PER3 polymorphisms and their association with prostate cancer risk in Japanese men

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Introduction
Prostate cancer (PCa) is one of the most common cancers affecting men globally. Although PER3 has been suggested as a risk factor for cancer development, there are few reports elucidating the relationship between PER3 and PCa. We investigated the association between PER3 polymorphisms (rs2640908 and VNTR) and susceptibility to PCa in the Japanese population.

Methods
Eighty three patients with PCa and 122 controls participated in this study. We analyzed rs2640908 and VNTR polymorphisms by using PCR-Restriction Fragment Length Polymorphism (PCR-RFLP).

Results
Some previous research indicated the association between various circadian genes and PCa. Wendu-Foyet et al. reported that pathway of 31 circadian genes including 872 SNPs significantly associated with PCa and this association was mainly supported by the circadian core-genes pathway which were NPAS2 and PER1 [12]. Yu et al. also indicated that NPAS2 as a part of circadian genes significantly associated with disease progression of PCa [13]. Other article showed that some circadian genes (PER1, PER2, CRY1, CRY2, CLOCK, NPAS2) were significantly associated with susceptibility to prostate cancer [14]. On the other hand, circadian genes could play a part of role of carcinogenesis or the tumor suppressor through cell proliferation and apoptosis [15]. These findings indicated there was a potential link between genetic variants in circadian genes and PCa. PER is one of the main mediators of the circadian rhythm, and has a function in the negative feedback loop where it is translocated from the cytoplasm to the nucleus to regulate their expression by an inhibit transcription [16, 17]. PER has three paralogs, PER1, PER2, and PER3. Disruption of the circadian genes, including that of PER3, affects carcinogenesis related cellular processes, including proliferation, cell cycle regulation, and apoptosis [18]. PER3 expression was decreased in chronic myeloid leukemia [19], and expression of PER3 in colorectal cancer tissue was lower than in healthy mucosa [18, 20]. Polymorphisms of PER3 have also been associated with various cancers [14, 21, 22].

The rs2640908 polymorphism is a PER3 single nucleotide polymorphism (SNP) associated with cancer
development [23-25]. The rs2640908 polymorphism has an association with patient overall survival in hepatocellular carcinoma and colorectal carcinoma. Another polymorphism of PER3 is a variable number tandem repeat (VNTR) consisting of 4-5 repeat 54-bp sequences in exon 18 encoding 18 amino acids [26]. Individuals with a variant of 5 VNTR repeats experience delayed sleep phase syndrome and extreme diurnal preference [27, 28]. A relationship between VNTR polymorphism and elevated levels of serum cytokine IL-6 has also been reported [29]. This suggested that PER3 polymorphisms could have an influence on the carcinogenesis through cell cycle regulation. However, the relationship between both rs2640908 and VNTR polymorphisms and PCa carcinogenesis within the Japanese population is yet to be studied in detail. Thus, the aim of this study was to evaluate the relationship between rs2640908 and VNTR polymorphisms and PCa carcinogenesis within the Japanese population.

**Methods**

**Study subjects**
A total of 83 patients with PCa and 122 healthy controls were recruited from the Japanese population. Patients were diagnosed at the University of Occupational and Environmental Health (UOEH) Hospital and University of Miyazaki Hospital in Japan, and all diagnoses were confirmed by histology. The control subjects were recruited from UOEH Hospital, University of Miyazaki Hospital, and a hospital located near UOEH Hospital. All subjects were surveyed with self–questionnaires that collected information on history of illness, occupation, and smoking status. Participants who had been exposed to carcinogenic agents, heavy metals, and radiation in their occupational history were excluded. The included subjects were classified into two groups according to smoking status. The non-smokers were grouped into the “Never” group, and both current and previous smokers were classified as “Smoker”. All participants were briefed about the study, and written informed consent was obtained from each participant. This study was approved by the Ethics Committee of the Faculty of Medicine, University of Miyazaki.

