CHAPTER 7

Genetic predisposition to DNA damage and its effects on genetic susceptibility to oral cancer

Introduction

The Micronucleus test has been used as a cytogenetic method since the 1970's. Metaphase and anaphase analysis of *in vivo* mammalian systems to study the effect of mutagenic or clastogenic potential of environmental agents were the early applications of cytogenetic preparations. Schmid and his coworkers (218) developed the bone marrow "micronucleus test" at the University of Zurich in 1971, for the rapid screening of large numbers of chemical compounds suspected to be potential mutagens. In the 1990's, Fenech and colleagues (219) developed the modified Cytokinesis block Micronucleus assay (CBMN assay), and applied it to genotoxicity studies in human populations. The use of micronucleus assay has thereafter been multifactorial.

The level of genetic integrity of human populations is increasingly under threat due to industrial activities that result in exposure to chemical and physical genotoxins. Other factors that can influence genetic damage include lifestyle factors (e.g., diet), various medical therapies, and climatic changes (e.g., increased exposure to ultraviolet radiation due to depletion of atmospheric ozone). It is therefore important to be able to (a) determine what is an acceptable level of genetic damage in a human population, (b) identify individuals who could be hypersensitive to selected genotoxins, (c) effectively screen new chemicals that are released into the environment, (d) determine the level of increase in genetic damage in a population following a major

accident, and (e) routinely monitor individuals who are occupationally exposed to agents that can be detrimental at the genetic level.

The MN methodology is simple and allows rapid assessment of cells, thus making it an economical procedure to implement on a large scale. MN assays can only be effective as quantitative biological dosimeters if one can identify those cells that have divided after exposure because only dividing cells can express micronuclei. This goal was achieved by the development of the cytokinesis-block micronucleus (CBMN) technique, which uses cytochalasin-B to stop dividing cells from performing cytokinesis, thus allowing cells that have completed one nuclear division to be recognized by their binucleate appearance (230,231). As a consequence, the CBMN assay has been shown to be more accurate and more sensitive than the conventional methods that do not distinguish between dividing and nondividing cells (231,232). The development of the CBMN assay has enabled direct and valid comparisons with chromosome aberration assays. It has now been shown conclusively that the CBMN assay can detect between 60 and 90% of acentric fragments (233,234), although the relative efficiency specific for chromatid deletions as opposed to chromosome deletions has yet to be defined. Furthermore, the CBMN assay combined with kinetochore or centromere detection has been shown to be an optimal procedure for measuring whole chromosome loss events (235-237). Further technical developments (e.g., chromosome painting) may also allow the measurement of unequal distribution of

chromosomes within daughter nuclei in cytokinesis-blocked binucleate cells. Spontaneous or baseline MN frequencies in cultured human lymphocytes provide an index of accumulated genetic damage occurring during the lifespan of circulating lymphocytes. The half-life and mean lifespan of long lived T-lymphocytes has been estimated to be 3 years and 4 years, respectively (238,239). The observed genetic instability may also reflect accumulated mutations in the stem cell lineage from which the mature lymphocytes originate. The type of mutations that could contribute to spontaneous micronuclei include (a) mutations to kinetochore proteins, centromeres, and spindle apparatus that could lead to unequal chromosome distribution or whole chromosome loss at anaphase, and (b) unrepaired DNA strand breaks induced endogenously or as a result of environmental mutagens, which may result in acentric chromosome fragments. (231)

Genetic variations or polymorphisms in DNA repair genes leads to functional differences in the respective protein. The presence of these polymorphisms may be associated with increased extent of DNA damage. Extensive work is being carried out to resequence DNA repair genes to identify variations that may be associated with defective function of their encoded proteins. [8] But studies that compare genetic polymorphisms with functional assays are the most valuable type of study to clarify the role of a defect in DRC with the development of cancer [8] because if genetic variations in DNA repair genes are a risk factor for malignancy, it should be reflected in the extent of actual DNA damage and repair. This can be elucidated through a number of *in vitro* cytogenetic assays.

