Original Research Article

Association of the +936 C/T Single Nucleotide Polymorphism of the VEGF-A gene with renal cell cancer in an Eastern Indian population

ABSTRACT:

Aims: Renal cell cancer is one of the major killer cancers affecting mankind. Various polymorphisms in different genes have been found to be associated with the disease. +936 C/T SNP in the VEGF gene has been reported to be associated with spread of metastasis in several parts of the world. In the present study, we decided to study its association with renal cell cancer in the Eastern Indian population.

Study design: A hospital based cross sectional study.

Place and duration of study: A tertiary care medical college & hospital in Kolkata, West Bengal having a study duration of one year from January 2018 to January 2019.

***Methodology*:** DNA was extracted from whole blood using phenol chloroform extraction method from 30 case and 40 control subjects. A section of the VEGF-A gene consisting of the base pair where the SNP occurs and small regions adjacent to it was then amplified from the genomic DNA by PCR. The PCR product was treated with restriction enzyme *Hin D III* and the restriction digestion pattern was analysed.

Results: In our results, the prevalence of the wild C and the mutant T alleles in the study group were found to be 60% and 40% respectively. The prevalence of the homozygous non-mutant (CC), heterozygous (CT) and the homozygous mutant (TT) genotypes were found to be 45%, 30% and 25% respectively.

***Conclusion*:** It is likely that there is a significant association between the +936 C/T SNP and renal cell cancer in the Eastern Indian population. Also, majority of the renal cell cancer patients from this region are prone to worse cancer prognosis and therefore may need a more active medical management including anti VEGF therapy. Further studies are required to confirm the association and to determine its nature.

Key words*: Renal cancer, VEGF, Single nucleotide polymorphis, Polymerase chain reaction, Restriction fragment length polymorphism, +936 C/T SNP of VEGF gene.*

1. INTRODUCTION

Angiogenesis is relatively an early and an essential event in carcinogenesis[1]. From the endothelial cells of blood vessels, newly formed cells help in growth of new blood vessels that help in maintaining the nutrient supply, oxygen support and disposal of waste materials for the rapidly growing tumour cells[2]. But formations of new blood vessels need the presence of cell growth factors in increasing amount. Increased syntheses of these growth factors are generally associated with the alterations in

the expression of their parent genes. Single nucleotide polymorphism (SNP) are the major contributors of such changes in the genetic structure that bring out altered expression of their parent genes. For increased stimulation of angiogenesis in cancer cells, growth factors like vascular endothelial growth factors (VEGFs) have been extensively studied. SNPs of VEGF are likely to be associated with several cancers like breast, colon, prostate and renal cell carcinomas[3-7]. Furthermore, these SNPs are also found to indicate the metastatic spread and prognosis of the overall disorder[8]. Different treatment modalities have been proposed to block the effects of their over-expression into corresponding proteins that finally help in improving the prognosis of the disease[9]. VEGF is a 34-46 kDa heparin binding dimeric glycoprotein that functions through its cognate receptors (VEGFR) which belong to the tyrosine kinase family of receptors[10]. The VEGF gene is present in the short arm (p) of chromosome number 6 in humans and chromosome 17 in mice (*mus musculus)*. Several isoforms of the VEGF gene are present many of which are directly linked with increased angiogenesis found in cancer cells. Polymorphic changes in these isoforms have been found to be linked with renal cell cancers. One such SNP is the *+936 C/T* mutation in the VEGFA (Vascular Endothelial Growth Factor A) gene which is found to have been strongly associated with Renal cell carcinoma (RCC)[3]. However, this association has not been consistent throughout the world showing considerable variation between different ethnic population groups from region to region. For example, one study in a Japanese population showed that different genotypes of the VEGF gene may affect the prognosis of RCC by altered genetic expression[11]. Whereas another study among the Japanese population suggested that three polymorphisms in the 3' – UTR region of the VEGF gene doesn't affect the risk of developing RCC or clinical parameters in RCC[12]. Yet another study in a Caucasian population showed that the -460 polymorphism in the VEGF gene is a risk factor of RCC[13]. Furthermore, one of the lacunae in the existing knowledge lies in the fact that not many studies have been carried out regarding the association of SNPs in the VEGF gene and renal cell carcinoma in the various ethnic groups of India. It has been proposed that the C allele in +936 position of the VEGF gene causes over-expression of the gene and thus increases the synthesis of Vascular Endothelial Growth Factor, a protein which helps in the formation of new blood vessels from the endothelium of existing ones (angiogenesis). The new blood vessels may provide an increased nutrient and oxygen supply to the cancer cells, thus facilitating tumor growth and may also help in metastasis, thus resulting in worsening of the prognosis of the disease. Hence, the +936 C>T mutation in the VEGF gene is supposed to play a protective role against cancer, as the C allele is replaced by T in this mutation. In other words individuals in whom this mutation is not present may be genetically predisposed towards cancer. For the present study, we hypothesized that there is an association between the *+936 C/T* polymorphism in the VEGF gene and renal cell carcinoma in the Eastern Indian population. To check the plausibility of the hypothesis, we carried out the present research project.

