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Different cDNA microarray patterns of gene expression reflecting changes during metastatic progression in adenoid cystic carcinoma

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Published: 19 December 2003

Received: 30 August 2003

World Journal of Surgical Oncology 2003, 1:28

Accepted: 19 December 2003

This article is available from: <http://www.wjso.com/content/1/1/28>

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Abstract

Background: The metastatic ability of tumor cells is determined by level of expression of specific genes that may be identified with the aid of cDNA microarray containing thousands of genes and can be used to establish the expression profile of disease related genes in complex biological system.

Materials and Methods: Salivary adenoid cystic carcinoma cell line and its high metastases adenoid cystic carcinoma clone were used as model systems to reveal the gene expression alteration related to metastasis mechanism by cDNA microarray analysis. The correlation of metastatic phenotypic changes and expression levels of 4 selected genes (encoding CD98, L6, RPL29, and TSH) were further validated by using RT-PCR analysis of human tumor specimens from primary adenoid cystic carcinoma and corresponding metastasis lymph nodes.

Results: Of the 7,675 clones of known genes and expressed sequence tags (ESTs) that were analyzed, 30 showed significantly different (minimum 3 fold) expression levels in two cell lines. Out of 30 genes found differentially expressed, 18 were up regulated (with ratio more than 3) and 12 down regulated (with ratio less than 1/3).

Conclusion: Some of these genes are known to be involved in human tumor antigen, immune surveillance, adhesion, cell signaling pathway and growth control. It is suggested that the microarray in combination with a relevant analysis facilitates rapid and simultaneous identification of multiple genes of interests and in this study it provided a profound clue to screen candidate targets for early diagnosis and intervention.

Background

Adenoid cystic carcinoma (ACC) of the salivary gland is

characterized by a prolonged clinical course, high rate of local recurrence and the delayed onset of distant

hematogenous metastases. Late distant metastases and local recurrences are responsible for a rather low long-term survival rate and poor treatment results [1]. However, the molecular mechanism behind the metastasis development is poorly understood, largely because the tumor metastasis is a complex process involving several distinct steps such as escape from primary tumor, dissemination through the circulation, lodgment in small vessels at distinct sites, penetration of the vessel wall and growth in the new site as a secondary tumor [2]. A possible breakthrough in understanding of tumor metastasis has emerged with the combination of a putative hypothesis and the development of high throughput cDNA microarray technology. It is hypothesized that the long-term development of metastasis induces genetic alteration and subpopulation of cancerous cells that characterize the metastatic cells with specific gene expression involved in temporal and spatial procession of metastasis [3]. On the other hand, examination of large-scale expression alteration of specific genes involved in tumor metastasis is made feasible by the recently developed cDNA microarray technology that allows the simultaneous analysis of the expression levels of thousands of genes [4].

Consistent with the above view, in this study, we used cDNA microarray technique to examine the differences in the gene expression profiles between a salivary adenoid cystic carcinoma cell line ACC-2 and a highly metastatic salivary adenoid cystic carcinoma clone ACC-M, which was screened from ACC-2 by combination of *in vivo* selection and cloning *in vitro* [5]. Since ACC-2 and ACC-M share identical genetic background except for different metastatic behavior, it is presumed that the differentially expressed genes ACC-2 and ACC-M as metastasis related genes, which play direct or indirect roles in the progression of metastasis. The results of the cDNA microarray analysis were further conformed by RT-PCR to determine the expression level of specific mRNA in the primary tumors and corresponding metastasis lymph nodes. Such a survey not only furthers an insight into the underlying mechanism of the adenoid cystic carcinoma metastasis, but also helps identify candidate marker for diagnosis and prognosis of ACC and molecular targets for metastasis intervention.