One technique adopted is the measurement of micronuclei [MN] in peripheral blood lymphocytes. Micronuclei originate from chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division. MN provides a measure of both chromosome breakage and chromosome loss. Chromosome abnormalities are a direct consequence and manifestation of damage at the DNA level. Chromosome breaks may result from unrepaired double strand breaks in DNA and chromosome rearrangements may result from misrepair of strand breaks in DNA. MN are expressed in dividing cells that either contain chromosome breaks lacking centromeres [acentric fragments] and/or whole chromosomes that are unable to travel to the spindle poles during mitosis. At telophase, a nuclear envelope

forms around the lagging chromosomes and fragments, which then uncoil and gradually assume the morphology of an interphase nucleus with the exception that they are smaller than the main nuclei in the cell and so called “micronuclei” (229). The cells containing micronuclei are potentially dead since they arise from disturbed genetic materials.

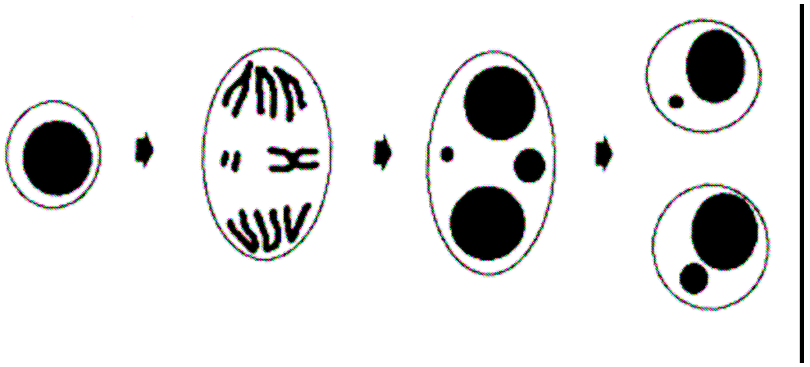


Fig 7.1 Diagrammatic representation of Micronucleus formation

Aim

The study was aimed to look into the overall distribution of polymorphisms in DNA repair genes prevalent in our populations and see whether the presence of any polymorphic variant was associated with increased extent of DNA damage

Specific objectives

To correlate the presence of DNA repair gene polymorphisms with the actual extent of *in vitro* DNA damage.

Subjects

The study population included 100 oral cancer patients being seen at the Head and Neck Clinic of the Regional Cancer Centre, Thiruvananthapuram. A total of 100 normal controls were also included. Controls were mostly from the same geographical area and socioeconomic background as the cases and included visitors and relatives of the patients. Control subjects were apparently normal and gave no history of any malignancy or any systemic disease. All controls were age and sex matched and also matched for habits. The study was approved by the Institutional Review Board and Human Ethics Committee of the Regional Cancer Centre. Informed consent was obtained from all subjects. Clinical pathological data were derived from patient records and pathology reports.

Cytokinesis Block Micronucleus assay

Ten ml of blood was collected by venipuncture in heparinized tubes from each subject and the assay was carried out within four hours of collection. The cytokinesis-blocked micronucleus assay was carried out as standardized by Fenech et al [229]. Briefly, lymphocyte cultures were set up by adding $1x10^6$ lymphocytes, which were separated from whole blood by Ficoll density gradient centrifugation to 10 ml of culture medium containing RPMI 1640, and glutamine. The cells were incubated for 72 hours and cytochalasin B was added after 44h for a final concentration of 4.5µg/ml. The cells were then harvested after incubation for another 22 hours as described by Fenech et al. (240). The slides were stained with Acridine orange/Ethidium bromide. Micronuclei were scored in 1000 binucleate cells following the criteria reported by Fenech.

