

SHORT COMMUNICATION

Preliminary findings of down-regulated genes in nasopharyngeal carcinoma

Edmund Ui-Hang Sim¹, Alan Kie-Leong Toh¹, and Thung-Sing Tiong²

¹Department of Molecular Biology, Faculty of Resource Science and Technology,
University Malaysia Sarawak, Kota Samarahan, Sarawak, Malaysia

²Department of Surgery, Faculty of Medicine and Health Science,
Universiti Malaysia Sarawak, Kuching, Sarawak, Malaysia

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Abstract. The cause and mechanism of nasopharyngeal carcinoma (NPC) progression are multifactorial and multigenic in nature. Despite the increasing number of genes found to be linked with NPC, the comprehensive list of associated genetic factors remains incomplete and the precise molecular pathways to this cancer are largely undefined. Here we show early evidence of possible association between several genes and the tumorigenesis of NPC. By employing the GeneFishing™ DEG Technique that allows the comparative analysis of expression profiles between normal and tumour nasopharyngeal biopsy tissues, we have identified 10 differentially expressed genes. These genes were down-regulated in tumours relative to normal control and have never been brought into the context of NPC tumorigenicity. Our findings represent preliminary yet novel clues of several associative genetic factors to neoplastic malignancy of the nasopharynx.

Keywords. Differentially expressed genes, Nasopharyngeal carcinoma

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a carcinogenic malignancy of the nasopharynx tissue – the neoplasm usually originating at the high recesses near the opening of the Eustachian tube in the fossa of Rosenmuller. As explained by Carlos *et al.* (1990), it represents epithelial cell cancer that generally arises from tumorigenic development of squamous cell (80% of all nasopharyngeal tumours) at the lateral or posterosuperior walls of the nasopharynx. Based on histopathological types, the World Health Organisation (WHO) 1991 classifies NPC as Type I – the keratinizing carcinoma, Type IIa – the non-keratinizing carcinoma, and Type IIb – the undifferentiated carcinoma. Type I is characterized by clear histological evidence of squamous differentiation and is the most common type. Types IIa and IIb have similar behaviour and contain undifferentiated squamous cell carcinomas and lymphoepitheliomas (carcinomas with lymphoid infiltration). To date, the molecular aetiology of NPC that correlates with mal-development of the cell/tissue of origin for each type of cancer is poorly understood.

The highest incidence of NPC is in South East Asia, predominantly among the Chinese (Marks *et al.*, 1998). In Malaysia, the population of Chinese descent represents the

highest risk group (Prasad and Rampal, 1992). However, in the East Malaysian state of Sarawak, studies by Devi *et al.* (2004) conducted from 1996 to 1998 revealed that the native population exhibits the highest age-standardized rates of NPC occurrence in the world. Literature, to date, has no evidence of established findings linking ethnicity and genetic susceptibility to NPC.

The occurrence of NPC can be associated with Epstein-Barr virus (EBV) infection (Raab-Traub *et al.*, 1983), especially for Type IIb (Teng *et al.* 1996). Recently, Lee *et al.* (2007) demonstrated that EBV tends to target and modulate differentially expressed genes in NPC cell lines, implicating an enhancer role of EBV in transforming epithelial cells to NPC. However, to date, the definitive mechanism of tumorigenesis as a consequence of EBV-NPC association is largely unclear.

Like most types of cancer, the occurrence of NPC is probably multifactorial in origin and multigenic in mechanism. Evidence for this can be found in the extensively

*Author for Correspondence.

