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RESEARCH ARTICLE

ROLE OF GENETIC POLYMORPHISMS IN DNA REPAIR GENES (XRCC1, XRCC2, XRCC3, XRCC4, XRCC5, XRCC6, XRCC7) IN HEAD AND NECK CANCER SUSCEPTIBILITY IN RURAL INDIAN POPULATION: A HOSPITAL BASED CASE-CONTROL STUDY FROM SOUTH-WESTERN MAHARASHTRA

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ABSTRACT

To identify the risk factors other than tobacco and alcohol for the development of head and neck cancer, functional polymorphisms of DNA repair genes including XRCC1 Arg194Trp in the exon 6, Arg280His in the exon 9, Arg399Gln in the exon 10, XRCC2 Arg188His in the exon 3, XRCC3 Thr241Met in the exon 7, XRCC4 codon 247, XRCC4 G1394T, XRCC4 intron7, XRCC5 2R/1R/0R, XRCC6 61 (C>G) and XRCC7 6721 (G>T) were studied among rural population of Maharashtra. The XRCC genes were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to elucidate the specific changes in the gene region. The result from our study showed that XRCC1 A399G in exon 10 (OR= 3.69; 95%CI= (2.34-5.51); p= <0.0001) XRCC4-2 (G1394T) OR=6.53; 95%CI=(4.71-9.05); p= <0.0001), XRCC5 (0R/0R) OR=1.97; 95%CI=(1.15-3.26); p= <0.0001) and XRCC7 (6721 T/T) OR=11.58; 95%CI=(7.44-18.02); p= <0.0001) genotypes significantly increased the risk of head and neck cancer. This study indicates that variant types of XRCC1, XRCC4, XRCCC5 and XRCC7 genes play a role in modifying genetic susceptibility of individual to head and neck cancer. Thus, the consistent findings from this case-control study suggest that selected DNA repair genes represent genetic determinants in oral carcinogenesis along with other risk factors in the rural Indian population.

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INTRODUCTION

Oral cancer is one of the most commonly diagnosed cancers all over the world increased markedly in last few years. Oral cancer specifies a subgroup of head and neck cancer (HNC) where cancer of the oral cavity, oropharynx and larynx constitute a major public health problem. Squamous cell carcinoma of head and neck (HNSCC) is the fifth most common type of cancer worldwide and second in the developing world (Parkin *et al*., 2002, Marcu and Yeoh, 2009). In the Indian subcontinent, HNC is the most common malignancy, accounting up to 40–50% of all malignant cancers (Ferlay *et al*., 2001).

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The incidence of HNC has significantly increased in the past 20 years and has become the fourth most common cause of cancer among men and third most common cancer among women in India. Overall survival in HNC is low in developing countries where the major risk factors of HNC are tobacco, alcohol or Human Papilloma Virus (HPV) infection (Miller and Johnstone 2001, Ogden, 2005, Warnakulasuriya *et al*., 2005, Marcu and Yeoh, 2009). Tobacco and alcohol account for more than 75% of HNC but specific carcinogenic mechanisms are unclear. The reactive oxygen species (ROS) derived from metabolites of tobacco and alcohol are known to cause oxidative damage to cellular DNA in the form of serious lesions like single strand breaks (SSBs), double-strand breaks (DSBs) the most serious DNA damage, which if not repaired or misrepaired, may result in genomic instability and cancer development (Khanna and Jackson 2001).

The genomic etiology of HNC is of great interest but largely unknown. It is believed that along with the environmental factors, the host factors including individual's genetic susceptibility are also likely to play a role in the development of this disease. Genetic susceptibility is related to chromosomal aberrations or genetic polymorphisms of various genes, including those involved in DNA repair pathway. Human DNA repair mechanisms are known to protect the genome from DNA damage caused by endogenous and environmental agents. Several kinds of DNA repair mechanisms play a pivotal role in maintenance of genomic integrity with various repair pathways such as base excision repair (BER), nucleotide excision repair (NER), double strand break repair (DSBR) and DNA mismatch repair (DMR). But it is not yet clear which DNA repair pathways or enzymes are most important for protection against HNC. The BER pathway is an important mechanism that repairs DNA damage resulting from chemical alterations of a single base (Lu *et al*., 2001, Hung, 2005). Number of X-ray repair cross complementing group (XRCC) genes are involved in repair steps have been extensively studied in the association with various human cancers (Mc Williams *et al* 2008, Yen at al 2008). The XRCC1 gene belonging to base excision repair pathway and plays an important role in maintenance of genomic stability and SSBs (Olshan, 2002, Caldecott, 2003). XRCC2, XRCC3 are DSB repair genes participate in homologous recombination repair pathway to maintain chromosome stability and DNA damage repair (Liu *et al* 1998, Pierce *et al* 1999, Thacker and Zdzienicka, 2004). The XRCC4, XRCC5, XRCC6, XRCC7 are DSB repair genes, involved in homologous recombination repair (HRR) pathway and non-homologous end joining (NHEJ) (Jackson, 2002, Helleday, 2003, Mari *et al*., 2006).