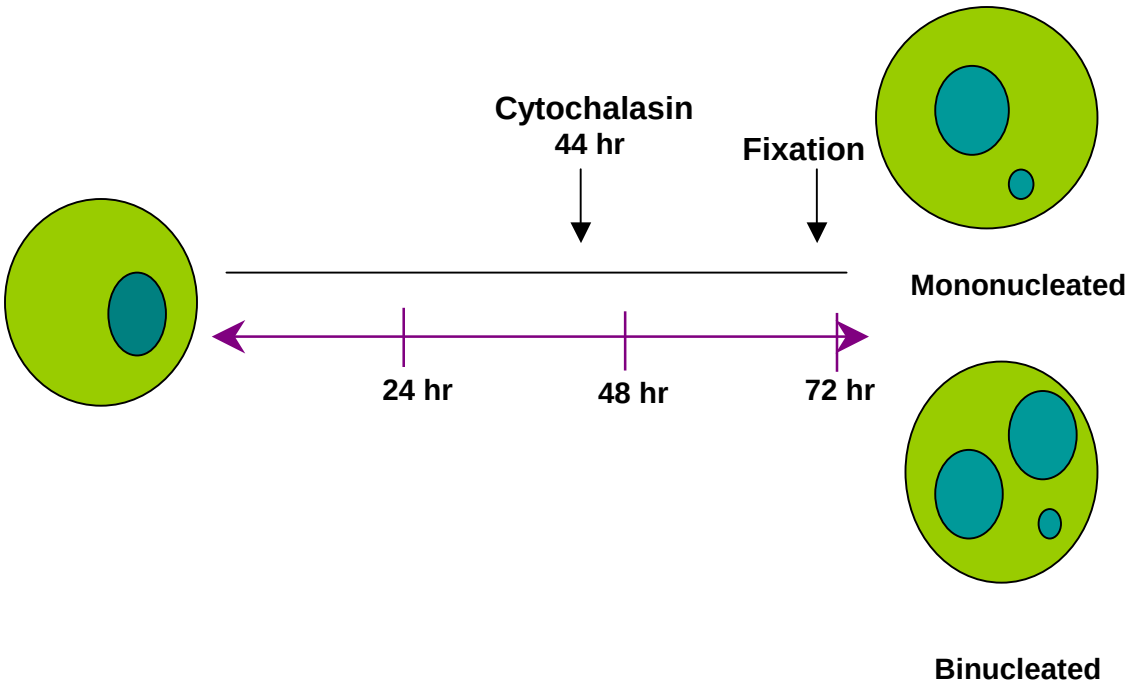


Fig 7.2 The cytokinesis blocked Micronucleus technique

Genotypic analysis

DNA was extracted from 4 ml of peripheral blood using the Genomic Prep Blood DNA Isolation kit [Amersham Pharmacia Biotech Inc, USA]. Polymerase chain reaction [PCR] followed by enzymatic digestion [Restriction Fragment Length Polymorphism] was used for the genotyping of XRCC1 *Arg*194*Trp*, *Arg*280*His*, *and Arg*399*Gln* polymorphisms, ERCC1 codon 118, XRCC3 codon 241, XPD codon 751 and ERCC4 codon 415 polymorphisms. Genotyping for XRCC1 [codons 194,280 and 399] and XPD [codon 751] were carried out by Polymerase Chain Reaction and Restriction Fragment Length Polymorphism[PCR RFLP] analysis as described earlier.

For the ERCC1 codon 118, the PCR reactions were started with a reaction volume of 50 µl containing 100 ng of genomic DNA, 0.4 mM dNTPs, 5 pmol of each primer, 1.25 unit of Taq DNA polymerase and 1 X PCR buffer, [50 mM KCl, 10mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$ and 0.1% Triton X-100]. The reactions were carried out in the following thermocycler conditions: denaturation at 94 °C for 4 minutes, 35 cycles of 30 seconds at 94°C, 45 seconds at 61°C and 30 seconds at 72°C, subsequently followed by a 10 minute extension period at 72°C.

PCR conditions for ERCC4 were, denaturation for 2 minutes at 96°C, followed by 25 cycles of denaturation at 96°C, annealing at 50°C for 15 seconds and extension at 60°C for 4 minutes. Negative controls in all PCR assays consisted of a similar reaction mixture with the template replaced with sterile water. The PCR products were visualized using a UV transilluminator after ethidium bromide staining.

For XRCC3 polymorphism, reactions were carried out in the following thermocycler conditions: denaturation at 94 °C for 2 minutes, 40 cycles of 15 seconds at 94°C, 45 seconds at 57°C and 45 seconds at 72°C, subsequently followed by a 5 minute extension period at 72°C.

The 199bp PCR products of ERCC1 were digested overnight by HpyCH4 enzyme (New England BioLabs, Beverly, MA) and led to *C/C* (21, 116 bp), *C/T* (21, 116, 137 bp), and *T/T* (137 bp) genotypes, with the control band of 62 bp for each genotype. The restriction enzyme used for *ERCC4/XPF Arg415Gln* genotype was *XmnI,* and the restricted products of *Arg/Arg*,

Arg/Gln, and *Gln/Gln* had band sizes of 96/284 bp, 96/284/380 bp, and 380 bp, respectively.

