deoxyribonucleic acid
MRNA      messenger-ribonucleic acid
PCR       polymerase chain reaction
PROGINS   progesterone receptor gene insertion in intron G
PSA       prostate-specific antigen
PSM       prostate-specific membrane antigen
TUR-P      transurethral resection of the prostate
1. Introduction:

1.1. Prostate cancer and risk factors

The prostate gland is a fibromuscular and glandular organ lying just inferior to the bladder (see Fig. 1). In the United States, prostate cancer has become the second most commonly diagnosed cancer in men (19). In 1995, prostate cancer was diagnosed in approximately 317,000 U.S. males and an estimated 41,400 Americans succumbed to prostate cancer (158). The incidence of prostate cancer has risen dramatically over the last decade and even more as can be explained by increased longevity (41). This may be partially explained by stage-migration as use of PSA has revolutionized prostate cancer diagnosis in many asymptomatic patients (30,90).

![Fig.1: Male human anatomy: localization of the prostate gland (143)](image)

Despite the high incidence of prostate cancer, there is surprisingly little knowledge about the etiology and pathogenesis of prostate cancer (88,161). Prostate cancer represents a heterogeneous disease entity with varying degrees of behavior, aggressiveness, patterns of metastasis and response to therapy (55). The cause of prostate cancer is likely to be a combination of environmental and genetic factors (18). Several factors have been found to correlate with an increased incidence of prostate cancer:
1.1.1. Family history and genetic factors:

There are substantial differences in the prevalence of prostate cancer among populations (115). Having a family history of prostate cancer increases a man’s own risk of developing prostate cancer (17). Among men with a positive family history for prostate cancer, there is a two- to threefold elevation in terms of relative prostate cancer risk (27,141). The relative risk was greater if a brother had prostate cancer (relative risk of 4.5) than if the father had prostate cancer (relative risk of 2.3). A family history of breast and/or ovarian cancer in a mother or sister was also positively associated with prostate cancer risk (relative risk of 1.7). Men with a family history of both prostate and breast/ovarian cancer were at an even increased risk of prostate cancer (relative risk of 5.8) (31). Genetic factors are especially important in younger patients as familial and inheritable forms of prostate cancer exist, too. These forms of prostate cancer may account for up to 43% of early-onset disease, but make up only a rather small proportion (9%) of all prostate cancer occurrences (28,74).

Therefore, the existence of prostate cancer susceptibility genes is likely. Genetic alterations involve changes in DNA sequence that may lead to aberrant gene products or proteins. Although accurate definitions are somewhat controversial, the term mutation as opposed to polymorphism is used by many to refer to changes in sequence which are not present in most individuals of a species and either have been associated with risk of disease or have resulted from damage inflicted by external agents. In contrast to mutations, which occur typically in less than one percent of the test population, polymorphisms are more frequently observed alterations (62,116).

A number of techniques are available for mapping and identifying genes involved in modifying susceptibility to prostate cancer (109). Genes identified as candidates either from mapping data or through knowledge of their function can be screened for polymorphisms, and, if polymorphisms are present, these can be tested for association with disease (160). If a disease gene is tightly linked to a genetic marker, then the alleles of the disease gene and marker may be associated with each other and a particular marker allele may be found more frequently among affected subjects than in the general population. These association studies are generally carried out by measuring the frequency of marker alleles in a group of cases and matched controls.
No single genetic locus appears to be responsible for a large proportion of hereditary prostate cancer cases (52,109). Rare, highly penetrant prostate cancer susceptibility genes (BRCA1/2) have been described (120). Familial susceptibility genes may be associated with a much lower risk but may be responsible for a greater proportion of cases (76). Penetrance is variable in these cases and environmental factors may be much more important (52). Polymorphisms in the prostate cancer susceptibility gene HPC2/ELAC2 have been shown to increase prostate cancer risk (117). Other polymorphisms in candidate genes, e.g. those which are not sufficient to cause prostate cancer on their own, have been shown to influence the development of prostate cancer and are risk factors in an epidemiological sense. Among these are the genes encoding for the vitamin D receptor (37), androgen receptor (111,133), IGF (insulin-like growth factor) (42,132), and genes for various enzymes involved in androgen metabolism (89). Furthermore, changes like hypermethylation of the GSTP1 gene promoter (91), mutations in the helix-loop-helix-leucine zipper gene MXII (46), mutations of PI3 kinase regulator gene PTEN (24,44), hypermethylation of cell-cell-adhesion molecule E-cadherin gene CDH1 (108,150), mutations of tumor suppressor gene p53 (15), possible overexpression of the HER-2/neu receptor tyrosine kinase gene in prostate cancer (151) and decreased expression of the metastasis suppressor gene KAI1 (43) have all been implicated with increased risk of prostate cancer.

1.1.2. Hormonal factors:
Hormone-related cancers account for almost 30% of all cancers diagnosed in the United States (65), including cancer of the prostate and testis in men and cancer of the endometrium, breast and ovary in women (128). Experimental evidence indicates that hormonal imbalances may be involved in tumorigenesis and/or contribute to their development (92). Steroid hormones like androgens, estrogens and progestins are involved in the development and/or progression of cancer (79). This is due to the fact that ligand-occupied steroid hormone receptors act as transcription factors thereby influencing the rate of cell division and degree of cell differentiation. The magnitude of the tissue response is correlated not only to the number of receptor-bearing cells but also to the receptor density per cell.
Male sex hormones, most notably testosterone and dihydrotestosterone, are heavily involved in growth and maintenance of normal prostate epithelium as well as in the development of prostate cancer (11,69). High levels of circulating testosterone and low levels of sex hormone binding globulin – even though both are within normal endogenous ranges - are associated with increased risk for prostate cancer (56). Serum levels of free testosterone were higher among prostate cancer patients than in controls (40). As prostate cancer growth depends on androgens, cancers often regress after androgen stimulation has been withdrawn (48,72) but nearly all patients ultimately develop androgen-independent prostate cancer and succumb to the disease (87). The mechanisms by which tumor cells finally escape androgen ablation and become androgen-independent are not well understood (144). More than 80% of androgen-independent prostate tumors show high levels of androgen receptor expression.