The polymorphisms in DNA repair genes are associated with differences in the repair efficiency of DNA damage and may influence an individual's risk of cancer. It is possible that these inherited polymorphisms of DNA repair pathway may affect the risk of HNC. Several functional genetic variants of DNA repair genes, particularly non-synonymous polymorphisms, have been identified in the XRCC genes which have shown a relationship with the susceptibility to multiple cancers and have provided meaningful results (Hiyama *et al*., 2008, Flores-Obando *et al*., 2010), but the associations between XRCC polymorphisms and individual susceptibility to some types of cancer examined in some of the studies with the results being contradictory. Also, the former observations were not consistent in terms of their roles in cancer susceptibility and the influence of the polymorphisms of XRCC genes on DNA repair capacity is still unclear. It is also unclear which DNA repair pathways or enzymes may be more important for protection against head and neck cancer. Therefore we focused on the reported polymorphisms with the greater allele frequencies of different DNA repair genes belonging to BER and DSB repair genes to evaluate their role in head and neck cancer. We performed a hospital based case control study with a relatively large sample size using a polymerase chain reaction based restriction fragment length polymorphism (PCR-RFLP) assay to genotype the polymorphisms of selected DNA repair genes in relation to head and neck cancer susceptibility in a rural population of South-Western Maharashtra from India. We determined the genotypic frequency of polymorphisms of the (i) XRCC1 gene at exon 6 exon 9 and exon 10, (ii) XRCC2 gene at 31479 (G-A), (iii) XRCC3 gene at exon 7, (iv) XRCC4 gene at codon 247 (rs3734091), G-1394T (rs6869366) and Intron 7 (rs1805377). Also the present study intended to investigate the associations between the XRCC5, XRCC6 and XRCC7 gene polymorphisms and the development of head and neck cancer in Maharashtrian population.

MATERIALS AND METHODS

Study subjects

This study was a hospital based, case-control study conducted in rural areas of South-Western Maharashtra from India. Study participants included 320 patients, who were newly diagnosed with head & neck cancer and 400 healthy cancer free age and sex matched individuals were selected as controls living in the same residential areas as the cases. Control subjects who were relatives of cases or had a prior history of cancer were excluded from the study. Patients diagnosed with oral cancer were recruited at the outpatient clinics of General Surgery and Department of Oncology at the Krishna Hospital & Medical Research Centre (KH&MRC) between the years 2012 to 2015. All cases ranged in age from 30 - 80 years (Mean± SD) 54.42 ±12.95 were recruited immediately after being diagnosed. Trained interviewers used a structured questionnaire to collect personal interview data from the participants regarding demographic factors (*e.g.* age sex and ethnicity) and known risk factors, including occupational history, dietary habits, smoking and drinking status and individual family history of diseases, including cancer. The study was approved by the Institutional Ethics Committee of the Krishna Institute of Medical Sciences University and written-informed consent was obtained from all participants.

Genomic DNA isolation from whole blood

Five milliliter (mL) of whole blood from patients and normal age matched controls was collected in sterile purple top vacutainer after receiving informed consent. Genomic DNA extraction was carried out from the peripheral blood sample using Purelink genomic DNA extraction and purification Kit (Invitrogen, Life technologies) following the manufacturer's instructions.

