

ORIGINAL ARTICLE

## Screening for XPD312 polymorphisms in human oral cancer: a preliminary study

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### Keywords

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**Abstract** Xeroderma pigmentosum-D (XPD) is one of the genes that play a role in the Nucleotide-Excision Repair (NER). Polymorphisms in XPD gene have been identified and reported to be associated with many types of cancer with two common single nucleotide polymorphisms (SNPs), namely, XPD312 and XPD751. The XPD312 polymorphism is at exon 10 codon 312 Asp to Asn (A→G) and the association of this polymorphism with oral cancer is very little known, especially, in Malaysia. The aim of this study was to screen for XPD312 gene polymorphisms in human oral cancer patients attending Hospital Universiti Sains Malaysia (HUSM), Malaysia. Blood samples were collected from 10 oral cancer and 10 normal healthy subjects with their consent. DNA was extracted using commercial DNA extraction kit and Polymerase Chain Reaction (PCR) was performed to amplify the XPD312 gene. The PCR products were digested using restriction enzyme, Sty I and analyzed on a 3% agarose gel for the detection of polymorphisms. This was followed by DNA sequencing to confirm the findings. In the current study, only homozygous wild type polymorphisms in the XPD312 gene was noticed in the oral cancer tissues as revealed by the restriction enzyme and DNA sequencing analyses.

### Introduction

Annually, more than 300,000 new cases of oral squamous cell carcinoma (SCC) are being diagnosed globally. Oral cancers are the 6<sup>th</sup> most common malignancy globally (Pisani *et al.*, 2002). It stands for 12% of all cancers in men and 8% of all cancers among women and is one of the ten most common cancers registered at the Institute of Radiotherapy and Oncology, Kuala Lumpur. Annually, 125 to 167 new cases of oral cancer were reported by the Division of Stomatology, Institute for Medical Research, Malaysia from 1989 to 1997 (Annual Report. Institute for Medical Research, MOHE; 1989-1997).

The overall survival rates of oral cancer patients have not changed for the last 4 to 5 decades (Mork, 1998; Walker *et al.*, 2003). Only approximately 50% of 5-year survival rate is achieved (Brunin *et al.*, 1999). It has shown strong evidence for the

contribution of both environmental and genetic risk factors in human cancers based on molecular epidemiology (Chen and Hunter, 2005). Smoking, alcohol consumption and betel nut chewing are the major environmental risk factors that predispose to oral cancer (Kerdpon *et al.*, 2001), in which a dose-effect relationship has been observed (Schmidt *et al.*, 2004).

The development of cancer is a multistep process which involves accumulation of DNA alterations, resulting in neoplastic transformation and uncontrolled growth. Genes involved in xenobiotic biotransformation, DNA repair, hormone metabolism, immune system regulation and development, apoptosis and cell cycle control play an important role in several common genetic polymorphisms (Nebert and Menon, 2001; Braakhuis *et al.*, 2004), which might modify the carcinogenesis process. Xeroderma pigmentosum-D (XPD) is one of the important proteins involved in the Nucleotide Excision Repair (NER) pathway. The NER pathway involves a

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multi-enzyme complex that is responsible for repairing a wide variety of DNA lesions. XPD polymorphisms are single nucleotide polymorphisms (SNPs) and there are two SNPs in XPD gene that are associated with cancer; XPD312 and XPD751. The XPD312 polymorphism causes G to A substitution at nucleotide position 23,591 of exon 10 codon 312; Asp to Asn amino acid exchange (Shen *et al.*, 1998), whereas XPD751 polymorphism changes A to C at nucleotide position 35,931 of exon 23 codon 751 and substitutes acid amino Lys to Gln (Shen *et al.*, 1998).

The identification of genes modulating cancer risk may have several implications, including the possibility of developing chemoprevention programs for highly sensitive individuals, allowing early intervention and the implementation of efficient prevention and treatment strategies. In this study, we screened for polymorphisms of XPD312 gene in DNA obtained from blood samples of oral cancer patients from Hospital Universiti Sains Malaysia.

## Materials and Methods

### Study design

Blood samples were collected from 10 oral cancer patients and 10 normal healthy subjects with their consent. The patients from Hospital Universiti Sains Malaysia who were confirmed of oral cancer based on the histopathological diagnosis by an experienced pathologist. However, based on the sample size calculation, 44 subjects were needed in each group to provide a statistical significance, but due to the dearth of time in conducting this research, only 10 samples could be employed under each group, which forms the limitation of this study. This study is a part of research project entitled, Oral Cancer and Precancer in Malaysia – Risk Factors, Prognostic Markers, Gene Expression and Impact on Quality of Life (IRPA RMK8MOSTI Number Project 06-02-03-0174 PR0054/05-05). This has been approved by the Human Ethics Approval from Research & Ethics Committee; vide reference, USMCK/PPSP ®/JK P&E 2004 dated 26 December 2005.