In some cancers of the prostate, androgen receptor levels are increased because of gene amplification and/or overexpression, whereas in others, the androgen receptor is mutated (162). Despite some controversy, an association between a polymorphism in the androgen receptor gene (CAG repeat) and prostate cancer has been made (60). Also, a polymorphism at codon 726 of the androgen receptor gene is six times more common in prostate cancer patients than in controls (106).

In addition to testosterone, the steroid hormones progesterone, estrogen and vitamin D as well as their cognate receptors are present and implicated in the development of normal prostate tissue and in the pathophysiology of prostate cancer (39). Nevertheless, most of the research correlating prostate cancer occurrence with hormonal factors has focused on androgens and their receptors rather than investigating the role of estrogen or progesterone.

1.1.3. Racial differences:
African-Americans in the United States have the highest prostate cancer rate in the world and nearly twice that of whites in the United States (121). In African-Americans, serum concentrations of complexed and free testosterone are 15 % and 13% higher than in Caucasians. This could explain the twofold difference in prostate cancer risk (121). More interestingly, African-American men demonstrate higher serum PSA levels than white males even after adjustment for patients' age and prostate volume in men without prostate
cancer and for cancer grade and stage in men with prostate cancer (1). The etiology of higher PSA levels in African-Americans is not understood yet and might be secondary to biological and/or environmental/socioeconomic factors (1).

1.1.4. Dietary factors:
Prostate cancer risk appears to be positively correlated with high intake of lipids of animal origin (8,119). This is judged from the observation that while native Japanese and Chinese males have a lower risk for prostate cancer, the risk observed in second- and third-generation Japanese-Americans and Chinese-Americans is similar to white American men (18). This may be secondary to changes in nutrition. Dietary lipid may be related to prostate cancer risk, although the specific lipid(s) responsible for this increased risk have not been identified (83). Given the diverse effects of fatty acids on cellular physiology and chemistry, the relationship between lipid intake and/or metabolism and cancer development is rather complex. However, an interaction between dietary factors and tumor development appears likely, as for example vitamins and minerals are acting as antioxidants and a variety of genetic factors influence lipid metabolism (83). In addition, obesity may be associated with increased risk not only for prostate cancer but also for a variety of other cancers. Obesity may influence the cancer risk secondary to alterations in the metabolism of steroid hormones, insulin, and insulin growth factor, the distribution of body fat and changes in adiposity at different ages (25). A positive association between plasma concentrations of insulin-like growth factor-I and prostate cancer risk was observed. Men in the highest quartile of insulin-like growth factor-I concentrations had a relative prostate cancer risk of 4.3 compared with men in the lowest quartile (33).

In the established prostate cancer cell line LnCaP, a close association between proliferation rate and content of the intracellular calcium pool was seen (32,93). Vitamin D has been shown to have antiproliferative effects in prostate cancer in-vitro (113). Therefore, vitamin D deficiency may increase the risk of initiation and progression of prostate cancer (148).

1.1.5. Age:
The incidence of prostate cancer increases with age. The highest prevalence is found in men in their seventh and eighth decade of life. As many as 40% of 70-79 year old males
may harbor prostate cancer (12). Increased age is a relative risk factor for advanced pathological findings in men with clinical stage B1 prostate cancer (7).

A summary of the risk factors discussed above is given in Tab.1.

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Increases individual risk</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of steroid hormones in serum</td>
<td>unknown</td>
<td>(11,69)</td>
</tr>
<tr>
<td>Age</td>
<td>unknown</td>
<td>(12)</td>
</tr>
<tr>
<td>Family history</td>
<td>two- to threefold</td>
<td>(27,141)</td>
</tr>
<tr>
<td>Race (African-American vs. Caucasian-American)</td>
<td>twofold</td>
<td>(121)</td>
</tr>
<tr>
<td>Diet, especially lipid intake</td>
<td>unknown</td>
<td>(8,119)</td>
</tr>
</tbody>
</table>

Table 1: Risk factors for prostate cancer

1.2. Clinical aspects of prostate cancer

The diagnosis of prostate cancer must be made early in order to effectively decrease prostate cancer mortality (85). In principle, prostate cancer patients can be stratified according to their risk of cancer progression and assigned to one of three groups:

a.) high-risk patients who will (or may have) already progressed to metastatic prostate cancer without appropriate forms of treatment and who may not be cured
b.) potentially curable patients at medium risk for disease progression and metastatic disease requiring antitumor therapy
c.) low risk patients with insignificant cancers likely not needing any form of treatment (127).

As a consequence of improved screening for prostate cancer, the number of patients with small, localized tumors (group c.) has increased over time (75). Therefore, screening of whole populations with PSA remains controversial (5,9).
It is now widely accepted that many patients with small, localized and well-differentiated tumors may never experience clinically manifest prostate cancer in their lives. Therefore, not every prostate cancer might warrant treatment. In contrast, patients falling into group b.) will have the greatest benefit from cancer screening. Nevertheless, even in patients who are at risk for cancer progression, treatment-induced morbidity may outweigh potential marginal survival benefits. This is due to the fact that many prostate cancer patients are diagnosed late in life, suffer from serious co-morbidities, and may die with instead from prostate cancer (71,99). Unfortunately, no molecular marker for biological aggressiveness and metastatic potential of prostate cancer is available to date which would allow to accurately predict the course of disease on an individual basis (47). Therefore, intense research effort is under way to determine genetic differences which could serve as individual risk predictors.

