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CANCER STEM CELL GENE VARIANTS AS A MODIFIER IN URINARY BLADDER CANCER PATIENTS

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ABSTRACT

Aim: Cancer stem cell surface markers are potent candidates for various cancers. Therefore, we investigated role of three cancer stem cell markers viz. *CD24, CD26* and *CD28* in risk prediction and prognosis of bladder cancer (BC) in North India. **Results**: We analysed *CD24, CD26* and *CD28* gene polymorphisms in 240 BC patients and 270 controls to find out combinations of genetic variants and confounding risk factors contributing to BC risk. Significant high risk in variant genotype of *CD24rs3838646CA/Del* (p=0.001), dominant model (p=0.027) as well as in allelic model (p=0.002) and reduced risk was seen in *CD26rs7608798T/C* (p=0.006, OR=0.572) and the dominant model, TC+CC also revealed reduced risk (p=0.043, OR= 0.695). No association was found based on tumor stage/grade and smoking habit of BC patients as well as those treated with BCG immunotherapy with any of the candidate gene variant. On performing haplotype analysis, we found significant association in one out of four sets with p=0.014; OR=1.615. Gene combination between *CD24rs3838646CA/Del* and *CD26rs7608798T/C* showed marginal association with BC risk. **Conclusion**: Our result suggests that CSC marker gene polymorphism could be used as a marker for risk prediction to BC. However, this study needs to be further validated in other ethnicities.

KEYWORDS: Urinary Bladder Cancer; Cancer Stem Cell Markers; BCG Immunotherapy; Cancer Susceptibility.

INTRODUCTION

Cancer is the biggest threat globally and is the second most common disease in India responsible for maximum mortality with about 0.3 million deaths per year. Bladder cancer is ninth most common cancer worldwide. (*Cancer Facts and Figures*, 2015). Bladder cancer incidence is about 4 times higher in men than in women. In males, it is the fourth most common cancer (4% of male total), whereas it is the 13th most common cancer in females (2% of female total) (Ferlay et al., 2013). As a general prevalence, in India, out of 1, 00,000 people 3.0 male and 1 female develop BC each year (Murthy et al., 2010).

Cancer stem cells having the property to self-renew, differentiate, proliferate and migrate and hence they play an important role in tumorigenesis and disease progression. In our recent study, we have reported two cancer stem cell markers CD44 and CD166 gene variants for risk association of BC. Out of five variants of CD44 gene, three variants were found to be associated with low risk of BC (Verma et al., 2017). Out of three variants of CD166 gene, one variant was found to be associated with high risk of BC among North Indians (Verma et al., 2016). With these results, in the present manuscript, we have tried to find out the association of a set of three cancer stem cell markers viz. *CD24*, *CD26*, and CD28 with bladder cancer risk in North Indians.

CD24 is a well-known cell surface marker and a potential prognostic marker in a wide variety of malignancies. *CD24* gene expression was associated with histone acetylation-independent of DNA methylation, suggesting its epigenetic regulation in breast cancer (Kwon et al., 2015). *CD24* was reported to play an important role in anoikis resistance in ovarian cancer cells, a property leading to metastasis in cancer cells (Li et al., 2015). *CD24* expression at early stages of the cancer process showed highly invasive/metastatic tumor in breast cancer tumors (Rostoker et al., 2015). *CD24* is highly expressed in ovarian, breast, prostate, bladder, renal, non-small cell carcinomas, and other human cancers (Kristiansen et al., 2003; Zheng et al., 2011). It is involved in cell adhesion and metastasis (Lee et al.,

2010). This indicates that *CD24* could be a significant marker in tumor prognosis and diagnosis. Based on its function, *CD24* acts as an alternate ligand for P-selectin, on platelets and endothelial cells (Aigner et al., 1998), through which their interaction facilitates the passage of tumor cells in the blood stream during metastasis. Additionally, it induces the adhesion and proliferation of tumor cells to fibronectin, collagen, and lamin (Zheng et al., 2011). The metastatic associations of *CD24* increase its importance as a prognostic factor and a new CSC marker (Lee et al., 2010). *CD24* was found differentially expressed on bladder cancer cells, *CD24* expressions correlated with lower cancer-specific survival in patients (Hofner et al., 2014). *CD24* showed the potential involvement in the development of malignant bladder cancer and in the recurrence of tumors. A high *CD24* protein expression also relates to the higher risk of bladder cancer recurrence (Liu et al., 2013). In the present study, we have studied *CD24, CD24rs552812045, CD24rs3838646 CA/Del* variants of *CD24* gene.

