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Reexploring the Possible Roles of Some Genes Associated with Nasopharyngeal Carcinoma Using Microarray-based Detection

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Abstract In gene expression profiling, nasopharyngeal carcinoma (NPC) 5-8F cells differ from 6-10B cells in terms of their high tumorigenicity and metastatic ability. Differentially expressed genes from the two cell types were analyzed by combining with MILANO (the automatic custom annotation of microarray results which is based on all the available published work in PubMed). The results showed that five genes, including *CTSD*, *P63*, *CSE1L*, *BPAG1* and *EGR1*, have been studied or mentioned in published work on NPC. Subsequently, we revaluated the roles of these genes in the pathogenesis of NPC by combining the data of gene chips from NPCs versus NPs and pooled cells from 5-8F, 6-10B and CNE2 versus NPs. The results suggested that the roles of *BPAG1* and *EGR1* are possibly different from those reported in previous NPC studies. These five genes are likely to be involved in the proliferation, apoptosis, invasion and metastasis of NPC. A reexploration of the genes will further define their roles in the pathogenesis of NPC.

Key words nasopharynx (NP); nasopharyngeal carcinoma (NPC); MILANO; differentially expressed gene

In many published reports, the genes thought to be involved in the pathogenesis of disease are categorized according to their up-regulation or down-regulation in differential gene expression profiling, whereas our conjectural results are sometimes contrary to the original hypotheses. Some experimental results are opposite to those reported in previous studies. In these situations, we have to question if the results reported by previous studies are correct.

Recently, we encountered a similar situation when we investigated the differentially expressed genes from nasopharyngeal carcinoma (NPC) cells using microarray detection. From the microarray data of NPCs versus NPs, and the pooled cells of 5-8F, 6-10B, CNE2 versus NPs and 5-8F versus 6-10B, it is possible for us to reanalyze the roles of the genes associated with the pathogenesis of NPC by integrating three sets of gene chip data.

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In this study, we reexplorated the possible functions of five genes, including *CTSD*, *P63*, *CSE1L*, *BPAG1* and *EGR1*. A revaluation of the genes will further elucidate their roles in the pathogenesis of NPC.

Materials and Methods

Specimen collection

Primary tumor and normal tissues were obtained with consent during biopsies for NPC at Hunan Cancer Hospital, Hunan Province, and Jiangmen Center Hospital, Guangdong Province, and were immediately stored in liquid nitrogen. Then, 102 specimens of squamous cell carcinoma with poor differentiation were strictly screened by frozen section diagnosis and Epstein-Barr virus (EBV) detection, and 32 specimens with EBV infection that contained more than 70% of cancer cells were finally chosen for further research.

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Cells and cell culture

5-8F (high tumorigenic and metastatic ability) and 6-10B (tumorigenic, but lacking metastatic ability) cells from colony lines of the NPC SUNE1 cell line [2] were provided by the Cancer Center of Sun Yet-Sen University, (Guangzhou, China). CNE2 cells were stored in our laboratory. These three types of NPC cells all originated from poorly differentiated squamous cell carcinoma of NP. These cell lines were maintained in RPMI 1640 supplemented with 10% NBCS at 37 °C in a humidified atmosphere of 5% CO_2 in air.

In situ NP transplantation of 5-8F and 6-10B in nude mice

Nude mice aged 6–7 weeks with a genetic background of BALB/c nu/nu were supplied by the Animal Center, Nan-Fang Medical University, Guangzhou, China. The mice were maintained in a barrier facility on HEPA-filtered racks. The animals were placed on a autoclaved laboratory rodent diet. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Animals under assurance number A3873-1. The mice were inoculated *in situ* nasopharynx with a single dose of 1×10^6 cells (5-8F and 6-10B). Growth and metastasis of tumor cells were monitored every other day with the help of a whole-body optical imaging system.