Table 1. Details of PCR and RFLP procedures and expected products

Gene	Primers Forward / Reverse	PCR conditions	PCR Product	Restriction enzyme	Restriction products
XRCC1 Arg194Trp cd 194 exon 6 *(*C26304T)	5'-gcc agg gcc cct cct tca a-3' 5'-tac cct cag acc cac gag t-3'	95^0C- 5 min, 30 cycles of 95^0C- 30 sec, 61^0C- 20 sec, 72^0C- 30 sec, 72^0C- 5 min	485 bp	1U of PvuII	Arg/Arg: 485bp Arg/Trp: 485 bp, 396bp, 89 bp Trp/Trp: 396 bp,89 bp
XRCC1 *Arg280His cd280* *exon 9* *(G27466A)*	5' cca gct cca act cgt acc 3' 5' atg agg tgc gtg ctg tcc 3'	95^0C- 5 min, 30 cycles of 95^0C- 30 sec, 61^0C- 30 sec, 72^0C- 30 sec, 72^0C- 5 min	257 bp	2U of RsaI	Arg/Arg: 241 bp Arg/His: 257 bp
XRCC1 Arg399Gln cd399 exon 10 *(*G28152A)	5'-cag tgg tgc taa cct aat c -3' 5'-agt agt ctg ctg gct ctg g- 3'	95^0C- 5 min, 30 cycles of 95^0C- 20 sec, 56^0C- 30 sec, 72^0C- 20 sec, 72^0C- 5 min	871 bp	2U of NciI	Arg/Arg:593bp, 461bp, 278 bp, 132bp Arg/Gln: 461bp, 278,bp,132bp, Gln/Gln:593bp,278bp
XRCC2 Arg188His cd188 Exon 3 (G31479A)	5'- agt tgc tgc cat gcc tta ca-3' 5'-tgt agt cac cca tct ctc tgc-3'	95^0C- 5 min, 30 cycles of 95^0C- 30 sec, 58^0C- 30 sec, 72^0C- 30 sec, 72^0C- 5 min	290 bp	1U HphI	Arg/Arg :290bp Arg/His: 290bp,148bp 142bp His/His: 148bp, 142bp
XRCC3 Thr241Met cd241 exon7 (C18067T).	5'-ggt cga gtg aca gtc caa ac-3' 5'-tgc aac ggc tga ggg tct t- 3'	95^0C- 5 min, 30 cycles of 95^0C- 30 sec, 53^0C- 30 sec, 72^0C- 30 sec, 72^0C- 5 min	455 bp	2U NlaIII	Thr/Thr : 315bp, 140bp Thr/Met: 315bp/210bp, 140bp, 105bp Met/Met : 210bp,140bp, 105bp
XRCC4-1 cd247	5'-gct aat gag ttg ctg cat ttt a-3' 5'-ttt cta ggg aaa ctg caa tct gt-3'	95^0C- 5 min, 30 cycles of 95^0C- 30 sec, 55^0C- 30 sec, 72^0C- 30 sec, 72^0C- 5 min	308 bp	1U BbsI	WT: C/C: 308 bp VT:A/A: 204bp, 104bp
XRCC4-2 G1394T	5'-gat gcg aac tca aag ata ctg a-3' 5'-tgt aaa gcc agt act caa act t-3'	95^0C- 5 min, 30 cycles of 95^0C- 30 sec, 53^0C- 30 sec, 72^0C- 30 sec, 72^0C- 5 min	300 bp	1U HincII	WT : T/T: 300 bp VT:G/G: 200bp, 100bp
XRCC4-3 Intron-7	5'-ttc act tat gtg tct ctt ca-3' 5'-aac ata gtc tag tga aca tc-3'	95^0C- 5 min, 30 cycles of 95^0C- 30 sec, 48^0C- 30 sec, 72^0C- 30 sec, 72^0C- 5 min	237 bp	1U Tsp509I	WT: G/G: 237 bp VT: A/A: 158bp, 79bp
XRCC5 2R/1R/0R	5'-agg cgg ctc aaa cac cac ac-3' 5'-caa gcg gca gat agc gga aag-3'	95^0C- 5 min, 30 cycles of 95^0C- 30 sec, 62^0C- 30 sec, 72^0C- 30 sec, 72^0C- 5 min	2R/2R: 266 bp 1R/1R:245 bp 0R/0R: 224bp		
XRCC6-61C>G	5'-tct cca ctc ggc ttt tct tcc a -3' 5'- tct ccc tcc gct tcg cac tc-3'	95^0C- 5 min, 35 cycles of 95^0C- 30 sec, 56^0C- 30 sec, 72^0C- 30 sec, 72^0C- 5 min	320 bp	1U BanI	WT C/C: 262bp, 58bp VT G/G:182bp,80bp,58bp
XRCC7 6721 G > T	5'-cgg ctg cca acg ttc ttt cc -3' 5'-tgc cct tag tgg ttc cct gg -3'	95^0C- 5 min, 30 cycles of 95^0C- 30 sec, 58^0C- 30 sec, 72^0C- 30 sec, 72^0C- 5 min	368 bp	1U PvuII	WT G/G: 368bp VT T/T: 274bp,94bp

Table 2. Distribution comparisons of selected demographic characteristics of head and neck cancer cases and healthy controls from rural areas of Maharashtra in India

Variable	Cases N=320		Controls N=400		*P*-Value based on $\chi 2$
Age (Mean ± SD) years	54.42 ±12.95		52.84 ± 12.94		
	No.	(%)	No.	(%)	
≤ 50	122	38.13	184	46.00	<0.05
51-60	86	26.87	100	25.00	
61-70	84	26.25	86	21.50	
>70	28	8.75	30	7.50	
Sex					0.44
Male	200	62.50	260	65.00	
Female	120	37.50	140	35.00	
Tobacco smoking Status					<0.001
Smokers Current	250	78.13	80	20.00	
Non smokers	70	21.87	320	80.00	
Alcohol status					<0.001
Drinkers	220	68.75	50	12.50	
Non-drinkers	100	31.25	350	87.50	
Diet					0.04
Vegeterian	90	28.13	140	35.00	
Non-vegeterian	10	3.12	20	5.00	
Mixed	220	68.75	240	60.00	
Education					0.73
High School	205	64.06	180	45.00	
High School graduate (12 y)	70	21.88	120	30.00	
College /Graduate	45	14.06	100	25.00	
Economic status					0.56
Middle	112	35.00	148	37.10	
Poor	208	65.00	251	62.90	
Family history of Cancer					
Yes	20	6.25	0	0.00	0.2
No	300	93.75	400	100	

After the quantitative and qualitative analysis of genomic DNA the final samples were kept in Tris-EDTA (T10E1) buffer (pH 8) at -20°C temperature until use.

Genotyping assays

Genotyping of XRCC genes (XRCC1, XRCC2, XRCC3 XRCC4, XRCC6 and XRCC7 were performed by PCR-RFLP methods with appropriate primer sets (Table 1). The primers were designed to amplify the regions of DNA that contain polymorphic sites of interest: *XRCC1* Arg194Trp in the exon 6 (C>T), *XRCC1* Arg280His in the exon 9 (G>A), XRCC1 Arg399Gln in the exon 10 (G>A), XRCC2 Arg188His in the exon 3 (G>A), *XRCC3* Thr241Met in the exon 7, XRCC4 codon 247, XRCC4 G1394T, XRCC4 intron7, XRCC5 2R/1R/0R, XRCC6 61 (C>G) and XRCC7 6721 (G>T). The *XRCC5* 2R/1R/0R polymorphisms were identified by polymerase chain reaction.

