Malaysian J Pathol 2006; 28 : 35 – 39

Mutational analysis of *p53* and *RB2/p130* genes in Malaysian nasopharyngeal carcinoma samples: a preliminary report

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Abstract

This study reports the results of mutation detection of tumour suppressor genes, *p53* and *RB2/p130* genes in Malaysian nasopharyngeal carcinoma (NPC) studied by PCR-CSGE analysis and direct DNA sequencing method. Frequent sites of mutation in both genes (exons 5-8 of *p53* and exons 19-21 of *RB2/p130*) were examined. Thirty-six NPC blood samples and three NPC cell lines were investigated for the presence of mutations. No mutation of *p53* and *RB2/p130* genes was identified in any of the blood samples. Nonetheless, there was an identical G → C nucleotide change at codon 280 of *p53* gene in all the cell lines. A larger study that includes biopsy tissues should be carried out to provide a more in-depth look into the pathogenesis of NPC in Malaysia.

Key words: PCR-CSGE assay, mutation, nasopharyngeal carcinoma

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is an epithelial malignancy characterized by geographic and population differences in incidence. The highest incidence is maintained amongst Southern Chinese (approximately 25-30 per 100,000 persons annually), especially those of Cantonese descent.[1] In Malaysia, the National Cancer Registry noted that NPC was the second most common cancer in men, with an incidence of 10.2 per 100,000 population in 2003.[2]

The oncogenesis of NPC involves accumulation of multiple genetic and epigenetic changes, which leads to the evolution of invasive clonal cell populations that possess growth advantages over normal cells. Major gene alterations often seen in NPC include gene deletions, hypermethylation and amplifications.[3]

p53 gene is the most frequently mutated gene in human cancers. Most of its mutations are found to be single-base substitutions.[4] Four mutation "hot spots" have been identified in exons 5 to 8, which coincide with the four most highly conserved regions of this gene.[5] Nevertheless, studies on *p53* in NPC are contradictory; some studies showed that p53 protein accumulation may be a common event in carcinogenesis,[6,7] while others stated that p53 overexpression or mutation did not seem to play a significant role in nasopharyngeal carcinogenesis.[5,8,9]

The retinoblastoma gene (RB) family consists of *RB/p105*, *p107* and *RB2/p130*. Sun *et al*[10] found no rearrangement of *p105* in NPC but another study[11] found 30% of Northern African NPC biopsies to contain a mutated *RB2/p130* gene.

To better understand the involvement of the *p53* and *RB2/p130* genes in Malaysian NPC pathogenesis, we performed mutational analyses of the two genes in 36 NPC blood samples and three NPC cell lines. The mutations were screened via PCR-conformation-sensitive gel electrophoresis method combined with direct DNA sequencing technique.

MATERIALS AND METHODS

Patient samples and cell lines

Blood samples from 36 Malaysian NPC patients were stored at –20^{0}C until DNA extraction. Three NPC cell lines (TW01, CNE1 and HONE1) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and incubated in a humidified atmosphere of 5% CO_2 at 37^{0}C.

DNA extraction

DNA was extracted from the blood samples and cell lines using QIAamp DNA Blood Mini kit (QIAGEN GmBH, Germany) and GENE ALL Tissue SV kit (General Biosystem, Korea), respectively, according to the manufacturers'

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instructions. The extracted DNA was stored at –20^{0}C until use.

Polymerase chain reaction (PCR)
Amplifications of exons 5, 6 and 8 of the *p53* gene were performed using published primer sequences.[12] The primers used for amplifying exon 7 were 5'-CCA GCC TGG GCG ACA GAG CGA GAT TC-3' (forward strand) and 5'-CCG GAA ATC TGA TGA GAG GTG GAT GG-3' (reverse strand). Each amplification was carried out separately in a 25-µL reaction volume containing 10-20 ng of extracted DNA, 0.1-0.3 µM of primers, 150-200 µM of each deoxyribonucleotide (dNTP), 2 mM of $MgCl_2$, 1X PCR buffer and 1 unit of *Taq* DNA polymerase (Biotools, Spain). The reaction mixture was heated up at 94^{0}C for 5 min, followed by 35 (exons 5, 6 and 8) or 45 cycles (exon 7) of amplification at 94^{0}C for 15 s, 55-61^{0}C for 15 s and 72^{0}C for 15 s. A no template control was included in each PCR to exclude contamination.

