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Genotypic analysis of *XRCC4* and susceptibility to cervical cancer

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ABSTRACT

Background: *XRCC4* encodes a DNA repair protein which maintains genome stability by repairing double-strand breaks by the error-prone method. Defects in the protein-encoding gene lead to impairment of DNA repair process and accumulation of DNA damage, a hallmark of cancer development. We hypothesised that variants in *XRCC4* are linked to cervical cancer.
Material and methods: Genotyping of *XRCC4* variants *viz*. intron3 DIP (rs28360071), intron7 DIP (rs28360017), G-1394T(rs6869366) and G-652T (rs2075685) was carried out in 246 women with cervical cancer cases and 246 control women.
Results: There were several links to cervical cancer: intron3 DIP (rs28360071) II genotype ($p = 0.002$) and I allele (odds ratio is 0.54–0.89) ($p = 0.004$), intron7 DIP (rs28360017) II genotype ($p = 0.003$) and I allele (odds ratio 0.68 [0.53–0.88]) ($p = 0.004$), and G-652T (rs2075685) genotype ($p = 0.044$) and the T allele (odds ratio 1.35 [1.03–1.77]) ($p = 0.032$). In combining data into haploviews, the DDGG allele combination had an odds ratio of 0.12 (0.04–0.39) ($p= 0.029$) and the IIGT combination an odds ratio of 3.08 (1.25–7.55) ($p = 0.01$) for cervical cancer.
Conclusion: Our results suggested that homozygous 'I' and 'T' genotypes in certain *XRCC4* sequences may be associated with the development of cervical cancer and so may be a useful biomarker to predict cervical cancer susceptibility

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Introduction

Cervical cancer is the third leading cause of female cancer and second most common between 15 and 44 years of age worldwide, with around 469.1 million women being at risk. The prevalence is estimated to be around 2.5 million, with about 569,847 new cases and 311,365 deaths in 2018 [1]. Epidemiological and clinical studies suggest that the aetiology of cervical cancer is a multifactorial process in which infection with human papillomavirus (HPV) takes the central place. Other risk factors for cervical cancer include HPV infection combined with smoking, having many sexual partners, early age at first intercourse, age at first pregnancy, parity, menstrual hygiene, early use of hormonal contraceptives, family history of cervical cancer, dietary factors and stress-related disorders. Cervical cancer begins in cells lining the cervix. However, these do not suddenly change into cancer instead the normal cells of cervix gradually develop pre-cancerous lesions and then transform into cancer, a process that may take 10–15 years [2].

The human genome is maintained by repair pathways that sense DNA damage and which may be exogenous and/or endogenous. If these pathways fail to repair DNA damage, the molecular machinery can sense defects and trigger apoptosis. However, when these DNA damaged cells are neither repaired nor undergo apoptosis, they may transform into tumours [3]. Due to defects in DNA repair, decreased genomic stability and integrity of genes play a critical role in cancer initiation and progression i.e. tumorigenesis. Two distinct DNA repair pathways are responsible for correcting double strand breaks (DSBs): homologous recombination (HR) and non-homologous end joining (NHEJ) [4].

The DNA repair gene, X-Ray Cross Complementary gene-4 (*XRCC4*), located on 5q14.2, maintains overall genome stability and is a member of NHEJ system which cooperates with ligase 4 to reverse the DNA DSBs and support V(D)J recombination [5–7]. XRCC4 protein plays a role in the Go/G1 phase of cell cycle in eukaryotes [8]. Polymorphism studies of *XRCC4* gene have been associated with different types of cancers such as bladder [9], breast [10], gastric [11], oral [12] and colorectal [13].

We hypothesised that any of four Single Nucleotide Polymorphisms (SNPs) of *XRCC4 viz*. intron3 Deletion/Insertion polymorphism (DIP) (rs28360071), intron7 DIP (rs28360317), G-1394T (rs6869366) and G-652T (rs2075685) are associated with cervical cancer. These polymorphisms are most frequent and subtle genetic variations in the human genome and have great potential for application to association studies in complex diseases.

