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RESEARCH

Study of the Differentially Expressed Genes in Pleomorphic Adenoma Using cDNA Microarrays

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Abstract Recent studies have determined that gene expression profiling using microarray technology can be used to identify tumor-related molecules. The objective of this study was to screen the differentially expressed genes between pleomorphic adenoma (PA) and the normal tissue adjacent to PA using cDNA microarrays and to further validate the differentially expressed genes by real-time PCR. In this study, we selected five pairs of PA and the surrounding normal salivary gland tissues. The total RNA was isolated from tumor and normal tissues and purified to mRNA. The mRNA was reverse-transcribed to cDNA with the incorporation of fluorescent-labeled dUTP to prepare the hybridization probes. The mixed probes were hybridized to Whole Human Gene Expression Microarrays by Agilent. Tumor-related genes were screened by analyzing the fluorescence intensity. As a result, a total of 447 genes were found to be differentially expressed between PA and normal tissue adjacent to PA. Among them, 185 genes were up-regulated and 262 genes were down-regulated in PA. By constructing a network from the differentially expressed genes, some genes, such as Gli2 and CTNNB1, were identified as being at the core of the network. In addition, differential gene expression was validated for 2 up-regulated genes, Gli2 and LOX, using real-time PCR and the results were consistent with those of the cDNA microarray analysis thus verifying the credibility of the microarray data. Therefore, our microarray data may provide clues for finding novel genes involved in the development of PA, and shed light on finding new targets for diagnosis and therapy of PA. Further characterization of these differentially expressed genes will be useful in understanding the genetic basis for PA.

Keywords cDNA microarray · Differentially expression genes · Gene chip · Pleomorphic adenoma

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Introduction

Pleomorphic adenoma (PA) is the most common benign neoplasm of the salivary gland. Although PA is usually found in the parotid salivary gland, it may also arise in the submandibular, sublingual and minor salivary glands. It occurs most often between the ages of 30 and 60 years and is found more commonly in females than in males. Although the tumor is usually benign, it has the tendency to recur when inadequately excised. Moreover, 2–17% of the tumors can progress to malignancy and give rise to carcinoma ex-pleomorphic adenoma, an aggressive malignancy that may metastasize and result in death. The biological behavior of neoplasm is thought to be associated primarily with genetic alterations in the tumor cells themselves. However, relatively little is known about the molecular mechanisms of PA. Therefore, it is necessary to study the differentially expressed genes in PA. cDNA microarray, a high-throughput tool for monitoring expression levels of thousands of genes simultaneously and dissecting signal transduction pathways, makes it possible to reveal a comprehensive relationship among the regulatory networks controlling various genes and pathways. This

Table 1 Primer sequences for real-time PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
GLI2	TGGCCGCTTCAGATGACAGATGTTG	CGTTAGCCGAATGTCAGCCGTGAAG
LOX	AAGAGTGAAAAACCAAGGGACA	TGGTAGCCATAGTCACAGGATG
GAPDH	ATCTTCCAGGAGCGAGATCCC	CGTTCGGCTCAGGGATGACCT

microarray technology has been widely used for comprehensive gene expression analysis and identifying genes involved in tumors. In the present study, we used cDNA microarrays to distinguish between PA and the normal salivary gland tissue based on differential gene expression patterns. As a result, 447 genes were found to be differentially expressed. These differentially expressed genes will contribute to an understanding of the genetic basis of PA and the elucidation of the mechanism of its biological behavior.

Materials and Methods

Tissue Samples

Tissue samples were obtained from five parotid gland pleomorphic adenomas identified by the Pathology Department at Ninth People's Hospital Shanghai Jiao Tong University School of Medicine in June 2009. Among the five PA patients, two were male, and three were female. The mean age was 42.6 years old (range 18–73). Surgical tissue specimens, including the tumor tissue and the surrounding normal salivary gland tissue, were collected at the time of resection and immediately frozen in liquid nitrogen. The tumors and normal tissues were pulverized and then stored at −80°C.