CD26 or Dipeptidyl peptidase IV (DPPIV) is a transmembrane glycoprotein that inactivates or degrades some bioactive peptides and chemokines. For this reason, it regulates cell proliferation, migration, and adhesion, showing its role in cancer processes. Numerous studies have revealed the alterations in DPP4 in cancer (Cordero et al.,2009; Erić-Nikolić et al., 2011, Cordero et al., 2011; Matic et al., 2012), expression seems variable, since the enzyme levels is increased in some tumors and decreased in others (Havre et al. 2008). Different DPP4 expression status has been observed in malignant tumors, including colon, renal, and ovarian carcinoma. Data mining of the published dataset (GSE31684) identified that DPP4 is significantly upregulated in urothelial carcinoma of the urinary bladder. Here in this study, we have studied *CD26*rs7608798T/C variant of *CD26* gene.

CD28, cell surface protein is expressed by a number of myeloma cells. CD28 may not function by directly stimulating the growth of myeloma cells, but rather may be involved in promoting metastasis (Shapiro et al., 2001). TCRs and costimulatory CD28 signaling domains have shown tremendous promise for adoptive cancer immunotherapy (Haynes et al., 2003; Hombach et. al., 2001). The interaction of B7 ligand and CD28 receptor leads to the co-stimulation and -inhibition of T-cell. (Zang et al., 2010). Upregulated expression of mRNA, as well as protein, was seen in TCC samples as compared to control samples, indicating that higher expression of B7/CD28 may be important during the progression of bladder cancer (Wu et al., 2015). In this study, we have studied CD28rs3116496T/C variant of CD28 gene in BC risk.

With all the background about the candidate genes, a case–control investigation was performed for 4 SNPs of *CD24*, *CD26*, and CD28 genes to analyze their contribution to risk prediction of BC and the associations between risk factors and bladder cancer clinicopathologic characteristics. Our investigations in the present paper are based on the hypothesis that *CD24*, *CD26*, and CD28 gene polymorphisms are associated with bladder cancer and may be effectively used in the risk assessment and genetic epidemiological analysis of bladder cancer.

MATERIAL AND METHODS

Ethics Statement

The study design was approved by the ethical committee of Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS) Lucknow (India). The authors followed the norms of World's Association Declaration of Helsinki. Duly signed informed consents were taken from each study subject. The cancer staging was done per the TNM classification system. (Colombel et al., 2008)

Study subjects

A total of 510 samples (240 confirmed bladder cancer patients and 270 healthy controls) were recruited in the present study. The patients were enrolled from outpatient department (OPD) of Urology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, a tertiary care center. All subjects in this study were of similar ethnicity from North India. The criteria for selecting patients: those with a previous history of other cancer, cancer metastasized to other site of body from another origin and previous radiotherapy were excluded, only histopathologically confirmed BC cases were included. At the same time, 270 healthy controls (Mean age = 54.5 years, M:F= 249:21) were taken from individuals who visited the hospital for their checkups, taking care they are unrelated to patients recruited and should be age and ethnicity matched. The criteria for selecting controls included no evidence of any personal history of cancer or other malignant conditions or any other chronic diseases.

The ratio of male: female among 240 patients was 211:29, with the mean age of 56.9 years. The ratio between male and female BC in the present study may be due to high prevalence in males (3:1). Duly signed consent forms were obtained from all the BC patients enrolled for the investigation.

Epidemiology Data Collection

Demographic characteristics data, such as gender, age, occupation history, tobacco history and other lifestyle factors, as well as clinicopathological examination were collected by conducting a personal interview based on a preformed questionnaire. Individuals who smoked for more than 5 years were considered as smokers. Individuals who never smoked were regarded as non-smokers. At the end of the interview, 5 ml of blood sample was drawn into coded EDTA vials. The demographic and clinical characteristics of the patients are demonstrated in Table 1.