**Genotyping**
Peripheral blood samples were collected from each subject, and genomic DNA was extracted by proteinase K digestion and phenol/chloroform extraction method [30]. Genotyping of the rs2640908 polymorphism was carried out using PCR-Restriction Fragment Length Polymorphism (PCR-RFLP). The sequence of PCR primers used for amplification were 5'-CTGTTTTAACACAGAAGTTGAAAGA-3' (forward) and 5'-GTCTGGATGGGATTGCCTGAC-3' (reverse). PCR was performed using a KAPATaq EXtra PCR Kit (NIPPON Genetics Co., Ltd., Tokyo, Japan) following manufacturer’s instructions. The thermocycler conditions were: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s, with final extension at 72°C for 7 min. PCR products were digested with AgeI restriction enzyme (New England Biolabs Inc., Ipswich, MA, USA) and incubated at 37°C overnight, before being resolved by 2% agarose gel electrophoresis. For genotyping, two digestion products of size 798 and 265 bp were expected for C/T genotype (homozygous wild-type), three products of size 1,063, 798, and 265 bp were expected for C/T genotype, and only a single product sized 1,063 bp was expected for T/T genotype. The electrophoretic image of each genotypes is shown in Figure 1. PCR genotyping for VNTR polymorphisms was similarly performed with primer sequences:

5’-CAAAAATTTATGACACTACAGAATGGCTGAC–3’ (forward) and 5’–AACCCTTGTACTTCCACATCAGTGCTCAG–3’ (reverse).

The thermocycler conditions were: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s with at final extension at 72°C for 7 min. A 4/4 genotype (homozygous for 4 repeats) had an expected product size of 581 bp, 4/5 (heterozygotes) had expected product sizes of 635 and 581bp, 5/5 (homozygous for 5 repeats) had an expected product size of 635 bp (Fig. 1). PCR products were resolved and visualized using 2% agarose gel electrophoresis.

**Statistical analysis**
A t-test was used to compare the continuous variables, and a χ² test was used to compare categorical data and to determine probability of allele frequencies being in Hardy–Weinberg equilibrium [31]. Odds ratio (OR) and 95% confidence interval (95% CI) were estimated using a multiple logistic regression analysis with adjustment for age and smoking status. Stratified analysis by smoking status was also performed. The level of statistical significance was set at P value < 0.05. All statistical analyses were performed using R ver. 3.6.1.

**Results**
Clinical characteristics of patient and control subjects, including age and smoking status, are summarized in Table I. The mean age was 69.2 years (SD: 10.4) for control group and 71.6 years (SD: 8.6) for the patient group. Incidentally, a significantly higher percentage of the control group (83.6%) was classified as “Smoker”. Participants who had been exposed to carcinogenic agents, heavy metals, and radiation in their occupational history were excluded. The included subjects were classified into two groups according to smoking status. The non-smokers were grouped into the “Never” group, and both current and previous smokers were classified as “Smoker”. All participants were briefed about the study, and written informed consent was obtained from each participant. This study was approved by the Ethics Committee of the Faculty of Medicine, University of Miyazaki.

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genetic variants, risk for PCa was significantly lower for T/T genotype (adjusted OR: 0.35, 95% CI: 0.15-0.81, P = 0.02), and C/T + T/T genotype (adjusted OR: 0.46, 95% CI: 0.24-0.88, P = 0.02) relative to the C/C genotype, with no significant difference observed for C/T genotype (adjusted OR: 0.52, 95% CI: 0.26-1.03, P = 0.06). In contrast, no significant association was observed between any of the VNTR variants and PCa.

Table III shows the analysis stratified by smoking status. For rs2640908 polymorphisms in the “Smoker” group, there was a significantly lower risk of PCa in T/T genotype (adjusted OR: 0.29, 95% CI: 0.10-0.77, P = 0.02) and C/T + T/T (adjusted OR: 0.47, 95%
This would affect cell proliferation, cell cycle regulation, and apoptosis, and apolipoprotein, and cell proliferation and apoptosis [37]. Deletion and reduced expression of PER3 results in upregulation of the estrogen receptors, and is associated with an increased recurrence of breast cancer [38], suggesting that the PER3 may also play a role in homeostasis for reproductive hormones.

