Differentially Expressed Homeobox Genes in Salivary Adenoid Cystic Carcinoma versus Normal Salivary Gland Tissue

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Abstract: The purpose of this study is to identify differently expressed homeobox genes in salivary adenoid cystic carcinoma (SACC) versus normal salivary gland tissue, and determine the effects of homeobox genes on oncogenesis and differentiation of SACC. Six paired tissue specimens of SACC and surrounding normal salivary gland tissue were obtained. Customized Oligo microarray was used to analyze differential homeobox gene expression. Data were scanned by Agilent Scanner. Real-time PCR was used for quantitative analysis of gene expression. The results showed that Homeobox genes TGIF and EVX1 were differentially expressed in SACC versus normal tissues. As regulators of cellular proliferation and differentiation, homeobox genes may be involved in SACC oncogenesis.

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1. Introduction

Adenoid cystic carcinoma of the salivary gland is one of the relatively common malignant salivary gland tumors. It has a strong infiltration capability and a high metastatic rate. The occurrence and development of adenoid cystic carcinoma is a complex process involving a variety of factors and steps, changes in the expression of various genes, and adjustments between the structure and function of relevant rerecording determinants.

The homeobox gene family is a large gene family which encodes transcription factors.[1,2] It has been confirmed that homeobox genes exist in all eukaryotic cells. Homeobox genes are expressed during specific growing stages of an organism and in specific tissues. Homeobox genes control cell mitosis and control the synthesis of various molecules and hormones involved in conveying information at a local and remote level to adjust the growth and differentiation of the embryo. Mutation in the sequence of certain homeobox genes can result in phenotype change in normal cells. Abnormal expression of homeobox genes may trigger uncontrolled cell proliferation and may prevent cells from differentiating and maturing, resulting in tumor. Recent studies have shown that disregulation of homeobox genes expression is not only involved in developmental deformity but also in the occurrence and development of tumors, including mammary cancer, malignant melanoma, cervical carcinoma, leukemia, and prostate cancer.[3-11]

Microarray technology[12,13] has been developing rapidly since the inception of the concept of DNA microarray by Fodor in 1991.[14] The purpose of this research is to compare the difference in homeobox gene expression between adenoid cystic carcinoma of the salivary gland and normal gland specimens by using microarray, and determine the relationship between homeobox gene expression and the illness itself.

2. Material and Methods

2.1 Patients and specimens

2.1.1 Salivary adenoid cystic carcinoma (SACC) and normal salivary gland tissues

Six patients with adenoid cystic carcinoma of the salivary gland who attended the Department of Oral and Maxillofacial Surgery, West China College of Stomatology, Sichuan University in June 2006 were enrolled in this study. Two patients were men and four were women. The average age was 50.7 years (range: 31 to 68 years). The degrees of the cases were as the follows: two cases were T_2; three cases were T_3; and one case was T_4 (UICC [15] TNM by stages). The patient's illness was diagnosed as salivary adenoid cystic carcinoma (SACC). All patients received extensive resection on the primary tumor focus with no other treatment such as chemotherapy or local radiotherapy.

Specimen processing: a 5×5×5-mm^3 piece of tissue from the primary focus of the SACC was cut with an aseptic scalpel and the unnecessary portion was discarded; a piece of normal gland tissue (of the same size) was cut 2-cm away from the edge of the primary tumor focus. The specimen was washed with DEPC solution and cleaned gently with aseptic

etamine. The specimens were divided into two parts. One part was fixed in formalin solution for further evaluation by a pathologist; the other part was treated with DEPC solution and immediately frozen in liquid nitrogen for RNA extraction. The process was carried out in aseptic conditions and within 10 minutes of specimen collection.

2.1.2 SACC cells

Two cell strain of SACC were used in this study: ACC-2 cells (low metastatic cell strain) and ACC-M cells (high metastatic cell strain). The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum.

2.2 RNA extraction, purification, and testing

Tissues and cell specimens were put it in ammonia, and fully grinded and mixed in the machine. The mixture was moved into a 15-mL RNase free centrifugal tube and centrifuged. The supernatant was removed and put into precooled Trizol buffer for total RNA extraction. The mixture was then transferred into a new tube and RNA was extracted with 1.5 mL, chloroform, precipitated with avantin, and then dissolved in RNAse-free purified water. To prevent DNA contamination, the RNA solution was treated with RNase-free DNAse. The quality and consistency of the total RNA was assessed by spectrophotometry; RNA was then purified on oligo-dT affinity column and the RNA quality was tested by electrophoresis on 1% agarose gel (normal electrophotogram clearly showed 28S, 18S, and 5S ribosomal RNAs).

2.3 Preparing custom homeobox gene microarray

The network of American National Biological Information Center was used to retrieve human gene data, and all the members of the homeobox family were found by using 'homeobox' as keyword in the database search. According to the relevant biological software design 232 oligonucleotide microarray probes were needed. Eight custom microarrays were made by using the Oligo microarray and the omni Grid 100 sample-application device (Gene Machine Company). There were 14 positive spots and 1 negative spot. The first 7 spots in the first and second ranks of each matrix within the microarray were quality control spots, which were identical in every matrix: every gene was tested 4 times repeatedly in the same microarray.

