



Investigating the frequency of 460C / T VEGF gene in prostate cancer patients in North West of Iran

Fatemeh Barkhori MEHNI¹, Safar FARAJNIA^{2*}, Ashraf FAKHRJOU³

¹Department of Biology, Faculty of basic sciences, Islamic Azad University, Ahar Branch, Ahar, Iran

²Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

³Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

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Abstract. Vascular endothelial growth factor (VEGF) is an important angiogenic factor and its role in tumor progression has been identified. Several polymorphisms of VEGF have been identified. Due to the potential role variants, VEGF460 C / T in prostate cancer, we hypothesized that these variants associated with prostate cancer risk. For this purpose, a normal control group of 50 healthy subjects and 50 patients with prostate cancer were studied. Polymorphisms of VEGF 460 C / T with the BSTUI restriction enzyme analyzed based on polymerase chain reaction. In this research, analyzes of variants VEGF460 C / T in term of T polymorphism revealed a significant difference.

Keywords: Vascular endothelial growth factor, Polymorphism, prostate cancer

1. INTRODUCTION

Prostate cancer is the most common malignancy among men in industrialized countries and is the second leading cause of cancer deaths in Europe and America. [1, 2]. The prevalence of cancer among various ethnic groups is statistically different. Reports indicate that prostate cancer is more common among Indians. In Iran, the death of prostate cancer among men is associated with a growing trend.

Prostate cancer cause is heterogeneous and includes genetic and environmental factors [4]. Incidence of death due to prostate cancer according to tumor grade, stage, age and ethnic and racial diversity is remarkable. Therefore, studying the cause of prostate cancer should explain that what social, environmental and genetic characteristics cause different form of this tumor [5-7]. Several factors are involved in the development of prostate cancer, that the genetic role of endocrine and environmental factors is dramatic. Genetic factors are responsible for 42% of cancer incidence [8]. In the search for relationships between patients with prostate cancer and natural controls, the role of genetic is important and various genes have been studied in this area [8-11]. It revealed that development or progression of prostate cancer with C / T460 polymorphisms for vascular endothelial growth factor [VEGF] can be related [12]. VEGF and it is highly expressed in human tumors, including tumors of the breast, non- stem cells of lung and prostate tumor and recently signaling pathway and VEGF factor is of interest to researchers as anticancer drug[13]. VEGF gene is located on chromosome 6 at 6p12.1 [14]. Coding region is about 14 kb and consists of 8 exons. Several polymorphisms are located in promoter region, the non-translated region [UTR] 5' and 3' [15].

The UTR 5 and UTR 3 region contains the regulatory elements that are sensitive to hypoxia and cause the variety in VEGF production in tissue distance which in prostate cancer tissue compared to normal tissue or benign prostatic hypertrophy [BPH] is far greater. VEGF and blood vessel cells production in great metastatic prostate cells than cells with few metastatic

* Corresponding author. Safar Farajnia *Email: farajnia@gmail.com*

have incremental processes that reflects the importance of VEGF in the progression of prostate cancer [16].

Cheng chieh Lin and et al conducted a study in Taiwan in 2003 stated that 460C / T polymorphism of VEGF gene could be a biological marker for prostate cancer [17]. In 2006, according to the study of Sfar and et al genetic variants in VEGF also creates the risk and the progression of prostate cancer as well [16]. Fokora and et al [2007] in a clinical study have observed a significant relationship between 460C/T polymorphism of VEGF gene and prostate cancer. In 2008, Langsenlehner and et al stated that polymorphisms and haplotypes of VEGF wouldnot change the risk of prostate cancer [18]. The aim of present study was to investigate the the frequency of C / T460 polymorphisms VEGF gene in prostate cancer patients in the North West of Iran.

Materials and Methods:

Collecting samples of study:

All of the subjects older than 40 years were selected. The collected samples were transferred to Tabriz research Institute of Pashmine and further investigation is done. In this study of 100 subjects, 50 healthy male who the lack of prostate cancer was confirmed in them were as control group and their peripheral blood samples obtained and as a patient group of 50 men with prostate cancer tissue pathology obtained. Based on Gleason grading, Gleason score determined for each tissue pathology.

