CHAPTER 6

Function of XRCC3 in Homologous Recombination Repair pathway and oral cancer susceptibility
XRCC3 and Homologous Recombination Repair

DSBs (Double-Strand Breaks) are perhaps the most serious form of DNA damage because they pose problems for transcription, replication, and chromosome segregation. Damage of this type is caused by a variety of sources including exogenous agents such as IR (Ionizing Radiation), UV (Ultraviolet Light) and certain genotoxic chemicals, endogenously generated ROS (Reactive Oxygen Species), replication of ss DNA (single-stranded DNA) breaks and mechanical stress on the chromosomes. DSBs differ from most other types of DNA lesions in that they affect both strands of the DNA duplex and therefore prevent use of the complementary strand as a template for repair. Failure to repair these defects can result in chromosomal instabilities leading to deregulated gene expression and carcinogenesis. To counteract the detrimental effects of these potent lesions, cells have evolved two distinct pathways of DSB repair, HR (Homologous Recombination) and NHEJ (Non-Homologous End Joining) (182). In the process of HR, a DSB is repaired by copying the missing information from the sister chromatid or homologous chromosome, resulting in the exact restoration of the DNA. In the NHEJ pathway, where the two DNA ends are connected without the need for longer stretches of homology, repair of a DSB is error prone and frequently leads to small deletions (183). The cellular decision as to which pathway to utilize for DSB repair is largely influenced by stage within the cell cycle at the time of damage acquisition (182). Because HR prefers the homologous sister chromatid as a template, HR repair is the main pathway for postreplicative repair during late S/G2-phase, whereas NHEJ is utilized in G1/early S-phase (184). Both pathways are highly conserved throughout eukaryotic evolution.
but their relative importance differs from one organism to another. Simple eukaryotes such as the yeasts S.cerevisiae and S.pombe rely mainly on HR to repair radiation-induced DNA DSBs. In contrast, in mammals the NHEJ pathway predominates in many stages of the cell cycle-particularly in G0 and G1-although HR is also of importance, particularly during S- and G2-phases (182).

HR-directed repair corrects DSB defects in an error-free manner using a mechanism that retrieves genetic information from a homologous, undamaged DNA molecule. The majority of HR-based repair takes place in late S- and G2-phases of the cell cycle when an undamaged sister chromatid is available for use as repair template (185). The Rad52 epistasis group of proteins, including, Rad51, Rad52, Rad54, Rad55, Rad57, Rad59, MRE11 (Meiotic Recombination-11) and NBS1 (Nijmegen Breakage Syndrome-1) function in the initial steps of meiotic recombination and are also involved in recombination processes in mitotic cells (186). The Rad52 protein itself is the initial sensor of the broken DNA ends. The initial steps in DSB repair by HR involve processing of DNA ends to produce 3’ ss-tails, the substrates for homologous pairing and strand invasion. Rad51 and Rad54, and other recombination factors act during the homologous pairing stage of the reaction, which follows exonucleolytic processing by forming a nucleoprotein filament along the 3’ ss-tails (187). Processing of the damaged ends ensues the production of 3’ ssDNA overhangs. The newly generated ssDNA ends are bound by Rad51 to form a nucleoprotein filament. Other proteins including RPA (Replication Protein-A), Rad52, Rad54, BRCA1, BRCA2 (Breast Cancer Susceptibility Proteins), and several additional Rad51-related proteins serve as accessory factors in filament assembly and subsequent Rad51 activities. The Rad51
nucleoprotein filament then searches the undamaged DNA on the sister chromatid for a homologous repair template. Once the homologous DNA has been identified, the damaged DNA strand invades the undamaged DNA duplex in a process referred to as DNA strand exchange. A DNA Polymerase then extends the 3' end of the invading strand and subsequent ligation by DNA Ligase-I yields a heteroduplexed DNA structure. This recombination intermediate is resolved and the precise, error-free correction of the DSB is complete.