The PCR amplification were carried out separately under different conditions in 20 micro liter (µL) reaction mixtures containing 1X PCR buffer (10 mili molar (mM) Tris-HCl (pH 9.0), 50 mM KCl 1.5 mM $MgCl_2$, 0.01% gelatin), 0.2 mM each dNTP, 10 picomole (pmol) of each primer listed in Table-1, 1U *Taq DNA* polymerase (GeNei, Merck Bioscience) and 100 nanogram (ng) of purified genomic DNA template. The reaction mixtures were subjected to PCR amplification with a Master Cycler Gradient PCR (Eppendorf). After performing PCR programme for each of the reaction, the PCR products were analyzed by agarose gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer. The agarose gels were stained with ethidium bromide (10 mg/mL) and visualized under UV Transilluminator and photographed in gel documentation system (BioRad Laboratories). After confirmation of DNA amplification, each PCR product was digested with an appropriate restriction enzyme as shown in table-1 for genotyping. Ten micro liters of the PCR products were digested at 37°C overnight with specific restriction enzymes in 20 µL reaction mixtures containing buffer supplied with each restriction enzyme.

After the overnight incubation, digestion products were then separated on a 2-3% low EEO agarose (GeNei) gel at 100 V for 30 min stained with ethidium bromide and photographed with Gel Documentation System (BioRad).

Statistical analysis

The associations between the *XRCC* genotypes and risk of HNC with or without smoking and drinking history were studied using odds ratio. Both the univariate and multivariate logistic regression analyses were employed to calculate the adjusted odds ratios (ORs) and 95% confidence intervals (CIs) to determine the cancer risk associated with genotypes. For each polymorphism the $\chi 2$ test was used to evaluate differences in the frequency distribution of selected demographic variables and the frequencies of allele and genotype of the XRCC polymorphisms between HNC cases and the controls.

Ethics and bio safety

The study protocol was approved by the Institutional Ethics and Biosafety Committee of Krishna Institute of Medical Sciences for the use of human subjects in research.

Table 3. The genotype frequencies of *XRCC* gene variants in untreated head and neck cancer patients and controls

GENE	Genotype	CASES (n= 320) (%)	CONTROL (n = 400) (%)	Odds' Ratio (95% CI)	*P* value	Adjusted Odds Ratio (95% CI)	*P* value
XRCC1 cd194	Arg/Arg	246 (76.9)	321 (80.2)	1		1	
ex-6	Arg/Trp	66 (20.6)	47 (11.8)	1.83(1.217-2.759)	0.005	0.67(0.27-1.63)	0.37
	Trp/Trp	8 (2.5)	32 (8.0)	0.32(0.14-0.20)	0.005	1.17(0.29-4.71)	0.82
	Arg/Trp+Trp/Trp	74 (23.1)	79 (19.8)	1.20(0.84-1.71)	0.3	0.64(0.34-1.22)	0.18
XRCC1 cd280	Arg/Arg	261 (81.6)	340 (85.0)	1		1	
ex-9	Arg/His	0 (0)	0 (0)	---		---	---
	His/His	59 (18.4)	60 (15.0)	1.28(0.86-1.9)	0.18	0.75(0.32-1.75)	0.50
	Arg/His+His/His	59 (18.4)	60 (15.0)	1.28(0.86-1.9)	0.18	0.84(0.41-1.69)	0.63
XRCC1 cd399	Arg/Arg	104 (32.5)	320 (80.0)	1		1	
ex-10	Arg/Gln	162 (50.6)	35 (8.8)	14.24(9.29-21.83)	0.0001	0.49(0.022-0.10)*	0.0001
	Gln/Gln	54 (16.9)	45 (11.2)	3.69(2.34-5.81)	0.0001	0.14(0.06-0.36)*	0.0001
	Arg/Gln+ Gln/Gln	216 (67.5)	80 (20.0)	8.30(5.92-11.65)	0.0001	0.09(0.05-0.16) *	0.0001
XRCC2	Arg/Arg	242 (75.6)	330 (82.8)	1		1	
cd88	Arg/His	70 (21.9)	32 (8.0)	2.98(1.90-4.67)	0.0001	0.20(0.08-0.50)*	0.0001
ex-3	His/His	8 (2.5)	38 (9.2)	0.28(0.13-0.62)	0.005	3.36(0.70-16.17)	0.12
	Arg/His+His/His	78 (24.4)	70 (17.2)	1.51(1.06-2.17)	0.02	0.56(0.31-1.04)	0.06
XRCC3	Thr/Thr	214 (66.9)	310 (77.5)	1		1	
Cd241	Thr/Met	84 (26.2)	29 (7.25)	4.19(2.65-6.62)	0.0001	0.19(0.08-0.45)*	0.0001
Ex-7	Met/Met	22 (6.9)	61 (15.25)	0.52(0.31-0.87)	0.01	4.30(1.12-16.51)*	0.03
	Thr/Met+Met/Met	106 (33.1)	90 (22.5)	1.75 (1.26-2.42	0.005	0.63(0.35-1.11)	0.11
XRCC4 cd247	C/C	240 (75.0)	310 (77.5)	1		1	
	A/A	80 (25.0)	90 (22.5)	1.14(0.81-1.62)	0.4	1.06(0.49-2.29)	0.87
XRCC4 G1394T	G/G	92 (28.8)	290 (72.5)	1		1	
	T/T	228 (71.2)	110 (27.2)	6.53(4.71-9.05)	0.0001	0.14(0.07-0.28)*	0.0001
XRCC4 intron7	G/G	210 (65.6)	272 (68.0)	1		1	
	A/A	110 (34.4)	128 (32.0)	1.11(0.81-1.52)	0.5	0.79(0.39-1.57)	0.51
XRCC5	2R/2R	90 (28.1)	314 (78.5)	1		1	
	1R/1R	204 (63.7)	40 (10.0)	17.79(11.78-26.86)	0.0001	0.03(0.01-0.08)*	0.0001
	0R/0R	26 (8.2)	46 (11.5)	1.97(1.15-3.36)	0.01	0.89(0.31-2.54)	0.83
	1R/1R+0R/0R	230 (71.9)	86 (21.5)	9.33(6.63-13.12	0.0001	0.087(0.05-0.15)*	0.0001
XRCC6	C/C	168 (52.5)	322 (80.5)	1		1	
	C/G	148 (46.25)	32 (7.7)	8.86(5.79-13.56)	0.0001	0.09(0.04-0.19)*	0.0001
	G/G	4 (1.25)	46 (11.8)	0.16(0.058-0.47)	0.005	7.94(1.05-59.88)*	0.04
	C/G+G/G	152 (47.5)	78 (19.5)	3.73 (2.68-5.19)	0.0001	0.20(0.11-0.35)*	0.0001
XRCC7	G/G	53 (16.6)	304 (76.0)	1		1	
	G/T	162 (50.6)	44 (11.0)	21.64(13.90-33.67)	0.0001	0.04(0.01-0.09)*	0.0001
	T/T	105 (32.8)	52 (13.0)	11.58(7.44-18.02)	0.0001	0.09(0.04-0.21)*	0.0001
	G/T+T/T	267 (83.4)	96 (24.0)	15.95(10.98-23.17)	0.0001	0.07(0.04-1.22)*	0.0001

