The Genetic Variants of NOTCH3 (6746T>C) and PSMA6 (-8C>G) As Possible Risk Factors of Psoriasis Development

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Abstract

Advances in genotypic technologies enable identification of possible associations between genetic variants of certain genes and increased risk of developing plaque psoriasis or psoriatic arthritis. The aim of the study was to analyze the NOTCH3 (6746T>C) (rs1044009) and PSMA6 (8C>G) (rs1048990) polymorphisms and their role in genetic susceptibility to psoriasis. The study included 158 psoriatic patients and 100 healthy controls. The frequencies of the NOTCH3 genotypes differed between the psoriatic patients and controls (p=0.050). No differences were found in the distribution of PSMA6 genotypes and alleles between the psoriatic patients and controls. The studied psoriatic patients presented a higher frequency of the CC genotype of PSMA6 compared to the healthy controls (8.8% vs 2%, respectively). Psoriatic arthritis was more frequent among patients with the CC genotype of PSMA6 (p=0.059). Simultaneous CC homozygosity of NOTCH3 and PSMA6 was significantly more commonly observed in the studied psoriatic patients than in the controls (p=0.032).

The obtained data suggest that genetic variants of NOTCH3 (6746T>C) and PSMA6 (-8C>G) genes may play significant roles in psoriatic patients. Further studies are necessary to unequivocally determine their role as genetic risk factors of psoriasis development.

Introduction

Owing to genotyping technologies, it has been possible to identify numerous single nucleotide polymorphisms (SNPs) which contribute to pinpointing plenty of genetic markers responsible for genetic susceptibility to psoriasis, a chronic, auto-inflammatory skin disease\(^1,2\).

The genetic background of psoriasis explains uncontrolled proliferation and abnormal differentiation of keratinocytes arising from disturbances in the innate and adaptive cutaneous immune responses. The interplay between keratinocytes and immune cells, including Th1, Th17, regulatory T cells (Tregs) and neutrophils, expressed by activation of the TNF\(\alpha\)–IL-23–Th17 proinflammatory axis, is known to cause changes in both the epidermis and dermis, i.e. epidermal hyperplasia, dermal inflammatory infiltration as well as elongation and increased permeability of blood vessels\(^3-5\).

The NOTCH signaling pathway, a highly conserved cell signaling system involving interaction between the NOTCH receptors and their ligands, has been shown to play an important role in the regulation of cell proliferation, differentiation, migration and apoptosis, and it is suggested to participate in the development of various skin diseases, including psoriasis, atopic dermatitis, hidradenitis suppurativa, Dowling Degos disease and Adams-Oliver syndrome\(^6\).

The NOTCH proteins form a family of four single-pass type 1 transmembrane receptors encoded by the NOTCH 1, 2, 3 and 4 genes. They transduce signals after activation by their transmembrane ligands, i.e. Jagged-1/-2 and Delta-like 1, 3 and 4. However, the outcome of NOTCH signaling is highly dependent on
the interaction of various cellular mechanisms, especially in the context of inflammation and malignancy\textsuperscript{6,7}.

Rooney et al.\textsuperscript{8} observed an upregulated Jagged-1 expression in psoriatic patients and activation of the NOTCH signaling pathway which led to hyperproliferation of the epidermal cells. Moreover, the Notch-1 receptor and its peptide ligand Delta-like 1 have been shown to regulate early keratinocytes differentiation\textsuperscript{9}. Apart from its effect on the epidermal cells, NOTCH is also important in the development of the T cells and their function\textsuperscript{10}. It has been proved that the NOTCH ligands are involved in polarizing the T-cell response towards producing Th1 and Th17 cells upon induction of the Delta-like ligands on the dendritic cells (DCs), however, when NOTCH interacts with the Jagged ligands, it activates production of Th2 cells\textsuperscript{10,11}. This suggests that the NOTCH signals can alter the balance of the CD4 T-cell differentiation into the Th1 or Th2 lineage.

NOTCH activation is also involved in different stages of blood vessels development, including vascularization and angiogenesis\textsuperscript{12,13,14}. Therefore, the NOTCH signaling pathway is supposed to play a role in psoriasis by modulation of keratinocyte proliferation, immune processes and angiogenesis.

