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SPECIAL REVIEW SERIES: Microdissection: a powerful tool linking structural and molecular sciences

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Gene expression profiling of oral squamous cell carcinoma using laser microdissection and cDNA microarray

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Abstract Cancer diagnosis and therapy are performed on the basis of clinical stage and clinicopathological findings; however, sensitivity to therapy and prognosis may not always be the same even when considering similar cancers because it is difficult to recognize adequate biological characteristics of a cancer when determining cancer therapy. To enable personalized medicine for cancer diagnosis and therapy, which may solve this problem, we used laser microdissection and cDNA microarrays to study the gene expression profile of oral squamous cell carcinoma. Moreover, to establish an objective evaluation with this system, we examined which type of gene expression profile corresponded to the biological characteristics of this cancer. We identified several genes that were up- or downregulated in the majority of cases and clarified genes sharing common behavioral profiles between metastasis-positive and metastasis-negative cases. It was suspected that the genes that were commonly up- or downregulated in the majority of cases were important for histogenesis and acquisition of invasion and proliferation capability and that the genes sharing common behavioral profiles between metastasis-positive and metastasis-negative cases played a large role in cancer metastasis. Using the expression profile of these genes, it may be possible to evaluate cellular state and metastatic potential and use them as tumor markers. Alternately, we showed averaged gene expression profiles in cases with or without metastasis; this may reveal a profile that could evaluate metastatic potential, which is an important element in the biological characteristics of cancer. In conclusion, our system using laser microdissection and cDNA microarray may contribute to cancer diagnosis and therapy and improvement in the quality of life of cancer patients.

Key words Laser microdissection · cDNA microarray · Oral squamous cell carcinoma · Gene expression profile · Personalized medicine · Metastasis

Introduction

Laser microdissection is a method for the procurement of targeted cells from a tissue section, and its use enables one to analyze both nucleic acid and protein from procured cells. The application of laser microdissection to oral cancer has been reported recently.[1–11] Most of the reports are concerned with genomic DNA analysis, such as mutation of p53,[8–10] detection of human papillomavirus 16,[5] and comparative genomic hybridization.[2] The majority of the remaining reports demonstrated zymography[1] or antibody arrays for proteomics[6] in combination with laser microdissection. Only a few reports were concerned with mRNA analysis using cDNA microarray and laser microdissection to analyze oral cancer.[3,7,11] Unfortunately, the number of samples in these reports was very small, only one[11] or five,[3,7] making it difficult to describe the trends in gene expression profiling of oral cancer.

cDNA microarrays allow an effective investigation of functional genomics.[12] They enable high throughput measurements of differential expressions of a multitude of genes, using a systemic and comprehensive approach. However, the application of tissue homogenates inevitably results in an averaging of the expression of different cell types, and the expression profile of a specific cell type may be primarily masked or even lost because of the bulk of surrounding cells. Therefore, selection of cancer cells using laser microdissection is indispensable in the objective evaluation of the biological characteristics of cancer cells using cDNA microarray.

In this study, we utilized cDNA microarray and a new laser microdissection technique, a laser pressure cell transfer method.[13–16] The new microdissection technique has several advantages: ease of handling of the original thin film, easier preparation before laser microdissection, and

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the use of toluidine blue stain to reveal genes expression profiles in oral squamous cell carcinoma (OSCC). Moreover, to identify important factors for histogenesis and acquisition of invasion/proliferation capability, we examined several genes that were up- or downregulated in the majority of cases and clarified genes which shared common behavior between metastasis-positive and metastasis-negative cases. Furthermore, to objectively evaluate the biological characteristics of a cancer from a pattern of gene expression profiles, we measured averaged gene expression profiles in cases with or without metastasis.

Materials and methods

Sample data

Specimens of primary OSCC from 11 patients were used in the present analysis (Table 1). Seven patients were male and 4 patients were female. The range of patient age was 42–80 years (mean, 69.5 years). Lymph node metastasis was detected at the time of surgery in 4 patients. Histologically, 9 OSCCs were moderately to well differentiated and 2 were poorly to moderately differentiated.