**Inclusion criteria:** All patients confirmed to have oral cancer based on histopathological diagnosis. For the control, only normal healthy subjects without any type of cancer were included.

**Exclusion criteria:** Patients with other types of cancer.

### DNA extraction

DNA was extracted from the blood samples using the commercial kit, QIAamp® DNA Mini kit (Qiagen, Germany) as per the manufacturer's instructions.

### XPD312 genotyping

XPD312 (rs1799793) genotyping was performed using PCR restriction fragment length polymorphism (PCR-RFLP) technique. The amplification of codon 312 of XPD gene was done using forward primer (5'-CAGCTCATCTCTCCGCAGGATCAA-3') and reverse primer (5'-GTCGGGGCTCACCCCTGCAGCACTTCGT-3') as published before (Jiao *et al.*, 2007) to amplify the specific region. The reactions contained 1xPCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.5 mM dNTPs (Promega, USA), 0.1 µM of each primer (Prologo, USA), 5.2% dimethylsulfoxide (DMSO), 1U Taq DNA polymerase (Promega, USA), 50 ng genomic DNA and ddH<sub>2</sub>O in a total volume of 50 µl. The following conditions were used to perform the amplifications: 94°C for 4 min; followed by 40 cycles of 94°C for 45 sec (denaturation), 62°C for 45 sec (annealing) and 72°C for 1 min (elongation); followed by 72°C for 4 min (final elongation). The PCR products were evaluated on a 2% agarose gel (Promega, Madison, WI, USA) and stained with SYBR® Green 1 (Cambrex BioScience Rockland Inc, USA) to confirm the band size (165 bp). After that, Sty1 restriction enzyme (New England Biolabs, USA) was used to digest 10 µl of PCR product for 4 hours at 37°C according to the manufacturer's instructions. Digestion products were evaluated on 3% agarose gels stained with SYBR® Green 1. Based on the presence of the appropriately sized DNA fragments, the genotypes can be concluded and grouped into Asp/Asp (165 bp fragment), Asp/Asn (165, 139 and 26 bp fragments) or Asn/Asn (139 and 26 bp fragments).

### DNA sequencing for confirmation

Prior to sequencing, the PCR products were purified using Novagen® kit (Novagen, Germany) according to the manufacturer's instructions and followed by cycle sequencing. The cycle sequencing reactions contained 4 µl of 5x BigDye® Sequencing buffer, 2 µl of BigDye® Terminator v3.1 (Applied Biosystems, California, USA), 2 µl of 10 µM forward or reverse primers each, 1 µl of pallet paint, 50 ng/µl of purified PCR product and ddH<sub>2</sub>O in 20 µl. The following

conditions were used to perform cycle sequencing: 96°C for 1 min (initial denaturation), followed by rapid thermal ramp (1°C/sec) at 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min and these steps were repeated for a total of 25 cycles. The samples were held at 4°C before proceeding to ethanol/EDTA precipitation method. For ethanol/EDTA precipitation procedure, the samples were transferred into 1.5 ml micro centrifuge tube. Afterwards, 5 µl of 125mM EDTA was added to each tube followed by addition of 60 µl of 100% ethanol and then mixed by inverting four times. The samples were then incubated at room temperature for 15 min, followed by centrifugation at 13,000 rpm for 45 min at 4°C. After that, all the supernatant was removed from the tube. Sixty microlitre of 70% ethanol was added into each tube and mixed well, followed by spinning at 13,000 rpm for 30 min at 4°C. After centrifugation, the supernatant was removed and dried for 30 min using Vacuum Regulator (Bio Rad, CA, USA). Then, the samples were resuspended in 20 µl of HiDi Formamide (Applied Biosystems, Warrington, UK). Finally, all the samples (patients and controls) were pipetted into 96 well plates and denatured for 5 min at 95°C. Then, all the samples were ice-chilled for 3 min before placing them in DNA sequencer model ABI Prism 3100 Genetic Analyzer (Applied Biosystems, California, USA).