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Materials and methods

We recruited 246 women with cervical cancer and 246 normal healthy age-matched control subjects enrolled from the outpatient unit of Department of Obstetrics and Gynecology, King George's Medical University, Lucknow, India. The study was conducted after due approval of Institutional Ethics Committee (No. 4135/R.Cell-13, dated 15 April 2013) and written consent from all subjects. After selection, subjects were counselled, and cervical biopsy was conducted by expert gynaecologists and sent for histopathological examination. Clinical details of patients and other risk factors *viz*. smoking status, parity, age at full term pregnancy, use of contraception, etc. were precisely recorded. Blood samples (3 mL) from all study subjects were collected in EDTA vials and stored at −20°C until further use.

The inclusion criteria for cases were histopathologically proven squamous cell carcinoma, all stages of cervical intraepithelial neoplasia. Women between 40 and 70 years and having symptoms such as vaginal discharge, pain in lower abdomen, menstrual irregularity, contact bleeding and cervical biopsy positive were included. Patients age >70 years, double malignancy, co-morbid conditions such as diabetes, tuberculosis, etc., cervical biopsy negative, already on follow-up and those not willing to participate in the study were excluded from the study. The healthy age matched, histopathologically negative for squamous cell carcinoma, all stages of cervical intraepithelial neoplasia with no previous history of any type of cancer were selected as control subjects for the study.

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) using salting out method [14] with slight modifications [15]. Deletion/insertion polymorphisms intron3 DIP and intron7 DIP were genotyped using polymerase chain reaction (PCR) amplification. *XRCC4* G-1394T and G-652T polymorphisms were genotyped in controls and cases using PCR and restriction fragment length polymorphism (PCR–RFLP). Details including the location of SNPs in respective genes, primer sequences and restriction enzymes with product sizes are presented in Table 1. PCR was performed in a 25 µl reaction mixture containing genomic DNA (50–100 ng), 10 pmol of each primer, 200 µM dNTPs, and 0.5 U of Taq DNA polymerase (MBI-Fermentas, USA) in a gradient Master Cycler (Eppendorf, Germany). The PCR products were digested with respective restriction enzymes, resolved on 2% agarose and 12% polyacrylamide gels.

Allele frequencies of alleles in all groups were compared in a 2 × 2 contingency table and genotype frequencies in a 2 × 3 contingency table using Chi-square test (χ^2) and Fisher's exact *t*-test. Hardy–Weinberg equilibrium at individual locus was assessed by χ^2 statistics using Statistical Package for Social Science (SPSS) version 21.0. All *p* values were two-sided and differences were considered statistically significant for $p < 0.05$. Odds ratio (OR) at 95% confidence intervals (CI) was determined to describe the strength of association by Logistic Regression Model. Haplotype analysis and Pairwise Linkage Disequilibrium (LD) based on 'D' statistics and correlation coefficient (r2) of frequencies was analysed using SHEsis [16].

Results

Genotyping of *XRCC4* intron3 DIP (rs28360071), intron7 DIP (rs28360317), G-1394T (rs6869366) and G-652T (rs2075685) were carried out cervical cancer cases and age-matched healthy subjects (Figure 1). The allele and genotype frequency distributions as well as carriage rates are shown in Table 2. All allelic and genotypic frequencies were found to be in Hardy–Weinberg equilibrium (HWE).

Table 1. Primer sequences, PCR conditions, amplicon sizes, restriction enzymes and allele sizes.

Reference SNP (rs number)	Positions	Primer Sequences	Annealing Temp. (°C)	Product Size (bp)	Restriction enzyme/ allele size
rs28360071	Intron3 DIP	F:TCCTGTTACCATTTCAGTGTTAT R:CACCTGTGTTCAATTCCAGCTT	51.5	139 109	-
rs28360317	Intron7 DIP	F1:ATACTGTGTTTGGAACTCCT for CCT-positive F2:ATACTGTGTTTGGAACTAGA for CCT-negative R:ATCCTATCATCTCTGGATA	54.5	239	-
rs6869366	G-1394T	F-GATGCGAACTCAAAGATACTGA R-TGTAAAGCCAGTACTCAAACTT	56.0	300	*HincII* TT 300 GT 300,200,100 GG 200,100
rs2075685	G-652T	F-GCTAGACACCACTCCAATAA R-GGCTACGTAGATTATGTGTG	55.5	326	*MboII* TT 326 GT 326,199,127 GG 199,127