RNA Isolation and Analysis

Total RNA was isolated from PA tissue and the normal tissue adjacent to PA by TRIzol reagent according to the recommended protocol. To ensure quality, total RNA was quantified by UV spectrophotometry, and its purity was assessed on a 2% agarose gel.

cDNA Probe Labeling and Hybridization

The message RNA was isolated and purified from the total RNA as described above. The purified mRNA from the tumor and nornal tissue was used to synthesize Cy3- or Cy5-conjugated dUTP-labeled cDNA probes, respectively, using an RNA Fluorescence Labeling Core Kit. Labeled Cy3 and Cy5 cDNA probes were dissolved in hybridization solution. The probes were denatured at 95°C for 5 min, dropped onto the center of the array surface and then covered with a coverslip without any bubbles. The slides were placed into a sealed cassette to hybridize in a 42°C water bath for 16 h. After hybridization, the microarray slides were washed with 0.1× saline sodium citrate (SSC), 0.2% sodium dodecyl sulfate (SDS) at 60°C for 2 min. The microarray slides were then washed with distilled water and spin-dried at room temperature. Subsequently, hybridized slides were scanned for Cy3 by 100% PMT and Cy5 by 10% PMT using an Agilent G2565AA and an Agilent G2565BA scanner. Thus, two separate images were generated for each slide.

cDNA Microarray Analysis

Raw signal intensities of gene expression data were processed and analyzed using ImaGene 3.0 software, and p-values were calculated. Raw data were exported into Excel. Differential gene expression analysis between PA and the normal tissue was subsequently conducted using the random variance model, which applies multiple testing corrections that determine the false discovery rate (FDR). Thus, FDR-adjusted differential scores and p-values for each gene between tumor and control samples were generated. Genes with an FDR-adjusted p-value of $p<0.05$ were considered to be differentially expressed genes.

Validation of Differential Expression of Genes by Real-Time PCR

Real-time PCR was employed to validate the relative change in expression of genes identified by cDNA microarray analysis. The RNA samples from the PA and the surrounding normal tissue were extracted using TRIzol reagent (Invitrogen). Then, the total RNA was reverse-transcribed into cDNA according to the instructions of the reverse transcription kit (TaKaRa). The real-time PCR was performed using ABI

Table 2 A partial list of the up-regulated genes in PA based on microarray analysis

Gene ID	Gene	*P*-value	FDR
5324	PLAG1	1.90E-06	2.05E-05
2247	FGF2	6.45E-05	0.000257
7042	TGFB2	0.000752	0.001843
1032	COL11a2	0.000255	0.00076
2487	FRZB	0.000178	0.00057
7465	WEE1	0.000429	0.001158
4015	LOX	1.00E-07	3.10E-06
2763	Gli2	0.000901	0.002136

Table 3 A partial list of the down-regulated genes in PA based on microarray analysis

Gene ID	Gene	*P*-value	FDR
51816	CECR1	6.07E-05	0.000245
1082	CGB	7.00E-07	1.08E-05
8755	ADAM6	1.64E-05	9.02E-05
2972	BRF1	1.00E-05	6.29E-05
1762	DMWD	1.20E-06	1.50E-05
79148	MMP28	6.09E-06	4.48E-05
9112	MAT1	1.20E-06	1.50E-05
27434	POLM	1.00E-07	3.10E-06

PRISM®7300 real-time PCR System according to the manufacturer's instructions. The total reaction volume was 20 μL (0.5 μL cDNA, 10 μL SYBR Premix Ex Taq, 0.4 μl ROX Reference Dye, 1 μL primer and 8.3 μL ddH_2O). The reaction conditions were as follows: activation at 95°C for 30 s, 40 cycles of denaturation at 95°C for 5 s, primer annealing and extension at 60°C for 31 s and ramping back to 95°C. Melting curve analysis of all samples was routinely performed to ascertain that only the expected products had been generated. mRNA expression levels of target genes were normalized to the expression of GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method [1]. The primers used for the identified genes, Gli2 and LOX, are presented in Table 1.

Results

Extraction of the Total RNA

OD of the total RNA was A260/A280 >1.8, and electrophoresis showed that the 18 s and 28 s bands were distinct. These data confirmed that the purity and quality of the total RNA met the standard.

Differentially Expressed Genes

In an effort to gain a better understanding of the mechanisms, clinical features and prognosis of PA at the molecular level, we have generated comprehensive gene expression profiles of five PAs. A total of 447 genes were found to be differentially expressed. Among these genes, 185 genes were up-regulated, and 262 genes were down-regulated. A partial list of the up-regulated and down-regulated genes of PA are shown in Tables 2 and 3.

Network of Differentially Expressed Genes

The differentially expressed genes between PA and the normal tissue adjacent to PA were analyzed using the KEGG database. The KEGG database can analyze the relationships between the gene expression products based on their interactions and construct a regulatory network from the differentially expressed genes (Fig. 1). Additionally, some genes at the core of the network, such as CTNNB1, Gli2, TP53, and WNT4, are also listed in Table 4.