Loss of HR leads to an inability to successfully traverse S-phase, and this is probably due to an inability to restart replication at sites where DNA replication forks have collapsed as a consequence of encountering endogenously generated lesions such as DNA ss-breaks (Ref.1). Such lesions are potential inducers of carcinogenesis through activation of proto-oncogenes, inactivation of tumor suppressor genes or loss of heterozygosity. Therefore, effective repair of DSBs is of great importance for the maintenance of genome stability and prevention of carcinogenesis (186).
Fig 6.1 The Homologous Recombination Repair Pathway

(Source: Applied Biosystems)
**XRCC3 gene**

The XRCC3 gene is located in the 14q32.3 region. The XRCC3 protein participates in DNA double-strand break/recombinational repair and is a member of a family of Rad-51-related proteins that probably participate in homologous recombination to maintain chromosome stability and repair DNA damage (188). For the XRCC3 gene, four coding SNPs (one synonymous and three amino acid substitutions) and 109 intronic SNPs have been described, but most of them have not been studied in relation to cancer risk. The prevalence of the XRCC3-241 polymorphism between different ethnic groups (the prevalence of Met/Met homozygosity was 4.6 percent in African Americans, 0.2 percent in Asians, and 12.4 percent in Caucasians; p < 0.001). The XRCC3-241Met variation is a nonconservative change, but it does not reside in the adenosine triphosphate binding domain, the only functional domain identified in the protein. (189)

The XRCC3 protein directly interacts with and stabilizes RAD51 and the closely related RAD51C (21–25). XRCC3 is a paralog of RAD51 and, similar to RAD51, it is essential for genetic stability (190-194). A polymorphism at codon 241 in the XRCC3 gene results in a Thr-to-Met amino acid substitution (88). Positive associations between XRCC3-241Met and malignancies have been made by several groups, including studies on bladder cancer and melanoma skin cancer (195). The variant XRCC3-241Met allele has also been associated with a higher level of DNA adducts compared with those individuals with the wild-type gene (196).
Aim

The study examined the genetic polymorphisms of DNA repair gene XRCC3 polymorphisms to see whether the presence of polymorphic variant increases the susceptibility to oral cancer by affecting DNA repair capacity of the HRR pathway.

Specific objectives

The present study was conducted with the following specific aims:

- To evaluate the frequency of XRCC3 polymorphic variants among the study subjects.
- To assess the effect of these gene polymorphisms on oral cancer risk

Materials and methods

Study population

The present study included 110 oral cancer patients, 44 subjects with hyperplastic leukoplakia and 40 with dysplastic leukoplakia, being seen at the Head and Neck Clinic of the Regional Cancer Centre, Thiruvananthapuram. A total of 110 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 all as far as possible 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.

**DNA Extraction**

DNA was extracted from the whole blood using the Genomic Prep Blood DNA Isolation kit (Amersham Pharmacia Biotech Inc, USA). The detailed procedure for genomic DNA extraction is given in Appendix 1 and explained briefly in Chapter 4.

**PCR analysis of XRCC3 Thr241Met polymorphisms**

The genomes of codon 241 were amplified in a PCR using the following primers

F 5'-GGTCGAGTGACAGTCCAAAC-3'

R 5'-TGCAACGGCTGAGGGTCTT-3'

The PCR reactions were done in 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 MgCl2 and 0.1% Triton X-100]. The 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. 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. Detailed protocol is provided in Appendix 1.
RFLP

The PCR products were digested at 37°C for 1 hour with 10 units of \textit{Nla}III [New England Biolabs] in 1 buffer supplied with the enzyme and supplemented with 100 ng/\textit{l} BSA. All \textit{XRCC3} PCR products contain an internal \textit{Nla}III site. After digestion, the \textit{Thr} allele gave a segment of 315 bp, while the \textit{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

Statistical analysis was done using the SAS software as detailed in Chapter 4.

Results

Genotype frequencies in cases and controls

This study analyzed the distribution of \textit{XRCC3} genotypes in 304 subjects, which included 110 cases of oral carcinoma, 84 cases of leukoplakia (44 hyperplasia and 40 dysplasia) and 110 normal controls. Table I shows the distribution of \textit{XRCC3} polymorphisms in the four groups of subjects.

Table 1 gives the Odds ratio (OR) estimates for the association between the genetic variants of \textit{XRCC3} gene and risk of oral cancer. A very small proportion of the subjects were homozygous for \textit{XRCC3} codon 241 polymorphism. There was no significant variation between distribution of the polymorphic variants between cases
Nla III RFLP ANALYSIS OF XRCC3 CODON 241 GENOTYPE

Lane 1: Thr/Thr wild genotype
Lane 2: Thr/Met heterozygous polymorphic genotype
Lane 3: Met/Met homozygous polymorphic genotype

XRCC3 GENE POLYMORPHISM

Codon 241 Thr

Codon 241 Met
and controls. No significant differences were observed between the crude ORs for oral cancer and the ORs obtained when adjusted for age and gender. Subjects with an XRCC3  *Met/Met*  and  *Thr/Met*  variants did not exhibit any statistically significant increase in risk of being a case (p value=0.60).