There are two polymorphisms described for PER3, rs2640908 and VNTR. At least one variant allele of the rs2640908 polymorphism is associated with a significantly lower risk of death among patients with hepatocellular carcinoma when compared to homozygous wild-type patients [24]. The T/T genotype of the rs2640908 polymorphism also has a protective effect in colorectal cancer compared to the C/C genotype [25]. We found a similar effect for PCa. However, we were unable to find a significant association between VNTR variants and PCa.

Disruption of the circadian rhythm is linked to an increased risk of several diseases, including cancer [18-20, 32]. Disruption can arise from different factors, such as shift work and exposure to light at night (LAN exposure) [33]. Shift work influences body temperature, energy allocation, and disrupts circadian rhythm, resulting in damage to health. The circadian rhythm is a physiological fluctuation that takes place in approximately 24-hour cycles, and is involved in the health and survival of most living organisms. Some reports have described auto-regulatory transcriptional and translational feedback loop mechanisms for circadian genes, with both positive and negative regulators [34]. Circadian genes have also been linked to carcinogenesis through DNA repair, apoptosis, and cell proliferation [15, 35, 36]. PER is a circadian gene, with 3 subtypes (PER1, PER2, and PER3) [14]. A previous study has suggested that heterozygosity of a PER3 polymorphism could be a risk factor for breast cancer among premenopausal women [26]. PER3 also plays an important role in regulating cell proliferation and apoptosis [37]. Deletion and reduced expression of PER3 results in upregulation of the estrogen receptors, and is associated with an increased recurrence of breast cancer [38], suggesting that the PER3 may also play a role in homeostasis for reproductive hormones.

In our study, no correlation was observed between VNTR polymorphisms and PCa. Varying relationships between VNTR polymorphism and carcinogenesis have been reported previously. The VNTR variant with 5 repeats is associated with increased risk of prostate cancer in men with high levels of insulin resistance [41] and with colorectal adenoma [21]. In contrast, a meta-analysis could not identify any significant relationships between VNTR polymorphisms and breast, prostate, and colon cancers [42]. The 5 repeats sequence of the VNTR polymorphism play an essential role as it is a phosphorylation site, and the 5/5 genotype plays a crucial role in the circadian process compared to the 4/4 genotype [43]. Heritable chronotypes may be polygenic, and variants of several genes may be required for full phenotypic expression [43]. That may explain why no association was observed between VNTR polymorphism and PCa in this study.

There relation between circadian genes and PCa was inconsistent. Mark et al. did not find the consistent association between 96 SNPs across 12 circadian-related genes and fatal prostate cancer risk using three patient cohorts [44]. On the other hand, the study on EPICAP study showed the evidence supporting hypothesis of a link between circadian genes and PCa [12]. Since circadian rhythms were produced by multiple molecular interactions of protein, and PCa was a complex polygenic trait, a single-SNP approach may not be sufficient to investigate the association between circadian genes and PCa [12]. Therefore, further investigation will be need to evaluate the relation between circadian genes and carcinogenesis of PCa with more samples and various genes including PER3 gene, especially for Japanese population.

We also evaluated the association between rs2640908 polymorphisms and susceptibility to PCa in smokers. We found that the T/T and C/T + T/T genotypes conferred lower risk of PCa compared to the C/C genotype within the “Smoker” group, but not the “Never” group. This result suggests that there is an association between smoking and PER3 polymorphisms, as previously observed in colon cancer [25]. Cigarette exposure changes DNA binding by modulating the redox potential of cells and tissues [45], and therefore affects transcriptional activity. Binding of transcriptional circadian genes, including BMAL1, CLOCK, and NPAS2, is dependent on the redox ratio [46]. Circadian genes and smoking may therefore interact in a synergistic manner. However, the possibility of association between gene polymorphisms and smoking remains controversial. Jin et al has advocated that genetic differences in risk tend to be smaller at high doses of carcinogens, including tobacco, when the environmental effect may overpower any genetic predisposition [47]. However, this was disputed by Kuroda et al, who reported that the Pro/Pro genotype of a TP53 polymorphism in smokers was significantly higher in patients with urothelial cancer compared with that in the control [48]. Polymorphisms for the metabolic genes GSTT1, GSTM1, and CYP1A1 are not associated with the smoking status in onco-hematological diseases [49].
Considering these contradictory findings, further studies are warranted to clearly elucidate the relationships between gene polymorphisms and smoking.

