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Microarray gene expression profiling of cell lines from primary and metastatic tongue squamous cell carcinoma: possible insights from emerging technology

Nadarajah Vigneswaran[1], Jean Wu[1], Peter Sacks[2], Michael Gilcrease[3], Wolfgang Zacharias[4]

[1]*Department of Diagnostic Sciences, University of Texas-Houston Dental Branch, Houston, TX, USA;* [2]*Division of Biological Sciences, College of Dentistry, New York University, New York, NY, USA;* [3]*Department of Pathology, University of Texas M.D. Anderson Cancer Center, Houston, TX, USA;* [4]*Department of Medicine and Department of Pharmacology and Toxicology, James Graham Brown Cancer Center, University of Louisville, Louisville, KY, USA*

BACKGROUND: To identify common gene expression patterns among two uniquely matched pairs of primary and metastatic oral squamous cell carcinoma (OSCC) cell lines derived from the same two patient donors.

METHODS: Two pairs of cell lines derived from the primary tumors and lymph node metastases of the same two patients were used to obtain microarray-based gene expression profiles. Reverse transcriptase-polymerase chain reaction and immunohistochemistry were used to confirm observed changes for some of the candidate genes.

RESULTS: Approximately 50% of the genes profiled were expressed in all four cell lines. Cluster analysis identified a group of 17 genes whose expression correlated inversely with metastatic progression. Only 10 common genes were differentially expressed in both pairs of primary and metastatic cells. A group of 28 highly expressed genes was common for both metastatic cell lines, among them some of the known metastasis-related genes such as laminin receptor, thymosin β-4 and β-10 and metallopanstimulin.

CONCLUSIONS: Groups of presumed metastasis-related genes are highly heterogeneous and vary significantly between the two patients. Thus, it is unlikely that the metastatic phenotype of these OSCC cells is acquired by de-regulation of a single gene or a group of few genes. Most likely, multiple combinations of differentially expressed genes are involved in facilitating metastatic spread of these oral carcinoma cell lines.

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Correspondence: Wolfgang Zacharias, Department of Medicine and Department of Pharmacology and Toxicology, James Graham Brown Cancer Center, University of Louisville, Delia Baxter Bldg. 321F, 580 South Preston Street, Louisville, KY 40202, USA. Tel.: 502 852 2579. Fax: 502 852 2356. E-mail: w0zach01@gwise.louisville.edu
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Introduction

For patients with oral squamous cell carcinoma (OSCC), the most detrimental event during oral cancer progression, and thus the biggest threat to survival, is the switch from a locally invasive stage to a metastatic tumor (1, 2). A stepwise accumulation of multiple genetic and epigenetic changes accompanies the gradual transition of normal oral epithelium to invasive OSCC to metastatic OSCC (3). These genetic aberrations result in the loss or activation of a number of critical genes, such as those involved in cell proliferation, differentiation, apoptosis, cellular adhesion and motility (4). However, little is known about genes that are specifically overexpressed or repressed during invasive and metastatic progression of OSSC (5–7).

Traditional gene-targeted studies that focus on one to a few genes at a time do not provide insight into the changes in global gene expression associated with tumor invasion and metastasis. Recent progress in high-throughput gene expression profiling (GEP) methods makes it possible to identify gene expression changes associated with the transition of primary to metastatic tumor. These methods include serial analysis of gene expression, differential display, subtractive hybridization, and representational difference analysis (8). However, with the recent development of cDNA and oligonucleotide microarrays, it is possible to compare relative expression levels of several thousand genes simultaneously in different disease states in a single experiment. The high-density synthetic oligonucleotide microarray approach has clear advantages over other methods, as it ensures a high degree of automated reproducible relative quantification of mRNA levels. Recently, oligonucleotide microarrays have been used to identify physiologically and pathologically relevant GEPs in organs in various developmental and pathological stages (9–14), including cancers of the oral cavity (15–19). Such high-density DNA microarray-based GEP can be used to identify differentially expressed genes between primary and metastatic oral cancer cells.

Describing the GEP-based metastatic phenotype is crucial for understanding the gene regulatory networks that convert OSCC cells to the metastatic state. It has been well documented in previous studies that malignant tumors of similar histology demonstrate great variation in their GEPs in different patients, which is mostly reflective of genetic heterogeneity among the patients (11). However, GEPs of primary/recurrent/metastatic tumors derived from the same patient are more similar (11). Therefore, comparing GEPs of clonally related paired samples of primary and metastatic OSCC cells can reveal subtle differences in their GEPs, thereby pinpointing the genes that are critical for the metastatic dissemination of OSCC (20). Determining the degree of diversity in the GEPs between primary and metastatic cell clones of the same patient is essential for their applications in tumor biology and clinical oncology.

The goal of our study is to characterize GEPs specific for metastatic phenotypes in two pairs of uniquely matched OSCC cell lines derived from the primary tumor and lymph node metastases of two oral cancer patients. From our data, we have identified expression patterns of genes that are commonly either up- or down-regulated during metastatic progression of these OSCC lines. We conducted this study as the two paired primary and metastatic cell lines were uniquely matched by being derived from the same donors, thus allowing to assess interpatient variability in addition to variability between primary and metastatic cells within each donor. Some of these newly identified genes may form the basis for new diagnostic and therapeutic approaches and provide insight into metastatic progression of OSCC.