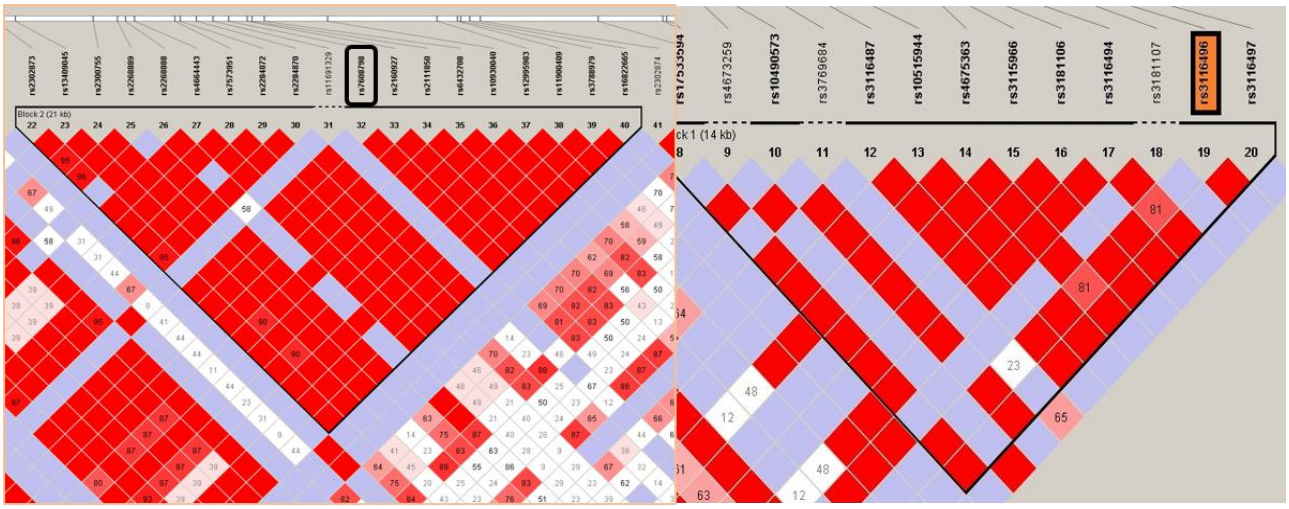
Clinical Data Collection

The clinical information about the recruited patients viz., tumor stage and grade, tumor size, interventional therapy, time of relapse, etc. were provided by the urologists in our department. The classification tumor stages were as per the American Joint Committee on Cancer's TNM staging system (Colombel et al., 2008). Of the 240 total patients enrolled in the study, 180 patients had non-muscle invasive bladder cancer (NMIBC) whereas other 60 patients had muscle invasive bladder cancer (MIBC). Patients with NMIBC at high risk (high grade, multiple and large tumor) were treated with intravesical *Bacillus* Calmette-Guerin (BCG) (n=86). The patients with non-muscle invasive BC of low risk were on cystoscopic examination and considered as non-BCG patients. Subsequently, all the patients were examined by cystoscopy after every 3 months in first and second years and later at bi-annual intervals if there was no tumor recurrence. BCG treatment consisted of 6 weekly instillation induction BCG (n=86). Since the number of patients receiving maintenance BCG was too low, we did not categorize the patients per BCG regime for statistical analysis. Blood sample was collected in EDTA from all subjects for genotyping at the time of enrollment and stored at -70ºC.

SNP Selection

The potentially functional polymorphisms within the *CD24*, *CD26* and *CD28* genes were selected by using the HapMap Project database (www.hapmap.org). We used certain criteria for the candidate gene polymorphisms viz., a minor allele frequency (MAF) greater than 10% in Caucasian population; located in the 3'UTR, 5'UTR, intronic/exonic regions of the genes which shows some biological significance. The SNPs were selected from the Haploview software 4.2 (Mark Daly's lab of Broad Institute, Cambridge, MA, Britain) (http://hapmap.ncbi.nlm.nih.gov/), based on the GIH population data of HapMap (HapMap Data Rel 27 PhaseII +III, Feb 09, on NCBI B36 assembly, dbSNP b126).

Tagger SNP rs7608798 was found to represent the known SNP in the haplotype blocks 3 in the *CD26* gene of GIH population and SNP rs3116496 of *CD28* gene also represents the haplotype block 3. In addition to these SNPs two SNPs of *CD24* gene, rs52812045 and rs3838646 is also included in this study. Basis of selecting these two SNPs of *CD24* gene was their functionality reported in various cancers and other diseases.



a) Linkage Disequilibrium Plot of *CD26* gene in HapMap-GIH Population

b) Linkage Disequilibrium Plot of *CD28* gene in HapMap-GIH Population

Figure 1: Linkage Disequilibrium Plots of a) *CD26* and b) *CD28* gene in HapMap-GIH Population.

Genotyping

Genomic DNA was extracted from venous blood by following standard salting out method.[14] Genotyping of *CD24rs52812045C/T* and *CD24rs3838646CA/Del* SNPs were carried out by using Taqman allelic discrimination assay. The primers and probes for the assay were procured by Applied Biosystems (Foster City, CA). Genotyping was performed with ABI 7500HT Fast Sequence Detection System (Applied Biosystems, Foster City, CA) using 96-well plates. Positive and negative controls were used along with every reaction plate, and 10% samples were randomly selected and checked in duplicates with 100% concordance. The results were reproducible with no discrepancy in genotyping. The polymorphism in *CD26rs7608798T/C* and *CD28rs3116496T/C* was genotyped by ARMS-PCR (Amplified Refractory Mutation System-Polymerase Chain Reaction) analysis. The primer sequence used for *CD26* (Higashibata et al., 2013) and *CD28* (Meyer et al., 2005) were adopted from a previous study. Genotyping was done on 10% Poly-Acrylamide Gel and visualized after staining with ethidium bromide. Positive and negative controls were used in each genotyping assay, and 10% of the samples were randomly selected and run

in duplicates with 100% concordance. The results were reproducible with no discrepancy in genotyping.