*: Indicates significant Odds Ratio ($p<0.05$)
p value determined based on $\chi 2$

Table 4. Stratification analysis of the demographic factors including age, tobacco smoking and alcohol drinking and distribution of genotypes with odds ratio of the XRCC genes in the patients with head and neck cancer and the control group from rural population of south-western Maharashtra

		Demographic Factors							
Gene	Genotype	Age (Cases/Control)		Sex (Cases/Control)		Smoking status (Cases/Control)		Drinking status (Cases/Control)	
		≤ 50 N=122/150	> 50 N=198/250	Male N=200//260	Female N=120/140	Smokers N=250/80	Nonsmokers N=70/320	Drinkers N=220/50	Non-drinkers N=100/350
XRCC1	Arg/Arg	86/80	160/170	148/180	98/92	190/62	56/286	174/48	72/290
Arg194Trp	Arg/Trp+	20/70	54/80	52/80	22/48	59/18	14/34	46/2	28/60
cd 194	Trp/Trp								
exon 6	OR	0.26	0.71	0.79	0.43	1.06	2.10	6.34	1.87
(C26304T)	(95% CI)	(0.14-0.47)	(0.47-1.07)	(0.52-1.19)	(0.24 -0.76)	(0.58 -1.95)	(1.05-4.17)	(1.48-27.08)	(1.12-3.15)
	P value	0.0001	0.1	0.2	0.004	0.8	0.03	0.01	0.01
XRCC1	Arg/Arg	85/90	176/190	162/200	99/100	204/60	56/278	177/47	84/300
Arg280His	Arg/His+	21/60	38/60	38/60	21/40	45/20	14/42	43/3	16/50
cd280	His/His								
exon 9	OR	0.37	0.68	0.78	0.53	0.66	1.65	3.80	1.14
(G27466A)	(95% CI)	(0.20-0.66)	(0.43-1.07)	(0.49-1.23)	(0.29-0.96)	(0.36-1.20)	(0.84-3.22)	(1.13-12.81)	(0.61-2.10)
	P value	0.0008	0.1	0.2	0.03	0.1	0.1	0.03	0.6
XRCC1	Arg/Arg	28/94	76/166	62/212	42/104	75/54	29/294	73/45	31/326
Arg399Gln	Arg/Gln+	78/56	102/84	138/48	78/36	140/26	76/26	147/5	69/24
cd399	Gln/Gln								
exon 10	OR	4.67	2.65	9.83	5.36	3.87	29.63	18.12	30.23
(G28152A)	(95% CI)	(2.71-8.05)	(1.78-3.94)	(6.37-15.16)	(3.14-9.14)	(2.24-6.68)	(16.48-52.26)	(6.90-47.59)	(16.71-54.69)
	P value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
XRCC2	Arg/Arg	86/112	156/190	160/222	82/108	190/66	51/282	177/44	65/328
Arg188His	Arg/His+	20/38	58/60	40/38	38/32	59/14	19/38	43/6	35/22
cd188	His/His								
Exon 3	OR	0.68	1.17	1.46	1.56	1.46	2.76	1.78	8.02
(G31479A	(95% CI)	(0.37-1.26)	(0.77-1.78)	(0.89-2.38)	(0.90-2.71)	(0.76-2.79)	(1.47-5.17)	(0.71-4.45)	(4.42-14.56)
	P value	0.22	0.4	0.12	0.1	0.24	0.01	0.21	0.0001
XRCC3	Thr/Thr	74/118	140/160	132/199	82/111	164/72	50/294	141/43	73/310
Thr241Met	Thr/Met+	32/32	74/90	68/61	38/29	85/8	20/26	79/7	27/40
cd241	Met/Met								
exon7	OR	1.59	0.93	1.68	1.77	4.66	4.52	3.44	0.34
(C18067T)	(95% CI)	(0.90-2.81)	(0.64-1.37)	(1.11-2.53)	(1.01-3.10)	(2.14-10.13)	(2.34-8.71)	1.47-8.01	(0.20-0.60)
	P value	0.1	0.74	0.01	0.04	0.0001	0.0001	0.004	0.0002
XRCC4-1	C/C	81/110	159/200	150/204	90/114	185/68	54/279	160/34	80/294
cd247	A/A	25/40	55/50	50/56	30/26	64/12	16/41	60/16	20/56
	OR	0.84	1.38	1.21	1.46	1.96	2.01	0.79	1.31
	(95% CI)	(0.47-1.51)	(0.89-2.13)	(0.78-1.87)	(0.80-2.64)	(0.99-3.85)	(1.05-3.85)	(0.41-1.54)	(0.74-2.31)
	P value	0.57	0.14	0.38	0.20	0.05	0.03	0.50	0.34
XRCC4-2	G/G	32/132	60/214	58/214	34/102	69/70	22/289	62/38	30/298
G1394T	T/T	74/18	154/36	142/46	86/38	180/10	48/31	158/12	70/52
	OR	16.95	8.84	11.38	6.78	18.26	20.34	8.06	13.37
	(95% CI)	(8.90-32.28)	(5.59-14.23)	(7.32-17.70)	(3.93-11.70)	(8.90-37.45)	(10.8-38.03)	(3.95-16.45)	(7.95-22.47)
	P value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
XRCC4-3	G/G	64/119	146/220	126/224	84/124	169/64	40/294	140/49	69/300
Intron-7	A/A	42/31	68/30	74/36	36/16	80/16	30/26	79/11	31/50