2.MATERIALS AND METHODS:

2.1. Study design:

The present study was undertaken as an observational, cross sectional, non interventional hospital

based study in a tertiary care hospital in West Bengal, India. During a period of 6 months, histopathologically diagnosed cases of renal cell carcinoma were selected from the Urology department using the method of convenience following the inclusion and exclusion criteria. All grades with or without metastasis were selected as the present study aimed to explore any probable relationship between the renal cell cancer and the *936 C/T* polymorphism in the VEGF gene in this region. Patients suffering from any other cancer, metabolic disorders like diabetes mellitus, thyroid disorders etc were excluded from the study. Patients having any other disorders related to microangiopathy or other angiopathic disorders were also excluded. Finally, only those patients giving consent for the study were included following the ethical guidelines. Following these inclusion and exclusion criteria 30 cases of renal cell cancers were selected within the study period along with 40 age and sex matched control subjects.

2.2. Ethical considerations:

The study strictly adhered to the ethical guidelines stipulated by Hensinki declaration for human studies and ICMR, India. All ethical protocols were followed as directed by the institutional ethical committee and the study was undertaken only after obtaining the institutional ethical clearance.

2.3. Study protocol:

Isolation of DNA from blood: 5 mL of venous blood was drawn from each of the patients and control subjects. DNA was extracted from the blood samples using the phenol chloroform extraction method followed by precipitation by 3 M sodium acetate and absolute ethanol. Briefly, blood samples were suspended in 1mL of the lysis buffer (320 mM sucrose , 5 mM MgCl2., 1% titron X-100. 10mM tris-HCl, pH = 7.5). and centrifuged at 10,000 rpm for 10 minutes to form pellets. Nuclear lysis buffer (composed of 400 µL; 10 mM tris-HCl, 10 mM EDTA, 50 mM NaCl, pH 7.5) containing proteinase K (0.1 mg/mL) was added to the pellets. The solution was incubated at 55^0 C for 3 hours with occasional shaking. The solution was then added with 1mL tris-saturated phenol (pH = 7.4) and centrifuged at 10,000 rpm for 10 min. Then the supernatant was extracted carefully which should be devoid of proteins. 1 mL of chloroform:iso-amyl alcohol (24:1) was added to the solution and it was centrifuged for 10 minutes at 10,000 rpm. The supernatant containing the DNA was then precipitated with 3 M sodium acetate and chilled ethanol. The extracted DNA was washed with 70% ethanol, dried and stored in TE (Tris-HCl EDTA) buffer at minus 20 degree centigrade. The OD260/OD280 ratio of the extracted DNA sample was found out to be ~ 2.1. Hence, it was concluded that the DNA sample was pure, as it is known that for a DNA sample to be pure the value of the ratio has to be >= 1.8.