Statistical Analysis

Power of the study was calculated by using Quanto software, version 1.0 (available from: http://hydra.usc.edu/gxe) The present study achieved 80% of the statistical power. The goodness-of-fit chi square test was used to analyze any deviation from the Hardy-Weinberg equilibrium in controls. A binary logistic regression model was used to estimate the risk as the OR at the 95% confidence interval. The statistical analysis was done using the Statistical Package for Social Sciences software, version 16.0 (SPSS, Chicago, IL), and $P<0.05$ was considered statistically significant. Haplotype analysis was done by SNP analyzer version 1.2A. Hardy-Weinberg equilibrium test was performed by Michael H. Court's (2005–2008) online calculator (http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20-%20HW%20calculator.xls).

Tests in bladder cancer patients and healthy unrelated controls did not show any significant deviation from HWE for any of the SNPs.

RESULTS

Characteristics of Study Subjects

The selected demographic characters of cases and controls are shown in Table 1. There was no significant difference between the patients and controls in accordance with age (p=0.138), and sex (p=0.105). However, there were more patients with a habit of smoking (70.4%) among the cases than among the controls (20.7%) (p <0.001) (Table 1).

Table 1: Demographic and clinical characteristics of Bladder Cancer Patients and Healthy Controls.

Variables	Cases n=240 n(%)	Controls n=270 n(%)	Chi square #p-value
Sex			
Female	29(12.1)	21(7.8)	0.105
Male	211(87.9)	249(92.2)	
Age (Years)			
Mean age ±SD	56.96±13.86	54.50±10.23	0.138
Smoking*			
Non Smokers	48(29.6)	214(79.3)	<0.001
Smokers	116(70.4)	56(20.7)	
Tumor Grade Stage			
TaG1	48(20.0)	-----	-----
TaG2-3+T1G1-3	128(53.3)	-----	
T2+	64(26.7)	-----	
Intravesical Therapy			
Non treated	83(47.7)	-----	-----
BCG Induction (BCG i+m)	86(52.3)	-----	
Event			
Recurrence	74(43.9)	-----	-----
Non-Recurrence	95(56.1)	-----	

#Student t-test was used to determine the p-value
Statistically significant values are shown in bold
BCG i+m, Bacillus Calmette-Guerin induction + maintenance.

Genotypic and Allelic Frequency of *CD24rs52812045C/T*, *CD24rs3838646CA/Del*, *CD26rs7608798T/C* and *CD28rs3116496T/C* gene Polymorphisms in Bladder Cancer

The observed genotype frequencies of four SNPs studied in healthy controls were in accordance with Hardy-Weinberg Equilibrium. The genotypic and allelic frequencies of *CD24rs52812045C/T*, *CD24rs3838646CA/Del*, *CD26rs7608798T/C and CD28rs3116496T/C* gene polymorphisms in context with bladder cancer risk among patients and controls are depicted in Table 2.

Table 2. Association of *CD24rs52812045C/T, CD24rs3838646CA/Del, CD26rs7608798T/C and CD28rs3116496T/C* gene Variants with Bladder Cancer Risk.

