Identification of Novel Tumor Suppressor Genes Down-Regulated in Recurrent Nasopharyngeal Cancer by DNA Microarray

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Abstract The nasopharyngeal cancer is a common cancer among southern Chinese. In order to better understand molecular mechanism of recurrent nasopharyngeal cancer (rNPC), we used DNA microarray to identify down-regulated tumor suppressed genes (TSGs) in rNPC, and bioinformatics to analyze their chromosomal localizations and molecular functions. Eight non-recurrent nasopharyngeal cancer (nNPC) and six rNPC tissue samples were selected, and Affymetrix Gene1.0 ST chips were used to construct the expression profiling of each tissue sample. Identify the down-regulated TSGs in rNPC by comparing expression profiling data of two type tissue samples. A total of five TSGs were identified to be down-regulated in rNPC. These five TSGs include SERPIN1, TPD52L1, FBLN1, RASSF6, and S100A2, and Signal Log Ratio were -2.2 , -2.3 , -3.5 , -3.9 and -6.9 respectively. Chromosomal localization analysis showed that S100A2, RASSF6, TPD52L1, SERPIN1, and FBLN1 were located on chromosomes 1q, 4q, 6q, 17p and 22q, respectively. Functional analysis showed that SERPIN1 and TPD52L1 belonged to enzyme activity genes, S100A2 and FBLN1 belonged to calcium ion binding genes, RASSF6 belong to protein binding genes. Five TSGs likely to be the candidate TSGs involved in rNPC, and may play important roles in occurrence of rNPC. Chromosomes 1q, 4q, 6q, 17p and 22q

may be considered as important region for screening TSGs that may relevant to rNPC. Those genes and chromosomal region need to be further studied.

Keywords Recurrent nasopharyngeal cancer · Tumor suppressor genes · DNA microarray · Gene expression · Chromosomal localization · Molecular function

Introduction

Nasopharyngeal carcinoma (NPC) is a unique malignancy that is particularly prevalent among the southern Chinese but it is rare in Caucasian. It has a significant geographical distribution, being most common among Asians, particularly the adult population of southern China and south-eastern Asia (prevalence: 15–50/100,000, 37) [1]. NPC differs from other squamous cell carcinomas (SCCs) of the head and neck in that recurrence can occur late. The clinical data showed 15–48% of the NPC patients have locally persistent, recurrent or new primary tumors in the previously irradiated volume or its vicinity [2, 3].

However, the mechanism of occurrence and recurrence of NPC is not fully clear. Environmental, dietary and genetic factors, as well as Epstein-Barr virus (EBV; human herpesvirus-4, HHV-4) infection are important etiological associations [1]. A variety of cellular proliferation pathways were up regulated in NPC, such as the Akt pathway, mitogen-activated protein kinases, Wnt pathway, and epidermal growth factor receptor signaling pathway. Genetic studies of endemic populations revealed the association of HLA antigen haplotype with NPC: HLA-2, HLA-B17, and HLA-Bw26 double the risk of the disease, and genomic and cytogenetic studies have shown multiple aberrations in

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chromosomes 1, 3, 9, 11, 12, and 14 [3]. Recent studies showed that higher incidence of loss of heterozygosity on chromosome 3p, 9p, 11q, and 14q, suggesting that tumor suppressor genes (TSGs) involved in the development of NPC [4–7].

In recent years, the gene chip technique, or DNA microarrays has been widely used to detect the gene expression differences in human tumor by parallel analysis. However, few studies have been done on mechanism of occurrence or genetic alterations of recurrent nasopharyngeal carcinoma (rNPC). Our studies by DNA microarrays find that 102 and 44 differentially expressed genes in primary nasopharyngeal carcinoma and rNPC, respectively. Most genes locates on chromosome 1, 3 and 7 (Data published in China, 2009 and 2010) [8, 9]. In present study, we used Affymetrix Gene 1.0 ST gene chips to screen TSGs in rNPC, and bioinformatics to analyze chromosomal localizations and molecular functions. This will be helpful in understanding the pathways involved in mechanism of occurrence of rNPC at molecular level. In addition, further studies on rNPC will be summarized in our next study.