Materials and Methods

Cell lines and specimens

Cell lines ACC-2 and clone ACC-M were previously established in the tumor biology laboratory of Shanghai Ninth Hospital in Shanghai Second Medical University. Cell line ACC-2 was derived from surgically excised primary tumor tissue from a histologically diagnosed patient with adenoid cystic carcinoma of the palate. The microscopic features of the tumor showed a dominantly solid pattern of ACC. The patient did not receive any therapy before sur-

gery. High pulmonary metastases clone ACC-M was selected from cell line ACC-2 after five repeated selection *in vivo* combined with an *in vitro* cloning technique and analysis of platelet aggregation activity [6].

Cells were cultured at 37°C in 5% CO₂ humidified incubator in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin and streptomycin.

Four pairs of fresh human specimens from the same patient were included in the RT-PCR analysis. In each pair, one specimen was from primary tumor tissue and the other from metastatic lymph node. Both of them were confirmed by histological examination. All specimens were collected after obtaining the informed consent from the patients. Tissues were frozen at -80°C immediately after separation, till analysis.

Total RNA extraction and mRNA isolation

Cells were grown to 80% confluence in culture bottles, and total RNA was extracted using standard Trizol reagent (Life Technologies, Gaithersburg, MD) following the manufacturer's protocol. Briefly, cells were first homogenized in Trizol solution and incubated for 20 minutes on ice, and then a 1/5 volume of chloroform was added to the homogenates. After vigorous agitation for 5 minutes, the inorganic phase was separated by centrifugation at 12000 g for 15 min at 4°C. RNA was then precipitated in one volume of isopropanol and centrifuged at 10000 g for 20 minutes at 4°C. RNA pellets were washed with 70% ice-cold ethanol and then dissolved in diethyl pyrocarbonate (DEPC)-treated H₂O. The amount and quality of total RNA were checked by electrophoresis on a 0.8% formaldehyde agarose gel. Then the polyA mRNA was purified using the Oligotex poly (dT) resin® (Qiagen, German) according to the manufacturer's instruction.

Synthesis and purification of labeled cDNA probe

1–2µg purified mRNA was labeled in reverse transcription using M-MLV reverse transcriptase (Promega, Madison, USA) in the presence of Easytides deoxyadenosin 5' triphosphate [α -³³P] (NEN® Life Science Product, Boston, USA). Before labeling, total RNA was treated with DNase (Boehringer Mannheim, Germany) and RNasin (Promega, Madison, USA) at 37°C for 15 minutes to remove contaminated DNA. For each labeling, the treated RNA was first incubated with 5 µl oligo dT (Life technologies, USA) at 70°C for 10 min. Then 6 µl 0.5× first strand reaction buffer, 1 µl 0.1 M DTT, 1.5 µl dNTP mixture containing dATP, dGTP and dTTP at 20 U/ml (Promega, USA), 10 µl α -³³P dCTP and 1.5 µl superscript reverse transcriptase were added and incubated at 37°C for 90 min. The labeled first strand cDNA probes were purified by

spin column (Clontech, CA) to remove the unincorporated nucleotides.

cDNA microarray preparation

Human cDNA clones picked from cDNA libraries were terminal sequenced. A cDNA array was assembled with a total of 7675 cDNA clones representing the same number of independent cDNA clusters. All cDNA fragments were amplified by PCR and verified by gel electrophoresis. PCR products were spotted onto two 8 × 12 cm Hybond[®]-N nylon membranes (Amersham Pharmacia, Buckinghamshire, UK) using an arrayer (BioRobotics, Cambridge, United Kingdom). Each spot carried ~100 nl in volume and was 0.4 mm in diameter, and each cDNA fragment was placed in two different spots (double-offset). Eight housekeeping genes encoding ribosomal protein S9 (RPS9), β-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase, hypoxanthine phosphoribosyltransferase 1, *M*_r 23,000 highly basic protein (RPL13A), ubiquitin C, phospholipase A2, and ubiquitin thiolesterase (UCHL1) were spotted and evenly distributed as hybridization intra-membrane control, each in 12 places. Hybridization data was considered invalid if among the 12 spots representing the same gene, the intensity of the darkest spot exceeded 1.5-fold of that of the weakest spot.

