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RESEARCH NOTE

Study on the *SFRP4* gene polymorphism and expression in prostate cancer

AISHWARYA NATARAJAN, SHAILE SUDHA BANDLA, MOHAN DAMODARAN, SANDHYA SUNDARAM and VETTRISELVI VENKATESAN*

Sri Ramachandra Institute of Higher Education and Research, Porur, Chennai 600 116, India
*For correspondence. E-mail: vettriselviv@sriramachandra.edu.in.

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Abstract. Prostate cancer is a heterogeneous disease and considered to be the most commonly diagnosed cancer. *SFRP4* gene acts as Wnt antagonist in the Wnt signalling pathway, thereby playing an important role in carcinogenesis. The aim of the present study was to investigate two single-nucleotide polymorphisms: c.958 C>A (rs1802073) and c.1019 G>A (rs1802074) in the *SFRP4* gene and its expression in prostate cancer. A sample size of 100 cases and 100 age-matched controls were recruited for the study. Statistical analysis revealed the heterozygous GA genotype of rs1802074 significantly increased in cases when compared to controls. Analysis of sFRP4 expression based on the genotypes showed a significantly increased expression for the heterozygous GA and homozygous AA genotypes in cases when compared to the controls. Fold change was calculated using $2^{-\Delta\Delta CT}$ method and the results showed that there were a 3.4 and 4.5 fold increase in the sFRP4 expression for GA and AA genotypes, respectively. Our results suggest that the rs1802074 polymorphism in *SFRP4* gene may be associated with the risk of prostate cancer.

Keywords. secreted frizzled related protein 4; prostate cancer; genotyping; haplotypes.

Introduction

Prostate cancer, referred to as malignancy of elderly people, is the second most common type of cancer among men and ranks fifth in cancer mortality rate worldwide (Banerjee and Kaviani 2016). The aetiology and pathological mechanism of prostate cancer remains unclear. Many signalling pathways are involved in the growth and progression of prostate cancer, of which the Wnt signalling pathway plays a pivotal role.

WNTs are a family of 19 secreted glycoproteins that are of prime importance in both the developing foetus and adults (Anastas and Moon 2013). Wnt signalling pathway plays a very crucial role in various biological processes including embryonic development, cell proliferation, cell fate specification and tissue homeostasis (Shi *et al*. 2007). There is abnormal activation of the Wnt signalling pathway in prostate cancer, and hence, the expression of Wnt ligands is altered (Kypta and Waxman 2012).

Secreted frizzled related proteins (sFRPs), containing five family members (1–5), are secreted glycoproteins that are commonly referred to as 'Wnt antagonists' (Surana *et al*. 2014). Studies have revealed that the sFRPs are transcriptionally inactivated in many tumours and possibly acts as a tumour suppressor gene (Rubin *et al*. 2006). The *SFRP4* gene located on chromosome 7 (7p14.1) is considered to be the largest member of the family and is shown to inhibit the canonical Wnt signalling pathway (Pohl *et al*. 2015).

SFRP4 gene has been implicated to play a major role in a variety of carcinomas. Studies have reported the downregulation and promoter hyper methylation of *SFRP4* gene in endometrial carcinomas, indicating its tumour suppressor role (Hrzenjak *et al*. 2004). High levels of *SFRP4* gene expression was detected in ovarian tumours (Drake *et al*. 2009). Another study reported increased levels of promoter methylation and decreased expression of *SFRP4* gene in bladder tumour samples (Urakami *et al*. 2006).

Previous literature clearly suggests that *SFRP4* gene plays a vital role in carcinogenesis. Although many studies have reported, the role of *SFRP4* gene polymorphisms and expression in various cancers, only limited literature exists regarding prostate cancer. Therefore, the present study was designed to investigate the association of two single-nucleotide polymorphisms (SNPs) in the

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SFRP4 gene and its expression with prostate cancer in the Indian population.

Materials and methods

Study population

This study comprised of histologically confirmed prostate cancer cases (n = 100) and controls (n = 100). The samples were collected from Sri Ramachandra Medical Centre, Chennai. The age of the patients ranged from 56 to 80 years and the mean age was 68 years. Relevant clinical and pathological data were collected from all the patients. The prostate specific antigen levels of the patients at the time of diagnosis were recorded and those ranging from 4 ng/mL were considered for the study. Gleason scores (GS) representing the pathological tumour grade was obtained by histopathological examination. Patients with development of secondary tumour were excluded from the study. The age of the controls ranged from 50 to 80 years with a mean age of 67 years and had no history of cancer. Ethical clearance was obtained from the Institutional Ethics Committee of Sri Ramachandra Medical College and Research Institute. Informed consent was obtained from each subject at the time of enrolment.

