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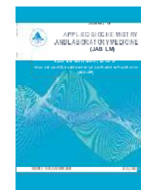
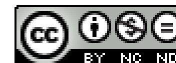


Original Article

Association of rs3025039C/T single nucleotide polymorphism of the VEGF gene with bladder cancer in a population of West Bengal.



Baidya Nabanita[1], Roy Suparna[2*], Bhattacharrya Swati[3].
1. Final year post graduate trainee, Dept of Biochemistry, Calcutta National Medical College & Hospital, Calcutta,
2. Associate Professor, Dept of Biochemistry, Jhargram Govt Medical College, Jhargram.
3. Professor & Head, Dept of Biochemistry, Calcutta National Medical College & Hospital, Calcutta .

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A B S T R A C T

Background:
Bladder carcinoma is the second most common malignant neoplasm of the genitourinary system. Genetic predisposition is one of the commonest predisposing causes for the bladder cancer. The SNP, particularly that of the rs3025039C/T has been reported to be linked with bladder cancer in various parts of the world. The present study was undertaken to find out any such association in our study region of West Bengal.
Methodology:
In a case control study, the prevalence of rs3025039C/T SNP was measured and compared in 75 matched cases and control subjects. The prevalence of allelic variation was assessed by restriction digestion of the PCR products of the isolated DNA from whole blood. Results were compared between the cases and controls using the chi square test and odds ratio analysis.
Results:
Association of the mutant T allele was found to be significantly more with the case group as evident from the Fisher's exact probability test in the genotype variation (P = .01) and odds ratio results (OR = 8.58, range = 1.95 to 37.7 at 95% CI) in the allelic variation analysis.
Conclusion:
The T allele of the rs3025039C/T SNP is more significantly associated with the bladder cancer in our study population. This allele also poses significant risk for the bladder cancer in population as evident from the odds ratio analysis.

INTRODUCTION

Being one of the commonest genitourinary cancers in men, urinary bladder cancer (BC) leads to massive pressure on health care system due to its constant increase in incidence day by day [1].

BC is the second most prevalent genitourinary cancer in males posing a huge health, financial and manpower problem for the society [2]. With the constant increase in environmental risk factors, the prevalence of all types of cancers have shown a significant rise in the world. It has been estimated that approximately 18.1 million cases of cancer existed with about 9.6 million mortalities in the year of 2018. In this huge burden of cancer throughout the world, BC is supposed to hold 10th and 17th position among all cancers in the context of incidence in the whole world and India respectively. In India BC consists of 3.9% of all malignancies along with a substantial mortality that accounts to be 19th in rank among all cancers [3-5].

As in many cancers, several major risk factors are responsible for occurrence of BC like long term smoking and use of tobacco in its variant forms, an increasing age, a male sex, chronic contact with chemicals like nitroso-ureas and long term consumption of drugs like cyclophosphamide[6]. But another seminal factor which is associated with BC is genetic factors like mutations and single nucleotide polymorphisms (SNPs) of various genes among which those for the vascular endothelial growth factor (VEGF) gene play perhaps of the most important role. Several mutations and SNPs of the vascular endothelial growth factor (VEGF) have been found to be linked with both the etiology and progression of the bladder cancer worldwide[3, 7]. Polymorphic variations in the VEGF gene give rise to different degrees of vascular growth in the tumor cells and so are associated with number of different types cancers. Association of VEGF polymorphisms has been found with several malignancies including the bladder cancer.

*Correspondence:
Dr. Suparna Roy, Associate Professor,
Dept of Biochemistry, Jhargram Govt Medical College, Jhargram.
Email: drsuparnaray.ray@gmail.com

Association of several polymorphisms of the VEGF gene has been found with breast cancer patients [16]. In this context, over-expression of VEGF is known to be highest in bladder cancer patient in comparison to healthy individuals and multiple single nucleotide polymorphisms (SNPs) particularly that of the rs3025039C/T, have been reported to be well linked with it[8, 9].

Environmental factors can be changed with a good knowledge for the predisposing risk factors. In contrast, it is difficult to alter the genetic factors of a person that is inherited from their predecessors. However, they are the most suitable factors for an early detection for the risk of cancers as they are present since birth.

Presently, the existing knowledge about the prevalence of this mutation is not conclusively available in our area. With this lacuna in the present knowledge and potential clinical importance of this association, we hypothesized that there is a significant association between the rs3025039C/T polymorphism of the VEGF gene and urinary bladder cancer in our study population.