Materials and methods

Cell lines

The study was approved by the Institutional Review Boards of the University of Texas Health Science Center at Houston and University of Texas M.D. Anderson Cancer Center as well as the University of Louisville. The following cell lines, established by one of the co-authors (P. Sacks, New York University, New York) (21, 22), were used. MDA686TU (686TU) and MDA686LN (686LN) cell lines were isolated concurrently from the primary tumor and lymph node metastasis of OSCC (tumor size: $5 \times 4 \times 4$ cm; tumor stage: T3N3B) involving the left tonsillar fossa and posterior portion of the tongue in a 49-year-old man. The patient underwent surgical resection of the primary tumor with left-sided radical neck dissection and postoperative radiation therapy. The patient died of complications 3 months postoperatively. The second set of cells, MDA1386TU (1386TU) and MDA1386LN (1386LN), were derived from the primary tumor and lymph node metastasis of OSCC (tumor size: $6 \times 4 \times 4$ cm; tumor stage: T4N3B) involving the hypopharynx of a 71-year-old man. The patient underwent surgical excision of the primary tumor with left-sided radical neck dissection and postoperative irradiation. The patient died of complications of a right-sided neck recurrence 3 years and 3 months postoperatively. The cell lines were obtained as passage 9 (686TU/LN) or passage 4 (1386TU/LN) and further cultivated for two additional passages to perform the experiments. Cells were grown as monolayers in a 1:1 mix of Ham's F12&DME medium containing 10% fetal bovine serum, and were harvested at 70–80% confluence for total RNA extraction.

Microarray procedure

Gene expression profiling analyses were done with the Affymetrix Microarray Analysis Suite instrument system (software version 4.01), using human HuFL6800 arrays, which contain probes for approximately 6800 known human genes. Cells were grown as monolayers to 70–80% confluence as described above. Total RNA was isolated from cultured cells using Trizol reagent, and the mRNA was purified from total cellular RNA with the OligoTex kit (Qiagen, Valencia, CA, USA). The cRNA sample preparation, hybridization, and data collection were performed according to the recommended Affymetrix protocols. Representative pools of biotin-labeled cRNAs obtained in this way were hybridized under standardized conditions to the microarrays. For each sample, the quality of the resulting cRNA pool with respect to representation and 5′-to-3′ bias was determined with Test3 arrays (Affymetrix) prior to entering the hybridization to the full-scale arrays. After applying a uniform global scaling procedure to the absolute analysis CHP files, using a global target intensity of 150 for all experiments, comparison data were obtained for the absolute analysis pairs 686LN vs. 686TU and 1386LN vs. 1386TU, with the TU sample being the reference in each comparison.

Data analysis

Cluster analysis was performed to collect genes that showed similar expression changes during metastatic progression. Genes with average difference values of 500 or above after global scaling were clustered into groups based on the similarities in their expression patterns using the GeneSpring 4.0 software (Silicon Genetics). Hierarchical clustering analysis was performed to generate a dendrogram for each cluster of genes based on their expression profiles. Heat maps were generated by setting the average value of expression level for each gene within the sample set to 1.0 and plotting the fold-change relative to 1.0. For interpretation of microarray data, it was assumed that fold-changes of ≤ 2 were of negligible biological significance. Genes with changes of twofold or more in their expression levels between the respective primary and metastatic cell lines were considered to be down- or up-regulated following metastatic progression. Also, the top 50% of expressed genes based on their average difference values in the absolute analysis CHP files were selected among all four cell lines, and explored for genes that are common in both metastatic cell lines. Genes identified from these analyses were grouped into categories of their putative functions, and genes with multiples roles were included in more than one category.

Reverse transcriptase-polymerase chain reaction

Double-stranded cDNA was generated using 1 μg of total RNA from 686TU and 686LN cell lines as a template and 2.5 μM Oligo $d(T)_{16}$ primer in a 20-μl reaction mixture; reverse transcription was carried out at 42°C for 1 h. Two μl of cDNA were amplified with gene-specific primers using the GeneAmp RNA-PCR kit (Perkin-Elmer). Identical primers, PCR conditions, and cycle numbers were used for each target gene, and the only variable among reactions to be compared is the source of the cellular RNA. The PCR cycle number was optimized for each gene-specific primer pair to ensure that amplification was in the linear range. In addition, as reference the amplification of β-actin was performed for the pair of samples and used as internal reference control. Thus, comparative semi-quantitative conclusions on relative expression levels within each sample pair under these conditions are acceptable for each gene. Aliquots of PCR products were analyzed on 1.5% agarose gels, and the ethidium-stained bands were quantitated by densitometry using AlphaImager™ 2000-Gel Documentation and Analysis System (Alpha Innotech, San Leandro, CA, USA).

Immunohistochemistry

Expression of vimentin, elafin, secretory leukoprotease inhibitor, basic fibroblast growth factor, and cystatin M were investigated in 686TU and 686LN cell lines by immunohistochemical staining. Briefly, cells were grown in microscopic slide chambers to 50–60% confluence, washed in PBS, and fixed in freshly-made cold acetone:methanol:formaldehyde (19:19:2 by volume) for 15 min. Slides were air-dried, washed in PBS, and treated with the following primary mouse monoclonal antibodies directed against vimentin (DAKO Corporation, Carpinteria, CA, USA; 1:100 dilution), human secretory leukoprotease inhibitor (R & D Systems, Minneapolis, MN, USA; 1:500 dilution), human elafin (Peptide International, Louisville, KY, USA; 1:100 dilution), human basic fibroblast growth factor (InnoGenex, San Ramon, CA, USA, 1:50 dilution), and cystatin M (kind gift from Dr Magnus Abrahamson; 1:500 dilution). Antibody binding was visualized using the avidin-biotin-peroxidase complex method as described below. Formalin-fixed paraffin-embedded tissue sections were obtained from the archival primary and metastatic lymph node tumors of 686TU and 686LN cell lines, respectively. Sections (5 μm thickness) were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol. Sections were pre-treated with 10% normal goat serum to block non-specific binding sites, and were incubated overnight at room temperature with the anti-elafin, anti-vimentin, or anti-cystatin M antibodies. Immunostaining was performed using the avidin-biotin method with appropriate secondary antibodies. Antibody-reactive sites were visualized with the chromogenic substrate 3-amino-9-ethylcarbazole (Sigma, St. Louis, MO, USA). The sections were counterstained lightly for 1 min with hematoxylin. Normal mouse or rabbit serum was substituted for mono- or polyclonal antibodies, respectively, as the negative controls.