The XRCC3 PCR products were digested at 37℃ for 1 hour with 10 units of *Nla*III [New England Biolabs] in 1_ buffer supplied with the enzyme and supplemented with 100 ng/_l BSA. All *XRCC3* PCR products contain an internal *Nla*III site. After digestion, the *Thr* allele gave a segment of 315 bp , while the *Met* allele gave the digested products of 210 and 105 base pair fragments. A control band at 140 bp was seen in all samples. The digested products were resolved on 3% agarose gels. 100 bp DNA molecular weight marker was used to assess the size of the PCR – RFLP products.

Data analysis

The analysis was performed using SAS software version 8.2.The 2x2 contingency cross-tabulation tables provide the distribution of cases and controls by genotype status for each set of genes. Normal genotype was considered as the referent group. PROC FREQ was used to obtain the results to meet the objectives. The CMH option provides adjusted odds ratio for 2x2 tables. PROC FREQ was further applied to compute the odds ratio estimate using the Mantel Haenszel and logit methods. The proc freq procedure presents both the Mantel Haenszel and logit estimate for the odds ratio. When any one of the cells in the contingency table was found to have zero counts, then value 0.5 was required to be added to each cell of the

contingency tables and thus logit estimate of the common odds ratio was reported. In cases where cell counts were non-zeros the Mantel Haenszel estimate was presented.

The chi-square or Fisher's exact test was used to measure the extent to which the observed data differed from those expected if the two odds of exposure are equal. Fisher's exact test was performed when the expected frequencies was less than 5 in any cell. For the confounding factor gender (strata level at female and male), stratum specific estimates and their confidence interval were estimated. The summarization of overall results of the study such that the confounding effect of exposure was removed was also calculated. The CMH option produces the Cochran-Mantel-Haenszel statistics (general association statistic). For this stratified 2×2 table after adjusting for gender, estimates of the common odds ratio and the Breslow-Day test for homogeneity of the odds ratios were also computed.

Results

Micronuclei in Cytokinesis Blocked Cells

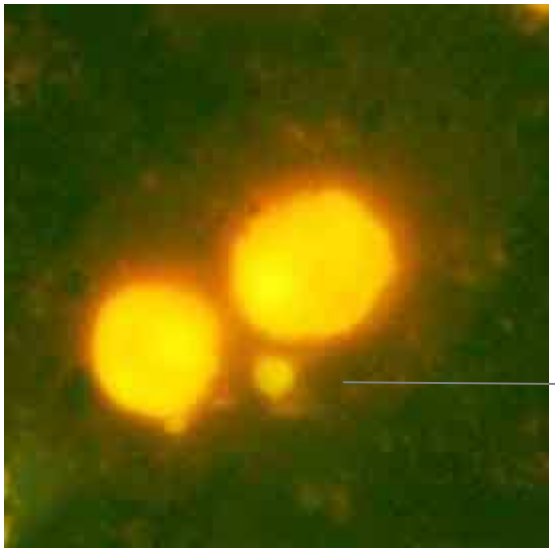
The incidence of MN in cytokinesis blocked lymphocytes of all study subjects was determined following the standard CBMN protocol. Micronuclei [MN] were scored in 1000 binucleated cells following the criteria reported by Fenech as per OECD guidelines. The standard mean obtained for cases and controls were significantly different with cases showing an increased mean of 11.8 [±1.6] from controls 4.6 [±0.7] (Table7.1)

Table 7.1 Mean Micronucleus in cases and controls

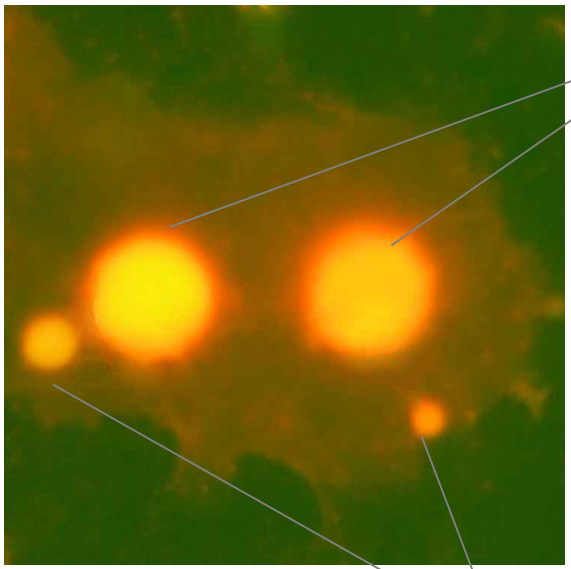
SUBJECTS	STANDARD MEAN	STANDARD DEVIATION
CASES	11.8	1.6
CONTROLS	4.6	0.7

