CHAPTER 8

DNA Repair Capacity (DRC) and its role as a phenotypic marker for oral cancer

Introduction to DNA Repair Capacity

Genetic instability, which drives tumorigenesis, is itself fuelled by DNA damage and by errors made by the DNA repair machinery. Cancer syndromes such as XP and Bloom's syndrome support the involvement of DNA repair processes in governing the amount of genomic instability that a cell experiences (269). DNA repair is a ubiquitous defense mechanism that is critical to maintaining the integrity of the genome and repairing the damage from exposure to exogenous environmental xenobiotics, as well as to endogenous damage (*e.g.,* from oxidative metabolism) or spontaneous disintegration of chemical bonds in DNA. There is quite substantial interindividual variation in DRC within a population. At the extreme end of this spectrum are patients with XP, who have a defect in NER, and who exhibit thousand-fold increased risks of skin cancer. There is a larger subgroup with reduced DRC who are likely to be at increased cancer risk, but are phenotypically normal. The challenge for molecular epidemiological research is to be able to identify this at-risk subgroup, who could be targeted for the most intensive behavior modification changes and screening interventions.

More than 80% of oral cancers are attributed to tobacco. However, only a fraction of smokers (15%) will develop oral cancer in their lifetime. One of the several cellular processes that could explain this interindividual variation in risk is DRC (270). It is believed that genetic variation further modifies an individual's susceptibility to the disease. Individuals with suboptimal DNA repair capacity, a genetically controlled phenotype, may be at increased risk

of developing cancer upon exposure to carcinogens that may have a lower carcinogenic potential in individuals with normal DNA repair capacity. Thus, developing functional assays that can quantify individual's DNA repair capacity is crucial for identifying high-risk subgroups in the general population. To date, a variety of assays have been developed to assess DNA repair capacity (271,-279). There are many assays that measure the efficiency of the multiple steps of excision repair individually; the ability to test the whole pathway is often needed for population studies, in which time, cost, and repeatability of measurements are major concerns. Therefore, measuring the expression level of damaged reporter genes using host-cell reactivation is the assay of choice. This assay uses undamaged cells, is relatively fast, and is an objective way of measuring intrinsic cellular repair. The host cell reactivation assay, an in vitro assay, has the capability to assess the repair efficiency of specific excision repair pathways by using relevant challenge mutagens. This assay has been implemented to quantify DNA repair capacity in studies of lung cancer (274,275), skin cancer (276, 277), head and neck cancer (278), and prostate cancer (279).

Genetic Influence on Phenotype Assays

Since damage and repair assays are labor intensive and not amenable to large-scale, high-throughput application in population studies, the long-term goal was to identify genotypes that predict the phenotypes of suboptimal DRC and mutagen sensitivity. Mohrenweiser and Jones (166) have pointed out

several lines of evidence that differences in DRC reflect genetic differences. DRC in lymphocyte subpopulations, from an individual, exhibit similar repair capacities. Furthermore, intraindividual variation in repair capacity in different subpopulations of lymphocytes is significantly smaller than is interindividual variation (280). There are also published studies showing that reduced repair capacity may aggregate in first-degree relatives of cancer probands (281-282). Ankathil *et al.* (283) showed that relatives of colorectal cancer patients tended to exhibit higher bleomycin-induced b/c.

DNA Repair Gene Polymorphisms

Extensive work is under way to resequence DNA repair genes to identify variations that may be associated with reduced function of their encoded proteins, rather than absence of function. Such polymorphisms could explain inter-individual differences in DRC (284-286). Although the variant alleles are likely to be associated with only modest risk, because they exist at polymorphic frequency, the attributable risks assume substantial relevance. As Berwick and Vineis (271) point out, studies that compare genetic polymorphisms with functional assays will likely be the most valuable type of study to clarify the role of a defect in DRC with the development of cancer. Hence this study focused on five genes from different repair pathways. It is likely that single nucleotide polymorphisms (SNP) in coding and regulatory sequences of genes in the repair pathway may result in subtle structural

alterations in DNA repair enzymes and modulate cancer susceptibility by affecting individual DRC.