## Results

DNA extracted from blood samples of 10 oral cancer patients and 10 normal healthy subjects were examined for polymorphisms in exon 10, codon 312 of the XPD gene. The DNA fragment for the exon 10 codon 312 of the XPD gene in the oral cancer samples of 165 bp has been successfully amplified (Figure 1). All the digested products of Sty I that were run on a 3% agarose gel for the detection of the polymorphisms showed the presence of bands, 165 bp (Figure 2) which indicated that only the homozygous wild type polymorphisms for XPD312 occurred in all the oral cancer samples studied in the present case. The sequencing (Figure 3), results of direct sequencing were in complete agreement with the PCR-RFLP, suggesting the presence of wild type XPD312 polymorphisms in all the current samples.

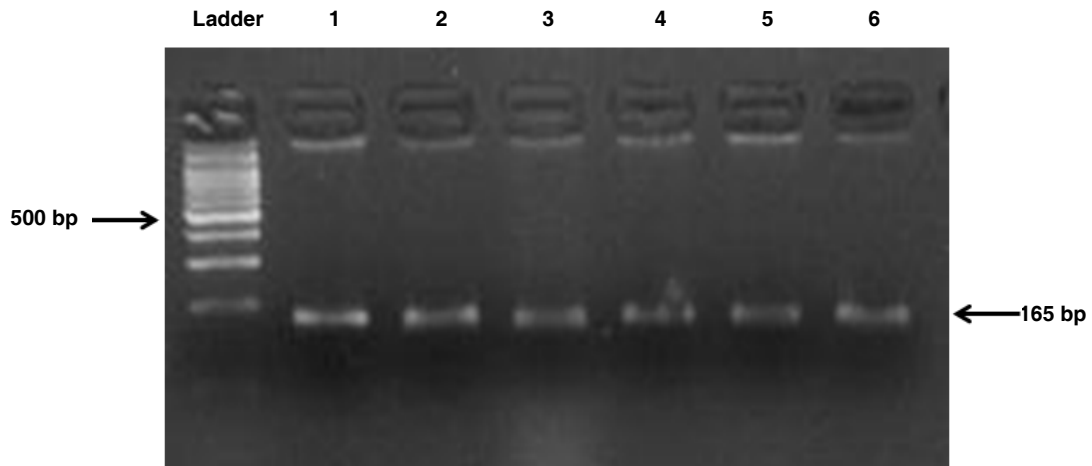
## Discussion

In this preliminary study, we identified the presence of only wild type polymorphisms of XPD312 gene in the 10 samples collected from the oral cancer patients. However, this is one of the earlier studies trying to screen the XPD polymorphisms in oral cancer patients in Malaysia.

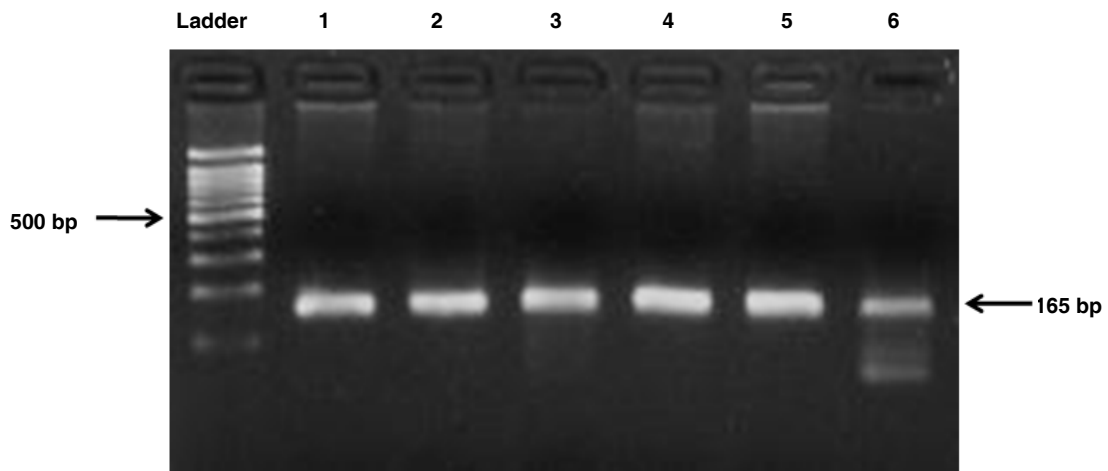
Oral cavity is the first place of contact between tissues and of genotoxic compounds by activities such as betel chew or smoking. Hence, studying polymorphisms of genes which are involved in carcinoma metabolism and DNA repair pathways (e.g. XPD312) are important in understanding the risk of oral cancer. Even though only one study had found the direct association of XPD312 with oral cancer (Flores-Obando *et al.*, 2010), most of the previous studies have not reported this association in oral cancer due to several limitations such as the selection bias; dental ailment in controls, which does not reflect the general populations, environmental control and the number of subjects involved (Majumder *et al.*, 2007). However, the contribution of other types of DNA repair genes polymorphisms; XRCC1 194Trp, XRCC3 241Met and XPD exon 6 but not XPD312 was reported to increase the risk of oral squamous cell carcinoma (OSCC) development in Thai population (Kietthubthew *et al.*, 2006). Nonetheless, there were reports on a significant association of XPD312 gene with bladder (Chang *et al.*, 2009) and lung cancer (Yin *et al.*, 2009) risk.