Fig7.3

CYTOKINESIS BLOCK MICRONUCLEUS ASSAY



Micronucleus (MN)



Binucleate cells

Micronuclei (MN)

Micronuclei were classified into two categories, based on their mean frequencies: [1] High [MN 16 and more] and Low [MN 15 and less]. The association between overall cancer incidence and MN frequency is described in Table7. 2. Significant increases were found for subjects in the high [OR 3.45: 95%CI 1.65-7.19] category when compared to the low group. Gender, smoking and alcohol did not seem to have an effect on a subject's risk for cancer, whereas, use of betel [OR 7.35; 95%CI 3.21-16.8: p value 0.0001] showed a more pronounced effect on oral cancer risk. The subjects were divided into two groups based on their mean age: more than and equal to 41 and less than 41. There was no significant association between age and risk of cancer.

Table 7.2

Risk of Oral Cancer Incidence by MN Frequency, Habits, Gender and Age

VARIABLES		CASES (n=100)	CONTROLS (n=100	OR (95%CI)	P VALUE
MN FREQUENCY	*Below 16*	68	88	3.45 (1.65-7.19)	0.001
	Above 16	32	12		
GENDER	*Male*	62	55	1.33 (0.75-2.34)	0.39
	Female	38	45		
AGE	*More than and equal to 41*	11	38	0.200 (0.96-0.42)	0.00
	Less than 41	89	62		
SMOKING	*Ever*	43	38	1.23 (0.69-2.16)	0.56
	Never	57	62		
ALCOHOL	*Ever*	28	37	0.66 (0.36-1.20)	0.22
	Never	72	63		
BETEL	*Ever*	39	8	7.35 (3.21-16.8)	0.000
	Never	61	92		

Association between MN and DNA repair gene polymorphism with age, gender and lifestyle factors

Analysis was done to determine the risk association of cases with polymorphic genotype, compared to the risk associated with control group having polymorphic genotype within different MN group among females. It was observed that in the MN group (Below 16) the p-values suggested no association between genotype status and outcome except for ERCC1 and XRCC194. The odds ratios (O.R) for the genes ERCC1, ERCC4, XPD, XRCC194, XRCC280, XRCC399 and XRCC3 obtained were 3.60, 1.23, 1.26, 3.15, 1.44, 2.64, and 1.44 respectively.

However, the odds ratios suggested that the odds of occurrence of abnormal genotype in case are greater than the odds of occurrence of abnormal genotypes in the control group for all genotypes.

In the MN group (Above 16) the p-values suggest that there is no association between genotype status and outcome except for ERCC1 and XRCC194. The odds ratios (O.R) for the genes ERCC1, ERCC4, XPD, XRCC194, XRCC280, XRCC399 and XRCC3 obtained were 0.03, 0.64, 3.06, 0.02, 0.36, 3.06 and 0.36 respectively. (Table 7.3)

Table 7.3a Distribution of abnormal genotypes status in case and control by genotypes for females

MN Group	Genotype	Genotype Status	Cases (N=100)	Controls (N=100)	Odds Ratio (95% CI)	p-value*
Below 16	ERCC1	Polymorphic	12 (12.00)	10 (10.00)	3.60 (1.16,11.13)	0.0231
		Wild	9 (9.00)	27 (27.00)		
	XRCC194	Polymorphic	12 (12.00)	11 (11.00)	3.15 (1.03, 9.61)	0.0403
		Wild	9 (9.00)	26 (26.00)		
	XRCC399	Polymorphic	15 (15.00)	18 (18.00)	2.64 (0.84, 8.30)	0.0923
		Wild	6 (6.00)	19 (19.00)		
Above 16	ERCC1	Polymorphic	5 (5.00)	8 (8.00)	0.03 (0.00, 0.53)	0.0016
		Wild	12 (12.00)	0 (0.00)		
	XRCC194	Polymorphic	4 (4.00)	8 (8.00)	0.02 (0.00, 0.41)	0.0005
		Wild	13 (13.00)	0 (0.00)		
	XRCC399	Polymorphic	11 (11.00)	3 (3.00)	3.06 (0.53,17.46)	0.3892
		Wild	6 (6.00)	5 (5.00)		

The analysis for determining the risk association with case with polymorphic genotype, compared to the risk associated with the control group having polymorphic genotype within different MN group among males was also done. In the MN group (Below 16) the p-values suggested that there is association between genotype status and outcome with respect to the genes ERCC1, XRCC194 and XRCC399 while there is no association with respect to the other genes. The odds ratios (O.R) for the genes ERCC1, ERCC4, XPD, XRCC194, XRCC280, XRCC399 and XRCC3 obtained are 2.50, 0.49, 2.80, 2.72, 0.43, 2.73, and 0.30 respectively.