Materials and Methods

Oligonucleotide Microarray Gene Chips

Affymetrix Gene1.0 ST gene chips were used to identify the down regulated TSGs in rNPC. This array contained about whole human TSGs from the Unigene GenBank of NCBI.

Patients and Tissue Specimens

A total of 14 human nasopharyngeal cancer specimens from patients who treated at our department from 2005 to 2009. Three cases of normal nasopharynx epithelium tissue specimens were from volunteers for external reference. All biopsies tissue specimens were immediately cryo-preserved in liquid N₂ after removal. Eight rNPC patients were diagnosed local recurrence (nasopharynx only) of nasopharyngeal carcinoma after first course radiotherapy and recurrence times ≥ 24 months (24–75 months). CT scans of chest, head, and pelvis were normal, and there were no palpable lymph nodes in the neck region or any findings suggesting distant metastases. Six rNPC patients were initial diagnosis of NPC without recurrence and metastases. All patients and tumor characteristics are summarized in Table 1. Clinical stage was determined according to the UICC TNM and WHO classification. The protocol was approved by hospital ethics committee, and consent was obtained from all patients.

Preparation of Messenger RNA

RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and concentration of RNA were determined by electrophoresis and spectrophotometry (Beckman DU800, Beckman, Fullerton, CA, USA). Before probe labeling, ribosomal RNA (rRNA) reduction procedure was performed, and RNA linear amplification system was used in vitro transcription. Briefly, the procedure included the following steps for linear amplification: (a) double-stranded cDNA was synthesized with random hexamers tagged with a T7 promoter sequence and column-purified, (b) double-stranded cDNA was used as a template and amplified by T7 RNA polymerase producing antisense cRNA, (c) antisense RNA was column purified and (d) second cycle of cDNA was synthesized from reversed transcription of the cRNA, DNA was labeled by terminal deoxynucleotidyl transferase (TdT) with the Affymetrix[®] proprietary DNA labeling reagent.

Microarray Hybridization and Data Analysis

Prepared the eukaryotic hybridization mix, and hybridized labeled target onto the Affymetrix Gene1.0 ST gene chips for 16 h. Each sample was washed and stained with Wash Buffer A (Non-Stringent Wash Buffer) and Wash Buffer B (Stringent Wash Buffer), 2× Stain Buffer 10 mg/ml Goat IgG Stock. The arrays were scanned by the Affymetrix GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA, USA). The primary signal data were normalized and corrected by using an internal reference gene (house-keeping gene). Data were analyzed by Gene Chip Operating Software (GCOS, Affymetrix, Santa Clara, CA, USA) and the intensity of fluorescence signals and their ratios were calculated. Data of fold change were calculated from the Signal Log Ratio (SLR). Genes with $SLR \geq 2$ or ≤ -2 were selected. Changes in genes expression of TSGs in rNPC were assessed by using Significance Analysis of Microarray (SAM) software.

Chromosomal Localization and Functional Category Analysis

Chromosomal localizations for the down-regulated TSGs in rNPC were obtained from National Center for Biotechnology Information (NCBI) Map Viewer (<http://www.ncbi.nlm.nih.gov/mapview/>) and European bioinformatics Institute (EMBL-EBI) genome site <http://www.ebi.ac.uk/Databases/genomes.html>). Molecular function analysis was applied by Gene ontology (GO) site (<http://www.geneontology.org/>).