Materials and Methods:

The present study was a hospital based case control study undertaken in the Urology and Biochemistry department of an urban teaching hospital. The study was conducted over a period of one and a half year after getting permission from Institute's Ethics Committee (IEC) with the following work plan:

i. Sample size calculation: As it was a hospital based case control study, all patients fulfilling the inclusion and exclusion criteria were selected for the study according to the method of convenience within the stipulated time period. The inclusion and exclusion criteria for the case subjects were as follows:

Inclusion criteria:

a. Histo-pathologically diagnosed patients of urinary bladder cancer attending the outpatient department (OPD) of the hospital and admitted in indoor section of the Urology department, CNMC&H, Kolkata. All ages along with both sexes were included.

Exclusion criteria:

a. Patients suffering from any other malignant disorder
b. Patients suffering from any type of chronic inflammatory disease
c. Patients suffering from any metabolic or endocrinologic diseases
d. Patients having addiction to smoking, alcohol or any drugs

Age and sex matched healthy control subjects were selected from the persons accompanying the patients after obtaining their consent. First degree relatives were excluded from the study to avoid any hidden effect of genetic linkage.

Procedural techniques in detail*:*

A. Separation of DNA from blood: Extraction of human DNA from whole blood by phenol chloroform extraction method[10]. 3ml of venous blood were collected from participants in EDTA vial. For each sample, 1ml of whole blood was taken from EDTA vial and kept in centrifuged tube and then mixed with 1ml of cell lysis buffer (10mmol Tris-HCl [pH 7.5], 5mmol $MgCl_2$, 1% Triton X-100, 320 mmol Sucrose). Then this mixture was centrifuged at 10,000 rpm for 15 minutes at 4-degree Celsius temperature. The supernatant was discarded and the pellet was taken. 500 µl nuclear lysis buffer (10mmol Tris- HCl [pH 7.5], 10 mmol EDTA, 50mmol NaCl, 2% SDS) and 10 µl proteinase K were added with pellet. The mixture was incubated at 37 ˚C overnight. On next day 1ml phenol (Tris saturated phenol at pH 7.4) was added with mixture and mixed well for 15 minutes by repeated inversion and then centrifuged for 7 minutes at 10000 rpm at 4 ˚ C. The transparent supernatant was slowly taken and poured into a new centrifuge tube. 1ml chloroform -isoamyl alcohol mixture (chloroform and isoamyl alcohol @ 24:1 ratio) was added. The mixture was centrifuged for 7 minutes at 10,000 rpm at 4 ˚C. The transparent supernatant was slowly taken and poured into a new capped sample cup. 10µl 3M Na acetate was added to the sample cup. 1ml chilled 95% ethyl alcohol(ethanol) was added slowly in sample cup. It was centrifuged at 10000 rpm at 20 ˚C for 10 minutes. The pellet was taken and dissolved in 50 µl TE buffer (Tris EDTA buffer). The solution of DNA was stored at minus 20 degree C for till further use.

Purification of the isolated DNA was done by observing the absorbance of the isolated sample for unpurified protein residues at 280 nm and at 260 nm for the purified nucleic acid. The ratio of absorbances at 260 nm and 280 nm was found to be greater than 1.8 and so the samples were supposed to be free of contaminating proteins. The integrity of the separated DNA was assessed by running them through 0.7% agarose gel.

B. PCR protocol:

We used 2x PCR master mix from Thermofisher, USA. The forward and reverse primers were obtained from the primer blast site of NLM, Pubmed. Among several primers available from the blast site, we selected the primer pair depending on their 3' complimentary value, Tm values and the amplicon size of their PCR products. The forward primer was 5'TAACCCCAGCCTTTGTTTTCCA3' whereas the reverse primer was 5'CCTCCCAACTCAAGTCCACA3'. A 2 minute pre-heating phase was followed by 30 cycles of 1 minute denaturation phase at 95^0 C, 1 minute annealing phase of 57^0 C and 1 minute extension phase at 72^0 C followed by a final extension phase of 5 minutes at 72^0 C. The mixture was preserved at 4^0 C till further use.