1.3. PROGINS
In 1995, a polymorphism in the human progesterone receptor gene was identified and named PROGINS (100,122). This polymorphism consists of three obligate structural aberrations: (1) Alu insert, (2) silent point mutation in exon 5 and (3) functional mutation in the hinge region (exon 4). As a consequence of this mutation, PROGINS encodes for a receptor protein with increased stability (123) and increased hormone-induced transcriptional activity. PROGINS was found to be an risk factor for ovarian cancer in BRCA1 and BRCA2 mutation carriers who were never exposed to oral contraceptives (100,122,123). In contrast, PROGINS lowers the risk for breast cancer in postmenopausal women (45,154).

1.4. Steroid receptors and cancer
Strong evidence for the implication of steroids and steroid receptors in oncogenesis and tumor growth exists in the female population (36,126). Estrogen-based contraceptives reduce the risk of ovarian cancer, but addition of a progestagen may counteract the increased risk of endometrial cancer (38). Estrogens used in postmenopausal hormone replacement therapy increase the risk of both breast and endometrial cancer, but addition of a progestagen may counteract the increased risk to the endometrium (38).
In human breast cancer, the presence and concentration of estrogen and progesterone receptors is of prognostic and therapeutic significance: tumors with high receptor levels
are more likely to respond to endocrine therapy than those expressing either low levels or no receptors at all (70, 77). Progesterone receptor levels were an independent prognostic factor for disease-free survival of patients with early stage breast cancer (114). In ovarian cancer, the presence of progesterone and androgen receptors but not estrogen receptors was associated with a significantly better survival rate (130, 136), while others have established estrogen receptors as a prognostic variable in human ovarian cancer (81). Progesterone receptor-positive but not estrogen receptor status was a significant prognostic variable of progression-free survival in stage III and IV epithelial ovarian cancer (58, 61, 64).

Despite the well-known effects of androgens, female sex steroids may well play a role in the development of prostate cancer. Several studies suggest an involvement of progesterone and estrogen and their receptors in the pathogenesis of prostate cancer. The proliferation rate of androgen-sensitive LNCaP cells, a human prostate cancer-derived cell line, can be stimulated at virtually the same order of magnitude by either androgens or by physiological concentrations of estradiol. Estradiol-mediated proliferation is paralleled by the expression of estrogen receptors and, at almost the same level, by progesterone receptors (29). Increased concentrations of progesterone receptors were detected in prostate biopsies taken from prostate cancer patients treated with estrogen (104). In the androgen-insensitive prostate cancer cell line PC-3 incubation with progesterone resulted in 20% growth inhibition, while 5-α-dihydrotestosterone had essentially no effect on cell proliferation (95). Progesterone showed competitive inhibition of testosterone-5α-reductase and specific binding of dihydrotestosterone by competition for the receptor in human skin homogenates (159). Progesterone and estradiol can even influence PSA expression as the hormone response elements of the PSA-promoter are not necessarily androgen-specific (131). Therefore, although PSA expression in LNCaP prostate cancer cells is predominantly influenced by androgens, it can be induced by progesterone and estradiol, while the antiandrogen hydroxyflutamide blocked not only androgen, but also progesterone induction of PSA (107). As a consequence of the promising results obtained in the treatment of breast cancer, the progesterone antagonist drug RU 486 (mifepristone) is under investigation as an antineoplastic agent for prostate cancer (156).
The multitude of effects of progesterone and its receptor and their involvement in hormone-dependent gynecological cancers was our rationale to investigate the role of the PROGINS polymorphism as a possible genetic risk marker for prostate cancer. We studied the PROGINS polymorphism by (1) determining its frequency in healthy males, (2) evaluating a potential association between PROGINS and the incidence prostate cancer, and (3) assessed whether PROGINS predicted certain variables characterizing the course of disease.
2. Materials and Methods:

2.1. Study design:
Patients (n=94) with histologically confirmed diagnosis of prostate cancer (American caucasians) were included regardless of age, stage or grade of tumor. For control, nucleated blood cells from 100 individuals (male German caucasians) were kindly provided by the Ulm University Medical Center Blood Bank (Ulm, Germany). In both groups, frequency of PROGINS was determined.

2.2. Buffy coat preparation:
Control samples: Blood was drawn once from each individual participating in this study. 10 ml heparinized blood was centrifuged (10 minutes at 1500-1800 rounds per minute), the plasma was removed and the buffy coat (white blood cell layer at the interphase between plasma and red blood cells) was transferred into a 2 ml freezing tube, filled up with PBS containing 10% DMSO and stored at -20°C until DNA isolation.

Prostate cancer samples: 8 ml of blood (Vacutainer cell preparation tube, Becton Dickinson) were drawn once from each prostate cancer patient enrolled in the study. To remove red blood cells, the sample was centrifuged (20 minutes at 1750 rounds per minute), the buffy coat at the bottom of the tube resuspended in plasma and the mixture was transferred to a sterile 15 ml tube. PBS was added (final volume 15 ml) and the white blood cells were pelleted by centrifugation (15 minutes at 440 rounds per minute). The supernatant was discarded, the cells were resuspended with 2 ml of guanidine lysis buffer (GLB, 4 M guanidine thiocyanate, 100 mM mercaptoethanol, 200 mM sodium acetate pH 4.0, 10 mM EDTA, 2% N-lauryl-sarcosine, freshly prepared every 2 weeks) and transferred to a 2 ml cryo tube. After complete lysis, the sample was stored at -80°C until the timepoint of further analysis.