The host cell reactivation assay technique

In this assay, lymphocytes are transfected with damaged nonreplicating recombinant plasmid harboring a *LUC(luciferase) gene*. To study tobacco-related cancers, a mutagen challenge is given, which is either a major chemical constituent of tobacco smoke or UV. These can irreversibly damage DNA by covalent binding or oxidation (287); such damages will be repaired by the BER/NER pathway that eliminates the widest variety of damage to the human genome, including UV-induced photoproducts, bulky monoadducts, cross-links, and oxidative damage . Because even a single unrepaired DNA adduct can effectively block LUC transcription , any measurable LUC activity will reflect the ability of the transfected cells to remove damage from the plasmids. For each determination of the LUC assay, LUC absorbance is measured by a luminometer. The LUC absorbances are recorded for the cells with undamaged (background reading) and UV-damaged (repair reading) plasmids, respectively.

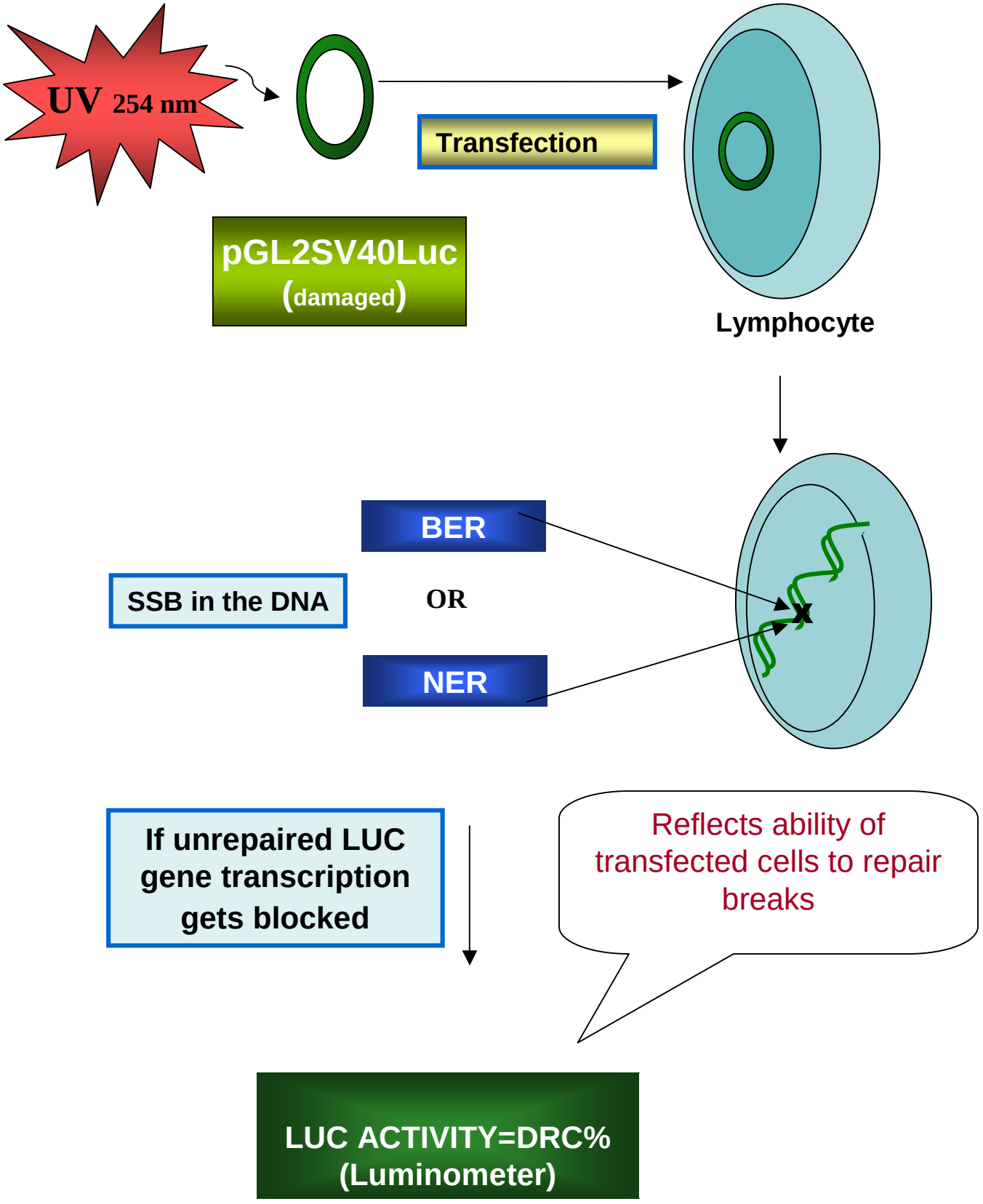
DRC (%) is calculated as a ratio of the damaged plasmid values to the undamaged plasmid values multiplied by 100%. The dose-repair curve for the LUC activity was first established by performing the LUC assay with three lymphoblastoid cell lines with different levels of DRC and primary