C. Restriction fragment length polymorphism (RFLP)technique: The amplified PCR product were then digested with 10U of the restriction enzyme Nla1. For each 10 µl PCR product sample, 2 µl 10X tango buffers is added, then 18 µl deionized water is added followed by addition of 1 µl restriction enzyme in capped sample cup. Then the mixture is incubated in sample cup for 37 ° C for one hour. After this submerge gel electrophoresis of restriction digested amplified PCR product and DNA ladder (100bp) were done by using 3% agarose gel (staining with 0.5 µl/ml of ethidium bromide) in 50×TEA buffer at 50 to 100 mA for 60 minutes. After this gel is visualized using UV light in the gel documentation system.

Results:

The present study was conducted among 75 histo-pathologically diagnosed patients of urinary bladder cancer following the method of convenience and 75 Age and sex matched healthy control to study a selected VEGF gene SNP in patients suffering from urinary bladder cancer.

The independent t test (P = .58) and Chi square tests revealed the case and control groups to be age and sex matched respectively (Fig 1 and Table 1).

Figure 1: Boxplot showing age distribution between case and control groups

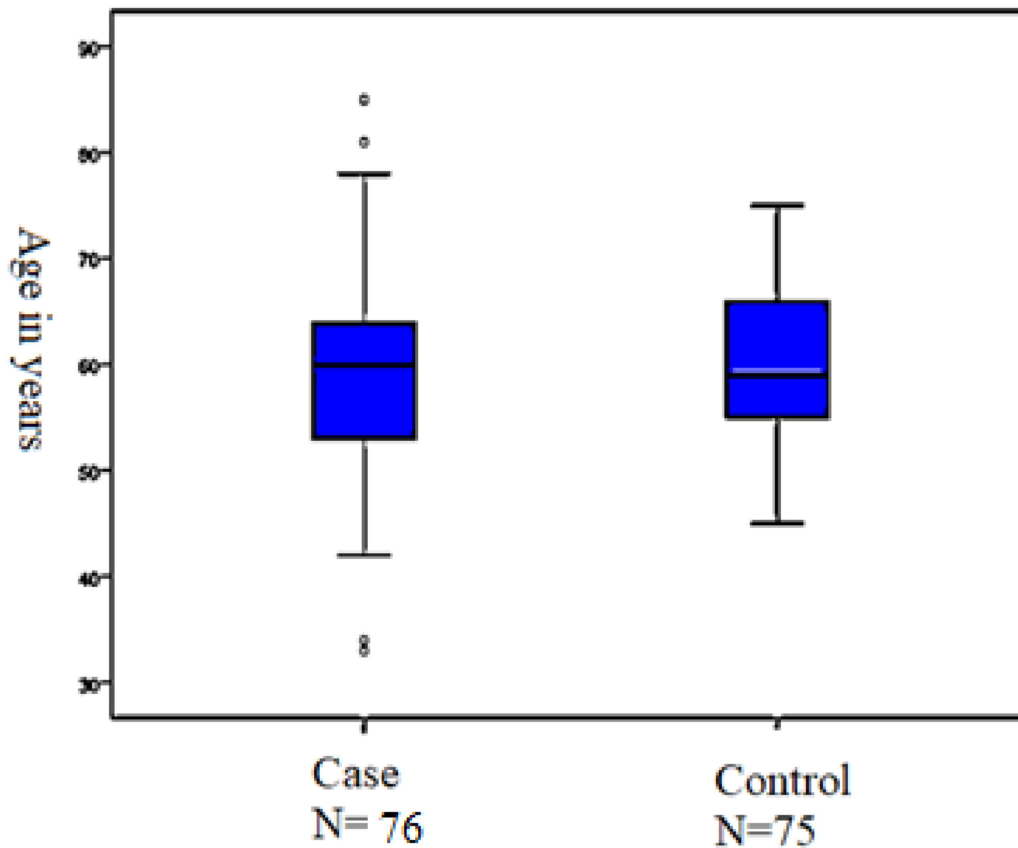


Table 1: Chi square test result showing the distribution of male and female subjects of the study population .

Sex	Case (n=76) n (row %)	Control (n=75) n (row %)	Total	Chi square value, df, p value
Females	10(55.6)	08(44.4)	18(100)	0.253*, 1, 0.615
Males	66(49.2)	67(50.8)	133(100)	

**P is considered to be significant at P >.05.*

The purity of our isolated DNA samples showed an $Abs^{960/980}$ ratio of 1.84 which was within the acceptable range. Results of the PCR showed no amplification for 13 samples out of total 150 DNA samples of case and control subjects. So further process of RFLP was carried out only in 137 samples out of which 74 were from case subjects whereas, 63 were from control population. The results of RFLP digestion is displayed in the Table 2. As some of the data were less than 5, Chi square test was replaced by the Fisher's Exact probability test for finding out the significance of differences in the genotype variations.

