

## Role of Genetic Variations in *CDK2*, *CCNE1* and *p27<sup>KIP1</sup>* in Prostate Cancer

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**Abstract.** *Background/Aim:* Our aim was to investigate possible influences of genetic variants in genes involved in the G<sub>1</sub>/S transition [cyclin-dependent kinase-2 (*CDK2*), cyclin E1 (*CCNE1*) and cyclin-dependent kinase inhibitor 1B (*p27<sup>KIP1</sup>*)] on the expression/activity of their corresponding proteins and to assess the functional impact of these variants on the risk of prostate cancer. *Materials and Methods:* We genotyped 530 cases and 562 healthy controls for two relevant single nucleotide polymorphisms (*CDK2* rs2069408 and *CCNE1* rs997669) by TaqMan genotyping assay. *p27<sup>KIP1</sup>* rs2066827 polymorphisms were studied by polymerase chain reaction-restriction fragment length polymorphism assay. In addition, the expression of *CDK2*, *CCNE1* and *p27<sup>KIP1</sup>* was evaluated by quantitative real-time-polymerase chain reaction and western blotting in 44 prostate cancer tissues and 31 benign prostatic hyperplasia tissues. *Results:* No association was found between *CDK2* rs2069408, *CCNE1* rs997669 or *p27<sup>KIP1</sup>* rs2066827 polymorphisms and an increased risk of prostate cancer development. Higher *CDK2* expression was

more prevalent in those with rs2069408 GG genotype than in AA carriers ( $p > 0.05$ ). We also noted reduced *p27<sup>KIP1</sup>* protein expression in those with the *p27<sup>KIP1</sup>* G109 allele. No difference was observed for *CCNE1* expression in relation to the risky genotype (CC). A significant association was detected between *CCNE1* mRNA overexpression and development of higher-grade carcinomas (Gleason score  $> 7$ ,  $p < 0.05$ ). *Conclusion:* Polymorphisms *CDK2* rs2069408, *CCNE1* rs997669 and *p27<sup>KIP1</sup>* rs2066827 have no significant impact on prostate cancer risk nor on the gene and protein expression of *CDK2*, *CCNE1* and *p27<sup>KIP1</sup>*, although high *CCNE1* expression was significantly associated with a higher tumour grade in patients with prostate cancer.

Prostate cancer is one of the most frequently diagnosed types of cancer, a leading cause of cancer-related deaths in men and displays racial disparity (1). It is a highly heterogeneous disease characterized by a complex etiopathogenesis in which familial, hormonal, and dietary factors play an important role (2, 3). Genetic and epigenetic alterations also participate in the initiation and progression of prostate cancer by modifying its morphological model and influencing its biological behaviour (4).

For the physiological development of the prostate, a balance has to be maintained between the factors regulating normal cell growth, proliferation and apoptosis (5). One of the characteristics of tumorigenesis is the occurrence of genetic variations in the genes which regulate the cell cycle and, in particular, the checkpoint genes (6). The G<sub>1</sub>/S transition phase defines the fate of a cell based on proliferative or apoptotic signals. The main key regulatory complexes of the G<sub>1</sub>/S transition phase are two cyclin/cyclin-dependent kinase (CDK)

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*Key Words:* *CDK2*, *CCNE1*, *p27<sup>KIP1</sup>*, G<sub>1</sub>/S transition, cell cycle, polymorphism, expression, prostate cancer, Slovak population.



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complexes, namely cyclin D–CDK4/6 and cyclin E–CDK2, which have been reported to trigger the G<sub>1</sub>/S transition by promoting phosphorylation of retinoblastoma protein family proteins (7-10). Under physiological conditions, cyclin E–CDK2 complex activity is associated with DNA replication and centrosome duplication during the G<sub>1</sub>/S transition in the cell cycle (11). This complex is primarily inhibited by CDK inhibitor 1B, p27<sup>KIP1</sup> (12, 13). In contrast, the cyclin E–CDK2 complex can phosphorylate p27<sup>KIP1</sup>, ultimately leading to its degradation (14). Overexpression of cyclin E, high activity of cyclin E–CDK2, and a deficiency of p27<sup>KIP1</sup> have been reported in several human cancer types (15-17).

We have hypothesized that common genetic variations, such as single nucleotide polymorphisms (SNPs), in the genes that control the cell cycle influence the expression or activity of the corresponding proteins and have a functional impact on the risk of prostate cancer. Previous studies have concentrated on the study of one of the p27<sup>KIP1</sup> polymorphisms, namely p27<sup>KIP1</sup>V109G (*rs2066827*), which occurs at codon 109 (T>G) and causes the substitution of glycine for valine. This substitution affects the normal function of the p27<sup>KIP1</sup> gene through altered transcription and leads to the dysregulation of the cell cycle and to the induction of tumorigenesis (18). The intron SNPs *rs2069408* in *CDK2* and *rs997669* in *CCNE1* have not been characterized functionally. However, there are no studies reporting association between *CDK2 rs2069408*, *CCNE1 rs997669* and p27<sup>KIP1 rs2066827</sup> polymorphisms and expression changes of the respective genes in prostate tumours. Thus, our aim was to analyse for the first time the way in which the selected SNPs influence the dynamic changes in gene and protein expression of *CDK2*, *CCNE1* and p27<sup>KIP1</sup> in tumour tissue of patients with prostate cancer and thus to determine any important roles that they might play in prostate carcinogenesis.