Table 1

DISTRIBUTION OF XRCC3 GENE POLYMORPHISM IN ORAL CANCER CASES AND CONTROLS

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Genotype status</th>
<th>Case (N=110) N (%)</th>
<th>Control (N=110) N (%)</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>XRCC3</td>
<td>Met/Met</td>
<td>2 (1.82)</td>
<td>1 (0.91)</td>
<td>2.31</td>
<td>(0.20,25.96)</td>
<td>0.600</td>
</tr>
<tr>
<td></td>
<td>Thr/Met</td>
<td>36 (32.73)</td>
<td>26 (23.64)</td>
<td>1.60</td>
<td>(0.88, 2.89)</td>
<td>0.122</td>
</tr>
<tr>
<td></td>
<td>Thr/Thr</td>
<td>72 (65.45)</td>
<td>83 (75.45)</td>
<td>1(referent)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

When individual risk of dysplastic leukoplakia subjects was studied as against normal population it was seen, XRCC3 polymorphic variant gave a risk of 11.3 (95% CI=1.08-108.4, p value= 0.03)(Table 2). In the case of hyperplastic leukoplakia against normal population it was seen that none of the polymorphic variant gave significant results.
Table 2
DISTRIBUTION OF XRCC3 GENE POLYMORPHISM IN DYSPLASTIC LEUKOPLAKIA CASES AND NORMAL CONTROLS

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Genotype status</th>
<th>Case* (N= 40) N (%)</th>
<th>Control** (N=110) N (%)</th>
<th>OR^A</th>
<th>95% CI</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>XRCC3</td>
<td>Met/Met</td>
<td>3 ( 7.50)</td>
<td>1 ( 0.91)</td>
<td>11.32</td>
<td>(1.12,114.2)</td>
<td>0.0370</td>
</tr>
<tr>
<td></td>
<td>Thr/Met</td>
<td>15 (37.50)</td>
<td>26 (23.64)</td>
<td>2.18</td>
<td>(0.99, 4.80)</td>
<td>0.0510</td>
</tr>
<tr>
<td></td>
<td>Thr/Thr</td>
<td>22 (55.00)</td>
<td>83 (75.45)</td>
<td>1(referent)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Cases include dysplastic leukoplakia
**Controls include normal population
^A OR =age, gender and habits adjusted Odds

All groups with lesions (hyperplasia, dysplasia and squamous cell carcinoma) were then grouped into cases and the normal population was ranked as controls to assess the cumulative risk of all these conditions together. It was observed that the XRCC3 polymorphic genotype did not show any significant results. Table 3 shows that the variant homozygous genotypes of these two codons were more frequent in cases than in controls. The homozygous Met/Met variant of XRCC3 gene had four fold increased risk (OR=4.05, 95% CI= 0.48- 34.2) of oral cancer as compared to the Thr/Thr wild genotype. The p values were however not significant.
Table 3

**DISTRIBUTION OF XRCC3 GENE POLYMORPHISM IN ORAL CANCER CASES AND NORMAL CONTROLS**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Genotype status</th>
<th>Case* (N=194) N (%)</th>
<th>Control** (N=110) N (%)</th>
<th>OR A</th>
<th>95% CI</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>XRCC3</td>
<td>Met/Met</td>
<td>6 ( 3.09)</td>
<td>1 ( 0.91)</td>
<td>4.05</td>
<td>( 0.48,34.25)</td>
<td>0.2490</td>
</tr>
<tr>
<td></td>
<td>Thr/Met</td>
<td>65 (33.51)</td>
<td>26 (23.64)</td>
<td>1.69</td>
<td>( 0.99, 2.88)</td>
<td>0.0534</td>
</tr>
<tr>
<td></td>
<td>Thr/Thr</td>
<td>123 (63.40)</td>
<td>83 (75.45)</td>
<td>1(referent)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Cases include dysplastic and hyperplastic leukoplakia and SCC
**Controls include normal population
ORA = age, gender and habits adjusted Odds