Table 2a: Fisher's Exact probability test showing distribution of study subjects according to rs3025039 C>T SNP genotypes

rs3025039 genotypes	Case (n=74) n (column %)	Control (n=63) n (column %)	df, p value
Heterozygus cut i.e both C and T alleles (CT)	10 (13.5)	02 (3)	**2, 0.01***
Homozygus cut (TT)	4 (5.5)	-	
Uncut (CC)	60 (81)	61 (97)	
Total	74 (100)	63 (100)	

P value considered to be significant at P ≤ 0.05 at 95% confidence interval (CI).

In Table 2B, allelic distribution of the mutant T allele and wild C allele have been represented. The significance of the strength of their association was measured by Chi square test while the risk ratio was measured by odds ratio calculation with its minimum to maximum interval at 95% confidence level. The chi square test showed that there was a significant association between the mutant T allele and bladder cancer patients (P = .001). As both the ratio and range of interval of the odds ratio are more than 1, so the risk of bladder cancer is strongly associated with the mutant T allele in our present study.

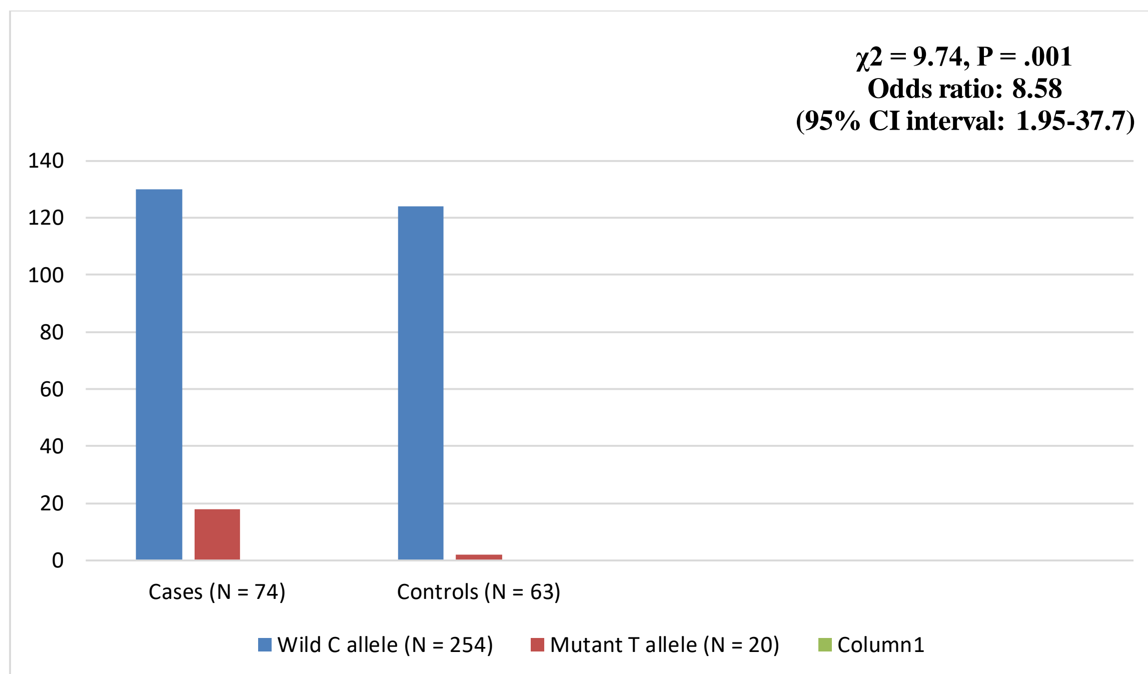
Table 2B: Chi square test and odd's ratio analysis to show the association and its risk for the mutant allele T of rs3025039 C>T SNP for bladder cancer in the in the case group**.**

	Controls	**Cases**	**Chi square (χ2)**	**Odds ratio with 95% confidence interval**	**Chi square value, P value**
Wild C allele	**124**	**130**	**7.67, P = 0.005**	**8.58 (1.95-37.7 at 95% CI)**	**9.74, P = .001***
Mutant T allele	**2**	**18**			

**P value considered to be significant at P ≤ 0.05 at 95% confidence interval (CI).*

The prevalence of bladder cancer with the wild C and mutant T alleles has been also depicted in Figure 2.