Results

Analysis of global gene expression in pairs of primary and metastatic OSCC cell lines

RNA transcript levels in the two primary and metastatic cell line pairs were determined using high-density oligonucleotide arrays which interrogate 6,800 genes in a parallel fashion. Transcripts for 3035 (45%) and 2855 (42%) of the 6,800 genes were detected in 686TU and 686LN cell lines, respectively. The number of genes expressed in 1386TU (3500 = 51%) and 1386LN (3671 = 54%) was greater than the number of transcripts detected in the 686TU/LN pair (Table 1). When comparing the distributions of gene expression patterns of all four cell lines, greater differences were seen between the two primary or metastatic cell lines derived from two different patients than between the primary and metastatic cell lines derived from the same patient (Fig. 1). A cluster analysis on the GEPs of both primary and metastatic cell lines was performed to identify genes with specific expression patterns related to metastatic pro-

Table 1 Summary of gene expression patterns and changes[a]

Absolute analyses	*686TU*	*686LN*	*1386TU*	*1386LN*	
Present (%)	3035 (45)	2855 (42)	3500 (51)	3671 (54)	
Comparison	*Changed*	*≥2-fold*	*≥5-fold*	*≥10-fold*	*≥20-fold*
686LN vs. 686TU (%)	288	154 (2.16)	74 (1.04)	29 (0.41)	11 (0.15)
1386LN vs. 1386TU (%)	915	588 (8.25)	142 (1.99)	76 (1.07)	39 (0.55)

[a]Percentages of detected genes in the four absolute analyses, and observed fold-changes of gene expression for the two comparisons analyses 686LN vs. 686TU and 1386LN vs. 1386TU.

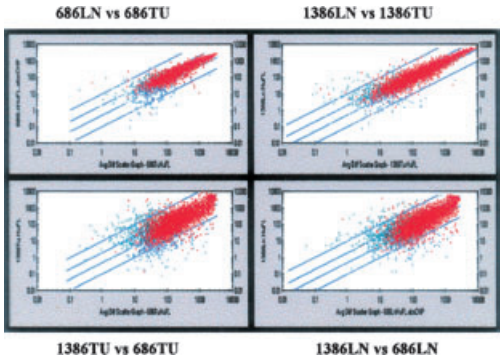

Figure 1 Distribution of fold-changes of gene expression as shown by scatter plots. The average difference values for the comparative analyses 686LN vs. TU, 1386LN vs. TU, 1386TU vs. 686TU, and 1386LN vs. 686LN are plotted for the baseline file on the *x*-axis, and the experiment file on the *y*-axis. The inside pair of lines includes up to twofold changes, the outside pair includes up to 10-fold changes. Different types of changes are displayed as follows: red: present to present; blue: present to absent; green: absent to present.

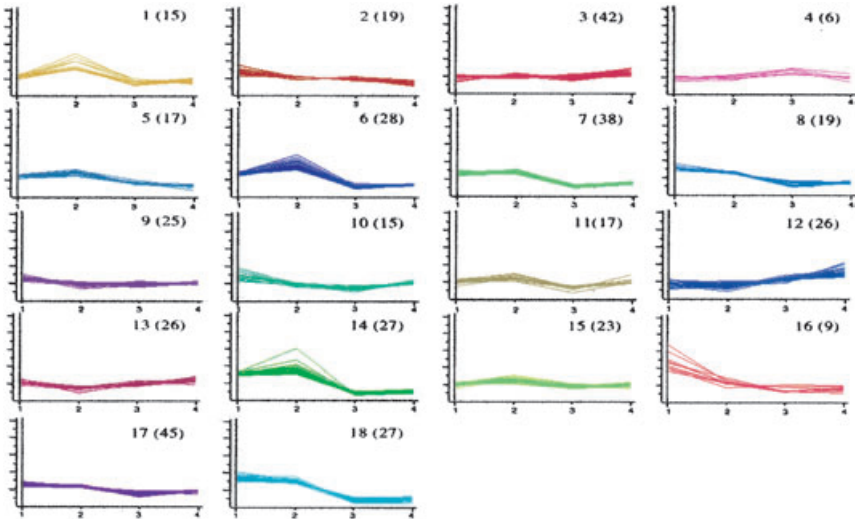

Figure 2 Gene expression profile clustering in comparison analyses 686LN vs. 686TU and 1386LN vs. 1386TU. Expression levels of 418 genes with average intensity values of > 500, which are common in the 1386TU vs. LN and 686TU vs. LN comparisons were grouped into individual clusters of similar behavior by the self-organizing map approach using the GeneSpring analysis software package. Sample numbering: 1 = 1386LN, 2 = 1386TU, 3 = 686LN, 4 = 686TU; numbers in brackets refer to the number of members in each cluster.

gression. Only those genes with target intensity values of 500 or higher ($n = 4019$) were included in the cluster analysis. Of the 4019 total genes, 584 can be grouped into 18 clusters (Fig. 2). One of the clusters (set no. 11) contained 17 genes whose expression profiles correlated inversely with metastatic progression of the oral cancer cell lines. This subset of genes was arranged by hierarchial cluster analysis (Fig. 3), which shows that all genes in this subset decrease in both LN vs. TU comparisons. Most of the genes found in this cluster were related to cellular metabolism or regulatory events, and have not been described previously as relevant for tumor metastasis.