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	OR	2.51	3.41	3.65	3.32	1.89	8.48	2.51	2.69
	(95% CI)	(1.44-4.38)	(2.11-5.50)	(2.32-5.75)	(1.73-6.36)	(1.02-3.48)	(4.56-15.77)	(1.23-5.11)	(1.60-4.52)
	P value	0.001	0.0001	0.0001	0.0003	0.03	0.0001	0.01	0.0002
XRCC5	2R/2R	24/114	66/176	51/200	39/102	69/68	21/274	66/40	24/314
2R/1R/0R	1R/1R + 0R/0R	82/36	148/74	149/40	81/38	180/12	49/46	154/10	76/36
	OR	10.81	5.33	14.60	5.57	14.78	13.89	9.33	27.62
	(95% CI)	(6.00-19.50)	(3.58-7.93)	(9.17-23.25)	(3.26-9.50)	(7.53-28.99)	(7.63-25.30)	(4.40-19.76)	(15.55-49.03)
	P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
XRCC6	C/C	46/86	122/180	95/208	73/92	135/62	33/283	124/46	44/320
61	C/G+G/G	60/64	92/70	105/32	47/48	114/18	37/37	96/4	58/30
C>G	OR	1.75	1.93	7.18	1.23	2.90	8.57	8.90	14.06
	(95% CI)	(1.06-2.89)	(1.31-2.85)	(4.51-11.42)	(0.74-2.04)	(1.62-5.20)	(4.79-15.33)	(3.09-25.59)	(8.17-24.17)
	P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
XRCC7	G/G	10/96	43/170	30/196	23/88	36/64	17/274	42/42	11/304
6721 G > T	G/T +T/T	96/54	171/80	170/44	97/52	213/16	53/46	178/8	89/46
	OR	17.06	8.45	25.24	12.18	23.66	18.57	22.25	51.66
	(95% CI)	(8.21-35.47)	(5.51-12.95)	(15.19-1.93)	(7.31-20.31)	(12.33-45.41)	(9.89-34.84)	(9.72-50.89)	(25.65-104.05)
	P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

RESULTS

Characteristics of the study subjects

During the study period 320 patients with oral cancer met the eligibility criteria for this study and 400 controls were selected to match these cases. The characteristics of age and sex matched cases and controls are presented in Table 2. The mean age in years was 54.42 (median: 55, range 30-80) for the cases and 52.84 (median: 53 range 28-75) for the controls. There were no significant differences between the cases and controls with respect to sex and ethnicity. An effort was made to achieve a frequency match on smoking and drinking status between cases and controls.

Association of polymorphisms in XRCC genes and smoking and drinking status

We investigated the association between the polymorphisms in XRCC genes and tobacco, alcohol consumption from rural Maharashtrian population. The distribution of *XRCC* genotypes and concordance of the seven polymorphisms are presented in Table-3.

XRCC1

The amplification of XRCC1 codon 194 resulted in the product of 485 bp. The PvuII enzyme was used to detect the *XRCC1* C26304T at codon 194 of exon 6. The PCR amplified products upon treatment with PvuII yielded wild-type (26304C) allele of single 485 bp fragment containing exons 5 and 6, and the polymorphic (T) allele produces 2 fragments of 396 and 89 bp. The PCR amplified product of 290 bp for XRCC1 (G27466A) at codon 280, showed the presence of 148 and 142 bp bands after RsaI digestion depicting homozygous wild and variant genotypes respectively. The amplification of XRCC1 codon 399 resulted in the product of 871 bp. The *Nci*I enzyme was used to detect the *XRCC1* G28152A at codon 399 of exon 10. The 28152A variant allele has lost an NciI site. The wild-type (28152G) allele, which has 2 NciI sites, produces 3 bands (461, 278 and 132 bp), and the variant A allele produces only 2 (593 and 278 bp).