Validation of Microarray Data for Selected Genes by Real-Time PCR

To validate the microarray data presented in this report, the relative mRNA abundance of two up-regulated genes, Gli2 and LOX, was quantified using real-time PCR. The amount of mRNA of the target genes relative to GAPDH was calculated using the formula $2^{-\Delta\Delta Ct}$, and the fold change in expression was calculated relative to the normal tissue

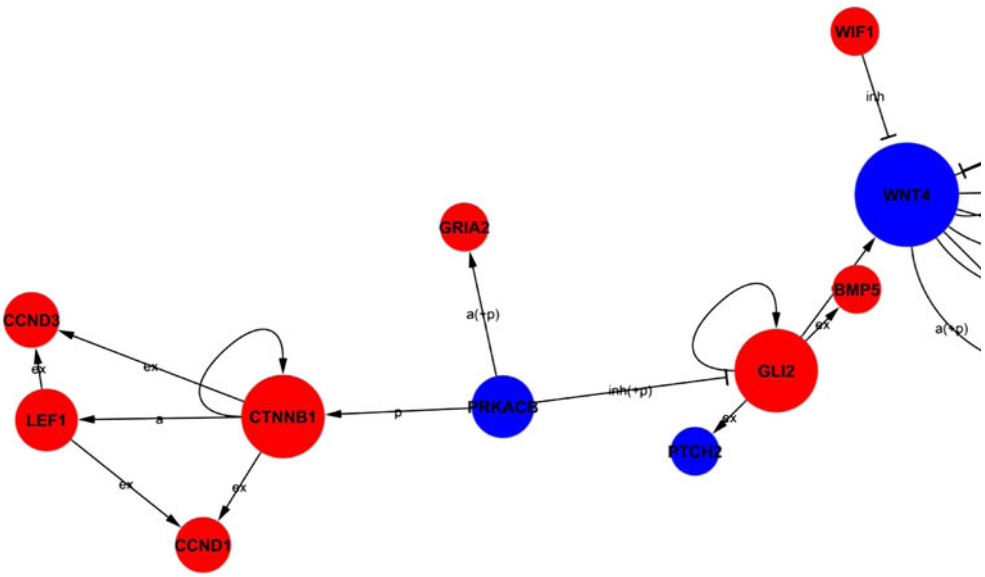

Fig. 1 Part of the regulatory network of the different genes. A point represents genes, a line represents the interaction relationship among genes, an arrow indicates activation and a flat arrow indicates inhibition. *Red*: up-regulated genes, *blue*: down-regulated genes, a: activation, b: binding, inh: inhibition, p: phosphorylation and ex: expression of genes

Table 4 The genes at the core of the network. Degree denotes the number of the genes that interact with other genes (that is the number of the edges in the network), Indegree denotes the degree that other genes regulate the gene, and Outdegree denotes the degree that the gene regulates other genes

Gene ID	Gene	Degree	Indegree	Outdegree	Style
54361	WNT4	11	3	8	down
5468	PPARG	10	0	1	down
2776	GNAQ	7	2	0	up
7157	TP53	6	1	5	up
1499	CTNNB1	6	1	3	up
2763	GLI2	6	1	3	up

adjacent to PA. As shown in Figs. 2 and 3, we observed some variation in the expression levels of Gli2 and LOX across samples based on the results from real-time PCR analysis, but the direction of regulation (i.e., up-regulation or down-regulation) remained consistent with the cDNA microarray-based observation, confirming the accuracy of our data.

Discussion

It is well-known that tumorigenesis and development is a complex and multi-stage process, including the changes of many oncogenes, tumor suppressor genes and expression disorders of signal transduction molecules [2–4]. Therefore, studying the alterations in gene expression and dysregulation of signaling pathways may be helpful in clarifying the mechanisms of tumor occurrence and development [5–7]. Recently, the cDNA microarray system, which is a high-throughput, highly automated and large-scale technology, has been used to identify differentially expressed genes involved in various tumors [8–11]. Our previous studies also showed that this technology has the potential to screen and identify the expression pattern of a large number of genes simultaneously [7, 9]. In the present study, we continued to use cDNA microarray technology to investigate the differentially expressed genes in five pairs of PA

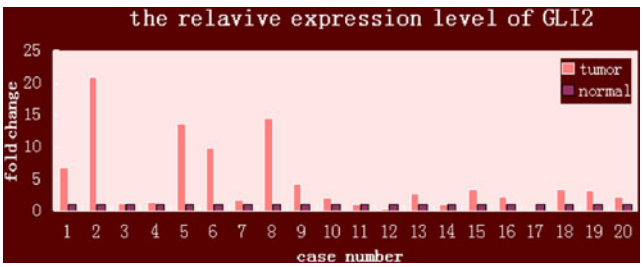

Fig. 2 Quantitative real-time PCR analysis of Gli2 in the PA and normal tissue samples