While the role of NOTCH1 in the skin is fairly well explained, there is still little information about a possible role of NOTCH3 in the pathogenesis of skin diseases.

NOTCH3, whose transcription can be directly activated by the intracellular domain of NOTCH1, is another regulator of keratinocyte differentiation which controls involucrin expression at the late phase of the process\textsuperscript{15,16}. The NOTCH3 receptor also participates in the regulation of T-cell differentiation and may be engaged in the autoinflammatory processes in psoriasis\textsuperscript{17}. Therefore, our study makes an attempt to find a possible association between the NOTCH3 polymorphism and genetic susceptibility to psoriasis. One of the polymorphisms in the \textit{NOTCH3} gene (\textit{locus}19p13.12) is observed in the exon 33 (6746T > C) (rs1044009) and resulted from the T (T allele) to C (C allele) substitution (GTG to GCG).

Similarly to the Notch pathway, the proteasome system, a large multiple subunit enzyme complex, also controls such processes as apoptosis, proliferation, differentiation and inflammation. PSMA6, a component of the 20S proteasome complex which is the main pathway for degradation of oxidatively damaged proteins, is coded by the gene located on the chromosome 14q13.2\textsuperscript{19}. There is a single \textit{PSMA6} (-8C > G) (rs1048990) nucleotide polymorphism in the exon 1 which has been found to be associated with diabetes, myocardial infarction and coronary artery disease also known as “oxidative stress conditions”\textsuperscript{20}. Therefore, since psoriasis is a systemic disease, it may be surmised that the \textit{PSMA6} polymorphism plays a role in the genetic susceptibility to psoriasis.

The presence of the G allele is associated with the PSMA6 transcriptional augmentation. An increased PSMA6 activity aggravates inflammation through activation of the nuclear factor-κB signaling pathway\textsuperscript{21}. In psoriasis, activation of the nuclear factor-κB signal induces expression of keratins 6 and 16
in keratinocytes, which leads to acanthosis and shortened turnover time in the epidermis\textsuperscript{22}. Both expression and activity of the 20S proteasome are also increased in the psoriatic skin cells\textsuperscript{5}.

A possible genetic interplay between the SNPs of \textit{NOTCH3} (6746T \textgreater{} C) and \textit{PSMA6} (-8C \textgreater{} G) in psoriasis has not been investigated yet. Therefore, the aim of this study was to analyze the \textit{NOTCH3} (6746T \textgreater{} C) and \textit{PSMA6} (-8C \textgreater{} G) polymorphisms and the role they play in genetic susceptibility to psoriasis in the Polish psoriatic patients.

**Material And Methods**

**Study groups**

Patients were recruited in the Chair and Department of Dermatology, Venereology and Pediatric Dermatology, the Medical University of Lublin (Poland) from May 2018 to May 2020. The inclusion criteria for patients were as follows: a) age \textgreater{} 18 years; b) confirmed psoriasis; c) unrelated individuals; d) Caucasian race.

Healthy subjects were recruited from a group of blood donors in the Regional Blood Donation and Blood Treatment Center in Kielce (Poland) and they had to fulfill the following inclusion criteria: a) age \textgreater{} 18 years; b) Caucasian race. Blood donors were excluded in the case of: a) HIV infection, syphilis, tuberculosis, hepatitis B or hepatitis C; b) condition that requires active medical intervention or monitoring to avert serious danger to the participant's health or well-being; c) race other than Caucasian.

The study was approved by the local Bioethics Committee of the Medical University of Lublin (KE-0254/35/2018) and was performed in accordance with relevant guidelines and regulations. All recruited patients and controls gave written informed consent to participate in the study.

**DNA isolation**

DNA isolation from peripheral blood was performed using a commercial kit (Qiagen, Germany) according to manufacturer's procedure. The concentration and quality of DNA was checked using NanoDrop device (Thermo Fisher Scientific, USA).