2.3.1. Amplification of the DNA by polymerase chain reaction:

A small segment in the DNA from each of the two chromosomes of chromosome 6 consisting of the base pair where the +936 C/T mutation occurs was then amplified by Polymerase Chain Reaction (PCR) and was treated with the restriction enzyme *Hin D III* to find out whether the mutation was present or not. The number of bands of the amplified segment(s) formed was then taken into note and accordingly the zygosity of the mutation was inferred i.e. whether the mutation is heterozygous, homozygous or not present at all in that particular case. The numbers of cases with homozygous non

mutant, heterozygous and homozygous mutant genotypes were noted down and the distribution of the genotypes and allelotypes in the study group was then determined from the previous data. The protocols was as follows:

2.3.2.Amplification of the VEGFA gen e in the extracted DNA by PCR:

Our aim for the PCR amplification was to amplify the VEGF gene containing +936 C/T polymorphism (SNP: rs3025039). For this we used a PCR reaction mixture of volume 25 µL with 2X PCR master mix obtained from Thermo fisher scientific. Forward and reverse primers were taken as *5'-AGGAAGAGGAGACTCTGCGCAGAGC-3'*and *TAAATGTATGTATGTGGGTGGGTGTGTCTACAGG-3'* respectively. 1 µL each of forward and reverse primers of the 10 µM working primer stocks, 1 µL of the template DNA and 9.5 µL of nuclease free water were then added to make the volume of the PCR reaction mix upto 25 µL. The microfuge was then loaded to a PCR Machine (Applied Biosystem) and the PCR program was run with **Initial denaturation** at 95^0 C for 5 minutes was followed by 35 cycles of **Denaturation** 95^0 C for 45 seconds, **annealing t** 59^0 C for 30 seconds and **extension at** 72^0 C for 45 seconds. After completion of 35 cycles, f**inal extension was** done at 72^0 C for 10 minutes with a holding possibility at 4^0 C for infinity.

2.3.3. Treatment of the PCR product with Hin DIII restriction enzyme:

The PCR product was digested using 10 units of the restriction enzyme as directed and the bands were observed in 3 percent agarose gel against a 100 bp DNA ladder for final interpretation.

3. RESULTS:

Fig 1 shows the restriction digestion pattern for each of these three categories.