Amplifications of *RB2/p130* exons 19 to 21 were also carried out using published primer sequences.[11] The PCR was performed in a final volume of 25 µL containing 10-20 ng of extracted DNA, 0.4 µM of primers, 200 µM of each deoxyribonucleotide (dNTP), 2 mM of $MgCl_2$, 1X PCR buffer and 1 unit of *Taq* DNA polymerase (Biotools, Spain). The mixture was initially heated up at 95^{0}C for 5 min, followed by 35 cycles of amplification at 95^{0}C for 1 min, 55-57^{0}C for 1 min and 72^{0}C for 1 min, with a final extension step at 72^{0}C for 5 min. Similarly, a no template control was included in each PCR run.

All the PCR products were visualized using ethidium bromide-stained 1.5% agarose gel.

Conformation-sensitive gel electrophoresis (CSGE)
Mutation screening was performed in a model S2 sequencing gel electrophoresis apparatus (Life Techniques Inc., USA), according to Ganguly *et al*.[13] with minor modifications. Briefly, 20 ng of PCR products were added with EDTA to a final concentration of 20mM, heated at 98^{0}C for 5 min followed by an incubation at 65^{0}C for 1 h to generate heteroduplexes. The samples were then mixed with an equal volume of loading buffer and electrophoresed using a 10% denaturing polyacrylamide gel in 1X TTE buffer at a constant 500V for approximately 4 h. The gel was stained with Syber Gold (Molecular Probe, USA) for 5 min and transferred to the gel documentation system for visualization. Samples with heteroduplexes were chosen for direct DNA sequencing.

DNA sequencing
DNA sequencing was performed using the Big Dye Terminator ver 3.1 (Applied Biosystems, USA) in an automated DNA capillary sequencer, ABI 310 (Applied Biosystems, USA). Sequenced data were compared against published *p53* and *RB2/p130* gene sequences in GenBank for the identification of mutations. The sequence of heteroduplexes was confirmed by using both forward and reverse primers.

RESULTS

Both the genes were successfully amplified in the 36 blood samples and three cell lines. Figure 1 shows representative amplifications of *p53* exons 5 to 8 and *RB2/p130* exons 19 to 21.

CSGE detected heteroduplexes in the *p53* gene in all the cell lines (Fig. 2). There was no observation of heteroduplex in the patients' samples. The site of the *p53* mutation in TW01, CNE1 and HONE1 was identical and located at exon 8. It involved a nucleotide change of sequence from AGA to ACA at codon 280, which resulted in the conversion of arginine to threonine in p53 protein encoded.

DISCUSSION

We have carried out a preliminary study of the mutation(s) of *p53* and *RB2/p130* genes in 36 Malaysian NPC blood samples and three NPC cell lines. The exons studied in the *p53* gene have long been established as the frequently mutated sites in other human cancers.[14] *RB2/p130* gene, which maps to the region 16q12.2-13, is also repeatedly altered in malignancies[15] and is regarded as a putative tumour suppressor gene.[16]

The prevalence of *p53* mutations in patients afflicted with NPC ranges from 0 to less than 30%.[1,9,17,18] Although these results including the present study's indicated that *p53* mutation is infrequent in NPC, its mutation was observed in cell lines established from primary NPC tumours. Our findings of a transversion mutation in all three cell lines at codon 280 (G→C) is in agreement with published reports.[5,8,19] The mutations have been suggested either to occur in the original primary tumours or, were acquired during *in vitro* establishment or growth of the cells in culture.[5,8]