Genotyping

About 2 mL of peripheral blood was collected from the subjects in an EDTA vacutainer. High molecular weight genomic DNA was extracted from the blood samples by salting out method and was stored at –20°C until further processing. In this study two sets of primers were used for each SNP and the optimum annealing temperature for the primers were standardized by performing Gradient PCR. A nested PCR was carried out in a total reaction volume of 20 μL containing 50 ng of genomic DNA, 25 pm of each primer (forward and reverse), 1x Taq buffer, 200 μM dNTP and 3 U of *Taq* DNA polymerase. The PCR cycling conditions were carried out as follows: 94°C for 5 min, 94°C for 1 min, 64°C for 1 min, 72°C for 1 min; 30 cycles and final extension 72°C for 5 min. Genotyping of SFRP4 c.958 C>A (rs1802073) and c.1019 G>A (rs1802074) polymorphisms were performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using the restriction enzymes *Hin*fI and *Ear*I respectively (Bahl *et al*. 2017). Representative samples from each genotype were subjected to sequencing to confirm the genotypes.

Gene expression

Prostate biopsy samples were collected from the subjects in sterile containers and RNA was isolated from the tissue samples by TRIzol method (Invitrogen). cDNA was synthesized from RNA samples using High-capacity cDNA reverse transcription kit (Thermo Fisher Scientific), according to the manufacturer's protocol. Quantification of SFRP4 was performed by real-time PCR using SYBR green chemistry (Sigma-Aldrich, USA) (Hirata *et al*. 2009).

Statistical analysis

Statistical analysis for polymorphisms was performed using the SNP stats online tool (Sole *et al*. 2006). The expected genotype and allele frequencies were calculated for the cases and controls. These frequencies were used to test for Hardy–Weinberg equilibrium. The association of the SNPs in *SFRP4* gene with prostate cancer was evaluated using odds ratio (OR) at 95% confidence interval (CI). Relative quantification of the *SFRP4* gene was calculated using $2^{-\Delta\Delta CT}$ method. Student t-test was used to calculate the level of significance for the fold change. Association between the clinical parameters and the genotypes of the polymorphisms among prostate cancer patients was estimated by one-way ANOVA method. P values < 0.05 were considered to be statistically significant for all the analysis.

Result

Genotyping

The genotype and allele frequencies were calculated for both the cases and controls. The association of *SFRP4* gene polymorphisms with prostate cancer is represented in table 1. The heterozygous GA genotype of rs1802074 showed a significant increase in the cases compared to the controls (OR = 4.708, 95%CI = 2.04–11.03, and P = 0.0005). Similar result was observed in the dominant model with OR = 4.6, 95%CI = 2.05–11.47 and P = 0.0001. This indicates that the heterozygous GA genotype is associated with the risk of prostate cancer. However, the rs1802073 gene did not show any association with prostate cancer. Haplotype analysis of *SFRP4* gene polymorphisms in cases and controls revealed a total of four haplotype combinations, among which the haplotypes CA (OR = 2.62, CI = 1.34–5.13, and P = 0.005) and AG (OR = 2.42, CI = 1.11–5.24, and P = 0.02) significantly increased in the cases when compared to the controls. The allele frequency observed in the present study was compared with the frequencies available in the HapMap data. Our result showed similar allele frequency with African population.

The genotypes of the *SFRP4* polymorphisms were compared with the clinical parameters including Gleason score, age at diagnosis. For Gleason score, the patients were grouped into two categories (low grade < 7 (less aggressive) and high grade ≥ 7 (more aggressive)). Genotype frequency distribution based on the tumour grade revealed no

Table 1. Association analysis of *sFRP4* gene polymorphisms with prostate cancer.

Genotype	Controls (n = 100)	Cases (n = 100)	OR (95% CI)	P value
rs1802073				
CC	54 (54%)	47 (47%)	Ref	–
CA	37 (37%)	47 (47%)	1.46 (0.82–2.61)	0.10
AA	9 (9%)	6 (6%)	0.77 (0.25–2.31)	0.32
CA + AA	46 (46%)	53 (53%)	1.32 (0.76–2.31)	0.16
CC + CA	91	94	Ref	
AA	9	6	0.6468 (0.21–2.31)	0.22
C allele frequency	0.72	0.70		
A allele frequency	0.28	0.30		
rs1802074				
GG	29 (29%)	8 (8%)	Ref	–
GA	68 (68%)	89 (89%)	4.708 (2.04–11.03)	0.00005
AA	3 (3%)	3 (3%)	3.495 (0.51–24.04)	0.33
GA + AA	71 (71%)	92 (92%)	4.6 (2.05–11.47)	0.0001
GG + GA	97	97	Ref	
AA	3	3	1.26 (0.2473–6.416)	0.39
G allele frequency	0.63	0.52		
A allele frequency	0.37	0.48		

Ref, reference genotype; NS, not significant.

significant difference for age and Gleason score for both the polymorphisms.