Mailing address: Department of Molecular Biology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia. Tel: 082-583041; Fax: 082-583160; Email: uhsim@frst.unimas.my

detected chromosomal addition/deletion in NPC cases. For instance, Loss of Heterozygosity (LOH) due to chromosomal gains in 1q13.3, 1q31-qter, 2q24-q31, 3q13, 5q21, 6q14-q22, 7q21, 8q11.2-q23, 12p11.2-p12, 12q14-q21, and 18q12-qter; and chromosomal deletions in 3p14-p21, 11q23-qter, 16q21-qter and 14q24-qter have been reported for NPC cases (Chien *et al.*, 2001). Studies on gene activities in NPC have also provided evidence to support the multigenic mechanism of NPC tumourigenesis. In 1999, Zhan and co-workers identified 9 known genes that are differentially expressed between primary culture of cells of normal human nasopharyngeal epithelium and a poorly differentiated NPC cell line, HNE1. These genes encode TRIP1 (TGF beta receptor interacting protein), TAF, ezrin, MHC II, actinin, Histone H1 zero, cytokeratin 13, Squalene synthetase, and RNA synthetase-like proteins. In studying the apoptotic rate of NPC cells, Li *et al.* (2000) revealed the dysregulated expression of *p16* and *bcl-2*. In addition, by using cDNA microarray analysis of NPC and control tissues, Li and co-workers (2001) demonstrated that 13 of 32 *p53*-regulated genes showed differential expression, among which are *MDM2*, *p21*, and *Bax*. A similar experimental approach by another group showed that 18 DNA repair-related genes were deregulated in NPC (Han *et al.*, 2002). Despite the increasing number of suspected causal genetic factors found to date, the complete repertoire of associated genes remains to be established. In addition, the molecular pathway(s) that explains the progression of NPC from early to invasive stages is poorly understood.

This study aimed to identify genetic factors that have possible association with tumourigenesis of NPC by performing comparative gene expression analysis between normal and tumour nasopharyngeal biopsy samples. The occurrence of differentially expressed genes formed the basis for identification of these factors. Our approach in using a modified differential display technique, the GeneFishing™ Differential Expressed Gene (DEG) system, has never been attempted on NPC studies before.

MATERIALS AND METHODS

The normal nasopharyngeal and NPC tissue biopsies were from patients admitted to the Serian Hospital, Kuching (Sarawak). These patients were presented with symptoms of swollen neck, and blood and mucus nasal discharge. Upon endoscopic examination of their nasopharynx, biopsy tissues were then taken using a specialised mechanical device, called the 'crocodile bite'. Verification of tumour status was via histological evaluation, which was further confirmed by the Fine Needle Aspiration Culture (FNAC) and swab test. In this study, a normal nasopharyngeal tissue (designated as N) from individual HS63; an NPC biopsy tissue (designated as IIa) of Type IIa from individual HS73; and an NPC biopsy tissue (designated as IIb) of Type IIb from individual HS66

were used.

Total RNA was extracted using the Trizol method. Basically, the biopsied tissues were finely minced and homogenised in Trizol reagent. Phase separation was facilitated by centrifugation after the addition of bromochloropropane. Total RNA at the top aqueous layer was precipitated and purified via isopropanol and 75% ethanol respectively. Dried total RNA pellet was dissolved in elution solution and kept at -80°C until use.

The GeneFishing™ Differential Expressed Gene (DEG) Analysis (Seegene, USA) method was conducted to compare differential gene expression patterns among the three (N, IIa and IIb) samples studied. The purpose of the comparative analysis was to identify differentially expressed genes as possible associative factors of NPC. The GeneFishing™ DEG Analysis procedure involved the Reverse Transcription (RT) and a two-stage PCR amplification steps. Specially designed primers, the Anneal Control Primer (ACP) were used in this system. Each ACP has a unique tripartite structure that consists of the core sequence, the regulator and the universal sequence. Our analysis was done in accordance with the procedures recommended by the manufacturer. In brief, about 5 µg of total RNA from each sample was used in the RT step. The oligo dT-ACP 1 primer was used to synthesize the first strand cDNAs. These were then used as templates for the two-stage PCR amplification process. For each PCR assay, one of the 20 arbitrary ACPs was used as the upstream primer, whereas the dT-ACP2 acted as the downstream primer. Hence, a complete GeneFishing™ DEG analysis would involve at least 20 reactions/tests (excluding replicates). Results of this analysis were observed using the Agarose Gel Electrophoresis assay. Basically, 3 µl of PCR products (of each reaction/test) were on electrophoresed on 2% agarose gel incorporated with ethidium bromide. The desired DNA fragments (based on bands with differential intensities) were extracted from the gel using the QIAquick Gel Extraction system (QIAGEN). This was done following the manufacturer's protocol.