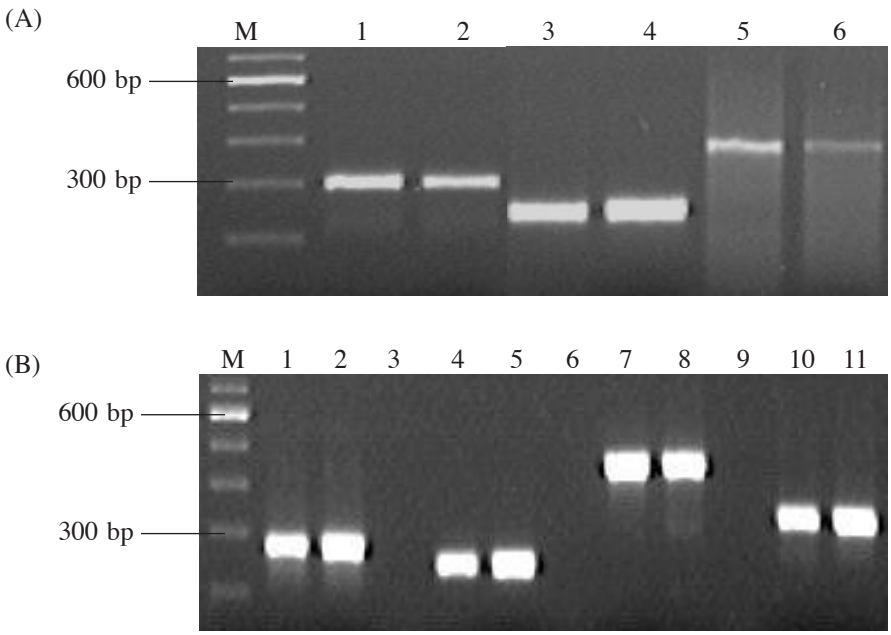

FIG. 1: PCR amplification results. (A) Lanes 1 and 2 show amplification of exon 5 (285 bp), lanes 3 and 4 of exon 6 (215 bp) and, lanes 5 and 6 of exon 7 (357 bp) of *p53* gene. (B) Lanes 1 and 2 show amplification of exon 8 (259 bp) of p53 gene, lanes 4 and 5 of exon 19 (250 bp), lanes 7 and 8 of exon 20 (446 bp) and, lanes 10 and 11 of exon 21 (325 bp) of *RB2/p130* gene. Lanes 3, 6 and 9 are blank lanes. Lane M indicates a 100-bp DNA ladder.

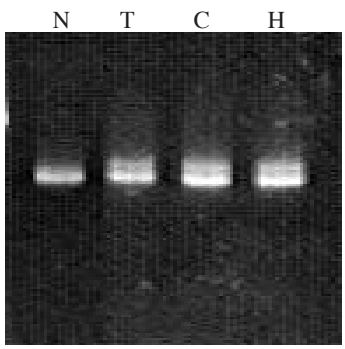

Fig. 2: Conformation-sensitive gel electrophoresis (CSGE) gel showing heteroduplexes for exon 8 of *p53* gene. N indicates a homoduplex for the control (a healthy individual), T, C and H indicate heteroduplexes for TW01, CNE1 and HONE1 cell lines, respectively.

The indication of a possible involvement of *RB2/p130* gene in NPC tumourigenesis was seen in the finding of 30% mutation prevalence in African NPC samples[11]. All the mutations caused a frameshift of the coding sequence at codons 928, 957 and 1079. *RB2/p130* mutations were also observed in non-small and small cell lung cancers, and Burkitt's lymphoma.[11,20,21]

All the quoted publications on mutation detection were investigated by PCR amplification followed by single-stranded conformational polymorphism analysis (SSCP) and direct sequencing technique. CSGE, on the other hand, was developed as a more powerful tool to screen large multi-exon genes for all possible sequence variations.[13] Aided by intronic-flanking primers, its sensitivity and specificity were reported to be approximately 100% in fragments of 200 to 500 bp[22] and over 95% in fragments up to 800 bp.[23] It has been described to be more sensitive than SSCP analysis in detecting sequence alterations in AT-rich, multi-exon genes.[24] In fact, its ease of use and practicality has also prompted its increasing application in discovering mutations

in different disease genes and single nucleotide polymorphisms (SNPs).[25,26,27]

One reason for discrepancy between our study and other reports is the type of NPC patients' samples used. All the quoted findings used NPC patients' biopsies or paraffin-embedded tissues. As hereditary or familial NPC is uncommon in Malaysia, we may have missed out the detection of mutations in both the genes by using blood DNA. In order to give a more precise report on the mutation spectrum of NPC in Malaysia, a larger study involving archival NPC tissues will be pursued.

In conclusion, present preliminary data found no detectable mutation of *p53* and *RB2/p130* genes in all the Malaysian NPC blood samples. On the contrary, there was a G→C nucleotide change at codon 280 of *p53* gene, which resulted in an amino acid conversion from arginine to threonine, in each of the cell line studied.

ACKNOWLEDGEMENTS

This study was supported by the Institute for Medical Research (IMR) research grant (IMR/PK/05/055). The authors accord their appreciation to the Director of IMR for his permission to publish the study findings and, the staff of the NPC Lab, University of Malaya (UM) and Molecular Pathology Unit, IMR, especially Dr Tan Eng Lai (UM), Ms Tan Lu Ping (IMR) and Ms Siti Ruhana Paidi (IMR) for their technical advice and support, and Dr Lee Han Lim (IMR) for his critical review of this paper.

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