## Materials and Methods

**Study population.** The Ethical Boards of the Jessenius Faculty of Medicine in Martin, Comenius University, approved the proposal of the present study. Briefly, 1,092 men (530 patients, 562 healthy individuals) who were recruited at the Department of Urology of University Hospital in Martin and the Jessenius Faculty of Medicine in Martin, Comenius University between May 2005 to May 2020 participated in the study. The patients with prostate cancer, healthy controls, and patients with benign prostatic hyperplasia (BPH) studied were all Caucasian. All of the cases were confirmed by histological diagnosis. The healthy controls had no previous history of any cancer and their prostate-specific antigen (PSA) levels were within the normal limit (<4 ng/ml). The clinical characteristics of the participants are summarized in Table I.

Informed written consent was obtained from all individuals prior to their inclusion in the study.

Whole blood collected from each individual was used for DNA isolation. Tumour tissues were obtained from 44 patients with histologically confirmed prostate cancer and 31 patients with BPH

Table I. Characteristics of the study groups.

	Healthy controls	Prostate cancer
Number	562	530
Age, years		
Mean±SD	64.4±10.5	67.2±8.6
PSA, ng/ml		
Mean±SD	2.95±0.7	45.9±5.8
Gleason score		
≤7	NA	277
>7	NA	135
Mean±SD	NA	7.3±0.06
Missing data	NA	118
Pathological stage		
pT1/pT2	NA	119
pT3/pT4	NA	119
Missing data	NA	292

NA: Not applicable; PSA: prostatic-specific antigen; pT: pathological T-stage; SD: standard deviation.

(controls) and were stored in mRNA-stabilizing solution (RNAlater®; Applied Biosystems/Ambion, Waltham, MA, USA) at –80°C until processed.

Genomic DNA was isolated from peripheral blood samples using a Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) and ethanol precipitation according to the manufacturer’s protocol.

**CDK2 (*rs2069408*) and CCNE1 (*rs997669*) genotyping with TaqMan assays.** The *CDK2 rs2069408* and *CCNE1 rs997669* polymorphisms were genotyped using the TaqMan SNP Genotyping Assay (Thermo Fisher Scientific, Waltham, MA, USA) in which a fluorogenic probe, consisting of an oligonucleotide labelled with both a fluorescent reporter dye [fluorescein amidite or 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein] and a quencher dye, was included in a typical polymerase chain reaction (PCR). Amplification of the probe-specific product cleaved the probe generating an increase in reporter fluorescence (19). The PCR was performed according to the manufacturer’s instructions (provided by Thermo Fisher Scientific) on a Viiia7 Real-Time PCR System (Thermo Fisher Scientific). The PCR program consisted of the following steps: Pre-read stage for 30s at 60°C, polymerase activation and DNA denaturation for 3 min at 95°C, followed by repetition of 40 amplification cycles for 15 s at 95°C, 1 min at 60°C and a final post-read step for 30 s at 60°C. The 10 µl PCR mixture contained the *CDK2* or *CCNE1* probe, SsoAdvanced Universal Probes Supermix (BioRad Laboratories, Hercules, CA, USA) and 1×TE buffer (AppliChem, Darmstadt, Germany). The genotypes were determined visually based on the fluorescent emission data of the dye component as depicted in the X-Y scatter-plot of the Viiia7 software (Thermo Fischer Scientific).

**p27<sup>KIP1</sup> (*rs2066827*) genotyping.** The polymorphic site of the p27<sup>KIP1</sup> V109G gene was determined by PCR–restriction fragment length polymorphism assays (20). The PCR primers used for amplification of a 454-bp region were: 5'-TGC AGA CCC GGG AGA AAG-3' (forward) and 5'-CTA ACC CCG TCT GGC-3'

(reverse). The PCR program consisted of the following steps: initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 15 s, 60°C for 30 s, 72°C for 1 min and a final 5 min extension step at 72°C. The 454-bp PCR fragment of *p27<sup>KIP1</sup>* was digested with *BglIII* restriction enzyme (10 U, 16 h, 37°C). The resulting fragments were separated on a 3% agarose gel with ethidium bromide. The *T* allele lacks a *BglIII* site that is present in the *G* allele. Therefore, digestion results in fragments of 76 and 377 bp (*T* allele) or 76, 116 and 262 bp (*G* allele). Individuals were classified as *TT*, *TG* or *GG*.

**RNA extraction, cDNA synthesis and quantitative real-time PCR.** Total RNA and cDNA were obtained and processed as described previously (21). A custom RT<sup>2</sup> Profiler PCR array (Qiagen GmbH, Hilden, Germany) was used for quantitative PCR analysis on a Vii7 Real-Time PCR System (Thermo Fisher Scientific) according to the manufacturer's protocol. Data were analysed according to the classic 2- $\Delta\Delta C_t$  method and normalized to the average of glyceraldehyde-3-phosphate dehydrogenase and  $\beta$ -actin expression in each sample (21).