Figure 2: Diagram showing the distribution of wild C allele and mutant T allele between the case and control group.



Discussion:

The present case-control study was conducted among 75 histo-pathologically diagnosed patients of urinary bladder cancer following the method of convenience and 76 Age and sex matched healthy control to study VEGF gene polymorphism rs3025039 C>T SNP in patients suffering from urinary bladder cancer.

The current study observed that the mean (SD) and median (Inter quartile range) age of the study population, among the case was 58.73 (9.57) years and 60 (52, 64) years; and among the control was 59.53 (7.42) years, and 59 (55, 66) years respectively with no significant difference between two groups. These findings are well congruent with the study done by S.S. Malik et al. [11] where a similar age group of 59.5 (±15.76) years and 56.5(±15.67) years respectively was observed in the BC patients and healthy control group.

The reason for a greater prevalence and mortality of bladder cancer in men is most probably due to the greater androgen levels in males that inhibit the tumor killing cells and CD8+ lymphocytes providing a better chance for the cancer cells to survive and proliferate [12]. The time of diagnosis besides primary care plays an important role in the development of urinary bladder cancer also. In the present study we observed that male population were predominant in both bladder cancer cases and control group respectively (93.6% and 91.5% were males) with no significant difference. The findings corroborate with the finding by S.S. Malik et al[11].

In the present study we found that rs 3025039 C>T genotypes were significantly more among the cases than controls (Table 2A and 2B) in spite of the fact that 81% of the cases and 97% of the control population belonged to the uncut wild allele. These results suggest an increased association and risk for BC among the study population of our region who have the mutant T allele of this SNP. Association of the mutant T allele of the rs3025039 SNP with BC has been reported in some earlier studies e.g that of Garcia-Closas et al [13] in Spain in 2007. This significant association was reported in that study independent of the stage, severity and histopathological classification of BC as the authors did not evaluate the correlation of VEGF polymorphisms with these factors. Although the present study included all grades of bladder cancer for analyzing the association of the concerned SNP with BC, it did not evaluate the association of the mutant T allele with different types and histopathological grades of this

cancer and hence reports a straightway association of the T allele of rs3025039 SNP of the VEGF gene with BC.
The SNPs studied above increase the activity of the VEGF gene resulting in higher amount of its cognate protein that stimulates increased angiogenesis in the tumor cells, thus helping in its growth and spread. Furthermore, increased levels of VEGF in the tumor tissues along with an increased micro vessel density have been reported in recent studies which strongly support the hypothesis of overactivity of VEGF in pathogenesis of cancers including that of the urinary bladder[14, 15]. Zhang et al. [15] suggested that VEGF expression levels were significantly associated with tumor stage, tumor grade and lymph node metastasis (all $P < 0.05$). Keeping in track with these hypotheses, association of VEGF polymorphisms has been found with several malignancies including the bladder cancer. Association of several polymorphisms of the VEGF gene has been found with breast cancer patients [16]. Furthermore, patients having mutant allele of the VEGF rs3025039 C/T genotype were reported to be more susceptible to breast cancer in a case-control study in the Caucasian population [17]. All these findings strongly indicate the role of VEGF gene mutations in initiation and maintenance of carcinogenesis that includes several organ systems including the urinary bladder more frequently.
Thus, the findings of the previous studies and the current one by the present researcher strongly suggest the role of rs 3025039 C>T SNP of the VEGF gene in the pathogenesis of urinary bladder cancer. Replacement of the cytosine by the thymine in the mutated gene results in increased levels of VEGF in the tumor tissues leading to an increased micro vessel density. Reports of recent studies also strongly support the hypothesis of overactivity of VEGF in pathogenesis of cancers including that of the urinary bladder[14].
In conclusion, the outcome of the present study reiterates the importance of the genetic linkages associated with common cancers. Bladder cancer is a highly lethal malignancy and the increasing trends of bladder cancer are alarming, especially in the developing countries including India, and thus, there is a strong need to identify and implement effective prevention and treatment strategies. As the genetic linkages are present from very birth, so an early detection of those which are potentially dangerous can save the fatal outcome in a lot of cases. We suggest that an early genetic screening for the rs 3025039 C>T SNP in our region should be considered in the susceptible groups for bladder cancer so that a lot of morbidity and mortality can be saved.

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Conflict of interest: Nil

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