According to Table 1 Gleason score of 50 patients, 3 subjects [6%] in the group between 3-4, 10subjects [20%] between 5-6 in the group, 25 subjects [50%] in the group between 7-8 and 12 subjects [24%] were in the group between 9-10.

Table 1. Gleason score of patients with VEGF460C / T gene.

Gleason score	Frequency	Frequency percentage
3-4	3	6
5-6	10	20
7-8	25	50
9-10	12	24

Through using the DNG-Plus salt, DNA extract obtained from blood samples. Extracting DNA phases from the blood was in order; preparing white plate from whole blood, adding DNG-Plus salt, adding isopropanol 100% drying and putting in 37degree water bath, adding 50 ml of distilled water. In order to extract DNA from paraffin-embedded tissue, Micronics sections prepared. Then in order Paraffin removal, solving the tissue by adding 200 microliter of DS solution, Adding 20 micro liters of proteinase K and 220 of MS solution, producing sedimentary DNA and washing. Finally the extracted DNA was stored at -20 ° C.

Quality and quantity of extracted DNA through loading the sample on a 1% agarose gel and using spectrophotometer assessed.

Designing PCR and RFLP primer:

For amplification of target DNA by PCR, primers were designed that was complementary for target sequences in the promoter region of the VEGF gene. The applied primers to amplify the VEGF gene promoter region designed in order direct primer forward [f]:5-TGT GCG TGT GGG GTT GAG C3 and reverse primer with sequence REVERS[R]:5-GTG AGG TTA CGT GCG GAC AG3. In each reaction of PCR, the presence of MIX solution

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[PCR·MGCLZ·DNTPS·DW buffer] Forward and Revers primers, DNA template, Taq DNA Polymerase is essential. Taq DNA Polymerase is specific enzyme for PCR and its optimum temperature is 72 degrees Celsius. According to PCR protocol, in a Micro-tube 88 microliter of mix solution with 4 microliter Revers primer, 4 microliter forward primer, Taq enzyme added and after the microcentrifugation, was divided in 4 micro-tubes of 0.2 ml. One of the micro-tubes considered as negative controls and DNA added to next micro-tubes and after defining the program, placed into thermocycler device. The required DNA for each PCR reaction considered 50-100 ng. The PCR reaction was prepared in cold condition on Ice pack. PCR thermal cycling program for VEGF gene amplification the initial Denaturation temperature 95 ° C for 4 min, 38 cycles 3 phase Polymerase include:

- 94 ° C for 1 minute Denaturation
- 60 ° C for 1 min for primer binding to the template [Annealing]
- 72 ° C for 30 seconds for polymerization [Extention]

The final amplification [final extention] at 72 ° C for 5 min

For PCR analysis of VEGF gene of a 2% agarose gel is used, because the amplified fragment size is small [175 bp]. In order to prepare the agarose gel 2%, weigh 2 g of agarose powder and dissolved in 100 ml of TAE 1x buffer. Then 7 ml of PCR product with 2 ml of the Sample Loading buffer was removed and run into wells of the gel [electrophoresis].

After the run up to the end of the gel, the PCR products were stained in ethidium bromide solution and finally through photographing the gel the presence of amplified fragment examined. Through RFLP technique, amplified fragment by PCR using restriction enzymes cut and according to size of the generated fragments desired polymorphisms investigated. In this technique, the product of PCR 175 bp with 2 units of Bstul and reaction buffer [R 10x] [thus; 2units of Bstul, 2ul reaction buffer [10x] and distilled water [DW] 17u combined. 10 ul of PCR product added to the above composition. Mixing ingredients together and doing microcentrifugation. After covering the micro-tube cap put it in incubation for 1 h at 37 ° C. Then the RFLP product, DNA ladder and one of the samples of PCR product as indigestive next to each other and in related wells is run. Finally through the photographing of gel and generated polymorphism and due to enzyme digestion is examined.

Table 2. Applied Primers for VEGF gene.