**Western blot analysis.** In brief, 14 prostate cancer tumour tissues were used to analyse the correlation between the *CDK2 rs2069408*, *CCNE1 rs997669* and *p27<sup>KIP1</sup> rs2066827* polymorphisms and protein expression levels. Isolation of proteins from the tumour tissues and determination of protein concentrations were carried out as previously described (21). Protein samples (30  $\mu$ g/ $\mu$ l) were separated by 1D electrophoresis in a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The separated proteins were then transferred onto nitrocellulose membranes by semi-dry transfer. Any binding of non-specific proteins was removed by washing the membranes with 2% bovine serum albumin (Tris solution, 0.05% Tween 20, 10% sodium azide) for 1 h. Subsequently, the membranes were incubated with primary antibodies overnight at 4°C, namely mouse monoclonal anti- $\beta$ -actin (1:1,000, CST-3700S) and anti-*CCNE1* (1:750, CST-4129), rabbit monoclonal anti-*CDK2* (1:500, CST-2546) and rabbit polyclonal anti-*p27* (1:1,000, CST-2552) all from Cell Signaling Technology (Danvers, MA, USA). As a next step, the membranes were washed with 50 mM Tris-Cl, pH 7.5, 150 mM NaCl and 0.05% Tween 20 (TBS-T solution) and incubated with secondary antibodies (1:5,000; Santa Cruz Biotechnology, Dallas, Texas, USA) for 1 h. The membranes were then washed again with TBS-T solution and incubated in Clarity Western ECL Substrate (BioRad Laboratories) for 2 min in the dark. After analysis of the membranes with Chemidoc XRS (BioRad Laboratories), the intensities of the individual bands were quantified using Quantity One software (BioRad Laboratories). The intensities of the spotted bands were normalized to the congruent intensities of  $\beta$ -actin bands.

**Statistical analysis.** Data were analysed with R [1], ver. 4.0.5. The fold change (FC) in expression was computed using the standard formula. Normality of FC values was assessed by the quantile-quantile plot (qqPlot) with the 95% confidence band constructed by bootstrapping. Symbol was used to suggest a transformation to bring FC close to normality. Logarithmic transformation (with base 2) of FC values was used and normality of the log-transformed FC values was assessed by qqPlot. Log-transformed FC data were explored using boxplots, overlaid with swarmplot of the data points. The null hypothesis of the equality of the population means of log-FC values in the two subpopulations was tested by two-sample, two-sided *t*-test. In the genomic association study, the Haldane exact test was used to test the Hardy–Weinberg equilibrium, the chi-squared test

with simulated *p*-values was used in the general, dominant, recessive and multiplicative models, and the Cochran–Armitage test of trend was used in the additive genetic model. The median-unbiased estimate and mid-point exact confidence interval were used to estimate the odds ratio and its 95% confidence interval. Findings with *p*-values below 0.05 were considered statistically significant.

## Results

Firstly, in our case–control study, we investigated the association of the three selected SNPs, namely *CDK2 rs2069408*, *CCNE1 rs997669* and *p27<sup>KIP1</sup> rs2066827*, with prostate cancer risk in 530 patients with prostate cancer and 562 healthy controls. The distribution of genotypes and allelic frequencies of *CDK2 rs2069408*, *CCNE1 rs997669* and *p27<sup>KIP1</sup> rs2066827* polymorphisms among patients and healthy controls were all in accordance with the Hardy–Weinberg equilibrium ( $p > 0.05$ ). The *CDK2 rs2069408* genotype in 0.8% of healthy controls, the *CCNE1 rs997669* genotype in 0.7% of cases and the genotype of *p27<sup>KIP1</sup> rs2066827* in 0.5% of healthy controls and 4% of cases were not determined because of the low concentration of DNA. The genotype-specific odds ratios for individual SNPs in selected regulatory genes of the cell cycle are shown in Table II. Overall, we found no significant association with susceptibility to prostate cancer for *CDK2 rs2069408*, *CCNE1 rs997669* and *p27<sup>KIP1</sup> rs2066827* polymorphisms in codominant, dominant, and recessive genetic models.

Furthermore, we analysed possible associations between *CDK2 rs2069408*, *CCNE1 rs997669* and *p27<sup>KIP1</sup> rs2066827* polymorphisms and clinicopathological characteristics. We categorized the cases into groups according to the PSA level (<10 and  $\geq 10$  ng/ml), Gleason score ( $\leq 7$  and  $> 7$ ) and pathological T-stage (pT1/pT2 and pT3/pT4). As shown in Table III, among those individuals with the *CDK2 rs2069408 GG* genotype and a Gleason score  $> 7$ , the risk of prostate cancer was non-significantly reduced in comparison with individuals with the *AA* genotype and a Gleason score  $\leq 7$  ( $p > 0.05$ ). No significantly increased risk of prostate cancer was seen in association with *CCNE1 rs997669* and *p27<sup>KIP1</sup> rs2066827* mutant genotypes (*CC* and *GG*, respectively) and clinicopathological parameters ( $p > 0.05$ ).

We selected 14 tumour tissues to confirm alterations in the expression of *CDK2*, *CCNE1* and *p27<sup>KIP1</sup>* associated with *CDK2 rs2069408*, *CCNE1 rs997669* and *p27<sup>KIP1</sup> rs2066827* polymorphisms in prostate tissues by western blot analysis (Figure 1). The frequency distribution of *CDK2 rs2069408*, *CCNE1 rs997669* and *p27<sup>KIP1</sup> rs2066827* homozygous, heterozygous, and mutant genotypes was 5, 5, and 4, respectively. We found that *CDK2* protein levels were higher in individuals with the *AG* and *GG* genotypes than in those with the *AA* genotype. In the *TC* and *CC* genotypes for *CCNE1 rs997669*, the protein levels were not significantly different from those of the *TT* genotype. The protein levels

Table II. Association between cyclin-dependent kinase-2 (*CDK2*) *rs2069408*, cyclin *E1* (*CCNE1*) *rs997669* and cyclin-dependent kinase inhibitor 1B (*p27<sup>KIP1</sup>*) *rs2066827* genotypes and prostate cancer risk.