lymphocytes from three donors. Assays repeated four times with one repair proficient cell line and one repair-deficient cell line indicated that the LUC assay could easily distinguish repair-proficient from repair-deficient cell lines with relatively small coefficients of variation (5.4% and 7.2%, respectively). The DRC phenotype by the two independent CAT and LUC assays in parallel showed that they were highly correlated, with a correlation coefficient of 0.651 (*P*= 0.0001) suggesting that these two assays are comparable (288).

Briefly, the frozen lymphocytes of each patient are thawed and processed to ensure a cellular viability of _80%. The cells are then stimulated so that they take up the plasmids (289-290). The number of viable, large lymphoblasts in the culture for each sample is counted to calculate the blastogenic rate [(number of lymphoblasts/number of lymphocytes stimulated) X 100]. Duplicate transfections with either untreated plasmids or damaged plasmids were always performed. Both lymphocytes and skin fibroblasts from patients who have basal cell carcinoma but not XP have lower excision repair rates than individuals without cancer (291). Consequently, the repair capacity of lymphocytes can be considered a reflection of the overall repair capacity of an individual and a quantitative measurement of the DRC of the host cells.

Fig 8.1

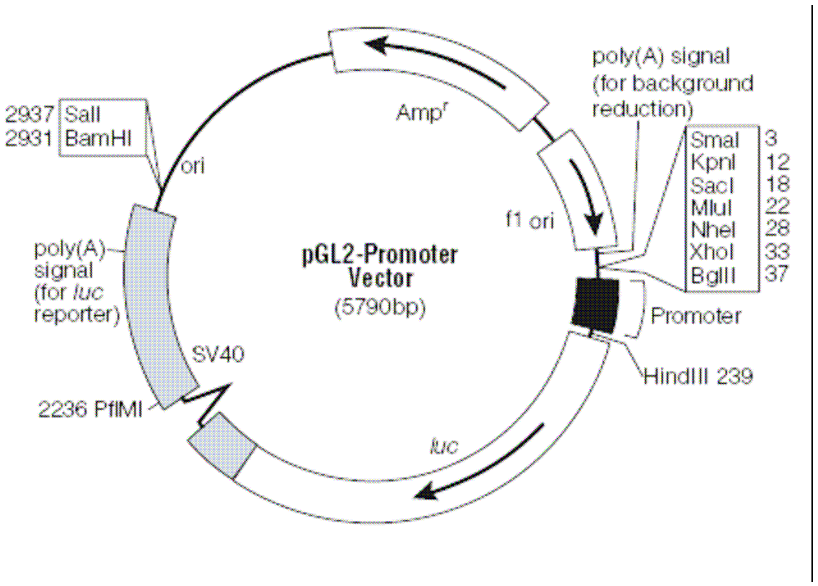
ASSESSMENT OF DNA REPAIR CAPACITY BY THE HOST CELL REACTIVATION ASSAY



pGL2SV40LUC plasmid vector

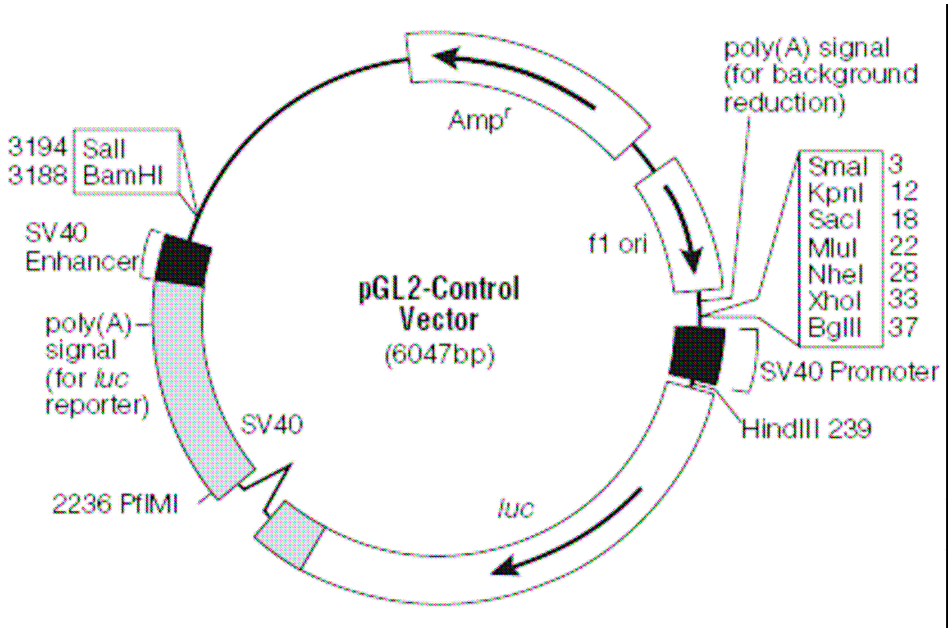
The pGL2-Promoter Vector contains the SV40 promoter upstream of the luciferase gene. DNA fragments containing putative enhancer elements can be inserted in either orientation and upstream or downstream of the promoter-*luc* transcriptional unit.