**Genotyping of NOTCH3 6746T > C polymorphism**

The polymorphism was assessed by PCR-restriction fragment length polymorphism (RFLP). Each PCR mix (25µl) contained 150ng genomic DNA, and PCR buffer (Clontech Laboratories, USA), dNTPs mix (0,25mM), HD polymerase (Clontech Laboratories, USA) and primers (10µM of each). The mix was heated to 94°C for 5min and underwent 35 cycles of amplification: denaturation 98°C for 10s, annealing 64°C for 10s, elongation 72°C for 20s. The final elongation took 5min at 72°C. The PCR reaction was performed using an Applied Biosystems 9700 Thermal Cycler. The following primers were used in PCR reaction:
• -forward 5'-CTT ACC TGG CAG TCC CAG G-3’
• -reverse 5'-AGT GGC AGT GGC TGG GCT AG-3’

The PCR products were digested with *MwoI* (HpyF10VI) restriction enzyme (Thermo Fisher Scientific, USA) for 16 hours at 37°C. RFLP products were analyzed on 3% agarose gel and stained with SimplySafe (Eurx, Poland) and visualized in G:Box (Syngene, Great Britain). T or C alleles were identified by the presence of 203bp (TT genotype) or 158bp (CC genotype) fragments, respectively. Heterozygous TC genotype showed the presence of two bands – 158bp and 203bp (Fig. 3a). An independent PCR analysis was carried out for each sample.

**Genotyping of PSMA6 -8C > G polymorphism**

For analysis of *PSMA6* polymorphism PCR-RFLP method was applied according to validated protocol of Bachmann et al. 2010\(^{28}\). *PSMA6* gene fragment length of 100 bp was amplified in PCR reaction using following primers:

• -forward 5'-CTG GTG CGG GAG CTA CGG G-3’
• -reverse 5'-AAT GGT AAT GTG GCG GTC AAA AC-3’

Each PCR mixture (25µl) contained 100ng genomic DNA and PCR buffer (Clontech), dNTPs mixture (0.25mM), HD polymerase (Clontech) and primers (10µM of each). The touchdown PCR method was used. The mixture was heated 95°C for 5min and underwent 14 cycles of amplification: denaturation 95°C for 30s, annealing 64.5°C for 20s (-0.5°C/per cycle), elongation 72°C for 20s. After 14 cycles the mixture underwent 20 cycles with constant temperature 57.5°C. The denaturation and elongation temperatures and times were the same as above. The final elongation taken 5min at 72°C. The PCR reaction was performed in a Applied Biosystems 9700 Thermal Cycler.

The PCR product was digested with *RsaI* (Thermo Fisher Scientific) for 16 hours at 37°C producing two fragments of 50 bp or one fragment 100 bp for presence of G or C allele, respectively. RFLP products were analyzed on 3% agarose gel and stained with SimplySafe (Eurx, Poland) and visualized in G:Box (Syngene, Great Britain) (Fig. 3b). An independent PCR analysis was carried out for each sample.

**Statistical methods**

The data were statistically analyzed using Statistica 13.1 software (STATSOFT, Tulsa, OK, USA).

Mean values (M) and standard deviations (SD) were estimated for continuous variables while absolute numbers (n) and relative numbers (%) for categorical variables.

The allele frequencies were assessed on the basis of the genotype distribution. Hardy–Weinberg equilibrium was evaluated in the controls by a $\chi^2$ test.
Fisher's exact test was used to compare genetic models between patients and controls. If significant difference was detected, odds ratio (OR) was estimated.

In correlation analyses between genetic models and psoriasis features Mann-Whitney’s U test or Fisher’s exact test were used.

The significance level was set at $p \leq 0.05$ in all statistical tests.

**Results**

**Characteristics of the patients**

In the study, 158 psoriatic patients and 100 healthy subjects (controls) were analyzed. Table 1 shows characteristics of the studied psoriatic patients. The mean age of the onset of psoriasis was $23.3 \pm 12.3$ years. In 86.71% of the patients, psoriasis developed at or under the age of 40 years (Type 1 psoriasis), while in the remaining 13.29% patients it started when they were over 40 (Type 2 psoriasis). The mean psoriasis duration was $23.3 \pm 12.5$ years. 35.44% of patients suffered from psoriatic arthritis. In 74.68% of the patients, psoriasis was moderate or severe. Almost a half of the studied patients (49.63%) had a positive family history of psoriasis.