2.3. DNA extraction from blood: samples taken from the control group:
Genomic DNA was isolated from white blood cells (stored at -20°C) by using a modification of the method developed by Miller (101). The buffy coat was thawed at room temperature, transferred to a 15 ml polypropylene centrifugation tube and resuspended in 10 ml of nuclei lysis buffer (155 mM NH₄Cl, 10 mM NH₄HCO₃, 0.1 mM EDTA pH 7.4), followed by vigorous shaking and centrifugation (10 minutes at 1200 rounds per minute).
The supernatant was discarded and the pellet was resuspended in another 10 ml of the same nuclei lysis buffer. These steps were repeated two more times. Finally, 3 ml of 10 mM Tris buffer (pH 8.0, 400 mM NaCl, 2 mM EDTA, 10% dodecyl sulphate (SDS)) and 50 µl of Proteinase K (Sigma, St. Louis, 1mg/ml) were added and the lysate digested overnight (37°C). Proteins were precipitated by the addition of 1 ml 6 M NaCl and vigorous shaking followed by centrifugation (10 minutes at 2000 rounds per minute). The DNA-containing supernatant was transferred to another 15 ml tube containing two volumes of 70% EtOH. Precipitated DNA was subsequently transferred to a 1.5 ml microcentrifuge tube, washed with 0.5 ml 70% EtOH, and recovered by centrifugation at room temperature (10 minutes at 10,000 rounds per minute). Finally, DNA was washed in 100% EtOH before being centrifuged at room temperature (10 minutes at 10,000 rounds per minute). DNA was air-dried for about 30 minutes and redissolved in 100 to 200 µl TE-buffer (10 mM Tris (pH 8.2) and 0.2 mM EDTA (pH 7.5)) and stored at 4°C.

2.4. DNA extraction from blood: samples taken from prostate cancer patients:
For a previously performed study, RNA had been isolated by applying a standard phenol-chloroform-isoamyl alcohol (25:24:1) extraction procedure to the buffy coats of the prostate cancer patient group. From this procedure, 1.5 ml tubes containing the sample DNA (stored at -80°C) were available. For extraction of DNA, the samples were thawed and phase separation was achieved by vigorous centrifugation (12 minutes at 10,000 rounds per minute). The aqueous phase containing DNA (approximately 450 µl) was transferred into a 1.5 ml tube containing 2 volumes (900 µl) of 100% EtOH and 10% (150 µl) sodium acetate (pH 5.2) in order to precipitate DNA. The DNA was pelleted by centrifugation (10 minutes at 10,000 rounds per minute), the supernatant was discarded, the pellet was washed once in 500 µl of 70% EtOH, finally recovered by centrifugation (10 minutes at 10,000 rounds per minute) and air-dried for about 20 minutes. The DNA was dissolved in 20 µl of TE-buffer (10 mM Tris (pH 8.2), 0.2 mM EDTA (pH 7.5)) and stored at 4°C.

2.5. Quantitation of DNA
Concentration and purity of DNA isolated from control samples were determined photometrically (124) and the samples were adjusted to a concentration of about 100 ng DNA per µl. In case of DNA isolated from prostate cancer patients concentration and
purity were determined, but concentrations were not adjusted and were in the range of 30-50 ng per µl. Nevertheless, 3µl of the DNA-containing solution were always sufficient to successfully perform PCR analysis.

2.6. *In-vitro* amplification of DNA by the polymerase chain reaction (PCR)

Although relatively new, PCR amplification has already been extensively applied in a multitude of research fields, among them diagnosis of genetic disorders (16). With this technique, the amplification of minute amounts of nucleic acids, even as little as a single molecule of DNA up to a billionfold, is possible. Due to this extreme sensitivity, precautions were taken to guard against contamination of the reaction mixture with trace amounts of DNA which could serve as an unwanted template. Moreover, a positive and negative control were included in each run. PCR consists of 3 steps (Fig.2): first, the double-stranded DNA is denatured into single strands by heating (denaturation). Second, the temperature is lowered to allow annealing of a short oligonucleotide (primer) to complementary sequences in the single-stranded DNA. The DNA-bound primer serves as a specific starting point for the *in-vitro* DNA synthesis (annealing). Finally, the DNA polymerase synthesizes a new complementary strand (primer extension), thereby leading to progressively increasing amounts of DNA with a desired sequence. This increase occurs as long as deoxyribonucleoside triphosphates are available as substrates for the DNA polymerase.

This repeated amplification of target sequences requires the use of a specific heat-resistant DNA polymerase. When the above steps are repeated, the newly synthesized DNA fragments serve as additional templates, and within a few cycles the predominant product is a single species of DNA fragment whose length corresponds to the distance between the two original primers. In practice, 20 to 30 cycles are required for effective DNA amplification (4).

2.7. Detection of intron G via PCR

The genomic DNA obtained from either healthy controls or prostate cancer patients was analyzed for the presence of PROGINS using PCR. For each sample, 20 µl of the reaction mixture containing 100 ng DNA in 2 µl of TE buffer (template), 2 µl (20 µM) containing equal amounts of all four deoxyribonucleoside triphosphates (dNTP’s), 0.4 µl of sense and antisense primers specific for PROGINS, 2 µl of 10x concentrated reaction buffer (5 mM
KCL, 10 mM Tris-HCl (pH 8.3), 0.15 mM MgCl₂), 0.2 units of Taq polymerase enzyme, and sterile water was set up.

The sequences of the oligonucleotides were:

Intron G sense primer: 5’-TGA AGT TGT TTG ACA AGC TGT TGG-3’
Intron G antisense primer: 5’-GCC TCT AAA ATG AAA GGC AGA AAG C-3’.

The PCR was performed for 34 cycles in a thermocycler (MJ Research PTC-100) under the following conditions: 1 minute at 94°C (denaturation step), 1 minute at 60°C (annealing step) and 1 minute at 72°C (extension step), with an initial denaturing step at 94°C for 3 minutes and a final extension step at 72°C for 5 minutes.
Fig. 2: Technique of PCR (124). The scheme illustrates the 3 steps of PCR (denaturation, annealing, synthesis of new strand in the 5' to 3' direction) as well as the increasing amounts of target DNA-fragments produced throughout the PCR cycling.
2.8. Gel electrophoresis of DNA
For analysis of PCR products, samples were submitted to electrophoresis using 2% agarose gels and TE buffer. For visualization of DNA, the gels were stained with ethidium bromide (0.5 µg per ml), destained with water and subsequently evaluated under UV illumination (104). The presence of one band at either 174 bp or 497 bp was indicative for the homozygous state of wildtype and mutated DNA respectively, while the presence of two bands (174 bp and 497 bp) indicated the heterozygous state (Fig. 3).