Fig 8.2 a



The pGL2 promoter plasmid vector map

Fig 8.2 b



The pGL2 control vector map

The pGL2-Control Vector contains the SV40 promoter and enhancer sequences,

resulting in strong *luc* expression in many types of mammalian cells. This plasmid is useful in monitoring transfection efficiency in general and is a convenient internal standard for promoter and enhancer activities expressed by pGL2 recombinants.

Aim

This part of the work was aimed to understand the role of DNA repair capacity in individuals and observe whether DRC is affected by the presence of SNPs in DNA repair genes.

Materials and Methods

Subjects

The study population included 50 oral cancer patients being seen at the Head and Neck Clinic of the Regional Cancer Centre, Thiruvananthapuram. A total of 50 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.

Cells: Human peripheral blood lymphocytes (PBLs) were isolated from oral cancer patients and normal control population by Ficoll gradient centrifugation and stored in liquid nitrogen. The cells were thawed and cultured in RPMI 1640, 20% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin.

Plasmids: The pGL2SV40promoter and pGL2SV40control reporter gene vectors (Promega) were used for transfection, containing the bacterial luc gene. pRTK luc gene containing the renilla luciferase gene was also used for measuring Renilla activity.

UV Treatment: For UV treatment, the plasmid DNA was pipetted into a sterile plastic 35-mm tissue culture dish placed on ice. Irradiation was performed with an unfiltered UV lamp (254nm) at a dose rate of 400J/cm^2 for 3 minutes and 20 seconds.

Transfection: Transfection was performed by using lipofectamine on 35-mm tissue culture dishes containing 1 x 10^6 cells plated 24 hr previously. 5 ug of plasmid DNA was used per plate. Cells were incubated with plasmid DNA for 45 minutes at room temperature. Cells were then fed with 20% RPMI and incubated for 48-72 hrs at 37^0C to allow repair of damaged DNA. Detailed protocol is given in Appendix 1.

Luciferase assay*: Luc* activity in crude cell lysates was measured using Dual Luciferase Assay Kit (Promega) and quantitated in a luminometer. DNA Repair Capacity (DRC %) was calculated as ratio of undamaged plasmid value to damaged plasmid value multiplied by 100.

Results

Genotype analysis

The first objective of the study was to determine the risk association of the case group having polymorphic genotype, compared to the risk associated with the group control having polymorphic genotype.

Table 1 presents the number and percentages of subjects in the case and control groups with wild, heterozygous polymorphic and homozygous polymorphic condition for each of the genes.

The odds ratio with respect to all genes showed that the odds of polymorphic genotype occurring in the case group are higher than it occurring in the control group. It was seen that there existed no association between the presence of genotypic variants in cases and controls in the genes XRCC1 (194,280 and 399 codons), XRCC3, ERCC1 and ERCC4. However, the odds ratio with respect to XPD showed that the risk of a subject carrying the polymorphic Gln/Gln variant to acquire cancer was 11.35 times higher with a confidence interval of (1.35, 95.46).

When the heterozygous polymorphic group was compared in cases and controls, it was observed that there existed an association between the genotype status and the outcome for each of the genotypes except ERCC4 (p=0.1595) and XRCC280 (p=0.4870).