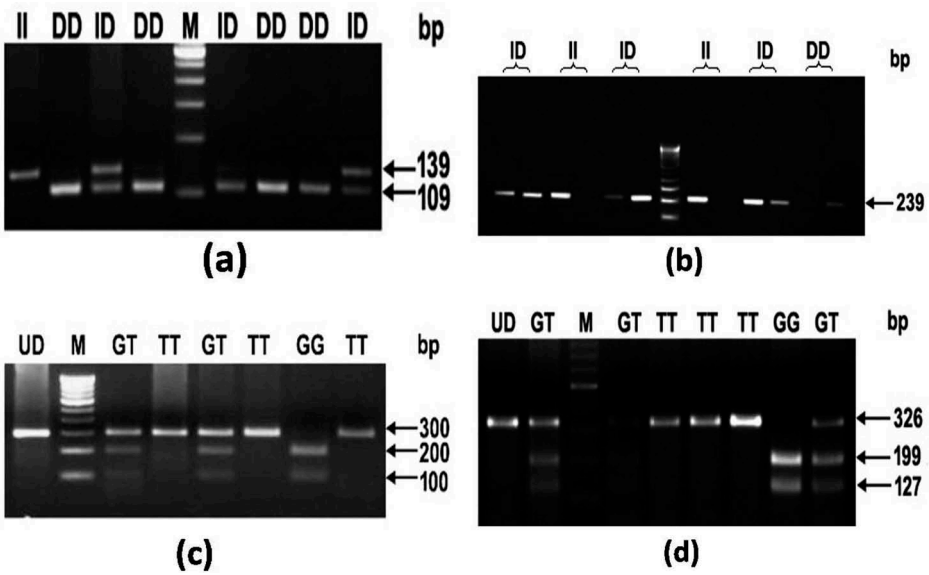

Figure 1. Agarose gels showing genotypes of *XRCC4* gene polymorphisms (a) insertion/deletion intron3 DIP; (b) insertion/deletion intron7 DIP; (c) *XRCC4* G-1394T; (d) *XRCC4* G-652T.

II: Insertion**; ID**: Insertion/Deletion; **DD**: Deletion; **UD**: Undigested; **M**: 100bp Marker.

Table 2. Genotypic and allelic frequencies of *XRCC4* polymorphisms, intron3 DIP, intron7 DIP, G-1394T and G-652T.

Genotypes	Controls n = 246 (%)	Cases n = 246 (%)	P-value
	Intron 3 DIP		
II	22 (9)	50 (20)	0.002
ID	146 (59)	135 (55)	
DD	78 (32)	61 (25)	
	Intron 7 DIP		
II	69 (28)	99 (40)	0.003
ID	132 (54)	117 (48)	
DD	45 (18)	30 (12)	
	G-1394T		
GG	28 (11)	23 (9)	0.368
GT	82 (33)	78 (32)	
TT	136 (55)	145 (59)	
	G-652T		
GG	142 (58)	110 (45)	0.044
GT	74 (30)	107 (44)	
TT	30 (12)	29 (12)	
Alleles	Controls; n = 492 (%)	Cases; n = 492 (%)	*p*-Value; OR (95% CI)
	Intron 3 DIP		
I	190 (39)	235 (48)	0.004
D	302 (61)	257 (52)	0.69 (0.54–0.89)
	Intron 7 DIP		
I	270 (55)	315 (64)	0.004
D	222 (45)	177 (36)	0.68 (0.53–0.88)
	G-1394T		
G	138 (28)	124 (25)	0.439
T	354 (72)	368 (76)	1.16 (0.87–1.54)
	G-652T		
G	358 (73)	327 (67)	0.032
T	134 (27)	165 (34)	1.35 (1.03–1.77)

95% CI: confidence interval, OR: odds ratio.