| Variable, parameter | IU /category | Psoriatic patients (N = 158) |
|---------------------|--------------|------------------------------|
| Age, Min-Max, M ± SD| years        | 20–84, 46.5 ± 14.1           |
| Gender, n (%)       | men          | 112 (70.89)                  |
|                     | women        | 46 (29.11)                   |
| Age of psoriasis onset, Min-Max, M ± SD| years| 1–63, 23.3 ± 12.3 |
| Psoriasis subtype, n (%)| at/under 40 years of age| 137 (86.71) |
|                     | over 40 years of age| 21 (13.29) |
| Psoriasis duration, Min-Max, M ± SD| years| 2–52, 23.3 ± 12.5 |
| Psoriatic arthritis, n (%)| yes| 56 (35.44) |
| Severity of psoriasis, n (%)| mild| 40 (25.32) |
|                     | moderate or severe| 118 (74.68) |
| Positive family history, n (%) (N = 135)| yes| 67 (49.63) |
Frequencies of genotypes and alleles of NOTCH3 and PMSA6 polymorphisms

Genotyping analyses were successful in all the studied subjects. Distribution of the NOTCH3 genotypes in the control group was in agreement with the Hardy-Weinberg Equilibrum (HWE) model ($\chi^2 = 0.706, p = 0.401$). Similarly, distribution of the PMSA6 genotypes was also in agreement with HWE ($\chi^2 = 0.756, p = 0.385$). The frequencies of the NOTCH3 genotypes differed between the patients and controls ($p = 0.050$) (Fig. 1). However, no differences were demonstrated in the distribution of PMSA6 genotypes and alleles between the psoriatic patients and controls (Fig. 2). The studied psoriatic patients presented a higher frequency of the CC genotype compared to the healthy controls (8.8% vs 2%, respectively). No statistical difference was found when the alleles distribution was analyzed.

NOTCH3 and PSMA6 polymorphisms and psoriasis in the analysis of genetic models

The relationship between NOTCH3 polymorphism and psoriasis was analyzed using five genetic models: dominant (TC + CC vs. TT), recessive (CC vs. TT + TC), additive (CC vs. TT), heterozygote (TC vs. TT) and allelic (C vs T). In the recessive model significant difference was demonstrated ($p = 0.001$). The CC homozygotes were more frequent in the patient group than in controls (8.86% vs. 2%, respectively). The individuals being CC homozygotes had 5-fold higher risk of the disease (OR = 4.76 95%CI 1.06–21.43). Similarly, a significant difference was observed in the case of the additive model of NOTCH3 polymorphism (OR = 4.968 95%CI 1.09–22.60, $p = 0.030$). In the allelic model, the C allele was found to be more prevalent in the psoriatic patients compared to controls (25% vs 18%, respectively), however, the result was close to statistical significance ($p = 0.066$).

The same genetic models were also used for analyses of the PSMA6 polymorphism in the psoriatic patients and controls: dominant (CG + GG vs. CC), recessive (GG vs. CG + CC), additive (GG vs. CC), heterozygote (CG vs. CC) and allelic (G vs. C). No association between the PSMA6 polymorphism and psoriasis was observed in any of the used genetic models.

Table 2 demonstrates the results of analyses of genetic models for both investigated polymorphisms.
Table 2
The analyses of relation between NOTCH3 and PSMA6 polymorphisms and psoriasis using genetic models

| Polymorphism | Genetic model | Genotypes/Alleles | Patients (N = 158) | Controls (N = 100) | p      |
|--------------|---------------|-------------------|-------------------|-------------------|--------|
| NOTCH3       | Dominant      | TC + CC           | 65 (41.14)        | 34 (34.00)        | 0.293  |
|              |               | TT                | 93 (58.86)        | 66 (66.00)        |        |
|              | Recessive     | CC                | 14 (8.86)         | 2 (2.00)          | 0.032  |
|              |               | TT + TC           | 144 (91.14)       | 98 (98.00)        |        |
|              | Additive      | CC                | 14 (13.08)        | 2 (2.94)          | 0.030  |
|              |               | TT                | 93 (86.92)        | 66 (97.06)        |        |
|              | Heterozygote  | TC                | 51 (35.42)        | 32 (32.65)        | 0.681  |
|              |               | TT                | 93 (64.58)        | 66 (66.35)        |        |
|              | Allelic       | C                 | 79 (25.00)        | 36 (18.00)        | 0.066  |
|              |               | T                 | 237 (75.00)       | 164 (82.00)       |        |
| PSMA6        | Dominant      | CG + GG           | 25 (15.82)        | 16 (16.00)        | 1.000  |
|              |               | CC                | 133 (84.18)       | 84 (84.00)        |        |
|              | Recessive     | GG                | 1 (0.63)          | 0 (0.00)          | 1.000  |
|              |               | CC + CG           | 157 (99.37)       | 100 (100.00)      |        |
|              | Additive      | GG                | 1 (0.75)          | 0 (0.00)          | 1.000  |
|              |               | CC                | 133 (99.25)       | 84 (84.00)        |        |
|              | Heterozygote  | CG                | 24 (15.29)        | 16 (16.00)        | 1.000  |
|              |               | CC                | 133 (84.71)       | 84 (84.00)        |        |
|              | Allelic       | G                 | 25 (8.00)         | 16 (8.00)         | 1.000  |
|              |               | C                 | 291 (92.00)       | 184 (92.00)       |        |