SFRP4 gene expression analysis

Data normalization was done using GAPDH as an internal control. Relative quantification of *SFRP4* expression showed no significant difference in the expression between the cases and controls. To determine the effect of the SNPs on the sFRP4 expression, the average delta *CT* values for each genotype were obtained and compared between the cases and controls (figure 1). Comparison of genotypes with expression revealed that the heterozygous GA and homozygous AA genotypes of rs1802074 showed an increased expression when compared to the controls. The student *t*-test showed that the upregulation of sFRP4 for GA (P = 0.04) and AA (P = 0.01) genotypes was statistically significant. Fold change was calculated to assess the quantity of the difference in expression among cases when compared to controls, results showed that there were a 3.4 and 4.5 fold increases in the sFRP4 expression for the GA and AA genotypes, respectively. No significant association was observed for the rs1802073 polymorphism.

Predicting the effect of polymorphisms on sFRP4 protein structure and function

The possible impact of the *sFRP4* gene polymorphism at the protein level was predicted through polymorphism phenotyping v2 (PolyPhen2). PolyPhen-2 is a tool available at http://genetics.bwh.harvard.edu/pph2/ (Adzhubei *et al.* 2013) and is used to predict the possible influence of the amino acid change on the structure and function of a human protein. The software prediction report is based on features of the sequence, phylogenetic and structural information characterizing the substitution. The PolyPhen-2 score ranges from 0.0 (tolerated) to 1.0 (deleterious). Variants with scores of 0.0 are predicted to be benign. Values closer to 1.0 are more confidently predicted to be deleterious. When we used this program, sFRP4 Arg340Lys (rs1802074) and Pro320Thr (rs1802073) gave a PolyPhen score of 0.00 which is judged to be benign.

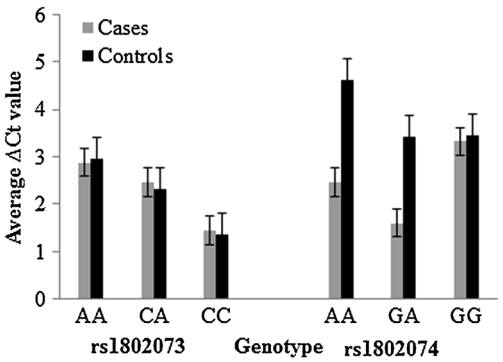

Figure 1. Expression of *sFRP4* based on genotypes.

Discussion

The increasing incidence of prostate cancer is one of the most serious oncological diseases among men. Because of the heterogeneous nature, mechanisms underlying the

disease onset and progression are not clear. This highlights the need to identify potential genetic markers for diagnosis and prognosis. Wnt signalling pathway is implicated in many biological processes in the developing embryo as well as adults. Altered expression of the Wnt proteins such as sFRP4 is implicated in various cancers including prostate cancer. The present study aimed to analyse the association of the two SNPs in the *SFRP4* gene: c.958 C>A (rs1802073) and c.1019 G>A (rs1802074) and its expression in prostate cancer. rs1802073 did not reveal any significant association between the cases and controls. For the second polymorphism (rs1802074), there was an increase in the number of heterozygous GA genotype (OR = 4.74, CI = 2.04–11.03) and dominant model (OR = 4.70, CI = 2.02–10.90) in the cases compared to the control group, indicating that the polymorphism is associated with prostate cancer. Previous studies have reported the association of rs1802073 and rs1802074 polymorphisms with renal cancer (Hirata *et al*. 2009) and lung cancer (Bahl *et al*. 2017). To the best of our knowledge, this is the first study to report the association of rs1802074 polymorphism with prostate cancer.

Expression analysis of the *sFRP* gene between the cases and controls showed no significant difference. However, the correlation analysis comparing the genotypes and expression between the cases and controls revealed a significant increase in the GA and AA genotypes of rs1802074 indicating that the genotype is associated with increased expression in cases. A study conducted on the Australian population revealed significantly higher *SFRP4* gene expression in tumour samples as compared to the normal samples (Horvath *et al.* 2007).

Wissmann *et al*. (2003) reported that the *SFRP4* gene was expressed in 69% of the patients and upregulated in 81% of the *SFRP4* expressing tumours. Another study by Horvath *et al.* (2004) revealed that loss of expression of the *SFRP4* gene is considered as a bad prognosis and may be associated with disease progression. A study conducted on the Norwegian population by Sandsmark *et al.* (2017) reported significant upregulation of the *SFRP4* gene in high grade tumour groups compared to low grade groups. However, our results did not show a significant association with tumour grade. Currently, there are no reports available on detailed molecular mechanisms involved in how these polymorphisms have an effect on sFRP4 protein. To understand the impact of this polymorphism, we performed the PolyPhen-2 analysis. Our results showed that, both the polymorphisms analysed is benign and does not cause any damaging effect in the protein structure. However, this data needs further validation in the experimental studies. Further functional studies on the role of *SFRP4* gene variation and expression will help in unveiling the molecular mechanism underlying the development and progression of prostate cancer.

To conclude, this is the first report documenting the association of rs1802074 polymorphism of the *sFRP4* gene with prostate cancer. Our results also demonstrated the association of this polymorphism with the upregulated expression of *sFRP4* in prostate cancer samples compared to the controls. Further studies are needed to prospectively examine the functional significance of this polymorphism.

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