Table 4

**DISTRIBUTION OF POLYMORPHISM WITHIN CASES AND CONTROLS**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Case(N=194)</th>
<th>Control(N=110)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>Odds of exposure N (%)</td>
</tr>
<tr>
<td>XRCC3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Met/Met</td>
<td>6 ( 3.09)</td>
<td>0.05</td>
</tr>
<tr>
<td>Thr/Met</td>
<td>65 (33.51)</td>
<td>0.53</td>
</tr>
<tr>
<td>Thr/Thr</td>
<td>123 (63.40)</td>
<td>-</td>
</tr>
</tbody>
</table>

*Cases include dysplastic & hyperplastic leukoplakia and SCC
**Controls include normal population
Within the case group, the results suggest that the odds of occurrence of homozygous polymorphic genotype is less than the occurrence of normal wild genotype for the gene XRCC3. Similarly, the odds of occurrence of heterozygous polymorphic genotype is less for the XRCC3 gene. Similar results were observed for the control group also.

Table 5

Combined homozygous and heterozygous XRCC3 gene polymorphism and risk of oral cancer

<table>
<thead>
<tr>
<th>Name of Gene</th>
<th>Type of Gene</th>
<th>Case (N=194) N (%)</th>
<th>Control (N=110) N (%)</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>XRCC3</td>
<td>Met/Met+ Thr/Met</td>
<td>71 (36.60)</td>
<td>27 (24.55)</td>
<td>1.77</td>
<td>(1.05, 2.99)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Thr/Thr</td>
<td>123 (63.40)</td>
<td>83 (75.45)</td>
<td>1(referent)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

On combining the homozygous and heterozygous variants of each codon, as the individual genotypes were few in number, XRCC3 gene exhibited nearly two-fold increased risk compared to the wild genotype. The risk estimates for combined analysis is given in Table 5.
**Effects of genotype and habits on oral cancer risk**

The combined effects of genotypes and covariates like smoking, betel quid chewing and alcoholism on estimates of risk are shown in Table 6. Smokers, alcoholics and betel quid users were classified into two groups: ever (users) and never (non-users). The XRCC3 did not modify the effects of betel quid chewing or alcohol, but exhibited a modest association with smoking (p=0.04).

**Table 6**

**Distribution of abnormal genotype status in case and control by genotypes among smokers**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Habit</th>
<th>Genotype Status</th>
<th>Case (N=194) N (%)</th>
<th>Control (N=110) N (%)</th>
<th>95% CI</th>
<th>Odds Ratio</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>XRCC3</td>
<td>Ever</td>
<td>Polymorphic</td>
<td>39 (20.10)</td>
<td>9 (8.18)</td>
<td>(1.01, 5.57)</td>
<td>2.37</td>
<td>0.0444</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wild</td>
<td>53 (27.32)</td>
<td>29 (26.36)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>Polymorphic</td>
<td>32 (16.49)</td>
<td>18 (16.36)</td>
<td>(0.70, 2.70)</td>
<td>1.37</td>
<td>0.3603</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wild</td>
<td>70 (36.08)</td>
<td>54 (49.09)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Discussion

XRCC3 gene participates in DNA double-strand break/recombinational repair and is a member of a family of Rad-51-related proteins that participates in homologous recombination to maintain chromosome stability and repair DNA damage (189).

The XRCC3-241Met variation is a nonconservative change, but it does not reside in the adenosine triphosphate binding domain, the only functional domain identified in the protein. The impact of this polymorphism on repair phenotype was previously studied in 80 healthy subjects (197); the XRCC3 241Met allele was associated with significant increases in chromosome deletions in x-ray-challenged blood lymphocytes (p < 0.05). Chromosome deletion is specific for abnormal repair of x-ray-induced DNA strand breakage. The overall frequency of aberrant cells associated with the variant was nonsignificantly higher than that in the wild-type genotype. On the other hand, the variant genotype had no effect on the repair of ultraviolet light-induced DNA damage in comparison with the wild-type genotype.

In a study of 133 nonsmokers, 93 former smokers, and 82 current smokers, the XRCC3 241Met variant was significantly associated with increased bulky DNA adduct levels among all volunteers as a group and among the nonsmokers (196). In blood samples taken from 435 newborns, the variant gene was not associated with an increase in the frequency of glycophorin A NN or NO mutations (197).
In the one study that investigated the XRCC3-241Met variant using a specific functional assay (198), the findings suggested that the increased cancer risk associated with the XRCC3-241 variant may not be attributable to an intrinsic homology-directed repair. However, such experiments cannot definitely rule out the involvement of other XRCC3 variants in linkage disequilibrium or possible genetic interactions between the XRCC3-241 variant and polymorphic alleles of other DNA repair genes that may lead to a homology directed repair defect. It is still possible that an extremely mild homology-directed repair defect would not be detectable in the assay or that XRCC3 acts within other cellular pathways.