For the genes ERCC1, XPD, XRCC194 and XRCC399 the odds ratio suggest that the odds of occurrence of abnormal genotype in case is greater than the odds of occurrence of abnormal genotypes in the control group. But for the genes ERCC4, XRCC280 and XRCC3, the odds of occurrence are less in case than compared to control groups.

In the MN group (Above 16) the p-values suggest that there is no association between genotype status and outcome with respect to all genes except ERCC1 and XRCC194.

The odds ratios (O.R) for the genes ERCC1, ERCC4, XPD, XRCC194, XRCC280, XRCC399 and XRCC3 obtained are 14.38, 1.22, 1.50, 14.38, 2.20, 1.20 and 2.20 respectively.

Table 7.3b Distribution of abnormal genotypes status in case and control by genotypes for males

MN Group	Genotype	Genotype Status	Cases (N=100)	Controls (N=100)	Odds Ratio (95 %CI)	p-value
Below 16	ERCC1	Polymorphic	27 (27.00)	17 (17.00)	2.50 (1.10, 5.65)	0.0268
		Wild	21 (21.00)	33 (33.00)		
	XRCC194	Polymorphic	28 (28.00)	17 (17.00)	2.72 (1.20, 6.17)	0.0157
		Wild	20 (20.00)	33 (33.00)		
	XRCC399	Polymorphic	27 (27.00)	16 (16.00)	2.73 (1.20, 6.23)	0.0156
		Wild	21 (21.00)	34 (34.00)		
Above 16	ERCC1	Polymorphic	8 (8.00)	0 (0.00)	14.38 (0.67,309.8)	0.0445
		Wild	6 (6.00)	5 (5.00)		
	XRCC194	Polymorphic	8 (8.00)	0 (0.00)	14.38 (0.67,309.8)	0.0445
		Wild	6 (6.00)	5 (5.00)		
	XRCC399	Polymorphic	9 (9.00)	3 (3.00)	1.20 (0.15, 9.77)	1.0000
		Wild	5 (5.00)	2 (2.00)		

No association was seen with age or lifestyle factors like smoking, alcohol and betel chewing when risk was compared with presence of Micronuclei and the polymorphic genotype.

Genotyping of DNA repair gene polymorphisms

The 100 oral cancer patients and 100 normal controls were genotyped for all the seven gene polymorphisms. The odds ratio are given in Table 7.4

Table 7.4 Distribution of abnormal genotype status in cases and controls by genotypes

Genotype	Case (N=100)	Control (N=100)	Odds Ratio	95% CI	p-value*
ERCC1					
Polymorphic	52 (52.00)	35 (35.00)	2.01	(1.14, 3.55)	0.01
Wild	48 (48.00)	65 (65.00)			
ERCC4					
Polymorphic	20 (20.00)	29 (29.00)	0.61	(0.32, 1.18)	0.13
Wild	80 (80.00)	71 (71.00)			
XPD					
Polymorphic	55 (55.00)	38 (38.00)	1.99	(1.13, 3.51)	0.01
Wild	45 (45.00)	62 (62.00)			
XRCC194					
Polymorphic	52 (52.00)	36 (36.00)	1.93	(1.09, 3.39)	0.02
Wild	48 (48.00)	64 (64.00)			
XRCC280					
Polymorphic	24 (24.00)	35 (35.00)	0.59	(0.32, 1.09)	0.08
Wild	76 (76.00)	65 (65.00)			
XRCC399					
Polymorphic	62 (62.00)	40 (40.00)	2.45	(1.39, 4.32)	0.00
Wild	38 (38.00)	60 (60.00)			
XRCC3					
Polymorphic	22 (22.00)	35 (35.00)	0.52	(0.28, 0.98)	0.04
Wild	78 (78.00)	65 (65.00)			

The Odds Ratios (OR) for the genes, ERCC1, ERCC4, XPD, XRCC194, XRCC280, XRCC399, and XRCC3 are obtained as are 2.01, 0.61, 1.99, 1.93, 0.59, 2.45, and 0.52 respectively.