Table 8.1 Distribution of abnormal and partially abnormal genotype status in case and control by genotypes

Genotype	Case (N=50)		Control (N=50)		Odds ratio	95 % CI	p-value*
	N	%	N	%			
ERCC118							
T/T	3	6	1	2	4.44	(0.44, 45.01)	0.3038
C/T	20	40	9	18	3.29	(1.30, 8.31)	0.0099
C/C	27	54	40	80			
ERCC4							
Arg/Gln	7	14	2	4	3.91	(0.77, 19.83)	0.1595
Arg/Arg	43	86	48	96			
XPD							
Gln/Gln	8	16	1	2	11.35	(1.35, 95.46)	0.0105
Lys/Gln	11	22	5	10	3.12	(0.99, 9.89)	0.0458
Lys/Lys	31	62	44	88			
XRCC194							
Trp/Trp	1	2	0	0	4.32	(0.17, 109.79)	0.4167
Arg/Trp	20	40	8	16	3.62	(1.40, 9.33)	0.0061
Arg/Arg	29	58	42	84			
XRCC280							
Arg/His	6	12	3	6	2.14	(0.50, 9.07)	0.4870
Arg/Arg	44	88	47	94			
XRCC3							
Thr/Met	12	24	3	6	4.95	(1.30, 18.81)	0.0117
Thr/Thr	38	76	47	94			
XRCC399							
Gln/Gln	4	8	1	2	5.47	(0.58, 51.42)	0.1665
Arg/Gln	16	32	8	16	2.73	(1.04, 7.21)	0.0386
Arg/Arg	30	60	41	82			

Since, the homozygous polymorphic subjects were less, the homozygous and heterozygous polymorphic group were combined and then assessed for risk of oral cancer among cases and controls. It was observed that variant

genotypes of ERCC118 (OR=3.41, p value= 0.005), XPD (OR=4.49, p value=0.002), XRCC1 194codon (OR=3.80, p value= 0.004), XRCC1 399 codon (OR=3.04, p value= 0.015) and XRCC3 (OR=4.95, p value= 0.011) exhibited nearly 3 to 4 fold increase in risk compared to the wild genotype.

Table 8.2 Distribution of polymorphic genotype status in case and control by genotypes

Genotype	Case (N=50)		Control (N=50)		Odds ratio	95 % CI	p-value*
	N	%	N	%			
ERCC118							
Polymorphic	23	46	10	20	3.41	(1.40, 8.29)	0.0057
Wild	27	54	40	80			
ERCC4							
Polymorphic	7	14	2	4	3.91	(0.77, 19.83)	0.1595
Wild	43	86	48	96			
XPD							
Polymorphic	19	38	6	12	4.49	(1.61, 12.55)	0.0027
Wild	31	62	44	88			
XRCC194							
Polymorphic	21	42	8	16	3.80	(1.48, 9.75)	0.0042
Wild	29	58	42	84			
XRCC280							
Polymorphic	6	12	3	6	2.14	(0.50, 9.07)	0.4870
Wild	44	88	47	94			
XRCC3							
Polymorphic	12	24	3	6	4.95	(1.30, 18.81)	0.0117
Wild	38	76	47	94			
XRCC399							
Polymorphic	20	40	9	18	3.04	(1.21, 7.60)	0.0153
Wild	30	60	41	82			

DNA repair phenotype of the study population

The study included 50 oral cancer patients and 50 normal healthy persons. DRC was measured in lymphocytes of both oral cancer patients and normal controls using a luminometer following the standard protocol as developed by L Grossman et al. (ref). Based on the mean values, DRC was divided into two groups: DRC% above 50.0 and DRC% below 50 (both groups being exclusive of each other). The former were considered to be as “normal” repair capacity, whereas, the latter was categorized as “subnormal/suboptimal” DNA repair capacity. It was observed that mean DRC was less in cases than in controls. (Table 8.3)

Table 8.3 Mean DRC in cases and controls

Subjects	Number (N)	Mean	Standard deviation
Case	50	57.95	24.7
Control	50	71.72	15.2

When risk was assessed for the subnormal repair group when compared to the normal group using chi square analysis (Table 8.4), it was observed that

there was a 5 fold increase in risk for the subnormal group to acquire cancer in the case of XRCC1 194 codon (OR=5.26, 95% CI= 2.02-13.7, p value=0.001) ; 2 fold in the case of XRCC1 399 codon (p value=0.04); 10 fold and 3 fold respectively in the case of ERCC1 (p value=0.0001) and XPD/ERCC2 (p value=0.009)