Hybridization and image analysis

Prepared nylon membranes were prehybridized in 20 ml of prehybridization solution (6×SSC, 0.5% SDS, 5×Denhardt's, and 100 μg/ml denatured salmon sperm DNA) at 68°C for 3 hours. Overnight hybridization with the ³³P-labeled cDNA in 6 ml of hybridization solution (6×SSC, 0.5% SDS, and 100 μg/ml salmon sperm DNA) was followed by stringent washing (0.1×SSC, 0.5% SDS, at 65°C for 1 h). Membranes were exposed to phosphor screen overnight and scanned using an FLA-3000A Plate/Fluorescent Image Analyzer (Fuji Photo Film, Tokyo, Japan). Radioactive intensity of each spot was linearly digitalized to 65,536 gray-grades in a pixel size of 50 μm in an image reader and recorded using the Array gauge software (Fuji Photo Film, Tokyo, Japan). After subtraction of background chosen from an area where no cDNA was spotted, genes with intensities >10 were considered as positive signals to ensure that they were distinguished from background with statistical significance >99.9%. Normalization among arrays was based on the sum of background-subtracted signals from all genes on the membrane. On the basis of each gene's ratio of expression level between the ACC-M and ACC-2 (ACC-M/ACC-2), we divided those with significantly differential expression into two classes: up-regulated with the ratio over 3.0, down-regulated with the ratio below 1/3.

Semi-quantitative RT-PCR

Four selected genes, CD98, L6, RPL29, and TSH, were selected and investigated in this study to evaluate the difference of expression of them between primary ACC tumor tissue and ACC metastasis lymph node in vivo, thus confirming the reliability of our approach. 1 μg each of total RNA from 4 primary tumor tissue samples and 4 corresponding metastasis lymph node tissue samples were used as templates in a total volume of 20 μl reverse transcription reaction system. The cDNA were then transferred to a PCR master mixture containing 1×PCR Buffer, 1.8 mM MgCl₂, and 2.5 units of Taq polymerase, 1 μM gene specific primers, as listed in Table 1. Primers were designed using the Primer3 program in the Center for Genome Research at the Whitehead Institute http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi. To avoid problems associated with genomic DNA contamination primers were made to span an intron. PCR conditions were as follows: 1 cycle of 95°C for 10 minutes, 25–30 cycles of 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute, and final extension at 72°C for 5 minutes after the last cycle. Amplified fragments were visualized by ethidium bromide staining of the agarose gel and photographed under UV light.

Table 1: Primers used for RT-PCR

Gene	Primers	Product size
RPL29	5'ACATGGCCAAGTCCAAGAAC3' 5'TTAGGCCCTTTTGTGTGC3'	165
CD98	5'TCTTGATTGCGGGGACTAAC3' 5'GAGCCTTGCCTGAGACAAAC3'	197
L6	5'AGGGCCAGTACCTTCTGGAT3' 5'AAGCCACATATGCCTCCAAG3'	169
TSH	5'CGTGCCACTGCAGTACTTGT3' 5'TTGAAGCGTGTCAAAGTGGT3'	165

Results

Identification of genes differentially expressed between ACC-2 and ACC-M cells

The cDNA microarray technique was applied to analyze the expression patterns of ACC-2 and ACC-M cells. All tests were considered valid with the maximal variation of hybridization intra-membrane control signal intensity being 1.4-fold. Among relevant 7,675 genes and expressed sequence tags (ESTs) assembled in the cDNA microarray, 30 were found differentially expressed, with the difference signal intensity ratio of more than 3. Genes and ESTs were divided into two groups on the basis of the difference in signal intensity: Out of 30 differentially expressed genes or ESTs, 18 were up-regulated (signal intensity ratio more than 3) in ACC-M clone compared with ACC-2 cell line