2.4 Microarray hybridization

A direct cDNA labeling method was used. cy3 fluorescent label was used for the cDNA from the experimental group and cy5 was used for the cDNA from the comparator group. After labeling they were hybridized by using equivalent probes. The hybridized microarray was scanned, the data recorded, and the Cy3/Cy5 ratio was calculated. Genes with a ratio value of 0.5 to 2.0 were not considered to have a significant difference in expression; genes with a ratio ≥2 were considered upregulated, and those with a ratio ≤0.5 were considered downregulated.

2.5 Quantitative real-time polymerase chain reaction (PCR)

Cells (ACC-2 and ACC-M) and normal tissue specimens of salivary glands were used in the quantitative real-time PCR. Primers were designed based on mRNA sequences found in the Genebank. The sequences of the primers for each gene studied are listed in Table 1.

Table1.Primersequencesusedinreal-timePCR

Gene	Primer sequences (5'-3')	PCR product (bp)
EVX1	F: 5'-GCGGGTTTCCTTTCATCTTC-3' R: 5'-GCTGTCATCCTCCTGCTG-3'	112
TGIF	F: 5'-GGAGAGTCGGCTGTGAAG-3' R: 5'-AAGGATAGGCATTGTAACGG-3'	291

The real-time PCR amplification reaction was carried out by using 10.0 μL SYBR Premix Ex Taq, 0.8 μL of each specific primer, 1 μL cDNA template in a total volume of 20 μL. PCR was performed in a GeneAmp PCR SYSTEM 9600 (Perkin Elmer, Waltham, Mass). For signal detection, the GeneAmp PCR SYSTEM 9600 was programmed to perform an initial step of 2 minutes at 95 C, followed by 45 thermal cycles of 15 seconds at 95 C, 20 seconds at 62 C, and 20 seconds at 72 C. For quantification, a cycle threshold (Ct) value was used against a standard curve constructed after amplification of 10-fold serial dilutions of template copies. All reactions were performed in triplicate. Target gene mRNA levels were normalized to the mRNA level of a control/reference gene: 18s (endogenous house-keeping gene).

2.6 Statistical analysis

One-way ANOVA was used to analyze gene expression in normal tissue, SACC tissue, and cells by using the SPSS software version 12.0 (SPSS, Chicago, Ill). The alpha level was set at 0.05.

3. Results

3.1 Pathological analysis of clinical specimens

Specimen tissue sections were stained with HE. The cancer was proven to be SACC by clinical diagnosis. The tissues surrounding the cancer were normal salivary gland tissues.

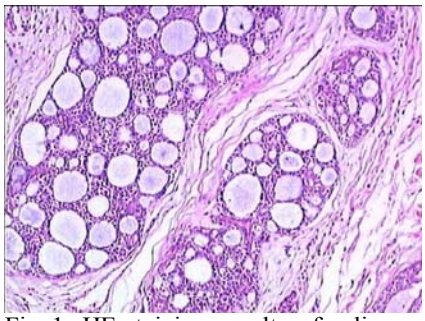

Fig 1. HE-staining results of salivary adenoid cystic carcinoma (300×)

3.2 RNA quality

Six groups of tissue specimens and two groups of cell specimens were evaluated. The total RNA were extracted and subjected to electrophoresis in 1% agarose gel. Clear zones of 28S, 18S, and 5S ribosomal RNA could be seen on the electrophoregram.

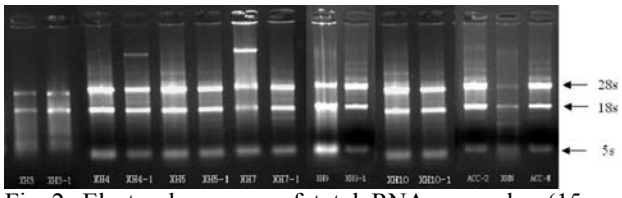

Fig 2. Electrophoregram of total RNA examples (15 specimens) : three zones as 28S, 18S and 5S can clearly be seen

3.3 Microarray results

The scanned image before microarray hybridization and the scanned image after hybridization showed that, in every group, the signal of the microarray was strong and the signals within each microarray was even. The correlation coefficients of two repeated points in two microarrays for the same sample were all above 80%.

3.4 Genes with differential expression

Eight paired specimens were studied. Data from the 8 hybridized microarrays were recorded and ranked by increasing ratio value. The ratio values were flagged as P.A or A, S/N≤2 or P≥0.05. Upregulated and downregulated homeobox genes on the microarray were selected, their sequence was retrieved, and the frequency of their appearance in the samples was counted (F, frequency) (Table 2).

Statistical analysis showed that, in tissue specimens, there were 9 upregulated homeobox genes whose frequencies were over 2. However, only one gene, TGIF, was upregulated both in cells and tissue specimens. Ten downregulated homeobox genes whose frequencies were over 2 were found in tissue specimens. Among them, EVX1 and IRX2 were downregulated both in cells and tissue specimens.