Grouping and functional annotation of differentially expressed genes

Comparison analyses revealed differential expression changes of 154 genes by greater than or equal to twofold and 74 genes by greater than or equal to fivefold between 686LN cells vs. 686TU cells (Table 1). Of the 74 genes with greater than or equal to fivefold change, 17 were up-regulated and 57 were down-regulated in 686LN compared with 686TU (Fig. 4). In the 1386LN vs. 1386TU comparison, 588 genes were differentially expressed by greater than or equal to twofold and 142 genes by greater than or equal to fivefold. Among the latter, 104 genes were overexpressed and 38 genes were repressed (Fig. 4). Qualitatively, the altered genes can be divided into those which changed while being clearly detectable in both samples (Fig. 4 group A plots) and those genes which were detectable in only one of the samples (Fig. 4 group B plots), thus being either induced from below baseline level or being down-regulated to below detection limit. While for the 1386TU/LN comparison, both group A (68 genes) and B (74 genes)

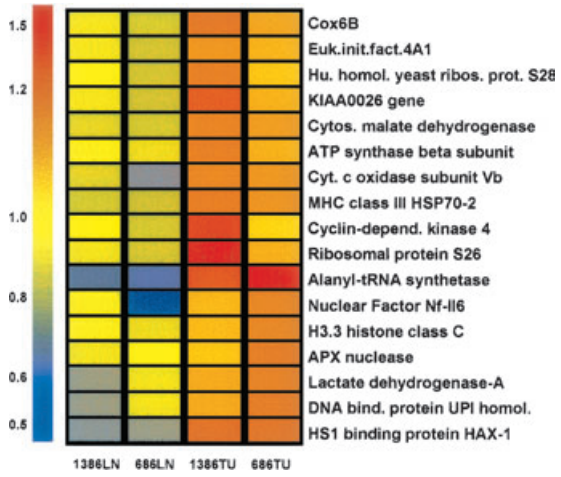

Figure 3 Representation of expression patterns (heat map) in cluster no. 11. The relative expression changes within each gene included in cluster no. 11, after scaling as described in Materials and methods, are displayed color-coded according to the color scale bar.

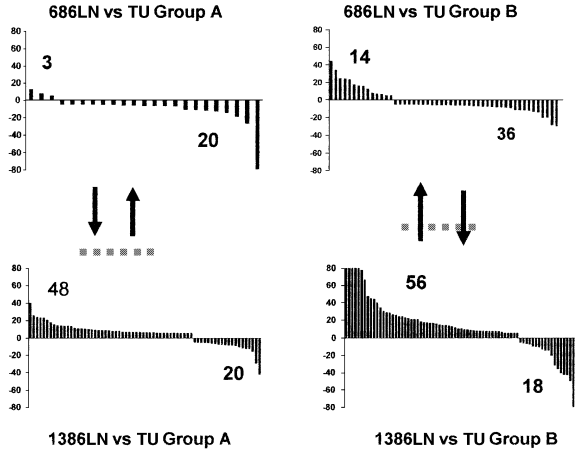

Figure 4 Comparative profile plots. Bar diagrams illustrating the fold-change distributions for group A genes (scored as present in both samples in a comparison) and group B genes (scored as present in only one of the two samples in a comparison). The inside numbers show the number of genes belonging to each subgroup.

genes are approximately evenly distributed, in the 686TU/LN comparison there are clearly more genes with the group B (50 genes) behavior than group A (23 genes) behavior. This again may reflect the heterogeneity of tissue samples derived from two individuals. Among these differentially expressed genes, we were able to identify 10 genes that were common for both 686TU/LN and 1386TU/LN pairs and showed similar patterns of differential expression. Five of these genes had decreased and five had increased expression in the metastatic cells (Table 2). These individual genes were assigned to broad functional categories according to their relevance to tumorigenesis and metastasis based on published reports related to these genes; no ribosomal and mitochondrial genes were included in this list.

Graphic illustration of the expression changes listed in Table 2 shows the concordant expression patterns among these genes (Fig. 5). There is good correlation of similar expression increases in all four LN vs. TU comparisons for RAB9 effector p40 protein (Z97074), H19 RNA (M320530), fibroblast growth factor-2 (M27968), and G-protein-coupled receptor kinase 5 (L15388), whereas there is only partial correlation for Zinc finger protein HTF10 (L11672) (brackets indicate GenBank accession numbers). Also, good correlation of decreased expression was seen in all four LN vs. TU samples for epidermal growth factor receptor (X00588), growth factor receptor-bound protein 10 (D86962), α1-collagen (M55998), and nucleosome assembly protein (D50370), but only moderate correlation for β2-integrin (M15395).