Gel extracted and purified PCR-generated fragments were cloned using the pGEM®-T Easy vector (Promega Corporation, USA) and transformed into JM109 competent cells. This was carried out following the manufacturer's protocols. Identification of positive transformants was via the blue-white colony (on agar culture plates) selection strategy. Recombinant pGEM-T Easy plasmids were isolated using the FastPlasmid Mini Kit (Eppendorf, USA), and performed according to manufacturer's instruction. Purified plasmids were sent to 1st Base Laboratories Sdn. Bhd. for DNA sequence acquisition. Sequence analysis was carried using the BioEdit Sequence Alignment Editor software (Version 5.0.9, freeware program from www.mbio.ncsu.edu/BioEdit/bioedit.html). Gene identification was done using BLAST analysis via the website provided by the National Center for Biological Information (<http://www.ncbi.nlm.nih.gov/>).

RESULTS AND DISCUSSION

Using the GeneFishing™ DEG technique we found 46 bands that showed the patterns of differential intensity among the samples studied, or between the normal (N) and NPC tumour (IIa and IIb) samples (Figure 1). Of these, gel extraction and purification were successfully carried out for 25 bands – isolated fragment thereof were cloned in pGEM®-T Easy system. Sequence data from 10 clones were of the quality amenable for further analysis. The BLAST analysis (via NCBI website) yielded information on identification of 10 different genes (Table 1). These 10 genes were observably down-regulated (Figure 1) in the tumours (IIa and IIb) compared to the normal control (N).

Of the ten genes reported in this study, two encode products that belong to the class of membrane receptors that are involved in cholesterol metabolism; two are ribosomal protein genes that code for the small ribosomal subunit proteins; one is a member of the Tumour Necrosis Factor receptor-associated factor (TRAF) gene family; two are enzyme-encoding (or enzyme domain-encoding) genes; and three are genes (of section of genes) whose functions are yet to be properly characterised. Our list of differentially expressed genes has not been reported by others using gene expression profile analysis approaches in identifying NPC-associated genes.

Comparing our data with that of similar studies by others, we observed that the TNF receptor-associated factor (TRAF) gene/protein family is commonly linked to NPC. Studies by Zeng *et al.* (2007) revealed the up-regulation of TRAF2 in NPC tumours relative to normal nasopharyngeal epithelial tissues, whereas ours showed the down-regulation of TRAF6 in tumours compared to normal controls. TRAF proteins are involved with intracellular signal transduction pathways – the mammalian TRAF2 is believed to regulate downstream targets via activation of the c-Jun N-terminal kinase pathway (Reinhard *et al.* 1997), and both TRAF2 and TRAF6 can also do so via the nuclear factor-kappa B (NFκB) signaling pathway (Cheng and Baltimore, 1996; Ishida *et al.* 1996). In these processes both factors interact with the cytoplasmic tail of a cell-surface glycoprotein, CD40, which in turn activates the relevant signalling pathways (Ishida *et al.* 1996). Since Zeng's group did not delve into in-depth studies on the role of TRAF2, and that our finding on TRAF6 is preliminary, the precise roles of TRAF gene family during the process of NPC are unknown.