Genetic Model	Genotypes	Controls n=270 n(%)	Patients n=240 n(%)	p value	OR*(95%CI)
	CD24rs52812045C/T				
Additive	CC	71(26.3)	70(29.2)	**Ref**	**Ref**
	CT	118(43.7)	115(47.9)	0.957	0.988(0.651-1.502)
	TT	81(30.0)	55(22.9)	0.124	0.689(0.428-1.108)
Dominant	CC	71(26.3)	70(29.2)	**Ref**	**Ref**
	CT+TT	199(73.7)	170(70.8)	0.470	0.866(0.588-1.278)
Multiple	C	260(48.1)	255(53.1)	**Ref**	**Ref**
	T	280(51.9)	225(46.9)	0.113	0.819(0.640-1.048)
	CD24rs3838646CA/Del				
Additive	CA/CA	139(51.5)	100(41.7)	**Ref**	**Ref**
	CA/Del	115(42.6)	105(43.8)	0.205	1.269(0.878-1.835)
	Del/Del	16(5.9)	35(14.6)	0.001	3.041(1.595-5.795)
Dominant	CA/CA	139(51.5)	100(41.7)	**Ref**	**Ref**
	CA/Del+Del/Del	131(48.5)	140(58.3)	0.027	1.485(1.046-2.109)
Multiple	CA	393(72.8)	305(63.5)	**Ref**	**Ref**
	Del	147(27.2)	175(36.5)	0.002	1.534(1.176-2.000)
	CD26rs7608798T/C				
Additive	TT	101(37.4)	111(46.3)	**Ref**	**Ref**
	TC	113(41.9)	71(29.6)	0.006	0.572(0.383-0.854)
	CC	56(20.7)	58(24.2)	0.799	0.942(0.598-1.486)
Dominant	TT	101(37.4)	111(46.3)	**Ref**	**Ref**
	TC+CC	169(32.6)	129(53.8)	0.043	0.695(0.488-0.989)
Multiple	T	315(58.3)	293(61.0)	**Ref**	**Ref**
	C	225(41.7)	187(39.0)	0.379	0.894(0.695-1.148)
	CD28rs3116496T/C				
Additive	TT	101(37.4)	96(40.0)	**Ref**	**Ref**
	TC	129(47.8)	108(45.0)	0.511	0.881(0.603-1.286)
	CC	40(14.8)	36(15.0)	0.840	0.947(0.557-1.609)
Dominant	TT	101(37.4)	96(40.0)	**Ref**	**Ref**
	TC+CC	169(32.6)	144(60.0)	0.548	0.896(0.627-1.281)
Multiple	T	331(61.3)	300(62.5)	**Ref**	**Ref**
	C	209(38.7)	180(37.5)	0.693	0.950(0.738-1.224)

Statistically significant values are shown in bold
*Age and gender adjusted Odds ratio; CI, Confidence Interval.

No significant differences were observed in the frequency distribution of *CD24rs52812045C/T* and *CD28rs3116496T/C* polymorphisms between bladder cancer patients and healthy controls, both at the genotypic and allelic levels. We found significant high risk in variant genotype, Del/Del of additive model of *CD24rs3838646CA/Del* (p=0.001**,** Adjusted OR=3.041), dominant model (p=0.027, Adjusted OR=1.485) as well as in variant allele, Del of allelic model (p=0.002, OR=1.534). A reduced risk was seen in *CD26rs7608798T/C*, heterozygous genotype, TC of additive model (p=0.006, Adjusted OR=0.572) and the dominant model, TC+CC also revealed reduced risk (p=0.043, Adjusted OR= 0.695) whereas at allelic level no association was seen.

Association of *CD24, CD26 and CD28* gene Variants at Genotypic Level with Smoking

We correlated the genotypes of all four polymorphisms i.e. *CD24rs52812045C/T, CD24rs3838646CA/Del, CD26rs7608798T/C and CD28rs3116496T/C* for the patients with smoking habits by using univariate analysis. For this analysis, we stratified patients as smokers and non-smokers.

No association was seen in any of the four variants of *CD24rs52812045C/T, CD24rs3838646CA/Del, CD26rs7608798T/C and CD28rs3116496T/C* with respect to smoking. (Table 3)

Table 3: Analysis of *CD24rs52812045C/T, CD24rs3838646CA/Del, CD26 rs7608798T/C and CD28rs3116496T/C* gene variants based on Smoking.

Genotype	Patients Non Smokers n= 48 n(%)	Patients Smoker n=116 n(%)	p – value	OR*(95% CI)
CD24rs52812045C/T				
CC	17(35.4)	25(21.6)	**Ref**	**Ref**
CT	23(47.9)	66(56.9)	0.092	1.951(0.897-2.247)
TT	8(16.7)	25(21.6)	0.142	2.125(0.777-2.815)
CD24rs3838646CA/Del				
CA/CA	18(37.5)	47(40.5)	**Ref**	**Ref**
CA/Del	20(41.7)	53(45.7)	0.969	1.015(0.480-1.145)
Del/Del	10(20.8)	16(13.8)	0.317	0.613(0.235-1.099)
CD26rs7608798T/C				
TT	20(41.7)	61(52.6)	**Ref**	**Ref**
TC	17(35.4)	30(25.9)	0.169	0.579(0.265-1.063)
CC	11(22.9)	25(21.6)	0.508	0.745(0.312-1.180)
CD28rs3116496T/C				
TT	16(33.3)	45(38.8)	**Ref**	**Ref**
TC	28(58.3)	53(45.7)	0.289	0.673(0.324-1.109)
CC	4(8.3)	18(15.5)	0.452	1.600(0.470-2.044)

*Age and gender adjusted Odds ratio; CI, Confidence Interval.