The XRCC3 Thr241Met (rs861539) variant has been shown to be functionally defective in suppressing duplication of the genome, which is thought to be important for maintaining genomic stability (199). Six previous studies in the USA, Italy and Sweden (200-204) including a total of 3112 cases and 3149 controls have evaluated its relationship with bladder cancer risk. The summary OR (95% CI) suggests a weak, though statistically significant, increase in risk with an OR of 1.17 (1.00–1.36).

The RAD51 paralogue XRCC3 also plays a central role in homologous recombination and is important for maintenance of the correct centrosome number in mammalian cells (205,206).

The XRCC3 Thr241Met variant has been also proposed as a low-penetrance cancer allele associated with breast cancer (207), lung cancer (208), acute myeloid leukaemia (209) and a reduced risk of cancer in the upper aerodigestive tract (210).
However, there are also reports that do not find a link between XRCC3 T241M and cancer (211,212).

The knock-out model of the HR repair gene $\textit{RAD51}$ is embryonically lethal (212,213) and $\textit{XRCC3}$ is also essential for genetic stability (190-194). Mutations in these genes are rare, demonstrating the absolute requirement of the encoded proteins. However, subtle differences in protein levels or protein activity resulting from polymorphisms are likely to be tolerated by cells, although these differences would be expected to have an effect when a high level of DNA damage is present.

Role of XRCC3 has also been observed in preventing accumulation of spontaneous DSBs in MCF7 cells, reduced proliferation and gH2AX accumulation in the nontransformed MCF10A breast cell line, following XRCC3 interference, suggesting that Xrcc3 is important for cell cycle progression not only in breast cancer cells but also in nontransformed mammary cells. The pattern of gH2AX expression in XRCC3-depleted MCF7 cells suggest that DSBs accumulate mostly during replication as a result of replication forks collapse. As such, deficiencies in the expression of wild type XRCC3, by reducing the capacity to repair spontaneous DSBs could contribute to breast cancer susceptibility in carriers of XRCC3 polymorphisms known to affect the DNA repair function. (215)

However, results from studies of the XRCC3 Thr241Met Single nucleotide polymorphism on the risk of various types of cancer have been inconsistent (216-219).
Most studies have suggested that the Met allele is related to an increased risk of cancer (217-219). The Thr to Met substitution results in an amino acid change from a neutral hydrophilic residue with a hydroxyl group to a hydrophobic one with a methyl sulfur group, which may result in a substantial change in the protein structure and function.

It has also been shown that the common and the variant XRCC3 alleles are functionally equivalent in the double-strand break repair pathway [220], which may explain the lack of association between XRCC3 Thr241Met polymorphism and cancer risk shown in several studies [221-222]. In the Caucasian population, there are inconclusive and conflicting results: several studies have found an increased risk for non small cell carcinoma and lung cancer [223], while other studies have shown a protective effect, once more for non small cell carcinoma and ever smokers [224,225]. Our study failed to show any positive association between polymorphism in the XRCC3 gene and risk of oral squamous cell carcinoma. No association was also seen between presence of polymorphic variant and lifestyle factors like smoking, alcohol and betel chewing and risk of oral cancer.

A statistically significant difference in the prevalence of the XRCC3-241 polymorphism has been observed between different ethnic groups; the prevalence of Met/Met homozygosity was seen to be 4.6 percent in African Americans, 12.4 percent in Caucasians and only 0.2 percent in Asians.
A significant increased risk of second neoplasms (all sites combined, as well as for upper aero digestive tract sites and for head and neck squamous cell cancers) was observed among XRCC3 241Met allele homozygotes (HR 2.65-3.44, P <.02). GAL Thomas J. In contrast, Berwick et al noted no associations between SNPs in XRCC3-Thr241Met, and survival outcomes in 120 patients with soft tissue sarcoma (226). No evidence of a modification of the effects of alcohol or smoking by the XRCC3 codon 241 SNP was also observed (227).