The frequency of *XRCC1* 26304 CC homozygotes was 76.9% in cases and 80.2% in controls whereas *XRCC1* 26304 TT homozygotes was 2.5 % in cases and 8.0 % in controls. The frequency of *XRCC1* 26304 CT heterozygotes was 20.6% in cases and 11.8% in controls. In codon 280, genotype frequencies for wild and variant genotypes were 81.6 and 18.4 per cent respectively in cases and 85 and 15 percent respectively in controls. The frequency of *XRCC1* 28152GG homozygous wild type alleles at codon 399 of exon 10 was 32.5%, 28152GA heterozygote alleles was 50.6% and for 28152AA homozygous alleles was 16.9% in the cases where that of the frequencies for the controls were 80.0, 8.8 and 11.2 % respectively (Table-3)

XRCC2

Table 3 displays the distribution of genotypes and frequency of alleles of the G31479A polymorphisms in patients with head and neck cancer and controls. All distributions of genotypes and alleles were in Hardy-Weinberg equilibrium. We did not find any significant difference in genotype or allele frequencies in patients with cancer and controls. We investigated the relationship between smoking and the risk for head and neck cancer, independent of genotype.

We also investigated the association between the polymorphisms and alcohol consumption (Table 4). The frequency of *XRCC2* 31479*GG* wild type alleles at codon 188 of exon 3 was 75.6%, 31479*GA* heterozygote alleles was 21.9% and for 31479AA homozygous alleles was 2.5% in the cases where that of the frequencies for the controls were 82.8, 8.0 and 9.2 % respectively (Table 3). Thus, the haplotype analysis according to wild type of G31479A showed a lack of association with head and neck cancer.

XRCC3

Allele frequencies and distribution of genotypes of *XRCC3* codon 241 are shown in Table 3. In frequency distribution of codon 241 of XRCC3 gene at C18067T, genotype frequencies for wild, heterozygote and variant genotypes were 66.9, 26.2 and 6.9 % respectively in cases where as the frequencies in controls were 77.5, 7.25 and 15.25 % respectively. XRCC3 Thr/Thr, Thr/Met and Met/Met genotypes did not show significant association with development of oral cancer (OR=0.52, 95% CI=0.31-0.87; p= 0.01 for 241 Met/Met).

XRCC4

The frequency of the genotypes of XRCC4-1, XRCC4-2 and XRCC4-3 between oral cancer and control groups are shown in Table 3. The distributions of all these polymorphisms were in Hardy-Weinberg equilibrium and were similar between oral cancer patients and controls. Also the joint effects of XRCC4-2 genotype and environmental factors, namely smoking, alcohol consumption on estimates of oral cancer risk are shown in Table-4. Among the XRCC4 polymorphisms investigated, only G1394T at XRCC4-2 seems to contribute to increased oral cancer risk. The G/G variant conferred an increased risk compared to the T/T wild-type genotype in the smoking stratification, with an increased risk of OR 6.53 (95% CI=4.71-9.05).

XRCC5

As shown in Table 3, the genotypes of 1R/1R, 1R/0R was more common (63.7%, 71.9%) and that of 2R/2R was less common (28.1%) among the cases than among the controls (10.0%, 21.50%, and 78.50% respectively). We found a statistically significant increased risk of head and neck cancer in group of heavy alcohol drinkers associated with 0R/0R and the combined 1R/1R and 0R/0R genotype. The genotype frequencies of these three polymorphisms among the controls were all in agreement with the Hardy-Weinberg equilibrium.

XRCC6

For the *XRCC6 (*61C > G) polymorphism, the frequencies of the CC, CG, and GG genotypes were 52.5%, 46.25%, and 1.25%, respectively, among oral cancer cases, and 80.5%, 7.7%, and 11.80%, respectively, among controls (Table 3). XRCC6 gene polymorphisms did not show association with increase in the risk of oral cancer when examined by stratifying potential confounding variables, such as age and gender.

XRCC7

For the *XRCC7 (*6721G>T) polymorphism, the frequencies of the GG, GT, and TT genotypes were 16.6%, 50.6%, and 32.8%, respectively, among oral cancer cases, and 76.0%, 11.0%, and 13.0%, respectively, among controls. However, the difference was statistically significant (P=0.0001) in cases than in conrrols for the variant TT and heterozygote GT types in cases than controls. In this study, we found that *XRCC7 (*6721G >T) polymorphism was associated with the risk of oral cancer (Table 4). Also, there were significant associations between the XRCC7 (6721G>T) variant genotypes and oral cancer risk stratified by variables age and gender