![Fig. 3: Detection of PROGINS by gel electrophoresis. The size of DNA fragments in base pairs is indicated on the both sides. Lane 1,4: wild type with one 174 bp fragment, lane 2,5: heterozygous state with both 494 and 174 bp fragments, lane 3,6: homozygous state with only one 494 bp fragment. Marker DNA consists of a mixture of DNA fragments of known sizes.](image)

2.9. Statistics:
The frequency of PROGINS in prostate cancer patients and normal control individuals was calculated. Comparison of the observed genotypic distribution of PROGINS between male prostate cancer patients and controls were made by using the χ² analysis. 95% confidence intervals were determined for the observed genotypic distributions of PROGINS in German and American caucasian males. To compare various correlations of factors with
PROGINS status, the Wilcoxon 2-sample test (normal approximation) or the Fisher’s Exact Test (2-tail) were used to analyze for any correlation between PROGINS and other well established prognostic factors for prostate cancer. The statistic evaluation and analysis was performed in collaboration with the Department of Biometry and Medical Documentation, University of Ulm, Germany.

Odds ratio or relative risk are commonly used in order to quantify the contribution of individual risk factors and compare the relative likelihood of an event occurring between two groups (135). The relative risk is easier to interpret and is consistent with general intuition (doubled risk corresponds to a relative risk of 2). Some designs, however, allow only for the calculation of the odds ratio. The odds ratio of an event are calculated as the number of events divided by the number of non-events. An odds ratio is calculated by dividing the odds in the treated or exposed group by the odds in the control group.
3. Results:

3.1. Frequency of PROGINS in prostate cancer patients and in healthy male controls:
Blood samples from 94 American caucasian prostate cancer patients and from 100 healthy German caucasian control individuals were tested for the presence of PROGINS. Out of 94 US Caucasian prostate cancer patients, 21 (22.3%) were positive for heterozygous or homozygous states of PROGINS (95% confidence intervals of frequency: 14.4%-32.1%), while 30 individuals (30.0%) tested positive for heterozygous or homozygous states of PROGINS out of the 100 healthy German Caucasian control individuals (95% confidence intervals of frequency: 21.2%-39.9%). Despite the reasonably high numbers of almost 100 individuals in both groups, confidence intervals for frequency of heterozygous or homozygous states of PROGINS overlapped, suggesting the absence of a statistically significant difference between the groups in terms of PROGINS status. Using the chi-square test, no difference in terms of PROGINS frequency could be observed between the 94 American caucasian prostate cancer patients and the 100 healthy German male controls (p=0.226).

3.2. Correlation of age at diagnosis of prostate cancer with PROGINS status:
The 21 prostate cancer patients showing heterozygous or homozygous states of PROGINS were on average 64.0 years old (mean age was 64.0 years, ranging from 49 to 76 years), whereas the remaining 73 patients with normal PROGINS status were on average 64.6 years old (mean age was 64.6 years, ranging from 42 to 82 years) at the time of diagnosis. Using the Wilcoxon 2-sample test (normal approximation), no significant difference in terms of age at diagnosis of prostate cancer exists between the two groups (p=0.89).

3.3. Correlation of PSA at the time of diagnosis of prostate cancer with PROGINS status:
For 72 prostate cancer patients the PSA concentration at the time of diagnosis was available. 15 individuals tested positive for heterozygous or homozygous states of PROGINS had a mean PSA value of 6.3 ng/ml, whereas in the group with normal PROGINS status (n=57), the mean PSA concentration was 10.0 ng/ml at the time of diagnosis. The Wilcoxon 2-sample test (normal approximation) revealed no difference in terms of the PSA value at the time of diagnosis of prostate cancer between PROGINS-positive or -negative prostate cancer patients (p=0.20).
3.4. Correlation of Gleason grade at the time of diagnosis of prostate cancer with PROGINS status:
For grading, the Gleason grade system is used which assigns one out of five different histologic patterns (1-5) to each of the two most representative areas of the tumor to characterize the degree of glandular differentiation under low-power magnification. For 61 prostate cancer patients the Gleason grade at the time of diagnosis of prostate cancer was known. 12 individuals tested positive for heterozygous or homozygous states of PROGINS were found to have a mean Gleason grade of 6.16, whereas 49 patients with normal PROGINS status showed a mean Gleason grade of 6.22 at the time of diagnosis of prostate cancer. The Wilcoxon 2-sample test (normal approximation) revealed no difference in terms of the Gleason grade at the time of diagnosis of prostate cancer between PROGINS-positive or -negative prostate cancer patients (p=0.88).

3.5. Correlation of prostate cancer grade with PROGINS status:
For 27 prostate cancer patients the prostate cancer grade was known. Amongst these, 6 individuals tested positive for heterozygous or homozygous states of PROGINS (4 individuals with grade 2 and 2 individuals with grade 3), whereas 21 patients demonstrated normal PROGINS status (15 individuals with grade 2 and 6 individuals with grade 3). Using Fisher’s Exact Test (2-tail), no difference in terms of histologic grade of prostate cancer between PROGINS-positive or -negative prostate cancer patients was observed (p=1.00).