Preparation of fluorescence-labeled probes and hybridization to arrays

The experiments were performed by Shenzhen Chipscreen Biosciences Limited, Shenzhen, China. Total RNA extracted by Trizol reagent was further purified using Qiagen RNeasy mini kit (Qiagen Inc., USA). Next, 20 μg of total RNA from the corresponding samples (NPC versus NP, three pooled NPC cell types, including 5-8F, 6-10B and CNE2 1:1:1 versus NP, and 5-8F versus 6-10B) were annealed to oligo$(dT)_{18}$ and reverse-transcribed in the presence of Cy3-dCTP and Cy5-dCTP. The resulting cDNAs were treated with 2 μl of 0.5 M NaOH, and then the pH was adjusted to neutral by 2 μl of 0.5 M HCl. The first-strand products of synthesis were finally purified by QIAquick PCR purification kit (Qiagen Inc., USA) and resuspended in 30 μl of hybridization solution. Human gene chips with approximately 8000 known or predicted genes were purchased from Shenzhen Chipscreen Biosciences Limited. The mixture of Cy3- and Cy5-labeled probes was hybridized against the chips in a humidified chamber with hybridization solution (7.5 μl of 4×hybridization buffer solution, 15 μl of 50% formamide and 7.5 μl of purified water) at 42 °C overnight, and then washed twice for 20 min each time in 0.1% SSC at 55 °C. The slide was dried and scanned with a Generation III array scanner (Amersham Pharmacia, France).

Scanning and data analysis

The scanned images were converted to digital data using Arrayvision 6.0. The ratio of the Cy3 intensity to Cy5 intensity of each spot was calculated after data normalization with LOWESS regression. This represents the relative gene expression level of the tested sample versus the control. Several different statistical methods were used to set the data selection criteria and all of them were based on hybridization intensity. One common data selection criterion was that the intensity should be more than the average signal value plus three standard deviations of the negative controls on the gene chip. The signal value of these negative controls were variable because of the various experimental conditions. Based on our previous experience with many duplication tests, we chose one signal intensity of 5E+08 for valid data selection. Theoretically, a ratio greater than 1.0 indicates that the expression level is higher in the tested sample than in the control and that the corresponding gene is up-regulated. A ratio lower than 1.0 shows that the expression level is lower in the tested sample than in the control and that the corresponding gene is down-regulated. We therefore chose our data selection criteria as the intensity greater than 5E+08 and a ratio greater than 2 or less than 0.5.

Bioinformatic analysis

Genes differentially expressed in 5-8F and 6-10B cells were analyzed by microarray literature-based annotation (MILANO). This program performs automatic searches in PubMed or the GeneRIF collection for articles containing co-occurrences of search terms and a list of genes (e.g. from a microarray experiment), and is used by pasting differentially expressed genes in the "Primary Search Term" field, and nasopharyngeal carcinoma search terms in the "Secondary Search Term" field. The output is a table containing the number of hits for each pair of search terms. Subsequently, the genes selected by MILANO analysis, together with their expression levels in NPC cells versus NP and pooled NPC cells versus NP, were further analyzed for their roles in the pathogenesis of NPC.

Semiquantitative RT-PCR

Purified total RNA was treated with RNase-free DNase I (TaKaRa, Japan). After removal of the DNase I, cDNA was

reverse-transcribed from 1 μg of RNA using oligo(dT)$_{18}$. Randomly selected differentially expressed genes (*PDGFRA*, *Bcl2A1*, *NK4*, *TGFB2* in NPC versus NP and *CTSD*, *BPAG1*, *P63*, *MVP*, *APOE*, *AKRIC1*, *AdOR3* and *UGT1A9* in pooled cells versus NP tissue) and the invariant housekeeping gene control, *ACGT*, were amplified in 25 cycles from 5% of synthesized cDNA. The primer pair designed for each gene spanned at least an intron to distinguish possibly amplified cDNA products from genomic DNA. Subsequently, 5 μl of each PCR reaction product was analyzed on 1.5% agarose gel, and the intensity of each band was quantitated with the Vilber gel documentation system (Vilber Limited, France). The signal for each gene from RT-PCR was normalized by the *ACGT* gene.