Genotype		Healthy controls, n (%)	Prostate cancer, n (%)	OR (95% CI)	p-Value
<i>CDK2</i> ( <i>rs2069408</i> )					
Codominant model	AA	219 (39.3)	191 (36.0)	1.00 (ref.)	
	AG	245 (44.0)	253 (47.8)	1.18 (0.91-1.54)	0.23
	GG	93 (16.7)	86 (16.2)	1.06 (0.75-1.51)	0.78
Dominant model	AA	219 (39.3)	191 (36.0)	1.00 (ref.)	
	AG+GG	338 (60.7)	339 (64.0)	1.15 (0.89-1.47)	0.28
Recessive model	AA+AG	464 (83.3)	444 (83.8)	1.00 (ref.)	
	GG	93 (16.7)	86 (16.2)	0.97 (0.70-1.33)	0.87
Allele	A	683 (61.3)	635 (59.9)	1.00 (ref.)	
	G	431 (38.7)	425 (40.1)	1.06 (0.89-1.26)	0.51
<i>CCNE1</i> ( <i>rs997669</i> )					
Codominant model	TT	192 (34.2)	201 (38.2)	1.00 (ref.)	
	TC	281 (50.0)	249 (47.3)	0.85 (0.55-1.09)	0.23
	CC	89 (15.8)	76 (14.4)	0.82 (0.56-1.17)	0.31
Dominant model	TT	192 (34.2)	201 (38.2)	1.00 (ref.)	
	TC+CC	370 (65.8)	325 (61.8)	0.84 (0.65-1.07)	0.16
Recessive model	TT+TC	473 (84.2)	450 (85.6)	1.00 (ref.)	
	CC	89 (15.8)	76 (14.4)	0.89 (0.64-1.25)	0.55
Allele	T	665 (59.2)	651 (61.9)	1.00 (ref.)	
	C	459 (40.8)	401 (38.1)	0.89 (0.75-1.06)	0.20
<i>p27<sup>KIP1</sup></i> ( <i>rs2066827</i> )					
Codominant model	TT	350 (62.6)	333 (65.4)	1.00 (ref.)	
	TG	189 (33.8)	158 (31.0)	0.88 (0.68-1.14)	0.35
	GG	20 (3.6)	18 (3.5)	0.99 (0.51-1.90)	>0.99
Dominant model	TT	350 (62.6)	333 (65.4)	1.00 (ref.)	0.37
	TG+GG	209 (37.4)	176 (34.6)	0.88 (0.69-1.14)	
Recessive model	TT+TG	539 (96.4)	491 (96.5)	1.00 (ref.)	
	GG	20 (3.6)	18 (3.5)	0.99 (0.51-1.90)	>0.99
Allele	T	889 (79.5)	824 (80.9)	1.00 (ref.)	
	G	229 (20.5)	194 (19.1)	0.91 (0.74-1.13)	0.42

CI: Confidence interval; OR: odds ratio; ref.: reference.

of the *p27<sup>KIP1</sup> rs2066827 TG* genotype were higher, whereas those for the *GG* genotype were reduced, when compared with those for *TT* genotype.

The expression of *CDK2*, *CCNE1* and *p27<sup>KIP1</sup>* mRNA was investigated by real-time PCR in prostate tumour tissues relative to that in BPH tissues. The relative *CDK2* mRNA expression values were not significantly different in prostate cancer tissues [median  $\log_2(\text{FC})$  value: 0.18;  $p=0.27$ ] from those in the BPH tissues. A similar non-significant difference was observed for *CCNE1* expression [median  $\log_2(\text{FC})$  value: 0.59;  $p=0.09$ ]. The relative *p27<sup>KIP1</sup>* mRNA expression was non-significantly reduced in prostate cancer tissues compared with BPH tissues [median  $\log_2(\text{FC})$  value:  $-0.96$ ;  $p=0.16$ ].

Furthermore, we detect an effect of the *CDK2 rs2069408*, *CCNE1 rs997669* and *p27<sup>KIP1</sup> rs2066827* polymorphisms on the relative *CDK2*, *CCNE1* and *p27<sup>KIP1</sup>* mRNA expression levels (Figure 2). Individuals with the *CDK2 rs2069408 GG* genotype presented a higher relative *CDK2* mRNA expression

than those with *AA* genotype [median  $\log_2(\text{FC})$  values: 1.46 and  $-0.05$ , respectively,  $p=0.27$ ]. A non-significant lower relative expression of *CCNE1* mRNA was observed in the *CC* genotype than in the *TT* genotype [median  $\log_2(\text{FC})$  values: 0.41 and 1.14, respectively,  $p=0.09$ ]. Additionally, no significant difference was seen between the median  $\log_2(\text{FC})$  *p27<sup>KIP1</sup>* values in the individuals who carried the *TG/GG* genotypes and those with the *TT* genotype [ $-0.87$  and  $-1.06$ , respectively,  $p=0.38$ ]. Relative *CDK2*, *CCNE1* and *p27<sup>KIP1</sup>* mRNA expression was also assessed according to clinicopathological features, such as PSA value, Gleason score and pathological T stage. The relative *CCNE1* mRNA expression was statistically significantly elevated in individuals with a Gleason score  $>7$  in comparison with Gleason score  $\leq 7$  [median  $\log_2(\text{FC})$  values: 1.09 and  $-1.47$ , respectively,  $p=0.014$ , Figure 3]. For all other clinicopathological factors, no statistically significant correlation was detected with any of the polymorphisms.