The odds ratio suggest that the odds of occurrence of polymorphic genotype in cases is greater than the odds of occurrence of polymorphic genotypes in the control group for all genotypes except ERCC4 (OR=0.61), XRCC280 (OR=0.59) and XRCC3 (OR=0.52) where the odds of occurrence is less in cases than compared to control groups

Further the p-values suggest that there is no association between genotype status and risk of oral cancer with respect to the genes ERCC4, and XRCC280 while association was observed with respect to other genes.

Association between MN and DNA repair gene polymorphisms

Presence of MN in cells indicates the extent of DNA damage in the cell. Abnormal number of MN may be related to the presence of polymorphisms in DNA repair genes and defective repair capacity of the individual. To see whether these two are correlated, we analyzed the risk of oral cancer for individuals polymorphic in the DNA repair genes XRCC1 and XPD. It was observed that carriers of the variant allele had more number of MN than the normal wild genotype in the case of XRCC 194 [OR 3.2, p value 0.02], XRCC 399 [OR 3.5, p=0.02] and XPD [OR 3.7, p=0.02] genes [Table 2].

TABLE 7.4 a

ASSOCIATION BETWEEN MN AND POLYMORPHISMS IN DNA REPAIR GENES XRCC1 and XRCC3

Genotype/Polymorphisms	MN		OR [A] (95% CI)	P Value
	Above 16	Below 16		
XRCC1 (194 codon)				
Arg/Trp+Trp/Trp	46	61	2.75 (1.47-5.14)	0.002
Arg/Arg	20	71		
XRCC1 (280 codon)				
Arg/His+His/His	14	45	0.53 (0.26-1.06)	0.09
Arg/Arg	52	89		
XRCC1 (399 codon)				
Arg/Gln+Gln/Gln	35	67	1.12 (0.62-2.03)	0.76
Arg/Arg	31	67		
XRCC3				
Thr/Met+Met/Met	14	43	0.57 (0.28-1.13)	0.13
Thr/Thr	52	91		

OR [A]= Age and gender adjusted odds

Table 7.4b

ASSOCIATION BETWEEN MN AND POLYMORPHISMS IN DNA REPAIR GENES ERCC1, ERCC4/XPF and ERCC2/XPD

Genotype/Polymorphisms	MN		OR [A] (95% CI)	P Value
	Above 16	Below 16		
ERCC1 (118 codon)				
C/T+T/T	31	56		0.54
C/C	35	78	1.23 (0.68-2.23)	
ERCC4				
Arg/Gln+Gln/Gln	11	38	0.50 (0.23-1.06)	0.08
Arg/Arg	55	96		
XPD (751 codon)				
Lys/His+His/His	31	62	1.02 (0.57-1.83)	1.000
Lys/Lys	35	72		

Discussion

The aim of this study was to determine the influence of polymorphisms in DNA repair genes on inherent micronuclei frequency in oral cancer patients and normal subjects.

Oral cancer is a serious health problem both in developing and underdeveloped nations as a consequence of long term tobacco and alcohol use. However, since not all exposed individuals actually develop cancer, variations in genetic susceptibility are equally important in the disease etiology. Each year after diagnosis between 3% and 5% of head and neck cancer patients develop a second malignancy and likewise such patients may be more genetically susceptible to cancer.

Multiple repair mechanisms have evolved in all organisms to minimize the consequences of cellular exposure to endogenous and exogenous agents that cause deleterious alterations in DNA (241) and a large number of low risk genes are known to modulate the carcinogenic process in humans. Amino acid differences (especially at conserved sites) in these enzymes could result in changes in repair proficiency. Therefore evaluating the functional relevance of these genotypes becomes important such that they can be used as tools for suggesting tumor response as well as in deciding personalized treatment.

The CBMN assay is a genotoxicity assay that provides concurrent information on a variety of chromosomal damage endpoints that reflect chromosomal breakage, chromosome rearrangements, and gene amplification. The frequency of micronuclei (MN) in peripheral blood lymphocytes (PBL) is being expansively used as a biomarker of chromosomal damage and genome stability in human populations especially in the past decade. Many studies have been done to investigate the effect of occupational mutagens on micronucleus frequency, but very few have considered the effect of genetic polymorphisms of genes involved in the DNA repair and MN frequencies.