There were some limitations to this study. First, our small sample may have induced a sampling bias and affected the results of the stratified analysis. Secondly, we had no information regarding the history of shift work, alcohol consumption, BMI, sleep time, clinical characteristics, smoking period and number of cigarettes. The lack of these data also induced information bias, and introduced the influence to our results. Especially, since circadian genes polymorphism could influence sleep condition and shift work tolerance [50], the interaction with circadian genes and sleep condition (sleep duration and sleep quality) could influence the carcinogenesis of PCa.

Therefore, additional study will be needed to evaluate the relation between PER3 gene polymorphisms and carcinogenesis of PCa with considering sleep condition. We could not use smoking condition (smoking period and number of cigarettes), but used the status of smoking. We found that the rate of smoking was higher in the control than in the patient group. We estimated the interaction with smoking and PER3 polymorphism (rs2640908) by multiple logistic regression analysis. Therefore, smoking condition (smoking period and number of cigarettes) could be important factor. It would be necessary to evaluate the relation between smoking condition and PER3 polymorphism stratified by smoking condition. Despite these limitations, we believe our findings provide a basis for future studies investigating the association between prostate cancer and circadian gene polymorphisms.

Conclusions

In conclusion, this is the first study that focused on associations between PER3 polymorphisms (rs2640908 and VNTR) and PCa risk. For rs2640908, the T/T and C/T + T/T genotypes had a significant protective effect against PCa in the Japanese population. However, no relationship between VNTR polymorphisms and PCa could be detected. Our finding suggests that the rs2640908 polymorphism may be a useful marker for prostate cancer and contributes to further understanding of the molecular mechanisms underlying PCa pathogenesis.

List of abbreviations

PCa: Prostate cancer.
SNP: Single Nucleotide Polymorphism.
VNTR: Variable Number Tandem Repeat.
PCR: Polymerase Chain Reaction.
PCR-RFLP: Polymerase Chain Reaction - Restriction Fragment Length Polymorphism.
CLOCK: Circadian Locomotor Output Cycles Kaput Gene.
PER: Period Gene.
CRY: Cryptochrome Gene.
PER1: Period 1.
PER2: Period 2.
PER3: Period 3.
IL-6: Interleukin-6.
UOEH: University of Occupational and Environmental Health.
AgeI: Restriction Enzyme.
χ² test: Chi-square test.
OR: Odds Ratio.
95% CI: 95% Confidence Interval.
SD: Standard Deviation.
LAN exposure: Light at Night Exposure.
IARC: The Agency for Research on Cancer.
DNA: Deoxyribonucleic Acid.
ESE: Exonic Splicing Enhancer.
mRNA: Messenger Ribonucleic Acid.
SR: Serine/Arginine-Rich.
BMAL1: Brain and Muscle Arnt - Like 1.
NPAS2: Neuronal PAS domain 2.
TP53: Tumor Protein 53.
GSTM1: Glutathione S - transferase Mu 1.
CYP1A1: Cytochrome P450 Family 1 Subfamily A Member 1.

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Conflict of interest statement

The authors declare no conflict of interest.

Authors’ contributions

TH contributed to experimental work and designed the experiments and analyzed the results. SM and TK contributed in the data collection. YK contributed on interpreted, reviewed the experiment design, and critically reviewed the manuscript. All authors reviewed the manuscript and approved the final draft.

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