3.6. Correlation of prostate cancer stage with PROGINS status:
For staging of prostate cancer, the TNM (tumor, node, metastasis) system proposed by the Union Internationale Contre le Cancer (UICC) was used. For 80 prostate cancer patients the prostate cancer stage was available. 17 individuals tested positive for heterozygous or homozygous states of PROGINS (TNM system: stage 1c: 4 individuals, stage 2a: 4 individuals, stage 2b: 4 individuals, stage 2c: 3 individuals, stage 3a: 1 individual, stage 3b: 1 individual) as compared to 63 patients with normal PROGINS status (TNM system: stage 1c: 12 individuals, stage 2a: 24 individuals, stage 2b: 13 individuals, stage 2c: 11 individuals, stage 3a: 3 individuals, stage 3b: no individual). Using Fisher’s Exact Test (2-
tail), no difference in terms of prostate cancer stage between PROGINS-positive or -negative prostate cancer patients was observed (p=0.47).
4. Discussion:

Prostate cancer is a complex disease and multiple genetic and environmental factors are likely to contribute to its development. From a clinical and genetic point of view the heterogeneous nature of prostate cancer, problems with confounding comorbidities in the population of prostate cancer patients, and problems in adequately staging the disease in patients not undergoing surgical therapy make the study of prostate cancer a challenge (63).

Even though significant progress was made in diagnosis and treatment of prostate cancer (10,110), to date no molecular markers indicating the biological aggressiveness and metastatic potential are available. Such markers could be useful to reliably distinguish the patients with prostate cancers that will remain clinically silent from the ones that will develop clinically apparent cancer which finally metastasize and ultimately take the life of the patient.

Several genomic alterations as well as proteins expressed by the tumor have failed to be useful as risk markers. Amongst them are prostate-specific membrane antigen (PSM) (67), PC-1 (112), PD-41 (13), telomerase (138), E-cadherin (149), BRCA1 (54), sex steroid hormone receptors (14,50,59) and expression of certain cell cycle-regulating proteins (98).

Studies on molecular genetic changes in prostate cancer are slowly improving our understanding of the molecular processes leading from a normal prostate epithelial cell to an adenocarcinoma cell with invasive and metastatic properties. In brief, genes are stretches of deoxyribonucleic acid (DNA) that produce a functional messenger-ribonucleic acid (mRNA) molecule which in turn is translated into a protein. Long stretches of noncoding DNA (introns) interrupt the relatively short segments of coding DNA (exons). The function of the intron-sequences is only partially understood, if at all. In addition, a gene is associated with regulatory sequences (promoter and silencer) that govern its expression (3). Large amounts of data regarding chromosomal aberrations in prostate cancer are available (23). Numerous genes including tumor-promoting genes like proto-oncogenes, tumor suppressor genes and genes encoding for transcription factors, growth factors and cell adhesion molecules seem to be required for prostate organogenesis.
and many of them may also be involved in prostate carcinogenesis (129). Polymorphisms in certain steroid hormone receptor genes (androgen receptor and vitamin D receptor gene) have been associated with increased risk for prostate cancer (60,106).

In an effort to identify a potential genetic risk marker for prostate cancer, the role of a polymorphism in the human progesterone receptor gene called PROGINS was investigated. PROGINS consists of a 320 base pair Alu-insertion in intron G of the human progesterone receptor gene with an associated mutational complex in exons 4 and 5 and leads to formation of a progesterone receptor with increased stability and increased hormone-induced transcriptional activity (123). An Alu-insertion is a type of sequence characterized by a short (about 300 bp long), interspersed, repetitive DNA element (137) which occurs in remarkably large numbers (about 300,000-500,000 copies) in the human genome and makes up about five to six percent of the total genome (155). Alu insertion transposable elements, e.g. DNA stretches that can multiply and spread from one site in a genome to other sites, cause the random duplication and relocation of DNA sequences (137). It is though that these elements have had a special evolutionary role in the generation of organismal diversity (3). Several examples of naturally occurring alleles are known to have apparent insertions of these elements (94,103,139). The movement of a transposable element can result in a new recognition site, for example, a new binding site for DNA-binding proteins can be generated (2,21). Therefore, DNA-sequence rearrangements are often observed to alter the timing, level, or spatial pattern of expression of a nearby gene without affecting the sequence of the protein or of the mature RNA molecule (2). PROGINS was found to be a risk factor for ovarian cancer (100,122,123) and to be associated with decreased risk for breast cancer (154), although others did not confirm this in smaller studies (86,97).

Investigating the genetic changes which lead to formation of an altered steroid receptor and the implications of the altered receptor in malignant transformation necessitates looking at the role of steroid hormones and their receptors in cellular biology.

Steroid hormones mediate complex signals involved in development, differentiation, and physiologic response of multicellular organisms to diverse stimuli (157). The steroid hormone progesterone counteracts the activity of other steroid hormones like estrogens,
thereby influencing complex regulatory circuits and suggesting interactions between members of the steroid hormone receptor family (78).

The effects of progesterone on different target cells are determined by the structure of the hormonal ligand, receptor subtype, type of hormone-responsive gene promoter, and availability of coactivators and corepressors modulating the cellular response (78). Upon entering target cells by simple diffusion, steroid hormones bind to and complex with specific nuclear receptor proteins which belong to a superfamily of ligand-modulated transcription factors (20,53,142). After binding to their respective receptor and travelling to the cell nucleus, a cascade of molecular events is induced by interaction of the ligand-occupied hormone receptor with specific hormone-response-elements present in the DNA (157). The resulting positive or negative regulatory effects are determined by cell type and promoter context (35). The presence of a specific intracellular hormone receptor defines a cell as target for the cognate hormone.

The human progesterone receptor belongs to the steroid-thyroid-retinoic acid receptor superfamily which include the receptors for steroids, thyroid hormone, vitamins A- and D-derived hormones, and certain fatty acids (53), while the majority of family members are homologous proteins called orphan receptors for which no ligands have been identified yet (74).

The human progesterone receptor is a single copy gene located on chromosome 11q22-23 (102). In terms of its genetic structure, the human progesterone receptor gene comprises 8 exons as well as 7 introns named A to G (see Fig. 4). Exon 4 translates into the hinge domain and amino-terminal part of the steroid binding domain, while exon 5 encodes for part of the steroid binding domain (102).
Fig.4: **Structure** of the human progesterone gene and receptor (102). The gene encoding for the progesterone receptor comprises 8 exons (E1-E8) and 7 introns (A-G). The protein consists of 4 function-related domains: 1) the amino-terminal domain, 2) the DNA binding domain, 3) the hinge domain and 4) the ligand-binding domain.