Table 2. Homeobox genes with differential expression between SACC and normal surrounding tissues

Differentially expressed genes	Frequency	Ratio (mean)	ACC-2	ACC-M
Upregulated		cy3/cy5 2.0		
DLX5 [a]	5	2.091	-	-
DBX1	4	2.119	-	-
EN1	4	5.193	-	-
IRX4	4	3.786	-	-
NKX6-1	4	4.119	-	-
DUXAP8	3	2.527	-	-
IRX3	3	7.617	-	-
ISL2	3	4.828	-	-
NKX6-2	3	4.914	-	-
TGIF	2	2.555	+	+
Downregulated		cy3/cy5 0.5		
EVX1 [a]	5	0.414	+	+
PITX1 [a]	5	0.166	-	-
DUXAP2	4	0.350	-	-
HOXB13	3	0.418	-	-
IRX2	3	0.227	+	+
ISL1	3	0.169	-	-
PITX2	3	0.305	-	-
PRRX1	3	0.315	+	-
SIX1	3	0.290	-	+
TLX2	3	0.325	-	-

[a]homeobox genes up-regulated or down-regulated in five microarray.

3.5 Quantitative real-time PCR

Quantitative real-time PCR results of the relative expression of EVX1 and TGIF in normal tissue and cells are shown in Table 3. There was no statistical difference in EVX1 expression between normal tissue and ACC-2. EVX1 expression was 200 times lower in ACC-M than in normal tissue (P<0.05) There was statistically significant difference in TGIF expression between normal tissue and ACC-M. TGIF expression was 8 times higher in ACC-M than in normal tissue (P<0.05)

4. Discussions

Genes which are differently expressed between malignant tissues and normal tissues may be involved in oncogenesis, cell multiplication and mitosis, and cell apoptosis. It may be closely related to malignant change and malignant progressing of adenoid cystic carcinoma of salivary gland tissue.

(1) DLX5 (distal-less homeobox 5) is a key differentiation factor which encodes a homeobox gene. This factor maintains the pluripotent differentiation potential and the self-turnover capability of embryonic stem cells. Reports have shown that this gene may be relevant to the pathogenesis of lymphatic leukemia.[16,17]

(2) DBX1 (distal-less homeobox) is the cognate frame of brain growth and a critical polyenergetic split determinant. It plays a critical regulatory role in the growing cycle of mammals.[18] Few reports have shown the relationship between DBX1 and oncogenesis of tumor.

(3) EN1 (engrailed homolog 1) plays an important role in the growing process of the rear somite in mammals. A study shows that transcription of the gene MENIN may be involved in the formation of multiglandular carcinoma, which is generally expressed in the form of medullary paraganglioma.

(4) EVX1 (eve, even-skipped homeobox, homolog 1) is located within chromosome No.7, and codes for a member of the even-skipped homeobox gene family. EVX1 protein may be an important transcription restraining determinant during embryonic development. So far, there is no relevant report on the involvement of EVX1 in the oncogenesis of human cancer.

(5) PITX1 (paired-like homeodomain transcription factor 1) encodes a member of the RIEG/PITX homeobox gene family. Members of this family are involved in organ growth and bilateral symmetry. Lord et al.[19] reported that the average level of PITX1 mRNA found in esophagus cancers associated with Barrett's esophagitis syndrome is much lower than that in Barrett's esophagitis syndrome (8.02) and normal esophagus (47, 46, $P<0.001$), which shows that PITX1 may play an important part in the process of occurrence and oncogenesis of esophagus Barrett's metaplasia-dysplasis – gland cancer.

In the present study, several homeobox genes, including DLX5, DBX1, PITX1 were differently expressed in SACC tissues vs normal salivary gland tissues, suggestion that these genes may be involved in oncogenesis in SACC. However, differential expression of these genes was not confirmed in cells. Of note, cells represent a 'single homogeneous component', while tissue specimens contain multiple cell types, including vessels, nerves, and extracellular matrix which may also be relevant to the tumor oncogenesis. These differentially expressed genes from tissue specimen may come from these different cell types within the tissue. The gene SIX1, which was differently expressed in ACC-M vs normal tissue but normal expressed in ACC-2 may be involved in the metastatic ability of cancer.

Two differentially expressed genes, TGIF and EVX1, were selected and their expression was evaluated using quantitative real-time PCR. TGIF was the only gene that was overexpressed in both SACC tissues and cells, although its frequency was relatively low. Several genes were downregulated in both SACC tissues and cells. Among them, EVX1 had the highest frequency. Cells (ACC-2 and ACC-M) were used to confirm EVX1 expression by real-time PCR because cells are more homogeneous; normal tissue was used as control. Real-time PCR confirmed that TGIF and EVX1 are differently expressed in SACC cells vs normal tissue.

In this study, we used microarray techniques and real-time PCR to identify the most relevant genes involved in SACC oncogenesis. TGIF and EVX1 were found to be differentially expressed in SACC vs normal salivary gland tissue. Further studies will be required to determine the mechanism and effects of these two genes on the oncogenesis of SACC.

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