Table 1 Tumor stage and recurrent month are list for all samples

Sample ID	WHO histological diagnosis	TNM	Gender	Age	Recurrent month
nNPC 1	WHOI	T1N ₀ M ₀	Male	52	
nNPC 2	WHOI	T1N ₀ M ₀	Female	52	
nNPC 3	WHOI	T1N ₀ M ₀	Male	36	
nNPC 4	WHOI	T1N ₀ M ₀	Male	56	
nNPC 5	WHOI	T1N ₀ M ₀	Male	53	
nNPC 6	WHOI	T1N ₀ M ₀	Male	59	
nNPC 7	WHOI	T1N ₀ M ₀	Female	67	
nNPC 8	WHOI	T1N ₀ M ₀	Male	39	
rNPC 1	rNPC	rT1	Male	56	24
rNPC 2	rNPC	rT1	Female	68	72
rNPC 3	rNPC	rT1	Male	45	28
rNPC 4	rNPC	rT1	Male	62	75
rNPC 5	rNPC	rT1	Female	61	35
rNPC 6	rNPC	rT1	Male	58	48

Results

Quality of RNA and RT-PCR

Total RNA of 17 tissue samples were qualified for PCR and hybridization: A260/A280 ≥ 2.0 , 2100 RIN ≥ 7.0 , 28S/18S ≥ 0.7 . The differential expression of genes identified by the hybridization method was confirmed by RT-PCR (Fig. 1).

Hybridization with Gene Chips

rNPC and nNPC tissue samples were scanned respectively after hybridization with the Affymetrix Gene1.0 ST gene chips. The results were collected and analyzed by bioinformatics. A total of five TSGs were identified to be down-regulated in rNPC. These five TSGs include SERPINF1, TPD52L1, FBLN1, RASSF6, and S100A2, and Signal Log Ratio (SLR) were -2.2 , -2.3 , -3.5 , -3.9 and -6.9 respectively (Fig. 2).

Chromosomal Localization and Functional Categories of Down-Regulated TSGs

Chromosomal localization analysis showed that analysis showed that S100A2, RASSF6, TPD52L1, SERPINF1, and FBLN1 were located on chromosomes 1q, 4q, 6q, 17p and 22q, respectively (Fig. 3). Functional analysis showed that SERPINF1 and TPD52L1 belonged to enzyme activity genes, S100A2 FBLN1 belonged to calcium ion binding genes, and RASSF6 belong to protein binding genes (Table 2).

Discussion

Recent advances in basic biological research and genomics have improved our understanding of the molecular basis of NPC development and progression. As a result, a variety of molecular tumor markers characterized in the laboratory have been studied in the clinic for their potential to predict

Fig. 1 Quality of RNA from all sample: nNPC: No. 1–8; rNPC:No. 8–6. A260/A280 ≥ 2.0 , 2100 RIN ≥ 7.0 , 28S/18S ≥ 0.7

Fig. 2 Signal log ratio (SLR) of five TSGs in rNPC

disease outcome or response to therapy in NPC patients. Our study is concentrated on patterns of TSGs expression of nNPC and rNPC, which different from previous research that focused on genetic aberrations between normal nasopharyngeal epithelial/cell lines and NPC. Because of relatively few cases of rNPC (within nasopharynx) without

metastasizes, we selected nNPC only on stage I (T₁N₀M₀) as control.

To our knowledge, there have been no previous cDNA microarray studies focused on chromosomal localization of rNPC. Previous studies revealed that genetic alterations in NPC were prevalent on 1q, 3p, 4q, 7, 9q, 11, 12q, and 18q

Fig. 3 The down-regulated TSGs in rNPC were distributed on chromosomes 1q, 4q, 6q, 17p, 22q

Table 2 Information and functional categories of five down-regulated TSGs

Gene symbol	Gene name	SLR	Genbank no.	Molecular function
RASSF6	Ras association domain family member 6	−3.9	BC058835	Protein binding
FBLN1	Fibulin 1	−3.5	AF126110	Calcium ion binding
S100A2	S100 calcium binding protein A2	−6.9	BC002829	Calcium ion binding
SERPINF1	Serpin peptidase inhibitor, clade F member 1	−2.2	M76979	Enzyme activity
TPD52L1	Tumor protein D52-like 1	−2.3	U44427	Enzyme activity

[7, 10–12]. Additionally, chromosome 3p plays an important role in carcinogenesis in NPC, and was a putative nasopharyngeal carcinoma susceptibility locus [6, 13].