In terms of function, the progesterone receptor protein (Fig. 5) consists of 4 function-related domains. These include a variable amino-terminal domain for transactivation of transcription; a DNA-binding domain for recognition of specific DNA sequences and protein-protein interactions; the hinge domain for conformational and structural changes which may play an important role in protein function (145), and at the C-terminal end, a ligand-binding domain, important for hormone binding, protein-protein interactions, and additional transactivation activity (84).
Fig. 5: Functional domains of steroid receptors and their functions: binding of ligand, dimerization, nuclear localization, specific DNA binding, transactivation or silencing of target genes, as well as interaction with modulating cofactors like heat-shock-protein (Hsp) and general transcription factor TFIIB (73,147).

Functionally, the ligand-occupied progesterone receptor ultimately allows for production of progesterone-inducible proteins by initiating a complex process. Binding of the ligand-occupied receptor to a progesterone-responsive element induces the binding of several enzymes (polymerases) to a promoter sequence upstream of the sequence to be transcribed.

Enhancers are particular types of promoter sequences with unusual properties in that they are able to alter transcription rates even when they are located several thousand basepairs away from their respective genes. Transcription involves initiating and elongating a RNA strand complementary to the DNA strand encoding for the gene of interest. RNA transcripts are further processed into mature RNA strands (splicing) which can be later used for protein synthesis in a process called translation (3). The regulation of gene expression is highly complex and can be achieved at different levels, e.g. at the level of the rate of transcription, mRNA turnover, mRNA processing or translation (152).

The progesterone receptor, like other transcription factors, can function as a transcriptional inducer (transactivator) as well as repressor (silencer) of transcription (118). Two distinct forms of the human progesterone receptor, the hPRB-isoform containing 933 amino acids
and the N-terminal truncated hPRA-isoform, containing 769 amino acids, exist. The role of these isoforms is currently unclear, but the existence of elaborate mechanisms regulating subtype-production and the observation that the ratio of these effectors varies among target tissues suggests that differential expression of the two isoforms may be critical for the appropriate cellular response to progesterone (35). Upon ligand (progesterone) binding, the progesterone receptor dimerizes, locates to the nucleus and binds to specific DNA sequences on target genes. Upon binding of agonist the receptor changes its conformation in the ligand-binding domain that enables recruitment of cofactors (134). Several steroid/nuclear receptor cofactors proteins like general transcription factor TFIIB (73) or heat shock protein (Hsp) (26) bind with the progesterone receptor to modulate its transcriptional activity (34). Cross-interference among nuclear receptors has been proposed to reflect the titration of coactivators that bind the receptors in a hormone-dependent manner (96).

In terms of receptor gene expression, intracellular amounts of progesterone receptors are regulated at the transcriptional as well as at the post-transcriptional level. Progesterone receptor expression is induced by estradiol at the transcription level and suppressed by progesterone at the transcriptional and post-transcriptional level (6,68). At the transcriptional level the estrogen receptor-mediated induction is inhibited through protein-protein interactions (125).

Based on the structure and function of the unaltered human progesterone receptor gene and protein, PROGINS seems to cause several alterations on a molecular level. While related sequence changes in the noncoding sequence of intron G of the human progesterone receptor gene do not translate into mutant elements of the progesterone receptor protein, these changes may influence various parameters of gene regulation. The PROGINS-associated mutational complex in exons 4 and 5 of the human progesterone receptor gene directly affects the receptor protein. While the alteration in exon 5 is silent, the point mutation in exon 4 caused an amino acid change from valine to leucine leading to a mutated progesterone receptor protein (82). The PROGINS-associated mutational changes have been shown to alter the progesterone receptor function in chloramphenicol acetyltransferase (CAT) assays, which are used to evaluate the transcriptional activity of DNA. As compared to the wild-type protein, the mutant progesterone receptor protein
demonstrated greater progesterone-inducible transcriptional activity in CAT assays while exhibiting the same steroid-hormone binding affinity (D.G. Kieback, personal communication). Interestingly, the highest transcriptional activity was achieved in-vitro by simulating the heterozygous PROGINS status. These results strongly suggest that heterozygous PROGINS constitution might lead to an overexpression of progesterone-inducible target genes even at physiologic levels of progesterone (123). Moreover, this mechanism may contribute to the process of malignant transformation and development of hormone-sensitive cancers. In conclusion, there is strong evidence that the presence of PROGINS in the heterozygous state alters timing, level, and/or spatial expression pattern of progesterone target genes, thereby exposing carriers to a constant and slightly increased expression of progesterone target genes (123). In case of ovary and breast cancer, one could speculate that these subtle changes in the hormonal environment are able to influence the process of malignant transformation ((82) and D. Kieback, personal communication). However, due to difficulties in identifying all potential progesterone target genes and the great complexity and multitude of biological effects of increased long term progesterone stimulation (126), the ultimate biological consequences remain to be elucidated.

While study populations with a similar genetic background are generally preferable, we do not believe that there exist significant confounding differences in terms of PROGINS status between male caucasian populations of German and North American origin as the frequency of PROGINS carriers in Austrian women is similar to those in women in North America and England (146). In addition, a previous study conducted by our group suggested an autosomal mode of inheritance. 443 German caucasian females and 120 American caucasian women were tested for PROGINS status. Out of the 443 German caucasian females, 99 (22.3%) tested positive for heterozygous or homozygous states of PROGINS (95% confidence intervals of frequency: 18%-27%), while 22 individuals (18.3%) tested positive for heterozygous or homozygous states of PROGINS out of the 120 American Caucasian women (95% confidence intervals of frequency: 12%-27%)(D. Kieback, personal communication). Here we demonstrate that the PROGINS polymorphism occurs in comparable frequencies in male and female caucasian populations of German and North American origin. 95% confidence intervals of frequency overlapped for all groups of German and American males and females. The rather identical
frequencies of PROGINS in German and American caucasian males and females indicate an autosomal mode of inheritance for PROGINS. PROGINS seems to be a fairly common and widespread genetic polymorphism that is stably inherited from generation to generation (57) as it does not seem to put heterozygous carriers at increased cancer risk before family planning has been completed.