Table 2: List of the up-regulated and down-regulated genes and ESTs in the microarray

Gene bank Accession No	Name	Chromosomal Localization	Ratio of ACC-M/ACC-2
Up-Regulated			
U05861	hepatic dihydrodiol dehydrogenase	10p15-p14	5.91
D17793	KIAA0119	10p15-p14	6.47
AB007870	KIAA0410	13q12.13	3.63
X16901	RAP30 subunit	13q14	3.18
U09848	zinc finger protein LNF139	7q21.3-q22.1	4.53
X84003	TAF1118	1p13.1	3.40
D90282	carbaryl phosphate synthetase I	2q35	3.40
U40490	nicotinamide nucleotide transhydrogenase	5p13.1-5cen	4.25
D84488	small GTP-binding protein	1q32	3.05
D14533	XPAC protein	9q22.3	3.12
X60221	H ⁺ -ATP synthase subunit b	1p13.2	3.12
A1436789	EST	?	3.13
W05842	EST	?	3.18
AA186737	EST	?	3.05
N6482	EST	?	4.36
A1254779	EST	?	3.50
AA582554	EST	?	3.40
AA478034	EST	?	3.10
Down-Regulated			
AB018010	CD98	11q13	0.22
Z49148	ribosomal protein L29	12q23.1	0.23
L13435	3p21.1 gene	3p21	0.31
U23143	mitochondrial serine hydroxymethyltransferase	12q12-q14	0.32
AW072071	EST	?	0.25
YO0483	gluthathione peroxidase	?	0.30
A1804930	EST	?	0.07
AJ249732	G8 protein	6p21.3	0.25
W20128	EST	?	0.32
A1005336	EST	?	0.25
S70585	thyroid-stimulating hormone (TSH)	6q12-q21	0.30
M90657	tumor antigen (L6)	3q21-q25	0.30

and 12 down-regulated (signal intensity ratio less than 1/3). Results are shown in table 2.

Semi-quantitative RT-PCR analysis

RT-PCR was performed on the primary tumor tissues and metastasis lymph nodes to give *in vivo* validation to the array results for the selected genes. The results were revealed in electrophoresis pattern shown in Figure 1, confirming the results of the cDNA microarray data. Expression level in ACC metastasis lymph nodes compared with ACC primary tumor tissues showed similarly down-regulation of CD98, RPL29, TSH and L6 in all the samples.

Discussion

Several approaches have been used to study the gene profile alteration that may occur in the development of ade-

noid cystic carcinoma and metastasis [7-11]. However, most studies were highly focused and do not provide insight into global gene expression pattern although they contributed greatly to our current understanding [12]. Focusing on gene expression patterns of a multitude of genes, referred to as genetic fingerprints, is proving to be a much more powerful approach than studying a small number of genes that may not hold much clinical significance individually [13]. Recently developed cDNA microarray technology makes it possible for us to analyze multi gene related process such as metastasis due to the wide spectrum of genes and high throughput analysis [14]. In this study, we have chosen human adenoid cystic carcinoma cell lines as a model system to study changes in gene expression that should be of importance in the pathogenesis of metastasis. Cell lines are important tools for *in vitro* studies of neoplasms, and are highly valuable