Primer Direction	Sequance		Connection Temperature
Forward	5'– TGT GCG TGT GGG GTT GAG CG – 3'		60
	5'–GTGAGGTTACGT GCGGACAG– 3'		Fragments Length
Revers	genotype	The location of section and identification	20 ·155 20 ·155 ·175 175
Enzyme	CC	$ \begin{array}{c} \downarrow \\ 5' \dots CG \vdots CG \dots 3' \\ 3' \dots CG \vdots GC \dots 5' \\ \uparrow \end{array} $	
Bstul	CT		
	TT		

Research Findings:

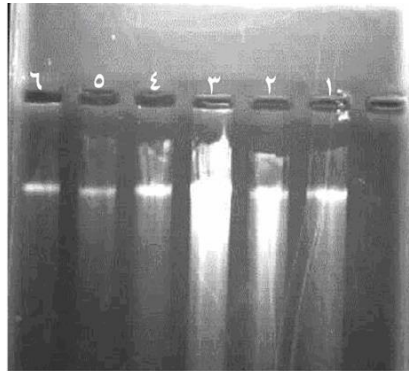


Figure 1. The result of extracted DNA quality of 6 blood samples on gel agarose 1%.

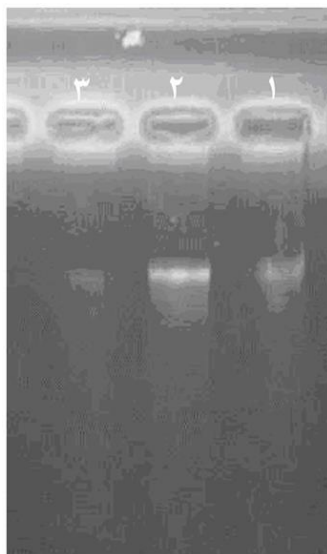


Figure 2. Extracting DNA result from tissue on gel agarose 1%, the concentration of extracted DNA in tissue was weaker than blood which related to differences in cell accessibility in blood and tissue.

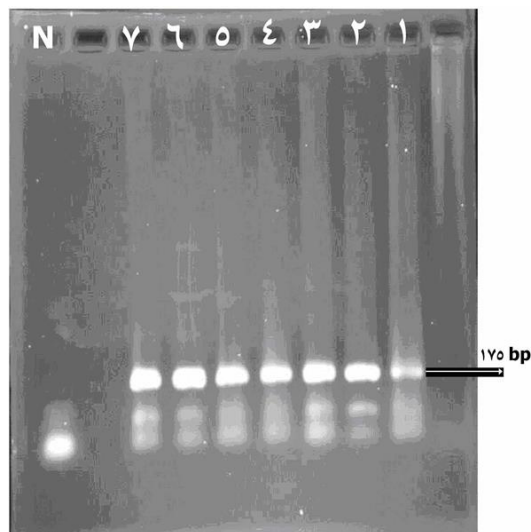


Figure 3. Indicate the PCR result of VEGF gene on gel agarose 2% in laboratory.

The proliferated fragment in PCR is about 170bp. Line 1 to 7 reveals the proliferated fragment and line N is considered as control that doesn't include the proliferated fragment.

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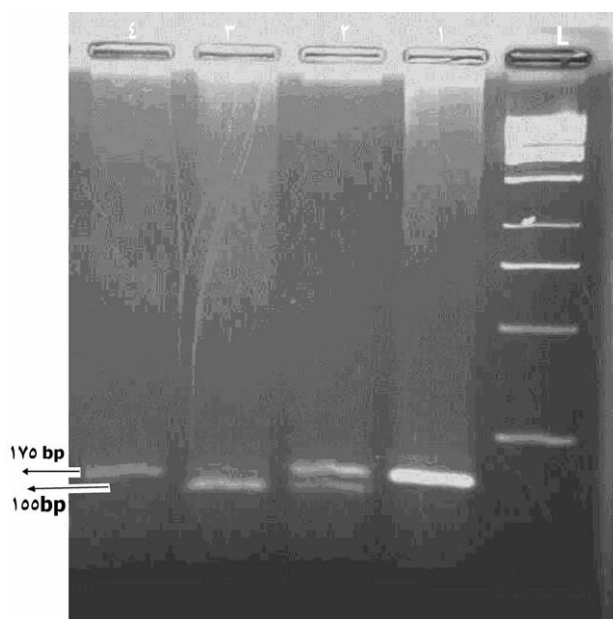


Figure 4. The result of conducting RFLP of VEGF gene on gel agarose 2.5%.