In order to identify potential metastasis-related genes that are highly expressed in both metastatic oral cancer cell lines, the top 50% of the highly expressed genes in 686LN ($n = 234$) and 1386LN ($n = 86$) cells were selected based on their target intensity (average difference) values. A total of 28 genes, excluding mitochondrial and ribosomal genes, were identified as highly expressed genes that are common for both metastatic cell lines (Table 3). The known functional category of each of these genes and their relevance to tumorigenesis and metastasis based on published reports were compiled (Table 3). Genes included in this list are laminin receptor (23–25), thymosin β-4 and β-10 (26, 27), and metallopanstimulin (28–30), which are known to be associated with metastatic phenotype.

Validation of microarray data

To substantiate the results of the microarray data, we performed reverse transcriptase-polymerase chain reaction (RT-PCR) to analyze the mRNA levels of 14 genes whose expression levels are higher ($n = 3$), lower ($n = 6$), or remained the same ($n = 5$) in 686LN cells compared with 686TU cells. As pointed out in the Methods section, the consistent RT-PCR conditions applied here allow relative comparisons among samples in a semi-quantitative way, using the respective β-actin product bands as reference. In addition, immunohistochemistry was performed to demonstrate possible reflections of mRNA changes on the expressed protein levels as well, although this technique clearly does not allow quantitative conclusions like array data. Confirmation by these techniques was done for one of the two pairs of cell lines in order to test for validity of the microarray data. For 12 of the 14 genes tested, the results of the RT-PCR analysis (Fig. 6) were in agreement with those from the microarray data (Table 4). Immunohistochemical analysis of the 686TU and 686LN cells using antibodies against selected genes (vimentin, elafin, SLPI, FGF-2 and cystatin M) confirmed the expression of all five of the genes tested at the protein level in both 686TU and 686LN cells (Fig. 7). Preliminary screening studies revealed that the antibodies to vimentin, elafin and cystatin M were reactive in archival formalin-fixed paraffin-embedded tissue, whereas the antibodies to FGF and SLPI were non-reactive in such specimens. Therefore, we were able to evaluate the expression patterns of vimentin, elafin and cystatin M in the archival tissue sections of the primary and metastatic tumors from which the 686TU and 686LN cells were derived (Fig. 8). Vimentin was not detected in the tumor cells of both primary and metastatic tumors, in contrast

Table 2 Common expression changes in the two LN/TU comparisons[a]

Gene name	*Accession no.*	*Expression level*	*Functional group*	*Oncogenesis*	*Metastasis*
Zinc finger protein HTF10	L11672	↑	2	X	
Nucleosome assembly protein	D50370	↓	2		
G-protein-coupled receptor kinase 5	L15388	↑	2		
Growth factor recept.-bound protein 10	D86962	↓	2	X	
RAB9 effector p40	Z97074	↑	5	X	
β2-Integrin	M15395	↓	4	X	X
Epidermal growth factor receptor	X00588	↓	3	X	
α1-Collagen	M55998	↓	4	X	
H19 RNA	M32053	↑	7	X	
Fibroblast growth factor-2	M27968	↑	3	X	X

[a]Differentially expressed genes (excluding the ribosomal and mitochondrial genes) common for the two LN vs. TU comparisons for the MDA686 and MDA1386 pairs. Expression level in metastatic cell lines is up-regulated (↑) or down-regulated (↓) relative to their respective primary cell lines. Functional group assignments, also used in Table 3, are: 1, immune/inflammation; 2, signal transduction/transcription; 3, growth/apoptosis/DNA repair and replication; 4, adhesion/migration; 5, metabolism/molecular chaperons; 6, structural and other proteins; 7, oncogene/tumor suppressor gene; 8, unknown/ESTs. Documented roles in either oncogenesis and/or metastasis are indicated in the last two columns.

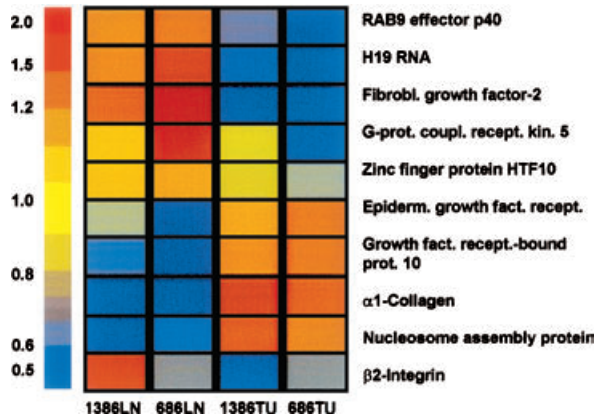

Figure 5 Concordant expression patterns in LN vs. TU comparisons. Relative expression levels of the genes listed in Table 2, after scaling as described in Materials and methods, are represented as heat map, color-coded according to the color scale bar.

Table 3 Highly expressed genes common in 686LN and 1386LN cells[a]

Gene name	*Accession no.*	*Functional group*	*Oncogenesis*	*Metastasis*
Elongation factor Tu	X03689	2		
P1 Cre recombinase	X03453	3		
Cyclophilin	X52851	5		
v-*fos* Transformation effector protein	M84711	3, 7		
Wilms tumor-related protein	M64241	7	X	
Csa-19	U12404	3		
Elongation factor-γ	M55409	2	X	
Calmodulin	M19311	2		
Laminin receptor	M14199	3, 4	X	
Ubiquitin	U49869	6	X	
Thymosin β-10	S54005	4	X	X
TGF-β induced gene product	M77349	2	X	
Profilin-1	J03191	4, 6		
Metallopanstimulin	L19739	2, 3	X	
α-Enolase	M14328	5		
Karyopherin α-4	AB002533	5		
Thymosin β-4	M17733	4	X	X
Annexin II	D00017	4, 6		
Metallothionine 2A	V00594	3, 5		
Ferritin light chain	M11147	5		
Cox-8	J04823	5	X	
Galectin-1	J04456	1	X	X
Calcyclin	BC009017	2		
β-Actin	X00351	4, 6	X	X
β-Tubulin	V00599	4, 6	X	X
G3PDH	M33197	3	X	X