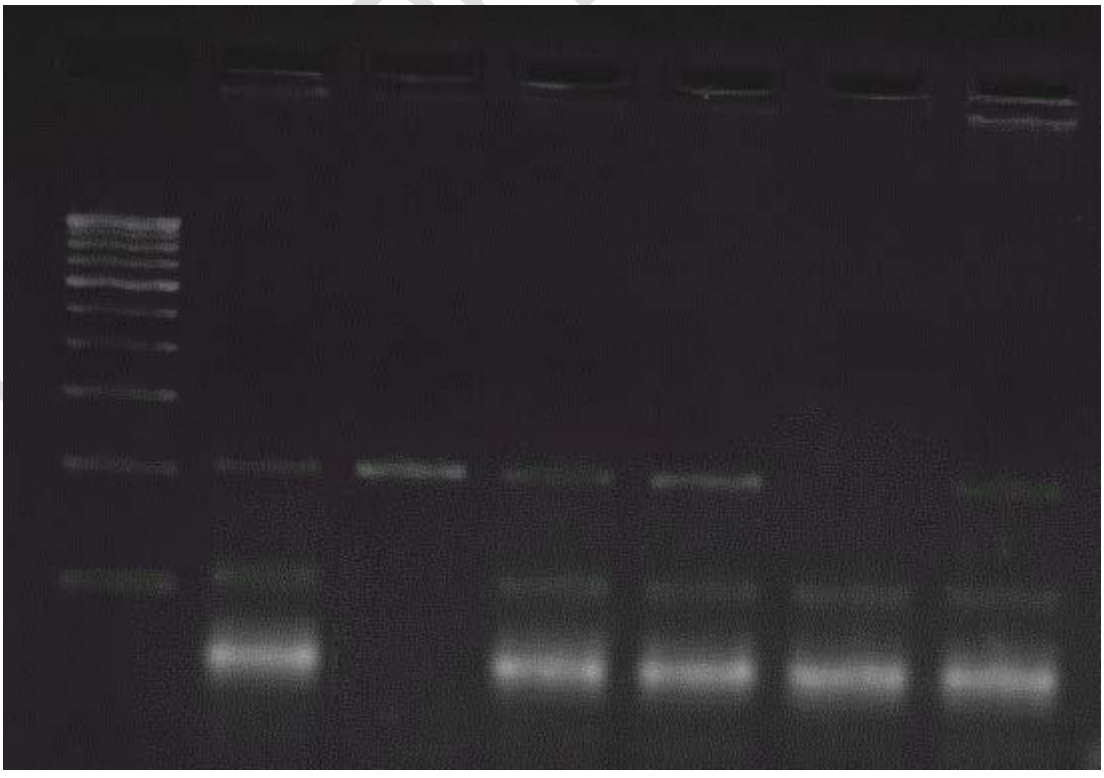

Fig 1: Lane 1 is a 100 bp DNA ladder; Lanes 2, 4, 5 and 7 show the Heterozygotes (CT) cut into fragments of lengths 207, 122 and 85 bp; Lane 3 shows the Homozygous mutant (TT) uncut and having a length of 207 bp; Lane 6 shows the Homozygous wild type (CC) cut into fragments of lengths 122 and 85 bp. (Lanes are numbered in the image from left to right)

The data obtained after PCR and restriction digestion were tabulated in the form of number of genotypes (CC, CT and TT) and allelotypes (C and T) for both case and control groups. The distribution of the genotypes in the study group of is shown in *Table 1* below:

Table 1: Distribution of the CC, CT and TT genotypes in the study group

	CC	CT	TT
Cases (n = 30)	15	9	6
Controls (n = 40)	8	16	16

Chi square = 7.36, *P* = .025

From the above table, the prevalence of each of these three genotypes in the study group were calculated to be CC: 50 %, CT: 30% and TT: 20%. While the same prevalence rates for the control group was found to be 20% for CC, 40% for CT and 40% for the TT genotype. A chi square value of 7.36 with P value of around .025 indicated that the increased prevalence of CC alleles in the renal cancer patients was significant statistically.

Table 2: Distribution of C and T alleles in the study group:

	C allele	T allele
Case allele (n = 30 x 2 = 60)	39	21
Control (n = 40 x 2 = 80)	32	48

Chi square value = 8.57, *P* = .003.

Table 2 shows that almost 65% distribution of the C allele in the case group against a 40% C alleles in the control subjects.

Figure 2: Area chart showing distribution of the C and T alleles in the study group