Association of *CD24rs52812045C/T, CD24rs3838646CA/Del, CD26rs7608798T/C and CD28rs3116496T/C* Genotypes with Tumor Stage /Grade of Bladder Cancer Patients

To study the association of polymorphisms of *CD24, CD26 and CD28* genes with tumor stage /grade, the BC patients were stratified into three groups based on their tumor stage/grade [TaG1 (low risk NMIBC), TaG_{2-3}+$T1G_{1-3}$ (High risk NMIBC) and T2+ (muscle invasive)]. TaG_1 was taken as a reference. We did not find any association of four gene variants *CD24rs52812045C/T, CD24rs3838646CA/Del, CD26rs7608798T/C and CD28rs3116496T/C* with any of the tumor stage/grade of BC patients. (Table S1)

Table S1: Association of *CD24rs52812045C/T, CD24rs3838646CA/Del, CD26 rs7608798T/C and CD28rs3116496T/C* polymorphism with tumor grade/stage in Bladder cancer patients.

Genotypes	n=48 n(%)	(b) n=128 n(%)	(c) n=64 n(%)	p-value (a-b)	OR* (95%CI)	p-value (a-c)	OR* (95%CI)	p-value (b-c)	OR* (95%CI)
CD24rs52812045C/T	**TaG1**	**TaG2-3, T1G1-3**	**T2+**						
CC	14(29.2)	34(26.6)	22(34.4)	**Reference**		**Reference**		**Reference**	
CT	22(45.8)	65(50.8)	28(43.8)	0.626	1.217 (0.553-1.675)	0.636	0.810 (0.339-1.138)	0.252	0.666 (0.332-1.135)
TT	12(25.0)	29(22.7)	14(21.9)	0.992	0.995 (0.398-2.188)	0.568	0.742 (0.267-1.062)	0.491	0.746 (0.324-1.217)
CD24rs3838646CA/Del	**TaG1**	**TaG2-3, T1G1-3**	**T2+**						
CA/CA	20(41.7)	52(40.6)	28(43.8)	**Reference**		**Reference**		**Reference**	
CA/Del	20(41.7)	56(43.8)	29(45.3)	0.841	1.077 (0.521-1.225)	0.932	1.036 (0.461-1.325)	0.905	0.962 (0.506-1.628)
Del/Del	8(16.7)	20(15.6)	7(10.9)	0.937	0.962 (0.365-1.533)	0.429	0.625 (0.195-1.205)	0.387	0.650(0.245-1.124)
CD26rs7608798T/C	**TaG1**	**TaG2-3, T1G1-3**	**T2+**						
TT	21(43.8)	63(49.2)	27(42.2)	**Reference**		**Reference**		**Reference**	
TC	13(27.1)	43(33.6)	15(23.4)	0.809	1.103 (0.499-1.637)	0.821	0.897 (0.352-1.289)	0.586	0.814 (0.388-1.707)
CC	14(29.2)	22(17.2)	22(34.4)	0.128	0.524 (0.228-1.204)	0.655	1.222 (0.507-1.946)	0.025	2.333 (1.110-2.907)

CD28rs3116496T/C	**TaG1**	**TaG2-3, T1G1-3**	**T2+**						
TT	26(54.2)	45(35.2)	25(39.1)	**Reference**		**Reference**		**Reference**	
TC	14(29.2)	63(49.2)	31(48.4)	0.113	2.600 (1.223-3.126)	0.510	2.303 (0.998-3.163)	0.715	0.886 (0.462-1.298)
CC	8(16.7)	20(15.6)	8(12.5)	0.449	1.444 (0.558-1.841)	0.945	1.040 (0.338-1.298)	0.500	0.720 (0.277-1.170)

*Age and gender adjusted Odds ratio; CI, Confidence Interval.

Modulation of *CD24, CD26 and CD28* Genotype Variants and Outcome after BCG Immunotherapy

For analyzing the association of *CD24rs52812045C/T, CD24rs3838646CA/Del, CD26rs7608798T/C and CD28rs3116496T/C* gene variants and the risk of recurrence in NMIBC patients, the further analysis was confined only to NMIBC patients (n=180). We analyzed the association of genotypes and risk of recurrence after giving BCG immunotherapy. The patients were grouped into BCG treated (n= 94) and non-treated (n= 86) because the patients with low grade tumors did not require BCG immunotherapy. None of the polymorphisms were associated with risk of recurrence. (Table S2)

Table S2: Association of *CD24, CD26 and CD28* gene polymorphisms with the risk of recurrence in NMIBC BCG treated patients.