Table III. Risk of prostate cancer associated with the cyclin-dependent kinase-2 (*CDK2*) *rs2069408*, cyclin E1 (*CCNE1*) *rs997669* and cyclin-dependent kinase inhibitor 1B (*p27<sup>KIP1</sup>*) *rs2066827* polymorphisms and clinicopathological characteristics of prostate cancer.

Genotype	PSA <10 vs. ≥10 ng/ml		Gleason score ≤7 vs. >7		pT1/pT2 vs. pT3/pT4	
	OR (95% CI)	<i>p</i> -Value	OR (95% CI)	<i>p</i> -Value	OR (95% CI)	<i>p</i> -Value
<i>CDK2</i> ( <i>rs2069408</i> )						
AA	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
AG	1.27 (0.85-1.92)	0.25	0.84 (0.54-1.33)	0.49	0.72 (0.40-1.28)	0.31
GG	0.95 (0.54-1.70)	0.88	0.68 (0.35-1.28)	0.27	0.91 (0.41-2.00)	0.84
<i>CCNE1</i> ( <i>rs997669</i> )						
TT	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
TC	1.23 (0.81-1.855)	0.35	1.03 (0.66-1.59)	0.91	0.86 (0.48-1.52)	0.15
CC	1.66 (0.94-2.96)	0.08	1.38 (0.76-2.49)	0.28	1.78 (0.81-4.05)	0.17
<i>p27<sup>KIP1</sup></i> ( <i>rs2066827</i> )						
TT	1.00 (ref.)		1.00 (ref.)		1.00 (ref.)	
TG	0.86 (0.56-1.30)	0.52	0.77 (0.48-1.21)	0.31	0.69 (0.37-1.27)	0.28
GG	1.32 (0.47-3.85)	0.62	1.37 (0.38-4.54)	0.76	1.25 (0.33-5.27)	>0.99

CI: Confidence interval; OR: odds ratio; PSA: prostatic-specific antigen; pT: pathological T-stage; ref.: reference.

## Discussion

Dysregulation of the cell cycle and alterations in regulatory proteins of the cell cycle, such as cyclins, CDKs and CDK inhibitors, lead to uncontrolled cell proliferation in many solid tumours. An understanding of the impact of the genetic alterations in these genes should therefore improve our ability to identify the interindividual variation in cancer development and progression. To our knowledge, no investigation has been reported the association of the *CDK2 rs2069408*, *CCNE1 rs997669* and *p27<sup>KIP1</sup> rs2066827* polymorphisms with prostate cancer risk and their influence on corresponding mRNA and protein levels and this led to our decision to assess their functional relevance. Our results show that *CDK2 rs2069408*, *CCNE1 rs997669* and *p27<sup>KIP1</sup> rs2066827* polymorphisms are not associated with an increased risk of prostate cancer development. Moreover, we found higher gene and protein expression of *CDK2* in the presence of the risky genotype (*GG*) of *rs2069408*, no altered *CCNE1* gene and protein expression in carriers of the *CCNE1 rs997669 CC* genotype, and reduced *p27<sup>KIP1</sup>* protein expression in those with *p27<sup>KIP1</sup> G109* allele. In addition, we identified a significant association between the relative expression of *CCNE1* mRNA and the development of higher-grade carcinomas (Gleason score >7).

The *CDK2 rs2069408* and *CCNE1 rs997669* polymorphisms have not yet been studied in prostate cancer. We detected no association between the *CDK2 rs2069408* polymorphism and prostate cancer risk. Several studies have also found no association between this polymorphism and breast and ovarian cancer risk in various populations (22-26).

With regard to *CCNE1 rs997669*, no association with prostate cancer was apparent in our study population. On the contrary, in a case-control study, Driver *et al.* reported an association of *CCNE1 rs997669* with breast cancer in a British population (23). Moreover, genome-wide association studies have identified *CCNE1 rs997669* as being associated with a risk for breast cancer and bladder cancer in Caucasian and Asian populations (27, 28).