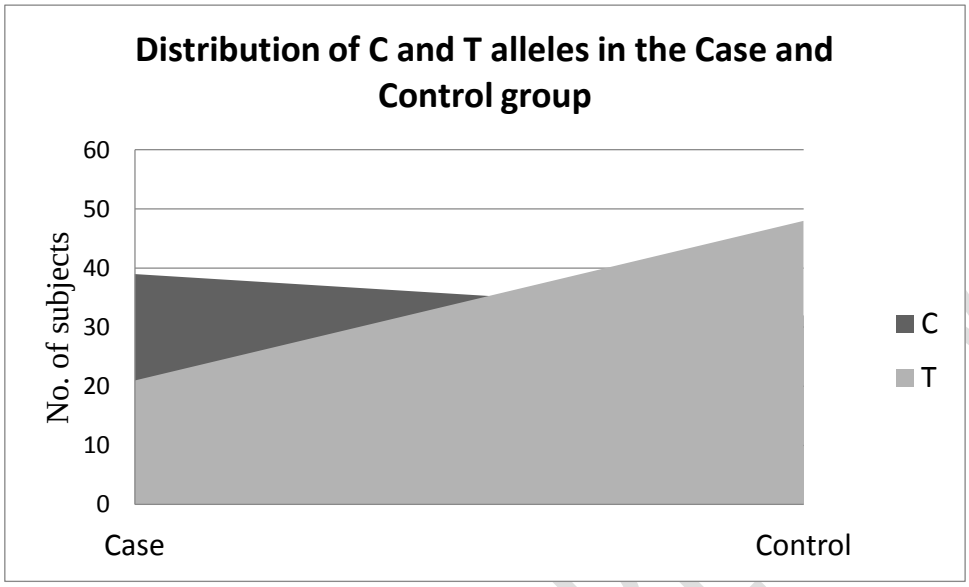

Figure 2 shows the overall distribution of C and T alleles in the case and control group as depicted by area chart that shows a distinctly more prevalent distribution of C alleles in the case group.

4. DISCUSSION:

The +936 C>T SNP may be present in both of the chromosomes of chromosome 6, any one of them or it may not be present at all. Depending on the alleles present on the two chromosomes in this position, the mutation may be classified into homozygous wild type or non-mutant (CC), homozygous mutant (TT) and heterozygous (CT) categories.

Thus, 50% of the present study group were found to possess the dangerous homozygous non mutant genotype (CC) which is reported to contribute to the risk of developing RCC, and 20% the safe homozygous mutant genotype(TT) which is found to be protective against RCC. The remaining 30% were found to be heterozygotes carrying the CT genotype i.e. equal numbers of the dangerous wild (C) and safe mutant (T) alleles. The prevalence of the non mutant C and mutant T alleles in the study group were found be 65% and 35% respectively. In comparison the distribution of the dangerous CC genotype and the C allele were much smaller in the non cancer control group (Table 1 and 2, figure 2). Thus it is likely that there is a significant association between the +936 C>T mutation and renal cell cancer in the Eastern Indian population.

Renal cell cancer is a multigenetic malignancy where various genetic factors play roles in its development and spread along with multiple environmental factors. But, like other cancers increased angiogenesis has been an important contributing factor in its metastasis and tumor growth. SNPs in the VEGF gene have been found to be associated with various forms of cancer including colorectal, breast, renal and prostate cancers (14-19). These polymorphisms generally cause altered expression

of the VEGF gene and thus causes increased or reduced synthesis of the Vascular Endothelial Growth Factor, a protein which plays a major role in angiogenesis. Angiogenesis usually promotes tumor growth and metastasis, as the newly formed blood vessels facilitate the transfer of the cancer cells from the tumor to various other locations throughout the body and also provides nutrition and oxygen supply to the cancer cells, thus helping them to multiply which results in tumor growth. Thus polymorphisms in the VEGF gene can affect an individual's genetic predisposition towards cancer. As increased angiogenesis plays an important role in facilitating metastasis and helping in tumor growth. The C to T mutation may act as an anti-cancer factor that restricts the excess VEGF mediated angiogenesis and spread of tumor. This explains their significant dominance in the control group (Table 1, 2 and figure 2). However, future studies involving longitudinal study designs are needed with larger sample size to establish the results of present study more conclusively. As the C allele in +936 position of the VEGF gene may play a role in worsening the prognosis of cancer, majority of the individuals from the Eastern Indian region who develop RCC have a risk of worse prognosis of the disease. Thus they may need a more active medical management, including anti VEGF therapy. Furthermore, we suggest that early screening for the +936C/T SNP is needed in renal cell cancer patients of this region to prevent worse prognosis in these patients.

5. ETHICAL CONSIDERATION: The study strictly adhered to the ethical guidelines stipulated by Hensinki declaration for human studies and ICMR, India. All ethical protocols were followed as directed by the institutional ethical committee and the study was undertaken only after obtaining the institutional ethical clearance.

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