When comparing genomic (chromosomal) locations of the differentially expressed genes (procured from GenBank database; refer to Table 1) with studies by others on chromosomal aberrations in NPC, we found a few interesting similarities (Table 2). This information provides further proof (albeit indirect evidence) on the involvement of chromosomal locus 3p25.3 (BC022834), 7q21.2 (NM_152789), and 9q22 (AL354862) with tumourigenicity and perhaps susceptibility to NPC. However, new information from our study that adds to the existing list of genomic locations associated with NPC

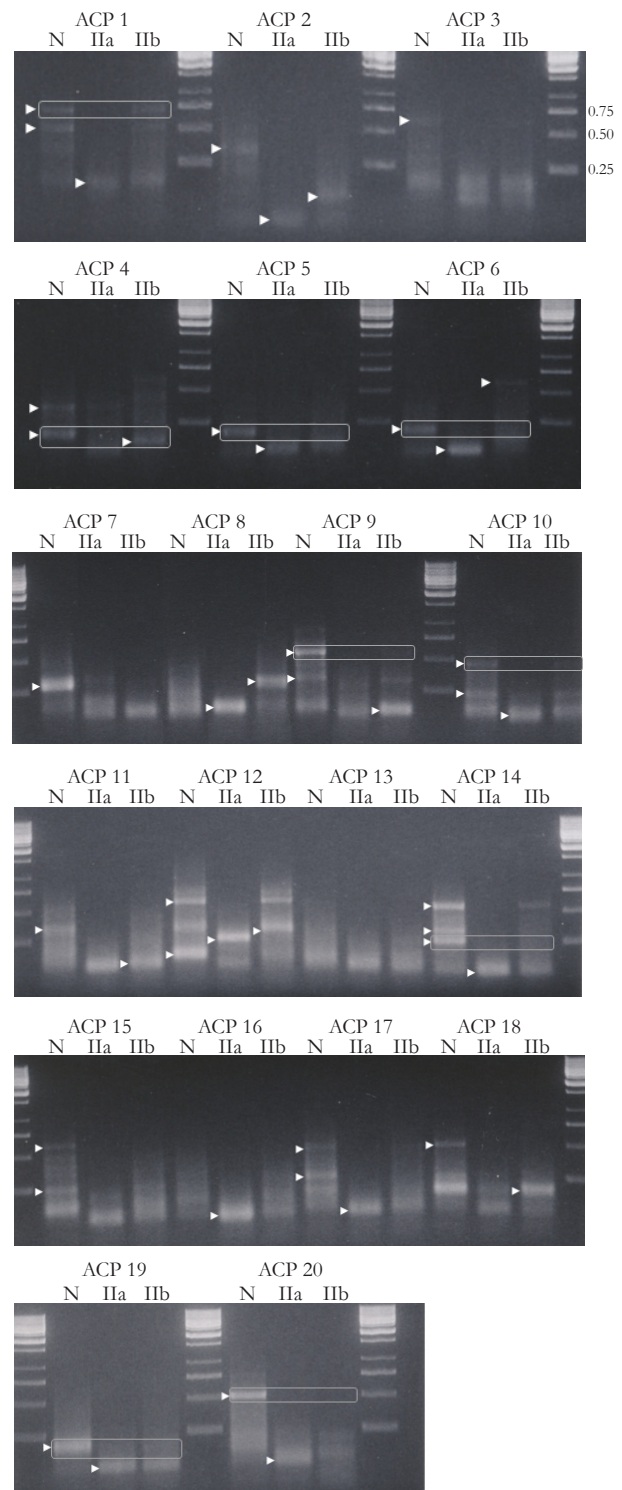


Figure 1. Agarose gel electrophoretic results obtained from the GeneFishing™ DEG analysis of total RNAs from biopsy samples of normal nasopharyngeal (N), WHO Type II (IIa), and Type IIb (IIb) carcinomas. The reference system is the GeneRuler™ 1kb DNA ladder (Fermentas); and the white arrows represent bands with differential intensity patterns between samples. Rectangular boxes indicate band patterns that correlate with down-regulated genes listed in Table 1.