In *XRCC4* intron3 DIP, insertion-deletion (ID) polymorphism was a 30 bp difference. Insertion/Insertion (II) genotype frequencies in cases were found to be higher in comparison to controls. Moreover, the prevalence of 'I' allele was significantly higher in cases as compared to controls (Table 2; Figure 1(a)). In case of genotype frequencies of *XRCC4* intron7 'ID' polymorphism, 'II' was higher in cases *vs*. controls. The

prevalence of 'I' allele was significantly higher among cervical cancer cases (Table 2; Figure 1(b)). The *XRCC4* G-1394T genotype and allele frequencies did not show significant association (Table 2; Figure 1(c)). The *XRCC4* G-652T polymorphism showed higher 'GT' genotype frequency in cases as compared to controls, while allelic frequency of G-652*T allele was significantly different (Table 2; Figure 1(d)).

Table 3. Haploview of SNPs in *XRCC4* intron3 DIP, intron7 DIP, G-1394T and G-652T showing association with cervical cancer.

Allele combination	Cases (freq. %)	Controls (freq. %)	*p*-Value	Odds Ratio [95% CI]
DDGG	3.0 (0)	25.4 (5)	2.900	0.12 [0.04–0.39]
DDGT	7.5 (2)	18.9 (4)	0.031	0.40 [0.17–0.94]
DDTG	58.6 (12)	79.4 (16)	0.087	0.73 [0.51–1.05]
DDTT	21.5 (4)	25.2 (5)	0.661	0.87 [0.48–1.58]
DIGG	31.4 (6)	41.6 (9)	0.269	0.76 [0.47–1.24]
DITG	77.9 (16)	78.3 (16)	0.842	1.04 [0.73–1.46]
DITT	45.3 (9)	29.9 (6)	0.046	1.62 [1.00–2.63]
IDGG	16.0 (3)	16.0 (3)	0.919	1.04 [0.51–2.09]
IDTG	48.1 (10)	41.6 (9)	0.376	1.22 [0.78–1.88]
IIGG	24.9 (5)	16.9 (3)	0.164	1.56 [0.83–2.94]
IIGT	18.9 (4)	6.6 (1)	0.010	3.08 [1.25–7.55]
IITG	67.1 (14)	58.8 (12)	0.318	1.21 [0.83–1.76]
IITT	37.7 (7)	34.8 (7)	0.620	1.13 [0.69–1.82]

95% CI: confidence interval; OR: odds ratio.

Haplotype analysis of *XRCC4* polymorphisms, intron3 DIP, intron7 DIP, G-1394T and G-652T showed 16 possible combinations. Out of 16 combinations 13 were relevant in present study population. The strongest link was the DDGG combination with an odds ratio of 0.12 (inverse 8.33) ($p<0.001$), followed by IIGT (odds ratio 3.08) ($p=0.01$) and weakly with DDGT with an odds ratio of 0.40 (inverse 2.50) ($p=0.03$) (Table 3; Figure 2).

Discussion

Functional polymorphisms in DNA repair genes affect their expression and confer susceptibility to cancer, its progression and severity [17]. Only 5–10% of all cancers are caused by inheritance of mutated genes and somatic mutations, whereas the remaining 90–95% are linked to lifestyle factors and environment [18]. *XRCC4* is involved in NHEJ in case of double strand breaks (DSBs). If DSBs cannot be repaired before the duplication of genome, they may result in irreversible cellular injuries which increase the possibility of cervical cancer as well as other types of cancers. Polymorphisms in *XRCC4* may bring about alterations in its normal expression and protein function and will probably affect the DNA repair mechanism thereby disturbing the cell cycle [5–8].

XRCC4 polymorphisms in intron3 DIP and intron7 DIP have been associated with risk of developing other cancers such as urothelial bladder [19], breast [20], prostate [21], hepatocellular