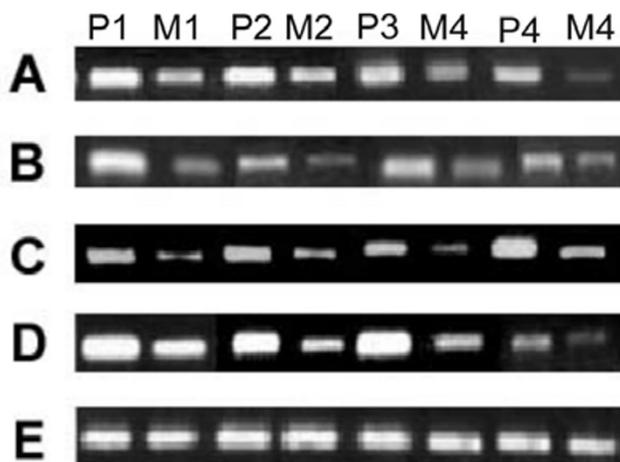


Figure 1

Semi-quantitative RT-PCR analysis of selected genes. Total RNA isolated from 4 primary tumor tissue samples and 4 corresponding metastasis lymph node tissue samples were used as a template for RT-PCR using the gene-specific primers described in Table 2. RT-PCR showing the down-regulation of (A) RPL29, (B) CD98, (C) L6 and (D) TSH. (E) GAPDH was used as an internal reference. P_i, primary tumor; M_i, metastasis tumor (i refer to the number of sample).

in studies requiring considerable amount of material [15]. On the other hand, since adenoid cystic carcinoma itself is heterogeneous in nature, cell lines can supply a homogeneous cell population exempted from the disturbance of non-parenchyma cells in ACC tumor, including stroma cells, adipose cells, endothelial cells, and infiltrating lymphocytes, and give a more accurate data interpretation.

In this study, cDNA microarray analysis of cell line ACC-2 and cell clone ACC-M revealed several differentially expressed genes. The reliability of our approach was further validated using RT-PCR on 4 paired specimens from primary tumor and metastatic lymph node. The difference of expression level of 4 selected genes disclosed by semi-quantitative RT-PCR was consistent with the results of cDNA microarray analysis. Among those differentially expressed genes, some of genes were already known as active participants related to tumor and metastasis.

CD98 is found under expressed by 4.5-fold in ACC-M clone compared with ACC-2 in our study. CD98, a heterodimeric type II transmembrane glycoprotein present in all established human cell lines and most malignant human cells, functions in amino acid transport, cell fusion and homotypic cell aggregation [16]. It is also an

important regulator of integrin activation and an oncogenic protein, playing a role in integrin signaling pathways [17-19]. It is known that integrin is key factor for cell invasion and migration, allowing the cancer cell to penetrate itself into the tissue and attach to the target tissue's basal matrix by binding to components of the extra cellular matrix [20]. Integrin-CD98 association and relative mechanisms to regulate cell adhesion, migration, and metastasis have been profoundly reported recently. Zent *et al* have shown that CD98 can associate with isolated cytoplasmic portions of some $\beta 1$ integrin isoforms [21]. Kolesnikova *et al* have recently shown that CD98 constitutively and specifically associated with various $\beta 1$ integrin subunits by reciprocal immunoprecipitation experiments [12]. They propose that by cross-linking CD98, it acts as a "molecular facilitator" in the plasma membrane, clustering $\beta 1$ integrins to form high-density complexes, thus subsequently lead to integrin activation and adhesion, integrin signaling, and anchorage-independent growth. In this context, our observation of down-regulated expression of CD98 in high metastasis cell clone might contribute to the elucidation of suppressive role of CD98 in molecular mechanism of metastasis although further investigation is needed to postulate relationship of the distribution of CD98 and the tumor-spreading pattern and differentiation and metastatic behavior in ACC.

Ribosomal protein L29 gene (Heparin/Heparan Sulfate Interacting Protein Gene) was shown to be down regulated in high metastasis cell clone ACC-M. HIP is a cell surface binding protein, highly expressed in most human epithelial cell lines, including uterine and fibroblastic cells [22]. By binding directly to heparin it functions as a cell-cell and cell-extracellular matrix adhesion molecule [23-26]. Recent studies suggested that HIP may play a role in cell migration and metastasis of melanoma and breast cancer cells [27,28]. Wang *et al* [29], reported Heparin/Heparan sulfate interacting protein (HIP) to be up-regulated in colorectal carcinoma, a significant inverse correlation between HIP levels and the presence of distant metastasis (Duke's stage D) was also noted. This indicated up-regulation of HIP to be an early event in carcinogenesis, and its increased expression may facilitate growth. Later in tumor progression, HIP may be down-regulated to decreased cell adhesion, favoring metastasis. Our data supports the concept of down-regulation of HIP in tumors with metastasis.