Frequency of inherent micronuclei were significantly more in oral cancer patients than in normal subjects and carriers of the variant allele had more number of MN in their cells than the normal wild genotype in the case of XRCC1 194 codon, 399 codon and the XPD genotypes.

In the present study, the frequencies of micronuclei were looked into and the numbers were significantly higher in oral cancer patients than in the control population. Cheng et al. reported similar results after evaluating the micronucleus frequency in 42 patients with lung cancer and 55 controls (242). The significantly higher spontaneous micronuclei levels observed in the cases suggest a higher background level of genetic instability in the cancer patients.

XRCC1 polymorphisms have shown to influence micronuclei formation significantly. This is conceivable, although the chromosome breaks detected by the CBMN assay are derived from double-strand breaks, studies have shown that XRCC1 mutants EM9 and EM11 cells showed a significantly reduced rate of single-strand breaks and double-strand breaks rejoining induced by ionizing radiation (243-245). It is evident that a longer lifetime of persistent single strand breaks would increase the likelihood that the breaks created in close opposition existed simultaneously and were converted to double-strand breaks (246,247). Therefore, the defective or variant XRCC1 protein may decrease the ability for single-strand breaks repair and inversely increase the transformation of single-strand breaks to double-strand breaks (248). XRCC1 is required for DNA strand break. Many studies have given direct proof of this by means of *in vitro* and *in vivo* models. Brem et al showed that reduced XRCC1levels partially inhibit DNA rejoining (as measured by the comet assay) and increase MMS-induced micronuclei formation. This is consistent with findings in XRCC1-deficient rodent cells, which exhibit delayed repair kinetics and increased rates of markers of genetic instability (249-253). Genetic instability as a consequence of DNA repair deficiency is regarded as a facilitating trait for carcinogenesis (254), and biomarkers of genetic damage such as micronuclei formation are considered to be causally related to early stages of chronic diseases, especially cancer (255).

The XPD protein is absolutely necessary in nucleotide excision repair. Once the DNA lesion has been recognized by specific proteins, the helicase activity of XPD, in concerted action with the xeroderma pigmentosum group B helicase, allows the opening of the double helix so that the damaged strand can be cut and the damaged piece of DNA removed. XPD activity is essential for life; total absence of the *XPD* gene results in embryonic lethality (256). Point mutations in the human XPD protein play a causative role in DNA repair-deficiency diseases (xeroderma pigmentosum, trichothiodystrophy, and Cockayne syndrome), which are characterized by high ultraviolet-light hypersensitivity, a high mutation frequency, and cancer-proneness, as well as some mental and growth retardation and probably aging (257). The very high cancer-proneness of xeroderma pigmentosum patients (including internal cancers) shows clearly the relevant association between DNA repair efficiency and cancer risk (258)

Various studies have compared genotype-phenotype relations using XPD gene and MN. A clear association was revealed between *GSTT1*-null and *XPD* polymorphisms and both spontaneous and BLM-induced MN frequencies, in a biomonitoring study involving 200 healthy volunteers, whereas the effect of the *XRCC1* polymorphism was marginally significant only with regard to spontaneous MN frequency. Genotype analysis did not reveal a clear association between the other studied SNPs (*GSTM1*and *XRCC3*) and MN frequencies. Other studies have addressed the question whether SNPs in *GSTT1*, *GSTM1*and DNA repair genes affect the

spontaneous level of DNA damage, regardless of specific genotoxic exposure (259,260) Various studies have also evaluated the performance of MN assay for the detection of chemically induced genotoxicity *in vitro* (261-263).

The effect of smoking on micronucleus frequency in peripheral blood lymphocytes has not been consistent across studies, which generally have been small and not properly designed to detect the effect of smoking as the main outcome measure (264-266). However, a pooled reanalysis of 24 databases (5,710 subjects, of which 1,409 were current smokers) from the HUMN Project revealed that micronucleus frequency was not influenced by the number of cigarettes smoked per day among subjects occupationally exposed to genotoxic agents, whereas a U-shaped curve was observed for non exposed smokers, with a significant increase of micronucleus frequency in individuals smoking 30 cigarettes per day (267,268). Our results showed no significant association for gene-environment interactions like smoking and alcohol and age, but positive interactions were observed for male and female, who were also carrying the variant allele.