While heterozygous PROGINS status was found to increase ovarian cancer risk in BRCA1 and BRCA2 mutation carriers who were never exposed to oral contraceptives (80,123) and to decrease risk for breast cancer by age 50 (154), this has not been confirmed by others analyzing less well defined collectives (86,97). In the present study, we were unable to demonstrate any statistically significant association between the PROGINS polymorphism and the presence or course of prostate cancer. We therefore conclude that PROGINS cannot be used to assess a genetic risk for prostate cancer. The heterozygous state of PROGINS did not seem to confer any protective or deleterious effects to its carriers in terms of prostate cancer. We certainly cannot rule out that progesterone is not involved in prostate cancer development in any way or that it is only contributing in a very minor way the effect of which would require very large sample numbers in order to become statistically significant.

In contrast to ovarian and breast cancer, PROGINS does not appear to be a suitable risk marker in prostate cancer. However, the role of PROGINS in ovarian and breast cancer remains an issue of controversial discussion (86,97,123,154). Interestingly, PROGINS is not suitable as a risk marker for endometrial carcinoma (D. Kieback, personal communication). Due to the extraordinary complexity of all aspects of steroid hormone and hormone receptor function, expression and interaction and due to the lack of appropriate in-vivo models (147), we can only speculate which of the molecular mechanisms associated with PROGINS seems to affect cancer risk and pathogenesis of certain malignancies. Unfortunately, and despite the implication of progesterone in the pathogenesis of prostate cancer in several studies (51,144,153,162), the role of progesterone and its receptor not only for the development and physiological function of the prostate gland but also for the
pathogenesis of prostate cancer is still largely unknown (79). Although the human prostate
gland contains significant concentrations of progesterone receptors, its function in prostate
tissue and prostate cancer remains unknown (22,105). Immunohistochemically,
progesterone receptors were found almost exclusively in stromal cells of benign and
malignant prostatic tissue and may indicate cases sensitive to progesterone therapy
(22,105). The expression of progesterone receptors in epithelial cells of the prostate is an
issue of discussion (66). Prostate carcinoma specimens taken from previously untreated
patients with a high degree of malignant involvement contained a significantly lower mean
concentration of progesterone receptors than BPH specimens (22,105). The mean
progesterone receptor content in cytosol of prostate cancer metastases was only one-fourth
compared with primary prostate cancer indicating dedifferentiation (49), while another
study did not identify any progesterone receptors in human prostate cancer metastases
(66). The changes in concentration and distribution of these steroid hormone receptors in
normal and diseased prostate tissue as well as their effects on the synthesis of growth
factors, growth factor receptors, and oncogenes need to be further investigated (140).

It remains unclear why PROGINS is not associated with hormone-dependent malignancies
like prostate cancer and endometrial cancer. Since PROGINS does not seem to be a
universal risk marker for all types of hormone-dependent cancers, this points to a tumor-
specific mode of action which makes any explanations even more difficult. A more
complete understanding of the role of progesterone and its hormone receptor not only in
prostate cancer but also in ovarian and breast cancer needs to be established.
5. Conclusion:

In an effort to identify potential genetic risk markers for prostate cancer, we have investigated the role of a polymorphism in the human progesterone receptor gene called PROGINS. It encodes for a progesterone receptor with increased stability and increased hormone-induced transcriptional activity (123,154). Epidemiological studies have shown that PROGINS modulates the risk for ovarian and breast cancer (123,154). We demonstrate that PROGINS is present in comparable frequencies in male and female caucasian populations of German and North American origin, suggesting an autosomal mode of inheritance. Despite of its implication as a risk marker for ovarian and breast cancer, PROGINS did not serve as a molecular risk marker for prostate cancer. In case of prostate cancer, changes in the human progesterone gene were not correlated with any of the variables characterizing the course of disease. In-vitro experiments with cells overexpressing either the mutated or the wildtype receptor suggest that individuals with heterozygous states of PROGINS may undergo constitutionally increased long-term expression of progesterone target genes. However, the exact mechanism of action of PROGINS remains unknown. This is mainly secondary to difficulties in identifying progesterone target genes causing malignant transformation, lack of knowledge on the significance and mechanism of action of progesterone in the prostate gland and the great complexity and multitude of biological effects of progesterone.
6. Summary:

*Purpose:* Recently, a polymorphism in the progesterone receptor gene was detected and named PROGINS. In comparison to the wildtype receptor, the receptor protein encoded by PROGINS shows increased stability. The purpose of this study was to evaluate PROGINS as a possible genetic risk marker for prostate cancer, since PROGINS has been shown to modulate the risk of ovarian and breast cancer.

*Materials and Methods:* Genomic DNA from 94 American caucasian prostate cancer patients and from 100 healthy male German caucasian control individuals were tested for their genetic constitution in terms of the PROGINS polymorphism by polymerase chain reaction.

*Results:* No association between the PROGINS polymorphism and the incidence of prostate cancer or with a variety of parameters characterizing the course of this disease was detected.

*Conclusion:* The PROGINS polymorphism cannot be used as a genetic risk marker for prostate cancer. Due to the great complexity and multitude of biological effects of progesterone, lack of knowledge on the exact mechanism of action of PROGINS and on the significance and function of progesterone in the prostate gland, more research is needed in these areas to comprehensively explain our data.
7. References:


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