Although XPD genes polymorphism roles seemed to be downplayed in its association with oral cancer, it was found that combination of XPD and *N*-acetyl transferase 2 genes (NAT2) polymorphisms increased the risk of oral cancer and leukoplakia. However, the association between the two at molecular level, biological function and expression level is yet to be studied (Majumder *et al.* 2007). The similar association was shown in lung cancer scenario where XPD312 and XPD751 were found to be in linkage disequilibrium (Spitz *et al.*, 2001). There was also a report associating the ASN/ASN (rare) type polymorphism with the risk of pancreatic cancer in chronic smokers (Jiao *et al.*, 2007) although, in general no significant association was noticed between the XPD polymorphisms and the risk of pancreatic cancer. These findings suggested that combination of two gene polymorphisms or gene polymorphism/environmental factor increased the risk of cancer.

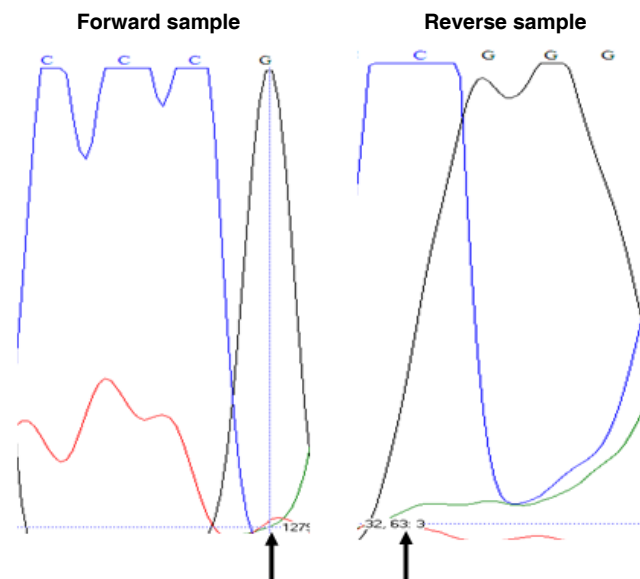
# Screening for XPD312 polymorphisms



**Figure 1** Agarose gel electrophoresis of the PCR products showing the size of amplified exon 10 codon 312 of the XPD gene. Lanes 1, 2, 3, 4, 5: Samples from oral cancer patients; Lane 6: Sample from normal healthy subject (control).



**Figure 2** Agarose gel electrophoresis of the PCR products after digestion with Sty I restriction enzyme showing the presence of wild type polymorphisms of the XPD312 gene. Lanes 1, 2, 3, 4, 5: Samples from oral cancer patients; Lane 6: Sample from normal healthy subject (control).



**Figure 3** Sequencing result confirming the presence of homozygous wild type polymorphisms in the XPD312 gene (arrows indicate the presence of G nucleotide in forward sample and C nucleotide in the reverse sample respectively).

Since single nucleotide polymorphisms (SNPs) by definition is the occurrence of gene polymorphisms in at least 1% of human population; the detection of XPD312 SNPs in all the ten samples was considered promising for the study to be extended with more number of samples to arrive at a conclusive result and to provide a better understanding of this gene in our population. Based on the earlier discussion, the study shall include other genes polymorphisms (such as Lys751Gln polymorphism) and cross related with demographic data as well as environmental factors (*i.e.* smoking and betel chew). Besides, the standardized procedures of collection and keeping of demographic data complete with environmental factors (*i.e.* smoking, betel chewing) and tissues samples by the IRPA group could make the analysis possible where some statistical correlation can be drawn upon to associate the SNP and/or environmental factors with oral cancer amongst Malaysian population.

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