TSGs are involved in protecting a cell from one step on the path to cancer. These genes have a dampening or repressive effect on the regulation of the cell cycle or promote apoptosis. Loss or reduction of suppressors may promote tumorigenesis of normal cell. Inactivation of tumor suppressors on 3p21.3 and 9p21 was demonstrated to be an early event during the development of NPC [14–16]. Additionally, chromosome 14 harbors tumor suppressor genes associated with NPC [17]. Many TSGs have been reported to relate to NPC, such as BLU, RASSF1A, TSLC1, PTPRG, P16, and DLEC1. In this study, none of TSGs were found in chromosome 3p.

Of interest, five candidate TSGs, included SERPINF1, TPD52L1, RASSF6, FBLN1, S100A2, were found among these four categories of genes in this study. We believe that this information may be important when trying to select candidate genes for the further study of the molecular mechanism of occurrence of rNPC.

SERPINF1 is a member of the serpin gene family, and involves in tumor cell proliferation and angiogenesis. Reduction in SERPINF1 expression levels may lead to tumor malignancy in VEGF-low ovarian tumors [18]. TPD52L1 is involved in cell proliferation and calcium signaling; it also interacts with the mitogen-activated protein kinase 5 (MAP3K5/ASK1) and positively regulates MAP3K5 and up-regulates the ASK induced apoptosis [19]. RASSF6 is a member of the RAS association (RA) domain family of RAS effectors/tumor suppressors that mediate some of the growth inhibitory properties of RAS. Allen et al. [20] found RASSF6 is a novel RASSF family member that demonstrates the properties of a Ras effector and tumor suppressor. FBLN1 is a secreted glycoprotein that becomes incorporated into a fibrillar extracellular matrix, and has been implicated in a role in cellular transformation and tumor invasion. FBLN1 mediates platelet adhesion via binding fibrinogen. It enhanced apoptosis of endothelial cells and tumor cells, and also inhibits tumor angiogenesis [21]. S100A2 protein encoded by this gene is a member of the S100 family of proteins containing 2 EF-hand calcium-binding motifs. These proteins are localized in the cytoplasm and/or nucleus of a

wide range of cells, and involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. Zhang et al. [22] found S100A2 was down-regulated in lymph node metastasis of squamous cell carcinoma of the head and neck (SCCHN), suggesting that instead of being a putative tumor suppressor, S100A2 may play a role in the metastasis of SCCHN. Thus it may be inferred that occurrence of rNPC is likely as a result of inactivation of TSGs. Accordingly, the development of rNPC may involved in abnormality of angiogenesis, cell proliferation/apoptosis, calcium signaling, tumor cell metastasis and invasion. Moreover, these five TSGs found in our study, located on chromosomes 1q, 4q, 6q, 17p, and 22q respectively, needed further study for confirmation.

Previous researches found genetic alterations in NPC that paved the way for us for further study in rNPC. Although the sample sizes of this study were small and our findings therefore require further validation in larger trials, such preliminary results may provide important clues to the understanding of the various gene networks implicated in rNPC carcinogenesis and may contribute to the selection of target TSGs for possible molecular diagnosis and therapy in the future.

Conclusions

We identify five differentially TSGs in rNPC by cDNA hybridization. These TSGs, including SERPINF1, TPD52L1, RASSF6, FBLN1, S100A2. They are belonged to enzyme activity, calcium ion binding and protein binding genes, and chromosomal localization showed the majority of genes located on chromosomes 17p, 6q, 22q, 4q and 1q. We suggest these TSGs may have a contribution to the mechanism of rNPC. The next study is needed to investigate roles of these predictor genes and select candidate genes in specific region of these four chromosomes that abnormalities occurred.

Key messages: This research is first to study TSGs in recurrent nasopharyngeal cancer. Ours result may contribute to the selection of target TSGs for possible molecular diagnosis and therapy in the future, and still warrant further study.

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