The exact mechanism by which the *p27<sup>KIP1</sup> rs2066827* polymorphism affects cancer susceptibility is not well understood. The *V109* allele has been hypothesized to alter the interaction between *p27<sup>KIP1</sup>* and its negative regulator *p38<sup>α</sup>* because it is located on the interaction surface and possibly leads to *p27<sup>KIP1</sup>* degradation and its reduced expression (29). Zhu *et al.*, using Polyphen2 bioinformatics tools, to show that the *p27<sup>KIP1</sup> rs2066827* polymorphism is predicted to be benign and will not affect the protein function of *p27<sup>KIP1</sup>* (30). During the past few years, several studies have focused on the effects of *p27<sup>KIP1</sup> rs2066827* polymorphism on prostate cancer risk within various ethnic populations (13, 17, 31-33). In the literature, both the *T* and *G* alleles of *p27<sup>KIP1</sup> rs2066827* have been shown to be associated with risk of but also protection against malignancy. One case-control study demonstrated that *p27<sup>KIP1</sup> rs2066827* polymorphism is associated with an increased risk of prostate cancer (31), whereas another study and a meta-analysis suggested that this polymorphism is associated with a reduced risk of prostate cancer (13, 17). In addition, other studies have reported no association between the *p27<sup>KIP1</sup> rs2066827* polymorphism and prostate cancer risk (30, 32, 33), which our results are in agreement with.

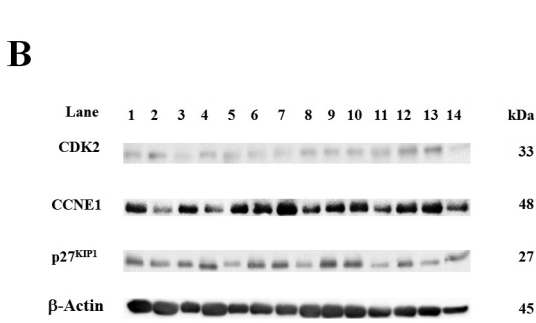
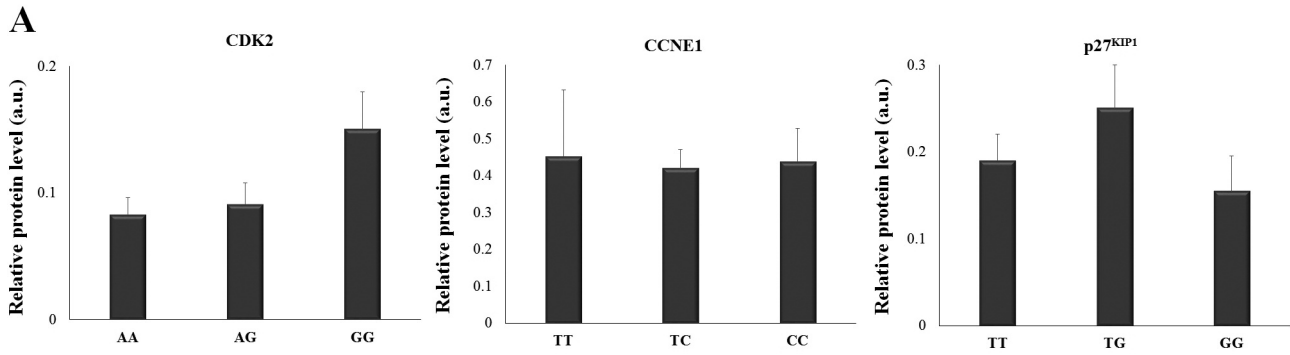


Figure 1. Association between alleles of cyclin-dependent kinase-2 (CDK2) rs2069408, cyclin E1 (CCNE1) rs997669 and cyclin-dependent kinase inhibitor 1B (p27<sup>KIP1</sup>) rs2066827 genes and their protein levels. A: Relative protein expression levels of CDK2, CCNE1 and p27<sup>KIP1</sup> were calculated densitometrically in reference to the expression level of β-actin. Values are given as the means±SEM. B: Western blot analysis of CDK2, CCNE1 and p27<sup>KIP1</sup> protein levels in prostate cancer tissues from individuals with different CDK2 rs2069408 CCNE1 rs997669 and p27<sup>KIP1</sup> rs2066827 genotypes. Individual genotype designations: Lanes 1 to 5, homozygous wild-type genotype (n=5); lanes 5 to 10, heterozygous genotype (n=5); lanes 11 to 14, homozygous mutant genotype (n=4). SEM: standard error of the mean.