[a]The list shows highly expressed genes (average difference >100) common in 686LN and 1386LN cells. Functional group assignments, also used in Table 2, are: 1, immune/inflammation; 2, signal transduction/transcription; 3, growth/apoptosis/DNA repair and replication; 4, adhesion/migration; 5, metabolism/molecular chaperons; 6, structural and other proteins; 7, oncogene/tumor suppressor gene; 8, unknown/ESTs. Documented roles in either oncogenesis and/or metastasis are indicated in the right two columns.

to the microarray, RT-PCR, and immunohistochemical results obtained in the cell lines. However, the stromal and endothelial cells found within these tumors were strongly positive for vimentin, serving as built-in positive controls (data not shown). In accordance with the microarray data, which showed reduced expression of elafin by 12-fold in LN cells, elafin was mostly negative in the metastatic tumor except for a few positive tumor cells in focal areas, whereas immunoreactivity for elafin was noted in more 60% of primary tumor cells (Fig. 8). Also in accordance with the microarray data, *in situ* hybridization and immunohistochemical studies for cystatin M in tissue sections revealed overexpression of this gene both at the mRNA and protein levels in the metastatic compared with the primary tumor (31).

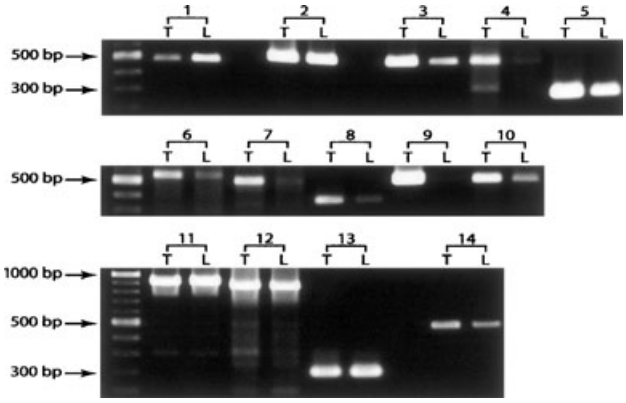

Figure 6 Validation of array data by RT-PCR. Gel electrophoresis of RT-PCR products obtained with gene-specific primers for selected gene targets. In order to remain within the linear range of the densitometric measurements, the amounts of reaction products loaded per lane varied, and the intensity values were then corrected for these varying loads. Lane numbers refer to those used in Table 4.

Table 4 Comparison of microarray and RT-PCR determinations[a]

Lane no.	*Gene name*	*Array*	*RT-PCR*
1	Cystatin M	44.7	4.0
2	Fibroblast growth factor 2	5.5	−1.2
3	Squamous cell carcinoma antigen	−10.2	−2.6
4	Elafin	−12.4	−8.0
5	β-Actin	1.4	−1.2
6	Cellular retinol-binding protein	−7.6	−3.9
7	Serine leukoprotease inhibitor	−27.6	−5.6
8	Small proline-rich protein	−5.3	−4.7
9	Retinoblastoma-binding protein 2	−11.5	−16.4
10	Vimentin	−80.1	−4.2
11	Cathepsin B	1.0	−1.1
12	Cathepsin D	−1.3	−1.2
13	Cathepsin L	1.2	1.1
14	Cystatin C	1.2	−1.5

[a]Fold-changes of 686LN vs. 686TU for selected genes are shown as determined by microarray and RT-PCR analysis. The values for array data are from the comparison CHP output files; values for RT-PCR were obtained by densitometric scanning of gene-specific RT-PCR bands after gel electrophoresis (lane numbers refer to Fig. 6).

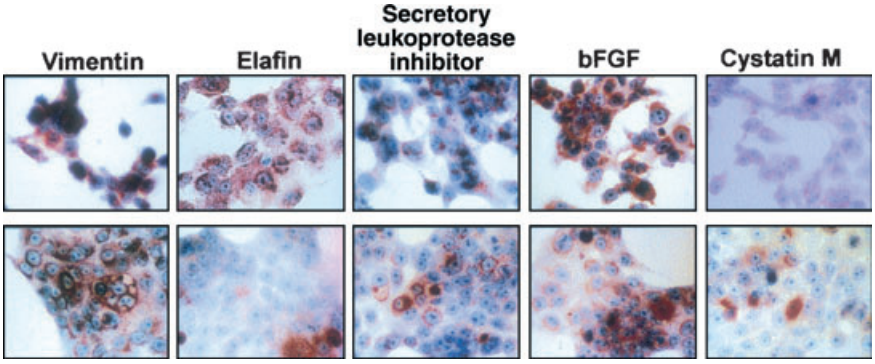

Figure 7 Immunohistochemical analysis of 686TU and 686LN cell lines. Protein expression was analyzed for elafin (a), basic fibroblast growth factor (b), secretory leukoprotease inhibitor (c), vimentin (d), and cystatin M (e). Top row = 686TU; bottom row = 686LN.