Results

Metastatic and tumorigenic ability of 5-8F and 6-10B cells

The results of the *in situ* transplantation of NP in nude mice showed that all 18 survivors inoculated with 5-8F cells displayed obvious metastasis, which included encephalic invasion, jugular lymphatic node metastases and pulmonary metastases as observed in human patients. However, most of the 20 nude mice inoculated with 6-10B cells did not show any sign of metastasis, with metastasis found in the lungs of two nude mice only (data will be shown in another paper). In addition, there was an obvious difference in tumorigenic ability between the two types of cells when they were inoculated into the subcutis of nude mice as reported previously [2].

Identification of genes associated with nasopharyngeal carcinoma in PubMed by gene chips and bioinformatics

There were 283 differentially expressed genes in 5-8F cells compared with 6-10B cells. These genes were subsequently analyzed and screened by MILANO, and five genes were finally found to be associated with NPC (**Table 1** and **Table 2**). Among these genes, *CTSD*, *P63*, *CSE1L* and *BPAG1* were up-regulated more than 2-fold, and *EGR1* was down-regulated less than 0.5-fold. In addition, we also determined the differential expression status of these genes in NPC tissues versus NP tissues and pooled NPC cells versus NP tissues.

Semiquantitative RT-PCR

Semiquantitative RT-PCR was used to confirm the differential expression of 12 genes chosen from gene chips of NPC tissues versus NP tissues and 5-8F versus 6-10B. We chose these genes only because they represent differential expression patterns and can be used as examples to confirm the reliability of the gene chips. As shown in **Figs. 1** and **2**, the genes display similar expression patterns to those of the gene chips. The results derived from the gene chips are therefore considered reliable.

Fig. 1 shows the gene-specific RT-PCR analysis of four genes detected as differentially expressed genes between NPC and NP in gene chip hybridization. *ACTG* (Actin γ-1 Hs.14376) was used as the internal control standard. The gene expression patterns are compatible to those shown in the gene chip data of *PDGFRA* (0.31), *TGFBR2* (0.25), *NK4* (6.26) and *BCL2A1* (4.11).

Fig. 2 shows the gene-specific RT-PCR analysis of eight genes detected using differential hybridization of gene chips between 5-8F and 6-10B. *ACTG* (Actin γ-1 Hs.14376)

Table 1 Differential expression changes of *CTSD*, *P63*, *CSE1L*, *BPAG1* and *EGR1* in 5-8F versus 6-10B, pooled NPC cells from 5-8F, 6-10B and CNE2 versus NP and NPC versus NP

	CTSD	*P63*	*CSE1L*	*BPAG1*	*EGR1*
5-8F/6-10B	12.44	6.79	6.22	5.63	0.18
Pooled cells/NP	N	N	N	0.40	0.71
NPC/NP1	N	N	N	2.32	0.40
NPC/NP2	N	N	N	0.94	0.50
NPC/NP3	N	N	N	7.14	0.24
NPC/NP4	N	N	N	2.18	0.45
NPC/NP5	N	N	N	6.33	0.36
NPC/NP6	N	N	N	4.06	0.36
NPC/NP7	N	N	N	1.93	0.22
NPC/NP8	N	N	N	1.10	0.68

N, signal value below 5E+08, not detectable.

Table 2 Genes associated with nasopharyngeal carcinoma found in Pubmed by MILANO analysis

Gene name	Article number
CTSD	1
P63	1
CSE1L	1
BPAG1	1
EGR1	2

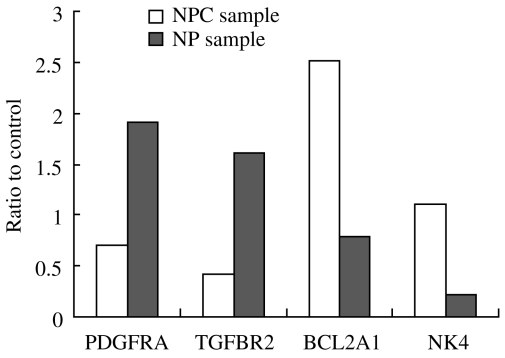

Fig. 1 Histogram identification of differentially expressed genes in NPC versus NP