Line L is containing DNA ladder. Lines 1 and 4 contain DNA 175 bp genotype TT And Line 2 contains DNA of 155bp and 175 bp which has heterozygous CT genotype. Line 3 contains DNA 155bp which has homozygous genotype CC.

Statistical Description:

In order to describe the data, descriptive statistics [frequencies and percentages] were used and Independent T- test [difference between the mean of two independent samples] used and through SPSS14 software statistically analyzed. In this study P values greater than 0.05 was not considered statistically significant.

VEGF460C/T gene frequency

According to the table of 100 subjects, 21 subjects [21%] had genotype CC, 41 subjects [41%] CT genotype and 38 subjects [38%] had the TT genotype.

Table 3. The frequency of each genotypes of VEGF460C/T gene.

Genotype	Frequency	Frequency percentage
CC	21	21
CT	41	41
TT	38	38

There is different between two groups of patient and healthy in terms of polymorphisms of VEGF 460C / T gene. To test this hypothesis, chi- square test used that the results are presented in table 4. According to the level of significance between the two groups, there is a significant difference. So the hypothesis accepted.

Table 4. The chi square test in polymorphism of VEGF460C/T gene between two group of patient and healthy.

Group	CC	CT	TT	Total	X2	P value
Healthy	10[20%]	25[50%]	15[30%]	50[100%]		
Patient	11[22%]	16[32%]	23[46%]	50[100%]	6.98	0.031

Different genotypes of VEGF460C / T gene in Gleason score of patients were not significantly different. To test this hypothesis, the statistical test of Cramer or phi used that the results presented in table 5. According to results there was no significant relationship between different genotypes and Gleason score.

Table 5. The frequency of Phi polymorphism of different group of Gleason score.

	3-4	5-6	7-8	9-10	P value
TT	1	4	9	9	0.21
CT	1	2	10	3	
CC	1	4	6	0	
Total	3	10	25	12	50

Discussion and Conclusion:

Vascular endothelial growth factor [VEGF], is a strong inducer of endothelial cells growth and its surface increases in several types of tumors [19]. Jackson and et al applied immunohistochemical staining and polymerase chain reaction to determine the wide expression of this gene. In addition, they confirmed that angiogenesis is important in the development of prostate cancer [20]. It is thought that tumor neovascularization can be controlled by chemical signals such as angiogenic factors [21]. VEGF is a cytokine that plays an important role in neovascularization and therefore is an important angiogenic factor. Based on these facts, VEGF may lead to the development and progression of prostate cancer [22]. VEGF plasma levels suggested as a prognostic factor in patients with prostate cancer [21, 23].

Cheng Chie Lin and et al conducted a study in Taiwan in 2003 stated polymorphism 460 C / T of VEGF gene would be biological marker for prostate cancer [17]. VEGF has undeniable and important role in angiogenesis and tumor generation in cancer and is the most abundant polymorphism observed in the C / T460 which located upstream of the VEGF gene in nucleic acid. Researchers are following to apply single nucleotide polymorphisms to identify disease genes that lead to prostate cancer. Polymorphism C/ T460 of VEGF gene is a superior candidate for further study leading to its association with prostate cancer in some studies to be confirmed. Many reports have shown that VEGF may combine with upstream signals or other growth factors to promote the growth and progression of prostate cancer. For example, it is known that the epidermal growth factor accelerates the expression of VEGF in the growth of prostate cancer significantly. In addition to the significant increase in VEGF levels in patients with prostate cancer, hormone refractory has been observed which indicate that VEGF is associated with tumor progression of cancer in some populations [21, 24, and 25].

Continued growth of the metastatic tumors is requiring a greater risk factor for increased neovascularization in favor of further growth of the tumor in vascular bed [26]. Our data are consistent with previous studies in which VEGF may be relevant prostate cancer [27]. In addition to the significant increase polymorphism C / T460 of VEGF gene was observed in patients with prostate cancer indicates that the polymorphism C / T460 VEGF gene associated with prostate cancer. Functional studies and genetic mapping are more likely to help to make relationship between the polymorphism 460C / T VEGF gene and prostate cancer more transparent.

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