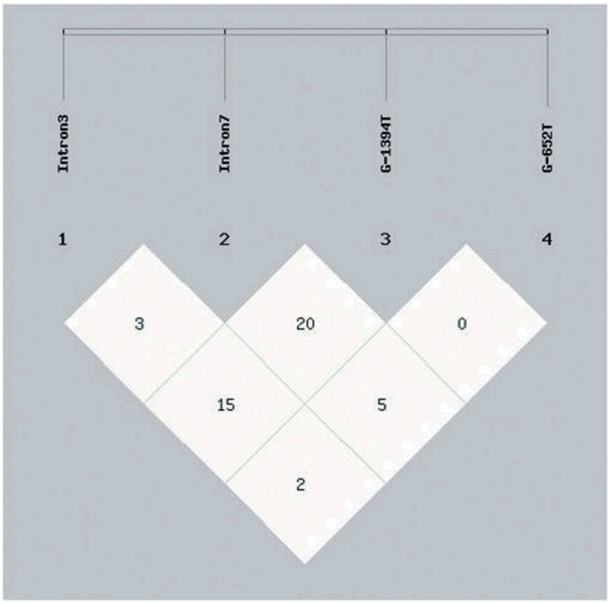

Figure 2. Haploview of SNPs viz. of *XRCC4* intron3 DIP, intron7 DIP, G-1394T and G-652T showing association with cervical cancer in North Indian population. Pairwise linkage disequilibrium (LD) (SHEsis, ver. Online).

[22,23], colorectal [13,24], thyroid and colon [25], lung [26], leukaemia [27] and multiple myeloma [28] cancers. We extend this literature by showing similar effects in cervical cancer. In detail, our results showed a significant association of *XRCC4* intron3 DIP, intron7 DIP and G-652T genotype frequencies with cervical cancer cases: significant association with 'I' allele of both intron3 DIP and intron7 DIP, and that individuals with 'I' alleles were more susceptible to cervical cancer. We speculate that women with 'II' genotype may not have enough capacity to remove all DSBs, thereby resulting in higher susceptibility to cervical cancer, and that the effect of carcinogens in women with 'II' genotype will increase with age and the DSBs remaining in their genome will significantly rise.

Two promoter polymorphisms were studied *viz* *XRCC4* G-1394T and G-652T since the promoter is thought to play a major role in regulating gene expression. In many studies, both 'T' and 'G' alleles have shown significant relation to many cancers including bladder [9], colorectal [13] and breast [10]. However, the *XRCC4* G-1394T polymorphism did not show any association with cervical cancer in the study population. The other promoter polymorphism G-652T was found to be significantly influencing genetic susceptibility to cervical cancer in the study population, as shown in case of prostate cancer [29]. Furthermore, this polymorphism showed no significant association with other cancers such that of lung [30], colorectal [13] and bladder [9]. This might be due to several reasons such as variations in ethnic groups, sample size, patient recruitment standards, geographical or environmental factors. The present study also supports the potential role of haplotype analysis of SNP combinations in that the IIGT* haplotype showed a significant three-fold risk for developing of cervical cancer, while the DDGG haplotype showed an odds ratio of 0.12, translating to a 88% reduction in the risk of cervical cancer.

Genetic polymorphic studies showed a considerable level of variation among various ethnic populations around the world. The results obtained from our study are consistent with several previous studies [19,20,24], however, similar genotyping analysis need to be performed in other ethnic populations in order to validate the association of *XRCC4* variants with cervical cancer. Once identified, individuals at risk will be able to take prior precautionary measures and avoid or delay the onset of disease. Future challenge is to understand the correlation of genotypic analysis with treatment outcome in cervical cancer cases are underway and will surely provide promising information for prediction of disease susceptibility.

This work is an advance in biomedical science as it links certain SNPs in *XRCC4* with risk of cervical cancer.

Summary table

What is known about this subject:

- Two distinct DNA repair pathways are responsible for double strand breaks (DSBs), homologous recombination (HR) and non-homologous end joining (NHEJ).
- The DNA repair protein, XRCC4 is a member of NHEJ system and maintains overall genome stability.
- Polymorphism studies of *XRCC4* gene have been associated with different types of cancers as bladder, breast, gastric, oral and colorectal.

What this paper adds:

- *XRCC4* intron3 DIP (rs28360071) II genotype and I allele, intron7 DIP (rs28360017) II genotype and I allele, and G-652T (rs2075685) genotype and the T allele are linked to cervical cancer.
- The *XRCC4* DDGG allele combination protects (odds ratio 0.12), whilst the IIGT combination promotes (odds ratio 3.1) cervical cancer.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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