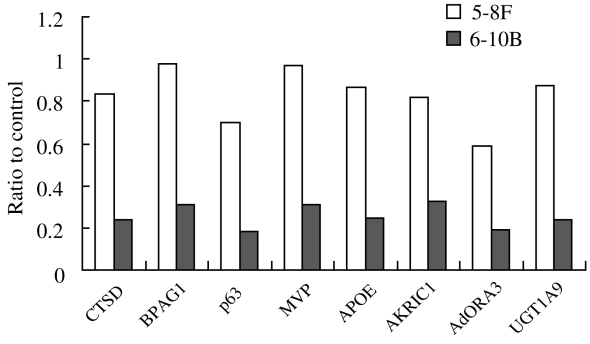

Fig. 2 Histogram identification of differentially expressed genes in 5-8F versus 6-10B

was used as the internal control standard. The gene expression patterns of *AdOR3* (4.82) and *UGT1A9* (12.45) are comparable to those shown in the gene chip data of *CTSD* (12.44), *BPAG1* (5.63), *P63* (6.79), *MVP* (6.77), *APOE* (7.13) and *AKRIC1* (5.23).

Discussion

Tumor metastasis is the main cause of mortality in cancer patients [3]. The transition from *in situ* tumor growth to metastatic disease depends on the ability of tumors at the primary site to invade local tissues and to cross tissue barriers. The primary tumor is composed of numerous heterogeneous subpopulations of cells, and only a small portion of cell subpopulations with metastatic potential in the primary tumor have the ability to invade and cause metastasis. This means that cells with high metastatic ability and cells with poor metastatic ability should be easily distinguished in gene expression profiling. Therefore, microarray analysis was performed on NPC 5-8F cells and 6-10B cells to detect the possible candidate genes associated with metastasis and other features of NPC. A total of 283 genes were discovered. In addition, MILANO analysis was performed on these 283 genes, and only five genes, *CTSD*, *P63*, *CSE1L*, *BPAG1* and *EGR1*, were found to be mentioned in previous NPC studies in Pubmed. We reanalyzed the roles of the five genes in the pathogenesis of NPC by combining the other two groups of microarray data.

Hemidesmosome (*HD*) is a transmembrane complex that mediates attachment of epithelial cells to the basement membrane. *BPAG1* is a major component of hemidesmosome. Lo *et al.* have demonstrated the down-regulation of *BPAG1* expression in NPC cells using differential gene display, which is consistent with our microarray analysis of pooled NPC cells from 5-8F, 6-10B and CNE2 versus NP tissues [4]. They suggested that loss of *HD* expression in NPC may be associated with the undifferentiated properties of NPC cells and may have prognostic significance. However, Herold-Mende *et al.* have found a distinct expression up-regulation with the onset of invasive growth by *in situ* hybridization in squamous cell carcinomas of the head and neck [5]. Results from our two other groups also showed the up-regulation of *BPAG1* in NPC tissues and 5-8F cells. We conclude that the cause of the low expression of *BPAG1* in NPC cells is that the tumor cells no longer interact with the tumoral extracellular microenvironment; that is, when NPC cells interact with their microenvironment, *BPAG1* expression will be induced and quickly up-regulated. Therefore, we think that the high expression of *BPAG1* in NPC tissues and 5-8F cells with high metastatic ability plays a role in facilitating tumor invasion and metastasis, which is contrary to the original report [4].