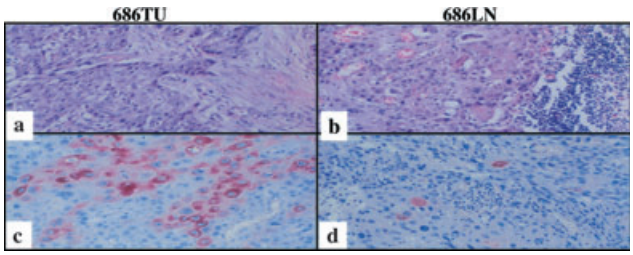

Figure 8 Immunohistochemical analysis of tumor tissues. The respective original tissue sections of primary and metastatic tumors, from which 686TU and 686LN cell lines were derived, were analyzed for protein expression (×200). (a) and (b): Hematoxilin and eosin stain; (c) and (d): immunohistochemical stain for elafin.

Discussion

Heterogeneity of expression profile changes

Microarray-based gene expression profiling allows detailed comparisons of regulatory changes in the paired specimens of OSCCs and their metastases. Based on only two cell line pairs, our data may not be representative of the entire parent tumor tissues or representative of metastatic spread in general. Yet they allow some interpretations and conclusions on the metastatic progression of these tumors *in vivo*, and indeed confirm a number of other published observations on metastasis-related gene expression changes. A striking feature of this study is that either the primary or metastatic tumor cells derived from the two different patients show marked variation in their patterns of gene expression. Cluster analyses of their gene expression profiles reveal that this variation is multidimensional; many different sets of genes show independent patterns of variation during metastatic progression of these two tumor lines. As demonstrated by the self-organizing maps of the genes expressed by these cell lines, only one (no. 11) of the 18 concordant gene clusters showed expression profiles related to metastatic progression which are similar in both pairs of cell lines. On the other hand, expression levels of >90% of the genes in cell lines derived from the primary and metastatic tumors of the same patient were more similar to each other than the two metastatic cell lines from two different patients. Genes identified as differentially expressed between primary and metastatic cell lines of the same patient also varied markedly between the two pairs of cell lines. The number of differentially expressed genes (greater than fivefold) between the 686TU/LN pair of cell lines was 74, which was remarkably lower than the total of 141 genes that were differentially expressed (greater than fivefold) between the 1386TU/LN pair of cells. Furthermore, during the metastatic progression in the 686TU/LN pair, more than 75% of the differentially expressed genes were down-regulated in the metastatic compared with the primary cell. Conversely in the 1386TU/LN pair, 74% of the differentially expressed genes (greater than fivefold) were up-regulated in the metastatic compared with the primary cell. It should be noted that the 686TU/LN pair of cell lines was derived from a high-grade squamous cell carcinoma in a younger patient, whereas the 1386TU/LN pair was derived from a long-standing low-grade squamous cell carcinoma in an older patient. The observed substantial differences in the GEPs between primary and metastatic cells of the1386TU/LN pair may indicate that this tumor is genetically more heterogeneous than the 686TU/LN pair. The biological events of metastatic progression represent the sequential emergence of a series of subpopulations of cells within the primary tumor that have additional selective growth advantage to establish a secondary tumor at a distant site (32, 33). This process, described as 'clonal evolution', results from the enhanced genetic instability inherent to malignant tumors (34, 35). Therefore, tumors that took longer to develop metastatic foci should have undergone a greater number of clonal evolutions leading to more genetic heterogeneity in both primary and metastatic tumors (32, 36). On the other hand, metastases that develop in a shorter time are genetically less heterogeneous, as only one or a few clones in the primary tumor metastasize (32, 36). Our findings are consistent with the above model. The 1386TU and 1386LN cells, which were derived from a low-grade oral SCC that took a longer time to undergo metastatic progression, were more heterogeneous in their GEPs than the 686TU/LN pair that was derived from a high-grade SCC that formed metastases in a shorter period.

Common themes among expression profile changes

We were able to identify 10 genes common to these differentially expressed genes in the 686TU/LN and 1386TU/LN pairs of cells. Among these genes, we found expression levels of fibroblast growth factor-2 consistently elevated in the two LN cells compared with their corresponding TU cells. Previous studies have shown that tumor-cell-derived FGF-2 plays an important role in angiogenesis, which is essential for metastatic growth (37). Integrin β_2 (CD18) expression was reduced by more than fourfold in both metastatic cell lines compared with their primary cells, which contradicts the conventional wisdom that integrin activation enhances tumor cell metastasis (38). However, a recent study comparing GEPs in the primary and metastatic variants of gastric cancer cells also reported down-regulation integrin β_4 expression related to metastatic phenotype (39). Another notable common gene that was down-regulated in metastatic cells was epidermal growth factor receptor gene. An interesting finding is the increased expression of H19 RNA by greater than or equal to fivefold in both metastatic cell lines compared with their primary counterparts. The H19 gene is highly expressed in embryonic tissue and in certain malignant tumors, but not in normal adult tissue (40, 41). In malignant tumors, H19 expression is inversely related to tumor cell differentiation (42). Whether increased expression of H19 RNA in the metastatic cell is a reflection of its poorly differentiated nature or is causally related to its metastatic phenotype remains to be determined.