DISCUSSION

In this hospital based case-control study we investigated the relationship between newly reported genotype polymorphisms of BER, DSBR, HRR genes and the elevated risk for oral cancer particularly in the users of tobacco and alcohol from the rural areas of Maharashtra. We determined the genotypic frequency of polymorphisms of the DNA repair pathway (i) XRCC1 gene at codon 194, codon 280, codon 399 (ii) XRCC2 gene at 31479 (G-A), (iii) XRCC3 gene at exon 7, (iv) XRCC4 gene at codon 247 (rs3734091), G-1394T (rs6869366) and Intron 7 (rs1805377). Also the present study intended to investigate the associations between the XRCC5, XRCC6 and XRCC7 gene polymorphisms and the development of HNSCC in Maharashtrian population. Comparable wild type genotype frequencies of XRCC1 codon 194, codon 280, codon 399 showed wide distribution in the Maharashtrian population in controls. The frequency of allele of the XRCC2 polymorphism at 31479 (G-A) did not show association with the oral cancer risk in South-western Maharashtrian population. Also, when we conducted a case-control study to investigate the relationship between the polymorphisms XRCC3 codon 241 and the risk oral cancer in a south western Maharashtrian population, we did not find association between the XRCC3 codon 241 polymorphism and smoking and drinking related oral cancer.

To the best of our knowledge, there are no reports concerning any *XRCC4* polymorphism in oral cancer risk. Therefore, the present study also planned to investigate the role of *XRCC4* gene polymorphisms, which has never been reported to be associated with oral cancer risk. Our findings suggest that the presence of the T allele of *XRCC4 (*G1394T) was associated with a higher susceptibility to oral cancer as compared to G allele which may be a useful novel marker in oral oncology for primary prevention and intervention. The polymorphisms *XRCC5 (*2R/1R/0R), *XRCC6 (*-61C>G) and *XRCC7*

*(*6721G>T) were also selected to investigate the associations between the polymorphisms and risk of HNSCC in a hospital-based case-control study in a South-Western Maharashtrian population from India. We found a significant association with the polymorphisms of *XRCC5(* 2R/1R/0R), *XRCC6 (*-61C>G) and the risk of HNSCC. However, there was no evidence for an association between the *XRCC7 (*6721G>T) variants and HNC. To the best of our knowledge, this is the first report that the XRCC4 (G1395T), *XRCC5* 2R/1R/0R, *XRCC6* -61C>G and *XRCC7* 6721G>T polymorphisms are associated with the risk of HNSCC.

There is an increasing evidence that reduced DNA repair capacity, resulting from genetic polymorphisms of various DNA repair genes, is associated with increased risk and susceptibility to various types of human cancers (Shen *et al* 2000, David-Beabes *et al*., 2001, Ratnasinghe *et al*., 2001, Hao *et al*., 2004, Zhi, *et al*., 2004, Zhang *et al*., 2005). The polymorphism in DNA repair genes has been extensively investigated for its associations with cancer risk and the results were conflicting in different types of cancer or different populations (Hu *et al*., 2005, Zeng *et al*., 2009, Wei *et al*., 2010, Saadat, 2010). Flores-Obando *et al* 2010 found no significant association between smoking, alcohol, human papillomavirus, ethnicity and DNA repair gene polymorphisms in XRCC1 and oral cancer.

Some reports have documented that the *XRCC2* –41657C>T variation was related to increased esophageal squamous cell carcinoma risk (Wang *et al.,* 2009). Significant association was found between the *XRCC3* 722C>T polymorphism, present in the coding region of the gene significantly increased the risk of HNSCC (Werbrouck *et al.,* 2008). The multiplicative effects of combined genetic variants for different DNA-repair pathways have been previously reported for lung cancer, breast cancer and glioma cancer (Smith *et al*., 2003; Kiuru *et al*., 2008). Only in single study does the research suggest that potential combination of *XRCC1*, *XRCC2*, *XRCC3* and *XRCC4* single nucleotide polymorphisms may have an impact on identification of an oral cancer high-risk population (Yen *et al* 2008). The frequencies of genetic variants were different from that reported in the Asians (Ratnasinghe *et al*., 2001).

Kumar *et al.,* 2012 found significant difference between patients and controls in respect of *XRCC1* Arg194Trp and *XRCC1* Gln399Arg SNPs in an Indian population, but no significant difference for *XRCC1* Arg280His. Very few studies from Northern and Southern India have reported the genetic polymorphisms in the DNA repair genes with respect to a variety of cancer risks including prostate, breast, oral and esophageal cancers (Sobti *et al*., 2007, Pachouri *et al*., 2007, Vettriselvi *et al*., 2007, Mitra *et al*., 2008 Mandal *et al*., 2012). However, very limited information is available on the association of genetic polymorphisms of DNA repair genes and their susceptibility to oral cancer from rural population of Maharashtra where the rate of tobacco and alcohol consumption is very high. Therefore in this study, we aimed to investigate the relationship between the development of oral cancer and genetic polymorphisms in XRCC genes with respect to tobacco, alcohol exposure from a pool of unexplored rural Maharashtrian population. Such genotyping analysis of DNA repair genes will enhance our ability to identify those individuals most susceptible to tobacco and alcohol induced carcinogenesis in the rural population of south-western Maharashtra. In conclusion, this study is the first time to report the combined effects of XRCC gene polymorphisms on the risk of head and neck cancer in Maharashtrian population. Our results showed that the XRCC1, XRCC4, XRCC5 and XRCC7 gene polymorphisms are associated with the risk of oral cancer. This analysis of correlation of DNA repair genes and HNC may provide a deeper insight into the association of genetic and environment factors with cancer risk in the rural unexplored population

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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