The protein encoded by the *EGR1* gene belongs to the *EGR* family of *C2H2*-type zinc-finger proteins. It is a nuclear protein and functions as a transcriptional regulator. It can be rapidly induced by growth factors to transduce the proliferative signal. The induction of *Egr1* by external stimuli is generally transient, but appears to be sustained in some prostate tumor cell lines and tumors, suggesting that *Egr1* stimulates tumor cell growth. In contrast, in breast, lung and brain tumors, *Egr1* expression is often absent or reduced, and when re-expressed, results in growth suppression. Re-expression of *Egr1* in tumor cells also leads to antiapoptotic activity, which would encourage tumor cell survival [6]. However, the role of *EGR1* is still unclear. *EGR1* induction is markedly augmented in cells

expressing mutant *p53* that contributes to enhanced transformed properties and resistance to apoptosis [7]. Nevertheless, NPC very rarely has *p53* gene mutations in primary tumors [8], which suggests that the induction of *Egr1* by mutant *p53* is nearly impossible. *EGR1* expression in our studies showed down-regulation in NPC versus NP, which is contrary to the microarray results obtained by Xie *et al.* [9]. The inconsistency between the two results might be caused by the different specimens that were selected or the different microarray systems used. In addition, *EGR1* also displayed down-regulation in NPC versus NP. These results suggest that *EGR1* might function as a cancer suppressor gene candidate in the pathogenesis of NPC.

CTSD, *P63* and *CSE1L* showed differential expression only in 5-8F versus 6-10B, and they could not be easily detected because of their extremely low expression in NPC versus NP and pooled tumor cells versus NP. The *CTSD* protein is a lysosomal aspartyl protease composed of a dimer of disulfide-linked heavy and light chains. This proteinase is a member of the peptidase C1 family that is involved in many physiological functions because of its proteolysic activity. *CTSD* has been extensively investigated for its roles in tumor invasion and metastasis [10,11], and its overexpression has been shown in breast cancer [12] and colon cancer [13]. In our study, higher expression of *CTSD* was found in 5-8F cells, which suggests that this gene is associated with the invasion and metastasis of NPC. *P63*, a member of the *p53* family of transmembrane proteins, is involved in the survival and differentiation of reserve/stem cells in different epithelia. High frequencies of gene gain have been detected for *TP63* in oral squamous cell carcinoma (OSCC) and human esophageal squamous cell carcinoma (EC-SCC) cell lines [14]. *TP63* transactivating isoforms, such as *TAp63/p73*, show TSG properties similar to p53, while isoforms lacking the N-terminal transactivating domain, such as *deltaNp63/p73*, induce a functional block against *p53* as well as *TAp63/p73* activities [15]. Semiquantitative RT-PCR analysis of mRNA from 25 NPC biopsies have shown that the dominant species expressed is invariably the truncated deltaN-isotype [8]. The high expression of *TP63* in 5-8F cells suggests that *TP63* in the *ΔN-p63* isoform acts as a suppressor of the wild-type *p53* function in NPC. *CSE1L/CAS*, the human homolog of the yeast gene *CSE1*, is believed to be an oncogene candidate. High frequencies of gain have been shown in NPC cells [16], medulloblastomas cells [17] and glioblastoma multiforme (*GBM*) [18], etc., and its overexpression has also been shown in prostate cancer [19]. *CSE1L* is thought to be involved in cell proliferation and apoptosis. *CSE1L/CAS* functions in the mitotic spindle checkpoint. It is also implicated in the nuclear to cytoplasmic reshuffling of importin alpha, which itself is necessary for the nuclear transport (export) of several proliferation-activating proteins, transcription factors, oncogenes and tumor suppressor gene products, such as *p53* and *BRCA1* [20]. The high expression of *CSE1L* in 5-8F cells shows to some extent that 5-8F cells with high tumorigenic and metastatic ability have stronger proliferative potential and resistance to apoptosis than 6-10B cells with low tumorigenic ability and no metastatic ability.

CTSD, *P63* and *CSE1L* cannot be easily detected in NPC versus NP and pooled tumor cells versus NP, which suggests that the high expression of these three genes is likely to occur mainly in highly malignant NPC cells and maintain its strongly malignant phenotype of cells.

In summary, the results of our study suggest that the roles of *BPAG1* and *EGR1* may be different from those reported in previous studies of NPC. The five genes are likely to be associated with the proliferation, apoptosis, invasion and metastasis of NPC. A reexploration of the genes will further define their roles in the pathogenesis of NPC.

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