Identification of metastasis-related genes

In order to identify genes that are highly expressed in the investigated metastatic oral SCC cells, we compared the top 50% of highly expressed genes in both 686LN and 1386LN cells. Many of the genes that are expressed in higher levels in these cells belong to the ribosomal and mitochondrial genes. Decreased intercellular adhesion, increased cell to matrix adhesion, and resistance to apoptosis are important phenotypical features necessary for metastatic dissemination of tumor cells. Several of these highly expressed genes specific for the metastatic cell lines have been reported to have a relationship with these phenotypes. For example, laminin receptor, which is highly expressed in both metastatic cell lines, interacts with the YIGSR sequence in the β1-chain of laminin, facilitating cell to matrix adhesion (43). It is also overexpressed in metastatic tumor cells of different tissue origin and has been shown to play a major role in tumor metastasis (23, 24). Treatment of tumor cells with monoclonal antibodies to the laminin receptor have been shown to block their attachment to laminin *in vitro* and to reduce metastasis *in vivo* (25). Thymosin β-4 and -10, which are found in higher levels in metastatic cell lines, play a pivotal role in tumor cell migration, essential for metastatic dissemination (26, 27). The β-thymosins are intracellular G-actin sequestering peptides, which prevent spontaneous polymerization of actin monomers and thereby increase the available pool of cytosolic actin monomer needed for tumor cell migration and metastatic spread (27). Interestingly, galectin 1, showing high expression levels in metastatic cell lines, has been reported in a number of GEP studies as being up-regulated in cancer cells with high metastatic potential (39, 44, 45). Galectins constitute a family of evolutionarily conserved carbohydrate-binding proteins with affinity for β-galactoside. They are involved in cell-to-cell and cell-matrix interactions, regulation of cell proliferation, pre-mRNA splicing, and, more importantly, apoptosis (46–48), and have been shown to enhance metastatic dissemination of tumor cells by protecting them from cell death in response to apoptotic signals (46). The DNA-binding protein metallopanstimulin, found to be elevated in both LN cell lines, is expressed in several neoplastic tissues (colon, breast, head and neck tumors), and its expression levels correlate with the grade of malignancy (28–30). However, some newly observed genes that are highly expressed in these metastatic cell lines include Wilms' tumor-related protein, P1 Cre recombinase protein and V-fos transformation effector protein. Whether these genes are involved in metastatic spread of oral cancer is a matter to be further investigated.

Verification of observed expression changes

We evaluated the microarray data at both mRNA and protein levels by analyzing expression of selected genes in 686TU and 686LN cells. We obtained overall good correlation between the quantitative microarray data and the semi-quantitative RT-PCR and more qualitative immunohistochemical findings for a selected group of genes. However, the correlation was not perfect, reflecting the inherent limitation of RT-PCR and immunohistochemisty for such quantitative analyses. We were able to confirm the differential expression of 12 of the 14 genes tested by RT-PCR. Our RT-PCR data validation was less exact for FGF-2 or vimentin. However, immunohistochemical studies confirmed expression some of these differentially expressed genes also at the protein level. It seems likely that some observed differences in the GEPs between the primary and metastatic cell lines could have been acquired in cell culture, and were not present in the original tumor tissue. In order to address this possibility, we examined the expression pattern of cystatin M, which is up-regulated by 40-fold in 686LN cells compared with its 686TU cells based on our microarray data. We examined the expression patterns of cystatin M in the original archival tissues of the primary and metastatic tumors from which 686TU and 686LN cells were obtained using both immunohistochemistry and *in situ* hybridization. We obtained excellent concordance between cystatin M expression patterns in the cell lines and in the corresponding original tumor tissues, both at mRNA and protein levels (31). On the contrary, microarray data revealed that vimentin mRNA was reduced by 80-fold in 686LN cells compared with 686TU cells; however, this was not reflected at the protein levels in these cell lines. Immunoreactivity for vimentin was detected in both 686TU and 686LN cells without any significant differences. Furthermore, both primary and metastatic tumor cells found in the original tumor tissue did not express vimentin as demonstrated by immuno-

histochemical staining. Therefore, it is possible that expression of vimentin in 686TU and 686LN cells might have been acquired *in vitro* during cell culture.

Conclusion

Our findings on a set of donor-matched OSCC cell line pairs are in agreement with previous reports that GEPs of primary tumors are mostly retained in their metastases except for a small proportion of genes (< 10%) whose expression pattern varies during metastatic progression. However, these differentially expressed genes reveal remarkable diversity from patient to patient, and the degree of diversity appears to vary depending on the age of onset of these tumors and the time spent to undergo metastatic dissemination. Interestingly, we observed a number of genes that are implicated in tumor invasion and metastasis (i.e. RBP2, SCC antigen, pro-IL-1, E-cadherin, maspin, cystatin M), which were differentially expressed in either one of the pairs of these cell lines but not in both pairs. These genes can be identified in the complete list of the differentially expressed genes that are available on request. On the contrary, some of the previously described metastasis-related genes, such as nm23, KiSS1 and MTA1, were not differentially expressed between primary and metastatic cell lines of both 686TU/LN and 1386TU/LN pairs.

Our data show that a more extensive analysis is warranted on additional donor-matched pairs of TU/LN cell line pairs and directly on clinical tissue samples in sufficient sample sizes. This will allow more rigorous correlations with clinicopathologic parameters and yield more general mechanistic conclusions. There is remarkable diversity in the number and functional groups of genes that are differentially expressed during metastatic progression of the oral SCC cell lines investigated. Therefore, it is unlikely that the metastatic phenotype of these oral SCC cells is acquired by up- or down-regulation of a single gene or a group of few genes. It is more likely that multiple combinations of differentially expressed genes are involved in facilitating metastatic spread of these tumor cells. Although we are far from identifying the different combinations of differentially expressed genes that can facilitate oral SCC metastasis, our findings will provide a framework for future studies.

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