p for Fisher’s exact test

Joint analysis of NOTCH3 and PSMA6 polymorphisms

Comparison of the impact of NOTCH3 and PSMA6 polymorphisms on the risk of psoriasis is demonstrated in Table 3. NOTCH3 and PSMA6 simultaneous CC homozygosity was significantly more
commonly observed in the studied psoriatic patients than in controls (OR = 4.76 95%CI 1.06–21.43, p = 0.032). The other combinations of genotypes of both polymorphisms did not differ in the studied groups.

**Correlations between analyzed polymorphisms and psoriasis clinical features**

Correlation of *PSMA6* G allele carriers (GG + CG) and CC homozygotes with psoriasis clinical features revealed that psoriatic arthritis was more frequent among patients with the CC genotype (p = 0.059). The other psoriasis clinical features did not correlate with the *PSMA6* polymorphism. Similarly, no statistical correlations were found between the *NOTCH3* polymorphism and psoriasis parameters (Table 4).
Table 4
Correlations of NOTCH3 and PSMA6 polymorphisms with psoriasis features

| Variable, category, parameter | NOTCH3 polymorphism | PSMA6 polymorphism |
|-------------------------------|---------------------|---------------------|
|                               | C allele carriers   | Wild-type homozgygotes | p  | G allele carriers | Wild-type homozgygotes | p  |
|                               | (CC + TC) (N = 65)  | (TT) (N = 93)        |     | (GG + CG) (N = 25) | (CC) (N = 133)         |     |
| Gender, male, n (%)           | 46 (70.77)          | 66 (70.97)           | 0.558 | 18 (72.00)        | 94 (70.68)              | 0.551 |
| Age of onset, M ± SD          | 22.9 ± 11.2         | 23.6 ± 13.1          | 0.727 | 23.7 ± 12.8      | 23.2 ± 12.3             | 0.855 |
| Psoriasis subtype, 40 + years old, n (%) | 6 (9.23)        | 15 (16.13)           | 0.154 | 3 (12.00)         | 18 (13.53)              | 0.567 |
| Psoriasis arthritis, n (%)    | 25 (39.68)          | 31 (33.33)           | 0.310 | 5 (20.00)         | 51 (38.35)              | 0.059 |
| Severity, moderate or severe, n (%) | 47 (72.31)       | 71 (76.34)           | 0.347 | 20 (80.00)        | 98 (73.68)              | 0.348 |
| Positive family history, n (%) | 27 (49.09)         | 40 (50.00)           | 0.528 | 13 (54.17)        | 54 (48.65)              | 0.396 |

* missing data in every column: 1, 22, 10 and 13, respectively. p for Fisher's exact test.

Discussion

It goes without saying that genetic, immune and environmental factors conspire with one another to trigger psoriasis. Our study aimed at investigating the genetic aspect of psoriasis etiology and it focused especially on the NOTCH3 and PSMA6 polymorphisms. We made an attempt to detect possible links between the NOTCH3 (6746T > C) and PSMA6 (-8C > G) polymorphisms and psoriasis in five genetic models: dominant, recessive, additive, heterozygote and allelic.