Table 8.4 Association between DRC and polymorphisms in DNA repair genes XRCC1, ERCC1 and XPD

Genotype/Polymorphisms	DRC (%) Above 50	Below 50	OR [A] (95% CI)	P Value
XRCC1 (194 codon)				
Arg/Trp+Trp/Trp	14	15	5.26 (2.02-13.7)	0.001
Arg/Arg	59	12		
XRCC1 (399 codon)				
Arg/Gln+Gln/Gln	17	12	2.63 (1.03-6.70)	0.049
Arg/Arg	56	15		
ERCC1 (118 codon)				
C/T+T/T	14	19	10.0 (3.64-27.50)	0.0001
C/C	59	8		
XPD (751 codon)				
Lys/His+His/His	13	12	3.69 (1.40-9.71)	0.009
Lys/Lys	60	15		

OR [A]= Age and gender adjusted odds

DRC and association with habits, age and gender

DRC was classified into two categories, based on their mean frequencies: [1] Normal (above 50%) and subnormal (less than 50%) The association between overall cancer incidence and DRC is described in Table 8.5. DRC was low in cases group compared to controls and carried a risk of 7 fold. (p=0.0001). Gender, smoking and alcohol did not seem to have an effect on a subject's risk for cancer, whereas, age showed a more pronounced effect on oral cancer risk. The subjects were divided into two groups based on their mean age: below 46 and above 46. There was seen a significant association between age and risk of cancer. (p value 0.0001). There were no betel quid users in the control group so it was not included in the analysis.

Table 8.5 Risk of Oral Cancer by DRC%, Habits, Gender and Age

VARIABLES		CASES (n=50)	CONTROLS (n=50	OR (95%CI)	P VALUE
DRC(%)	*Below 50*	22	5	7.07 (2.40-20.8)	0.0001
	Above 50	28	45		
GENDER	*Male*	34	35	0.91 (0.39-2.12)	1.000
	Female	16	15		
AGE	*Below 46*	4	25	11.5 (3.5-36.7)	0.0001
	Above 46	46	25		
SMOKING	*Ever*	19	13	1.74 (0.74-4.08)	0.28
	Never	31	37		
ALCOHOL	*Ever*	15	9	0.66 (0.36-1.20)	0.24
	Never	35	41		

Discussion

The host-cell reactivation assay is a direct measure of repair kinetics (4), unlike the cytogenetic assays that only indirectly infer DRC from cellular damage remaining after mutagenic exposure and recovery (271), and as such probably reflects general and nonspecific impairment of the DNA repair machinery. Peripheral blood lymphocytes (PBLs) are the tissue of first choice for molecular epidemiological studies that assay DRC (292). Host Cell Reactivation Assay involves measuring cellular ability to remove damage from plasmids transfected into lymphocyte cultures *in vitro* by expression of damaged reporter genes

This study observed that DRC was indeed less in oral cancer patients than in the control population. The risk seemed to increase with diminishing repair capacity. It was seen that as repair capacity diminished, the probability of being a case increased.

In a much larger case-control analysis, the mean DRC and SD for 764 lung cancer cases was 7.82% (2.82) compared with 8.79% (3.87) for the 677 controls (P =0.001). The adjusted univariate risk estimate associated with suboptimal DRC (defined as below the control median) was 1.50 (1.2–1.9); When evaluated by quartiles of DRC, the risks increased with diminishing repair capacity.

The next logical step was evaluating the functional relevance of these polymorphisms. We have evaluated the correlation between some of the DNA repair gene polymorphisms and our functional DNA repair data.

When genotype – phenotype correlations was studied it was noted that polymorphisms in DNA repair genes had a subtle effect on DNA repair capacity of an individual. The XRCC1 (BER pathway) and XPD and ERCC1 (both NER pathway) gene polymorphisms had a modest risk association with cancer outcome. In other words, the results suggested that these *XPD and XRCC1* polymorphisms had a dominant effect on DRC in cases and a smaller effect on DRC in controls.