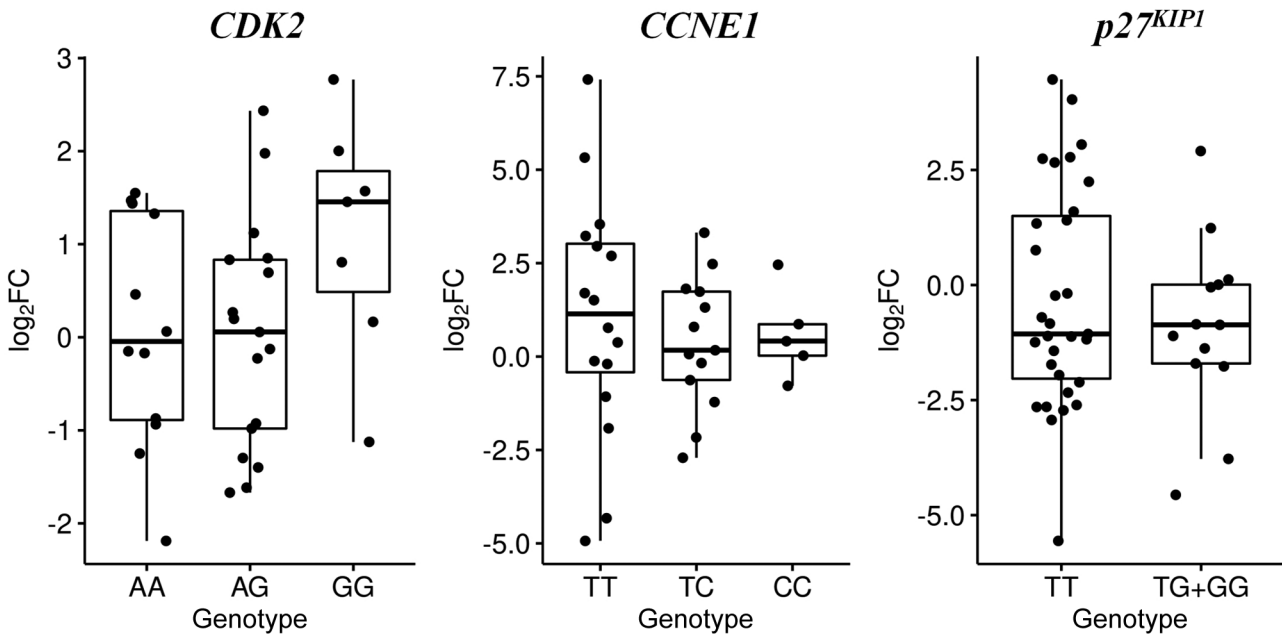


Figure 2. Log<sub>2</sub> fold change (FC) in the relative mRNA expression levels of cyclin-dependent kinase-2 (CDK2) rs2069408, cyclin E1 (CCNE1) rs997669 and cyclin-dependent kinase inhibitor 1B (p27<sup>KIP1</sup>) rs2066827 with respect to their genotypes among patients with prostate cancer. Boxplots depict the sample quartiles as the lower and upper edges of the box, and the sample median as a horizontal line inside the box. Whiskers denote the minimal and maximal values, unless there are outliers in the data.

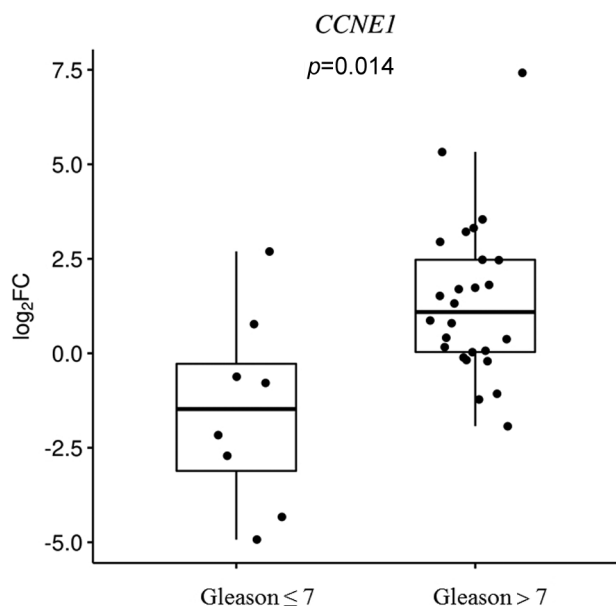


Figure 3.  $\log_2$  fold change (FC) in the relative mRNA expression of cyclin E1 (*CCNE1*) with respect to the Gleason score among patients with prostate cancer. Boxplots depict the sample quartiles as the lower and upper edges of the box, and the sample median as a horizontal line inside the box. Whiskers denote the minimal and maximal values, unless there are outliers in the data. *p*-Value was derived from two-sample, two-sided *t*-test.

Several possible causes can be proposed related to the inconsistency between our study and the other investigations, such as sample sizes and heterogeneous populations/ethnic variance. Moreover, the *p27<sup>KIP1</sup> rs2066827 G* allele might be functionally relevant or in linkage disequilibrium with alleles at other susceptibility loci.

The overexpression of CDK2 should cause abnormal regulation of the cell cycle and should influence human carcinogenesis (16, 34). However, CDK2 activity has been shown to be altered not by up-regulation but by its binding partners (cyclins and CDK inhibitors) or by alterations to post-translational modifications (35). Furthermore, the expression of the major regulator of CDK2 activity, namely cyclin E1, is frequently observed at high levels in solid tumours (36, 37). We have shown no altered relative *CDK2* and *CCNE1* mRNA expression in prostate tumour tissues compared with BPH. However, an increased expression of *CDK2* and *CCNE1* mRNA has been seen in several types of cancer, including primary hepatocellular carcinoma (38), advanced cervical cancer (39), breast (40) and ovarian (41) cancer.

Reduced expression of the CDK inhibitor, *p27<sup>KIP1</sup>*, is correlated with poor patient prognosis (42, 43). The translocation of *p27<sup>KIP1</sup>* to the cytoplasm is hypothesized to result in the loss of its suppressive function in cell-cycle