The results of our study showed a higher frequency of the NOTCH3 CC genotype in the psoriatic patients in comparison to healthy controls (p = 0.050), whereas the other investigated genotypes and alleles were similar in both studied groups. Compared with the healthy controls, the recessive (CC vs. TT + TC) and additive (CC vs. TT) models of the NOTCH3 polymorphisms were significantly different in the psoriatic patients. The CC homozygotes were more frequent in the studied patients than in controls and the individuals being CC homozygotes had a 5-fold higher risk of developing psoriasis. Also, the allelic model was more often observed in the studied psoriatic patients, and this finding turned out to be close to statistical significance (p = 0.066).
In the light of the study of Ota et al., who investigated Notch1, 2, and 3 on mRNA and protein levels and observed their decreased expressions in the psoriatic epidermis compared with normal epidermis, our study results may be supportive of a role of the NOTCH3 (6746T > C) polymorphism in the pathogenesis of psoriasis. According to Ota et al., a lack of NOTCH signaling causes abnormal differentiation of keratinocytes observed in psoriasis. Furthermore, Pan M et al. suggested that in psoriasis the activation of Notch signaling pathway may be regulated by miR-125b which plays a role in controlling of cell proliferation by downregulation of the Jagged-1 ligand expression.

Investigation of the other studied polymorphism, PSMA6 (-8C > G), brought to light no links between the PSMA6 genotypes and alleles and psoriasis. However, in our patients with psoriatic arthritis, the PSMA6 CC genotype was more frequently observed than in the carriers of G allele (38% vs 20%). The difference was found to be close to statistical significance (p = 0.059), while the other studied clinical features did not correlate with the PSMA6 polymorphism. This observation is quite surprising since the G allele was demonstrated to enhance the transcription of PSMA6, also in HEV cell lines. The authors suggested that some nuclear factor(s) may bind to this region and thus regulate transcription of PSMA6. In turn, altered expression of PSMA6 gene may increase inflammation through activation of NF-kB protein.

Similarly to our results, the PSMA6 CC genotype was previously demonstrated to be more common in the patients with the end-stage of renal disease than in controls (80% vs. 58%, respectively), which suggested a protective role of the G allele. In our study, the PSMA6 G allele carrier frequency was similar in the psoriatic patients and controls. Only one psoriatic patient was a GG homozygote, which may be a result of a limited number of the study subjects.

A Stuart et al.’s meta-analysis of two genome-wide association studies (GWAS) revealed three genomic regions associated with psoriasis, one contained NOS, another one contained FBXL19 and the third one contained PSMA6 and NFKBIA. All of them were associated with both plaque psoriasis and psoriatic arthritis. Among them only PSMA6, an encoding proteosomal subunit involved in MHC Class I antigen processing, was overexpressed in psoriatic lesions. Moreover, in the blood of psoriatic arthritis patients, Colmegna et al. observed elevated levels of anti-proteasome antibodies, which, according to them, might reduce the activity of proteasomes. Reduction of the 20S proteasome activity in the blood cells of psoriatic patients observed by Karabowicz et al. confirmed the findings of Colmegna et al.

Our study results appear to support the view that a reduced activity of proteasome may be somehow associated with the PSMA6 polymorphism.

We made an interesting observation that in the individuals who were both PSMA6 CC homozygotes and NOTCH3 CC homozygotes the risk of psoriasis was almost 5-fold higher than in the individuals with other combinations of genotypes (OR = 4.76, p = 0.032). The NOTCH3 polymorphism results in an amino acid dimorphism (Val/Ala) at residue 2223 of the intracellular domain. Since the intracellular domain of NOTCH3 is thought to be involved in signal transduction, this polymorphism has been suggested to be directly associated with the NOTCH3 function, i.e. keratinocyte and T-cell differentiations.
To the best of our knowledge, the present study analyzes for the first time the relation between PSMA6 polymorphism and psoriasis. However, the study has some limitations. First, to establish the role of PSMA6 polymorphism in psoriasis development, experimental studies are necessary. Second, the study is based on a limited number of psoriatic patients all of whom were Caucasian. This makes subgroup analyses challenging. Further studies on larger groups of patients are needed to confirm the present results which could shed more light on the genetic susceptibility to psoriasis.

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**Figures**
Figure 1

Frequency of genotypes and alleles of NOTCH3 polymorphism in psoriatic patients and controls

Figure 2

Frequency of genotypes and alleles of PMSA6 polymorphism in psoriatic patients and controls
Figure 3

Detection of 6746T>C NOTCH3 (a) and -8C>G PSMA6 (b) polymorphisms