Under expression of tumor-associated antigen L6 gene in high metastatic cell clone and metastasis lymph nodes is another finding in this report. Tumor-associated antigen L6 is a kind of cell surface antigen that is known to express in the lung, breast, colon, and ovarian carcinomas, mediating cell to cell and cell-to matrix interaction [30]. However, other research have shown that the expression

level of L6 was found to increase in metastatic-tumor-derived cells compared with primary-tumor-derived cells [31], thus leaving the controversy open, and requiring further investigations.

Thyroid stimulating hormone (TSH) has been reported to be associated with enhanced tumor proliferation and aggressiveness in follicular thyroid cancer cells [32]. Loss of TSH stimulation allows the lung metastasis the highest basal invasive potential [33]. Similar findings of decrease in TSH mRNA expression level had been observed in ACC-M cell line and metastasis lymph node, supporting the previous report of metastasis inhibiting role of TSH [33]. Our results show that a decreased transcription of this gene could be helpful for the tumor metastasis by decreased cell adhesion and loss of contact inhibition control.

It is clear from our results that high metastasis ACC cell clone has a genome-wide genetic expression profile that differs from primary ACC cell line. Having determined that such differences exist, the next question is to what extent are these gene expression alterations related to metastasis? Or whether there is genetic expression profile heterogeneity among tumors of a particular histopathologic grade and metastatic potential? Future studies are needed to directly compare gene expression profiles between different samples of same stage to affirm this possibility.

Analysis of cell lines using cDNA microarray also revealed several novel differentially expressed genes, which have not been previously reported to be related to tumor metastasis, for example, TAF1118 gene, zinc finger protein LNF139 gene, hepatic dihydrodiol dehydrogenase gene etc. The further validation and detailed mechanism of these genes in conferring the phenotype of high metastasis to a cell need more extensive investigation. The global molecular regulatory network would also shed a light on our understanding of the mechanism of pathogenesis of metastasis and potential gene therapy[34].

We report here an average 3-fold differential expression ratio. We attribute this result to the close genetic background of the ACC-2 and ACC-M cells as they were derived from the same parental source. ACC-2 was established from primary adenoid cystic carcinoma and ACC-M was screened out from ACC-2. We have set only those genes significant whose expression was changed by at least a factor of three. However, we think that this cutoff point is arbitrary and that there might exist some important enough genes whose expression changed less than a factor of three. It is necessary to complement the preliminary cDNA microarray results with some more sensitive confirmatory experiments for target genes. There might be

some false negative metastasis-related genes missed in this report especially for those with signal intensity ratio less than three. Hopefully, this technical problem could be prevented and the overall signal intensities could be adjusted by a newly introduced optimization strategy called directed application of capture oligonucleotides [35].

The measurement of gene expression can, therefore, provide information on regulatory mechanisms, biochemical pathways, cellular control mechanisms and potential targets for intervention and therapy in a variety of disease states [36]. Our results support the hypothesis that multiple specific genes may contribute to the development of distant metastases from adenoid cystic carcinoma cells. cDNA microarray analysis of the model system for differentially expressed gene involved in adenoid cystic carcinoma metastasis has revealed a variety of specific genes, including putative common metastasis-related genes, which provides a basis for rationally determining which pathways are appropriate for further study and which molecular targets are potential targets for gene therapy [37]. These findings will provide new insights to further explore the complicated molecular events of tumor metastasis. Studies of molecular mechanism regulating these changes would help to identify prognostic marker and treatment targets for cancer metastasis and allow more accurate risk stratification for clinical metastases.

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