Table 1. Differentially expressed genes in tumours of NPC (relative to normal control) identified via the GeneFishing™ Differential Expressed Genes (DEG) Analysis technique. Information of annotations on genes (names and chromosomal locations) was taken from GenBank database assessed via the NCBI website (<http://www.ncbi.nlm.nih.gov/>).

GenBank acc. no. (& chr. location)	Name of gene / gene product (based on GenBank annotation)	ACP primer type	Expression profile
NM_203346(2q36-q37.2)	high density lipoprotein binding protein (HDLBP), vigilin	1	Down-regulated in Type II compared to Normal and Type IIb
BC017905 (15q26.1)	abhydrolase domain containing 2 (ABHD2)	4	Down-regulated in Type IIa and IIb compared to Normal
NM_152789 (7q21.2)	family with sequence similarity 133, member B (FAM133B)	5	As above
BC022834 (3p25.3)	protein weakly similar to serine/threonine kinase Kp78	6	As above
NM_001013693 (1p36.12)	low density lipoprotein receptor class A domain containing 2 (LDLRAD2)	9	As above
NM_004651 (Xp11.23)	ubiquitin specific peptidase 11 (USP11)	10	As above
NM_001029 (12q13)	ribosomal protein S26 (RPS26)	10	As above, but more pronounced in Type IIb
AL354862 (9q22)	Human DNA sequence from clone RP11-83L6 on chromosome 9 Contains an olfactory receptor pseudogene, the SYK gene for spleen tyrosine kinase and a CpG island	14	Down-regulated in Type IIa and IIb compared to Normal
NM_001030 (1q21)	ribosomal protein S27 (metallopanstimulin-1), mRNA Kp78	19	As above
AY228337 (11p12)	TNF receptor-associated factor 6 (TRAF6)	20	As above

Table 2. Similarity in the genomic locations of the differentially expressed genes identified in this study with NPC-associated chromosomal aberrations from studies by others.

Results from this study	Findings from studies by others
3p25.3 (GenBank acc. no. BC022834)	3p25.3-p26.3; Deng et al., 1998
7q21.2 (GenBank acc. no. NM_152789)	7q21; Chien et al., 2001
9q22 (GenBank acc. no. AL354862)	9q21-q22; Huang et al., 1994 & Yang et al., 1999

is the chromosomal positions of 1p36.12, 1q21, 2q36-q37.2, 11p12, 12q13, 15q26.1 and Xp11.23 (Table 1).

Extensive literature search by us did not reveal direct association of the 10 differentially genes to cancers, except for the human ribosomal protein gene, *RPS27 (metallopanstimulin-1)*. The *RPS27* gene has been implicated in tumorigenesis of prostatic carcinomas (Fernandez-Pol *et al.*, 1997); malignant progression of hepatocellular carcinomas (Ganger *et al.*, 2001); and as a tumour associated antigen in breast cancer (Atsuta *et al.*, 2002). Although the role of ribosomal protein in cancer is not uncommon, prior to our findings there is no published report on association between ribosomal protein (RP) genes and NPC. Ongoing work by our group using RT-PCR technique coupled with appropriate statistical test (t-test), has established the significant down-regulation of *metallopanstimulin-1* in NPC samples (unpublished data). This analysis involved the use of 10 paired samples (tumours with their paired normal controls). Therefore, besides providing novel proof on the existence of NPC-associated RP

gene, this has empirically validated our results on RP genes despite the small sample size used in the GeneFishing™ DEG assay.

No doubt, a major limitation of this study is the small sample size (n=1 for each type of sample). In addition, except for *metallopanstimulin-1*, the differential patterns for the other 9 genes were not verified using Northern Analysis or RT-PCR strategy. Until more samples have been analysed, inferences regarding association of the 9 differentially expressed genes with NPC remain to be validated. In its present state, we claim our findings as preliminary. Despite this, our discovery provided information on possible NPC-associated factors that is brought into the context of NPC tumorigenesis for the first time. Further work will be required to establish their roles in this cancer.

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