Amino acid differences (especially at conserved sites) in these enzymes could have resulted in changes in repair proficiency. There are a variety of factors that modulate the path from genotype to phenotype including protein-protein interactions, post-translational modification, gene silencing, epigenetic regulation, and environmental factors. Furthermore, proteins involved in DNA repair pathways are often multifunctional, resulting in a variety of phenotypes

These data are consistent with some of the published small-scale studies looking at such genotype-phenotype correlations. Hou *et al.* (293) noted a significant trend for increasing aromatic DNA adduct levels with increasing number of variant alleles in exon 10 (P = 0.02) or in exon 23 (P =0.001). In addition, subjects with the combined exon 10 AA and exon 23 CC genotype showed a significantly higher level compared with those with any of the other

genotypes (*P* = 0.02). Hu *et al.* (294) reported that in both prostate cancer cases (*n* = 66) and controls (*n*= 54), those homozygous for the variant allele had lower DRC (8.7% and 6.4%, respectively) than those with the wild-type genotype (11.1% and 10.9%).

However, Moller *et al.* (295) reported no relationship of the *Lys751Gln* polymorphism with DRC (measured by the host cell reactivation assay or the comet assay) in 80 subjects, including 20 healthy subjects. Lunn *et al.* (296) reported that possessing the Lys/Lys 751 common *XPD* genotype was associated with increased risk of suboptimal DRC (as reflected in the number of

X-ray-induced lymphocyte chromatid aberrations). They found no association with the Asn312 allele. In another small sample of 76 healthy subjects, no association was noted between SCE frequencies or in the presence of polyphenol DNA adducts by *Lys751Gln* genotype (297).

In yet another study lower DRC, as measured by the LUC assay in 102 healthy non-Hispanic white subjects, was consistently associated with homozygosity of the *XPD* polymorphic alleles, representing a reduction of 10% in DRC compared with their wild-types. For the *Gln751Gln* genotype compared with the *Lys751Lys* genotype this difference was statistically significant(286).

XRCC1 (The X-ray repair cross-complementing) gene also seemed to have an adverse effect on DRC in cases in this study. The 194Trp codon was associated with 5 fold increase in risk of acquiring cancer (p=0.0001), whereas the 399Gln was associated with 2 fold increase in risk of oral cancer (p=0.049)

In a recent evaluation of 524 lung cancer cases and 524 controls, it was found that the allele frequencies were 0.07 and 0.38 for *XRCC1* exon 6 and exon 10 variant alleles, respectively, which are consistent with many other studies (298,299).The differences of allele frequencies detected among these studies might be because of different sample sizes and study populations. Lunn *et al.* (298) noted that individuals with the *399 Gln* allele were at significantly higher risk (OR, 2.4) for exhibiting detectable aflatoxin B1 adducts and higher glycophorin A variant frequency than 399Arg/Arg carriers.

Duell *et al.* (297) found elevated SCE frequencies and polyphenol adducts with *399 Gln/Gln* homozygous genotypes. Abdel-Rahman and el-Zein (300) evaluated functionality of the 399 Gln polymorphism in 47 volunteers by measuring 4-(methylnitrosamino)- 1-(3-pyridyl)-1-butanone-induced SCEs. They noted that individuals carrying the Gln allele had significantly higher numbers of SCEs in response to 4-(methylnitrosamino)- 1-(3-pyridyl)-1-butanone treatment compared with those with the Arg/Arg genotype. No differences were detected with the codon 194 genotype.

These findings are biologically plausible. The DNA repair-deficient cell line, EM-11, which has high background SCE frequencies, has a mutation in XRCC1 at codon 390 (88). This cell line is also sensitive to accumulation of single-strand DNA breaks after damage. The *XRCC1* codon 399 is located within the BRCT domain (amino acids 301–402) that interacts with poly(ADR-ribose), and that is present in many DNA damage response and cell-cycle checkpoint proteins. This region also has homology with yeast Rad4 repair-related genes. Because the role of *XRCC1* in BER brings together DNA polymerase , DNA ligase III, and poly(ADP-ribose) polymerase at the site of DNA damage, the exon10 variant could have an altered repair activity. The codon 194 polymorphism is in the linker region of the XRCC1 NH2-terminal domain separating the helix 3 and _-pol involved in binding a single nucleotide gap DNA substrate (301). Therefore, it is less likely to cause a significant change in repair function.

Whereas studies of actual protein function and related DNA repair phenotype are needed to confirm that these amino acid differences do modulate DRC, these data are indeed suggestive, and provide additional confirmation that these polymorphisms may be important in cancer risk assessment.

For a complex disease like cancer, multiple genes, each with a small effect, probably act independently or interact with other genes to influence the disease phenotype. Although these data suggest that the polymorphisms

have functional relevance, biochemical and biological characterization of the variants are needed to validate the findings.