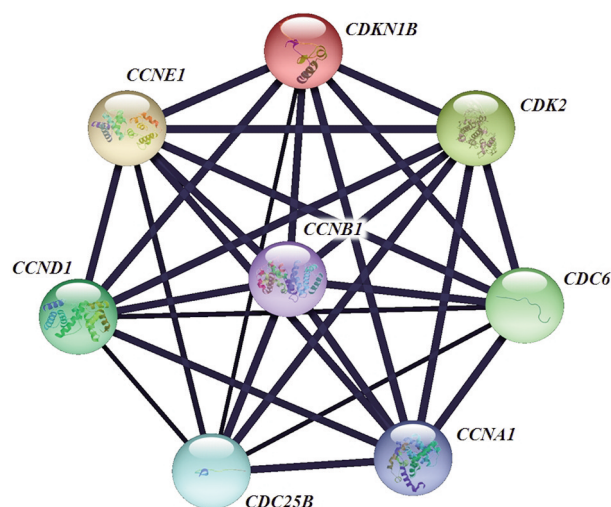


Figure 4. Interactions of human cyclin-dependent kinase 2 (*CDK2*), cyclin E1 (*CCNE1*) and cyclin-dependent kinase inhibitor 1B (*p27<sup>KIP1</sup>*) with other genes generated by String software analysis. *CCNA1*: Cyclin A1; *CCND1*: cyclin D1; *CCNB1*: cyclin B1; *CDC6*: cell-division cycle 6; *CDC25B*: cell-division cycle 25B.

progression (44, 45). However, other studies have been unable to identify a prognostic effect of reduced *p27<sup>KIP1</sup>* expression (46, 47). Zhu *et al.* used *in-silico* tools to show the expression of *p27<sup>KIP1</sup>* to be reduced in prostate cancer tissue compared with paracancerous counterparts, especially in less advanced prostate cancer (Gleason score = 6 or 7), but not in more advanced prostate cancer (30). Our results revealed a similar trend for *p27<sup>KIP1</sup>* mRNA levels in prostate tumour tissues when compared with BPH tissues. The differential expression of *p27<sup>KIP1</sup>* mRNA has been also reported in various types of cancer (45, 48, 49).

Common SNPs might affect the normal function of proteins that play key roles in the  $G_1/S$  transition by altering their transcriptional efficiency and protein levels, leading to cell-cycle dysregulation and cancer cell behaviour (29). In the present study, we evaluated whether *CDK2*, *CCNE1* and *p27<sup>KIP1</sup>* mRNA and protein expression in prostate tumours is influenced by the *CDK2 rs2069408*, *CCNE1 rs997669* and *p27<sup>KIP1</sup> rs2066827* polymorphisms. To the best of our knowledge, we report the first analysis of this association in prostate cancer. We found a higher expression of *CDK2* in the presence of the risky genotype (*GG*) of *rs2069408* at both the mRNA and protein levels. We detected no significant difference in *CCNE1* expression in carriers of the *CCNE1 rs997669 CC* genotype. Furthermore, our data suggest that the *p27<sup>KIP1</sup> rs2066827 G* allele is associated with reduced *p27<sup>KIP1</sup>* protein expression and no alterations in mRNA level. Han *et al.* observed that LNCap cells

transfected with the *p27<sup>KIP1</sup>* G allele exhibited a lower proliferative activity and expression level of p27<sup>KIP1</sup> protein than cells transfected with the V allele (17).

We observed no significantly increased risk of prostate cancer for *CDK2 rs2069408* and *CCNE1 rs997669* mutant genotypes by all clinicopathological parameters. Furthermore, our data do not suggest that the *p27<sup>KIP1</sup> rs2066827* polymorphism is associated with PSA level, Gleason score or pathological T stage, thus confirming the overall null association with the clinicopathological characteristics of prostate cancer, as reported previously (32, 33). Interestingly, we did find that *CCNE1* mRNA expression was significantly increased in carcinomas with Gleason scores higher than 7.

Moreover, we hypothesized that molecular mechanisms other than those of SNPs can affect alterations of regulatory proteins of the cell cycle in a cell-type and condition-specific manner such as by: a) Regulation of gene expression by transcription factor [*e.g.* FORKHEAD box transcription factor O1 (50)] or by microRNA (48); b) regulation of protein content by the control of proteolysis or mRNA translation (12); c) change in the cellular localization of cell-cycle regulators (51); and d) regulation of their expression by hormones [androgens and oestrogens (52)]. Because tumorigenesis and tumour progression are processes involving multiple genetic defects, other important genes that are involved in the *CDK2–CCNE1–p27<sup>KIP1</sup>* gene–gene interaction might not have been efficiently tagged and might play important roles in prostate carcinogenesis (Figure 4).

In summary, we observed no statistically significant overall associations of *CDK2 rs2069408*, *CCNE1 rs997669* and *p27<sup>KIP1</sup> rs2066827* polymorphisms with prostate cancer risk and no significant effects of these polymorphisms on the gene and protein expression of CDK2, CCNE1 and p27<sup>KIP1</sup>. Significant interaction was found between the relative *CCNE1* mRNA level and higher-grade carcinoma. Although the present study provides important insights into the role of genetic alterations in the regulators of the cell cycle in the development of prostate cancer, the strengths and limitations of our study should be mentioned. The major strength of our study is its large ethnically homogeneous sample size, which reduces allelic and genotypic heterogeneity found in case–control studies. We were however limited by the tissue samples available. Because this was a preliminary study, a more detailed understanding of the importance of polymorphisms in the regulation of the cell cycle and of gene–gene interactions can only be achieved by further studies.

### Conflicts of Interest

The Authors declare no conflicts of interest.

### Authors' Contributions

MKS, MH, JJ and JK conceived the idea for this study and wrote the article. MH, JJ, MV, and KHH contributed to the design of the research. RD and DE were involved in the data collection. PK and MG analysed the data. All Authors edited and approved the final version of the article.

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