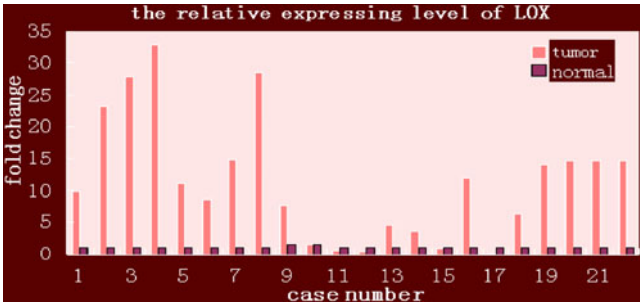

Fig. 3 Quantitative real-time PCR analysis of LOX in the PA and normal tissue samples

and normal tissue adjacent to PA. Consequently, a total of 447 differentially expressed genes were identified, indicating the complicated gene changes in PA. That would provide information useful in elucidating the genes responsible for PA. Among the 447 genes, 185 genes were up-regulated and 262 genes were down-regulated. On the basis of these differentially expressed genes, we constructed a signal transduction network using the KEGG database. Figure 1 shows part of the regulatory network including a series of oncogenes and tumor suppressor genes. Genes at the core of the network mainly consist of the up-regulated genes (CTNNB1, Gli2, TP53, GNAQ) and down-regulated genes (WNT4, PPARG). Some of the differentially expressed genes have previously been shown to be associated with PA, whereas some others seem to be novel in terms of their association with PA and are of potential interest for follow-up studies.

Overexpression of oncogenes is a key event in human tumors because the physiological functions of these genes are involved in the regulation of normal cell growth processes, including the cell cycle and nuclear transcription. We therefore selected two up-regulated genes, Gli2 and LOX, to validate by comparing the microarray data with real-time PCR analysis. In this microarray data, the mRNA expression levels of Gli2 and LOX were up-regulated in PAs compared with normal salivary glands and the fold change of was 11.70 and 30.43, respectively. Our consistent results demonstrate that cDNA microarray and real-time PCR are feasible methods for evaluating gene expression in PA.

Gli2, which is a total of 3,678 bases long and localized at 2q14, is a zinc finger transcription factor of the Hedgehog (Hh) signaling transduction pathway [12–14]. Studies report that the Hedgehog signaling pathway not only regulates cell growth, proliferation and differentiation, but also participates in tumor cell proliferation and diffusion. Therefore, the abnormal regulation of the Hedgehog signaling pathway plays an important role in tumor occurrence and development [15, 16]. Many studies have found that the abnormal activation of Gli2 could greatly enhance the transcription of controlled molecules downstream the Hedgehog signaling pathway, leading to a

variety of tumors [17–20]. In this study, we detected overexpression of Gli2 in PA using cDNA microarray and validated this by real-time PCR. Thus, up-regulated Gli2 gene expression may play an important part in PA pathogenesis. However, further experimental studies are still needed.

CTNNB1, cadherin-associated protein, is expressed on the surface of layered organs and mediates cell adhesion, signal transduction and tumor metastasis. Under normal circumstances, the CTNNB1 gene, which encodes the β-catenin protein involved in cell-cell adhesion and the WG/WNT signaling pathway, is highly and ubiquitously expressed. Researchers have reported that CTNNB1 mutations are associated with the genesis of tumors, such as endometrial cancer, breast cancer, ameloblastoma, odontogenic tumors, and desmoplastic fibroma [21]. In this study, we found that the expression of CTNNB1 in PA was up-regulated by 2.6 times that of the corresponding normal salivary gland. Recently, it has been found that CTNNB1 is the chromosome translocation partner of PLAG1 in 25% of all PA of the salivary glands. PLAG1,a crucial oncogene in PA of the salivary gland, is consistently rearranged and overexpressed with 8q12 translocations [22]. In the specific case of t(3:8)(p21;q12), the CTNNB1 gene on chromosome 3p21 is involved as translocation partner of PLAG1. As a result of the translocation, the constitutively active CTNNB1 promoter drives ectopic expression of the otherwise developmentally regulated PLAG1 gene and vice versa [23].

In summary, we identified a large number of differentially expressed genes in PA using cDNA microarrays. Some of the differentially expressed genes have previously been shown to be associated with PA, while some others seem to be novel in the context of PA. However, further characterization of these differentially expressed genes will be useful in understanding the genetic basis for PA, and their functions should be confirmed in vivo and in vitro.

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