Genotypes	**BCG Treated Patients**			
	No Recurrence N=49	**Recurrence N=37**	**p-value**	**HR* (95%CI)**
	n (%)	**n (%)**		
CD24rs52812045C/T				
CC	10(20.4)	15(40.5)	**Ref**	**Ref**
CT	26(53.1)	16(43.2)	0.366	0.799(0.453-1.339)
TT	13(26.5)	6(16.3)	0.801	1.087(0.569-1.707)
CD24rs3838646CA/Del				
CA/CA	18(36.8)	19(51.4)	**Ref**	**Ref**
CA/Del	20(40.8)	12(32.4)	0.104	0.649(0.385-1.093)
Del/Del	11(22.4)	6(16.2)	0.135	0.597(0.303-1.174)
CD26rs7608798T/C				
TT	21(42.9)	18(48.6)	**Ref**	**Ref**
TC	19(38.8)	14(37.8)	0.960	0.986(0.568-1.712)
CC	9(18.3)	5(13.6)	0.571	1.187(0.657-1.943)
CD28rs3116496T/C				
TT	15(30.6)	13(35.1)	**Ref**	**Ref**
TC	24(49.0)	20(54.1)	0.646	0.890(0.541-1.465)
CC	10(20.4)	4(10.8)	0.201	0.327(0.126-0.846)

Association of *CD24* Haplotypes with Bladder Cancer Risk

Haplotypes are more powerful discriminators between cases and controls in disease association studies. It may manifest in risk prediction and association of disease as compared with an analysis of single nucleotide polymorphism. Having this in mind we examined the effects of *CD24* gene variants by constructing haplotype sets, taking combination C/CA as a reference as these two alleles were wild alleles from the two candidate SNPs. We found significant association with risk of bladder cancer in case of C $_{CD24rs52812045C/T}$ Del $_{CD24rs3838646 CA/Del}$ combinations (C/Del p=0.014, OR= 1.615), after applying Bonferroni correction the p value became marginally significant (pc=0.056). (Table 4)

Table 4: Haplotype analysis among *CD24rs52812045C/T* and *CD24rs3838646CA/Del.*

Haplotype Combination	**Controls n (%)**	**Patients n (%)**	**p Value**	**OR* (95% CI)**
C $_{CD24rs52812045C/T}$ CA $_{CD24rs3838646 CA/Del}$	195(36.2)	166(34.7)	**Ref**	**Ref**
T $_{CD24rs52812045C/T}$ CA $_{CD24rs3838646 CA/Del}$	197(36.6)	138(28.9)	0.203	0.823(0.609-1.111)
T $_{CD24rs52812045C/T}$ Del $_{CD24rs3838646 CA/Del}$	82(15.2)	86(18.0)	0.265	1.232(0.854-1.777)
C $_{CD24rs52812045C/T}$ Del $_{CD24rs3838646 CA/Del}$	64(12.0)	88(18.4)	$^{\#}$0.014	1.615(1.102-2.368)

*Age and gender adjusted Odds ratio; CI, Confidence Interval.

Statistically significant values are shown in bold. $^{\#}$After applying Bonferroni correction pc=0.056

Gene-gene interaction in between *CD24rs3838646CA/Del* and *CD26rs7608798T/C*

To find out combined effect of these SNPs, gene-gene interaction analysis was done. Gene combination between *CD24rs3838646CA/Del* and *CD26rs7608798T/C* showed marginal association with BC risk (CA/CA $_{CD24rs3838646\ CA/Del}$ +TC $_{CD26rs7608798T/C}$ P=0.052; Del/Del $_{CD24rs3838646\ CA/Del}$ +TT $_{CD26rs7608798T/C}$ p=0.051) (Table 5).

Table 5: Gene-gene interaction in between *CD24rs3838646CA/Del* and *CD26rs7608798T/C.*

Combinations	Controls N (%)	Patients N (%)	p value	OR* (95%CI)
CD24rs3838646 CA/Del* and *CD26rs7608798 T/C				
CA/CA $_{CD24rs3838646\ CA/Del}$ +TT $_{CD26rs7608798T/C}$	55(20.4)	52(21.7)	**Ref**	**Ref**
CA/CA $_{CD24rs3838646\ CA/Del}$ +TC $_{CD26rs7608798T/C}$	55(20.45)	29(12.1)	0.052	0.558(0.310-1.004)
CA/CA $_{CD24rs3838646\ CA/Del}$ +CC $_{CD26rs7608798T/C}$	29(10.7)	19(7.9)	0.299	0.693(0.347-1.384)
CA/Del $_{CD24rs3838646\ CA/Del}$ +TT $_{CD26rs7608798T/C}$	41(15.2)	45(18.8)	0.607	1.161(0.658-2.049)
CA/Del $_{CD24rs3838646\ CA/Del}$ +TC $_{CD26rs7608798T/C}$	51(18.9)	31(12.9)	0.139	0.643(0.358-1.155)
CA/Del $_{CD24rs3838646\ CA/Del}$ +CC $_{CD26rs7608798T/C}$	23(8.5)	29(12.1)	0.397	1.334(0.685-1.595)
Del/Del $_{CD24rs3838646\ CA/Del}$ +TT $_{CD26rs7608798T/C}$	5(1.9)	14(5.8)	0.051	2.962(0.996-3.802)
Del/Del $_{CD24rs3838646\ CA/Del}$ +TC $_{CD26rs7608798T/C}$	7(2.6)	11(4.6)	0.329	1.662(0.599-2.612)
Del/Del $_{CD24rs3838646\ CA/Del}$ +CC $_{CD26rs7608798T/C}$	4(1.5)	10(4.2)	0.118	2.644(0.781-2.956)

*Age and gender adjusted Odds ratio; CI, Confidence Interval
Statistically significant values are shown in bold

DISCUSSION

CD24 promotes tumor cell proliferation and changes the adhesive properties of tumor cells (e.g., by promoting their adhesion to P-selectin, fibronectin, collagens I and IV, and laminin). Additionally, cell spreading, motility, and invasiveness are strongly increased upon *CD24* expression. Several case-control studies have been performed to investigate the putative association of *CD24* gene polymorphisms with cancer development and progression, although the results are controversial. In the present study, we performed a case control association among 240 BC patients and 270 unrelated healthy controls. In our study *CD24rs52812045C/T* did not show any association and risk to bladder cancer. There are other studies which supports our study viz. *CD24rs52812045C/T* did not show any risk to breast cancer in German population (Buck et al., 2013) and in Chinese population (Zhou X, 2014). Contrary to our results *CD24rs52812045C/T* gene also found to be a risk predictor in some studies. It is found associated with risk to pancreatic ductal carcinoma in Israeli population (Shamai et al., 2015) and with risk to colorectal liver metastasis in Austrian population (Stremitzer et al., 2015). *CD24rs52812045C/T* is also studied in diseases other than cancer like oral lichen planus; no risk (Kaplan et al., 2015), multiple sclerosis; no risk (Goris et al., 2006) and Inflammatory bowel disease; high risk (Lisiansky et al., 2014). The varied behavior of the candidate SNP may be due to nature of disease or ethnicity variation.

In the present study *CD24rs3838646Del/CA* gene found associated with risk to bladder cancer among North Indians. Supporting our study *CD24rs3838646Del/CA* gene found associated with risk to pancreatic ductal carcinoma in Israeli population (Shamai et al., 2015). Whereas there are studies reporting no association of *CD24rs3838646Del/CA* to breast cancer in Chinese population (Zhou X, 2014) and German population (Buck et al., 2013).

The present study found association of *CD26 rs7608798* with no risk to bladder cancer among North Indians. *CD26 rs7608798* is associated with high risk to prostate cancer in Japanese population (Higashibata et al., 2013). Another study in myocardial infarction revealed its association with higher risk to the disease among Europeans (Aghili et al., 2012). Ethnicity variation and nature of disease may lead to a varied behavior of association as well as risk prediction of the candidate molecule.

The etiology and pathogenesis of bladder cancer depend on multiple factors. A good understanding of patient's genetic background could help in bladder cancer prognosis, diagnosis and may be treatment. In this case-control study, we genotyped *CD28rs3116496T/C* SNP in bladder cancer patients and found no significant association with the disease. There have been many studies which report similar results as of the present study like *CD28rs3116496T/C* showed no risk to melanoma in German population (Bouwhuis et al., 2010), it also revealed no significant association with cervical squamous carcinoma in Polish population (Pawlak et al., 2010), also no association was seen with cervical cancer in Swedish population (Ivansson et al., 2010). Whereas there are studies which show involvement of *CD28rs3116496T/C* with high risk to many cancers. *CD28rs3116496T/C* is found associated with risk to sporadic breast cancer in Han Chinese population (Chen et al., 2012). It is also associated with high risk to renal cell carcinoma in Polish population (Tupikowski et al., 2015). *CD28rs3116496T/C* also had studied on diseases other than cancers like it showed

high risk of acute kidney allograft rejection in Polish population (Pawlik et al., 2014).

To the best of our knowledge the present study reported here is the first study from Northern India to show the association between cancer stem cell markers and bladder cancer. These results further need to be validated at transcriptional as well as translational level. The tissue level expression is warranted for more studies to conclude the candidate SNPs as prognostic and diagnostic biomarkers.

CONCLUSION

Our results suggested that *CD24rs3838646Del/CA* and *CD26 rs7608798* perhaps could be used as a predictive marker for high risk to BC and reduced risk to BC respectively, in North Indian population if worked extensively at functional level along with the polymorphic study in larger sample size and different ethnicity.

DISCLOSURE

Authors have no conflicts of interest in this work.

AUTHOR'S CONTRIBUTION

AV had done the work, drafted the manuscript and interpreted the data. RK provided the samples and provided relevant clinical data. Inception and planning of the study was done by RDM and manuscript bought to final stage by her.

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