Preparation of frozen fixed samples and laser microdissection

Tissues sampled from resected materials were embedded in OCT compound and frozen in isopentane cooled in liquid nitrogen. The frozen blocks were sliced by a cryomicrotome at a thickness of 8 μm, and each tissue section was affixed to a slide to which an original thin film (Meiwa Shoji, Tokyo, Japan) had been attached by silicone adhesive (GE Toshiba Silicone, Tokyo, Japan).

Slice samples were stored at −40°C until use. The sliced sample was quickly fixed in 100% methanol for 3 min, and then returned to room temperature and stained with 1% toluidine blue solution. A Laser Microbeam System (P.A.L.M., Bernried, Germany) with a 337-nm nitrogen laser was used for laser microdissection. We procured a few hundred cells from cancer tissues and normal mucosal epithelia in each OSCC case for analysis of gene expression.

RNA extraction from microdissected samples

Total RNA was independently extracted from each population of laser-microdissected cells. Briefly, the microdissected cells within the cap were covered with 200 μl buffer solution, 4 M guanidine thiocyanate, 25 mM sodium citrate, and 0.5% sarcosyl, and the capped tube was vortexed. After the addition of 20 μl 2 M sodium acetate, 220 μl water-saturated phenol, and 60 μl chloroform-isoamyl alcohol, the tube was centrifuged at 10 000 *g* at 4°C for 30 min to separate the aqueous and organic phases. The aqueous layer was transferred to a new tube; 2 μl glycogen and 200 μl isopropanol were added, and this was centrifuged at 10 000 *g* at 4°C for 30 min. After removing most of the supernatant, the pellet was washed with 70% ethanol. After the pellet was centrifuged and air-dried, mRNA was resuspended in RNase-free water.

cDNA synthesis and amplification

Total RNA was reverse-transcribed using the SMART PCR cDNA Synthesis Kit (Clontech, Franklin Lakes, NJ, USA). Then, 4 μl total RNA, 1 μl cDNA Synthesis (CDS) Primer, and 1 μl SMART II oligonucleotide were mixed and incubated at 70°C for 8 min. After a short spinning time, 2 min on ice, and 2 min at 42°C, a master mix containing 2 μl 5 × buffer, 1 μl dithiothreitol (20 mmol/l), 1 μl deoxynucleosi de triphosphate (dNTP) (10 mmol/l), and 0.5 μl RNase H^- Moloney murine leukemia virus reverse transcriptase (PowerScript; Clontech) were added and incubated at 42°C for 1 h. Afterward, cDNA was mixed with 38.5 μl TE buffer and purified by Atlas NucleoSpin Extraction Kits (Clontech). Next, 400 μl buffer NT2 was added to the cDNA to load a column. According to the manufacturer's protocol, columns were washed three times. For elution, 50 μl elution buffer was applied, incubated for 2 minutes, and centrifuged.

From the eluted cDNA, 2 μl was used to measure absorbance at 260 nm. For the polymerase chain reaction (PCR)-based amplification, 42 μl cDNA was mixed with 5 μl 10 × buffer, 1 μl PCR primer (10 μmol/l), 1 μl dNTP (10 mmol/l), and 1 μl Advantage 2 polymerase mix. PCR conditions were 95°C for 1 min, followed by 22 cycles at 95°C for 15 s, 65°C for 30 s, and 68°C for 3 min. The resulting PCR product was

Table 1. Clinicopathological findings of eleven oral squamous cell carcinomas (OSCCs)

	Sex	Age	Location	Differentiation	Lymph node metastasis
Case 1	Male	76	Tongue	Poor to moderate	Positive
Case 2	Male	76	Tongue	Well	Negative
Case 3	Male	71	Tongue	Well	Negative
Case 4	Male	72	Gingiva	Moderate	Negative
Case 5	Female	42	Oral floor	Moderate	Negative
Case 6	Female	79	Tongue	Moderate	Negative
Case 7	Female	66	Cheek	Moderate	Negative
Case 8	Female	69	Gingiva	Poor to moderate	Positive
Case 9	Male	80	Gingiva	Poor to moderate	Positive
Case 10	Male	63	Gingiva	Moderate to well	Positive
Case 11	Male	71	Cheek	Moderate to well	Negative

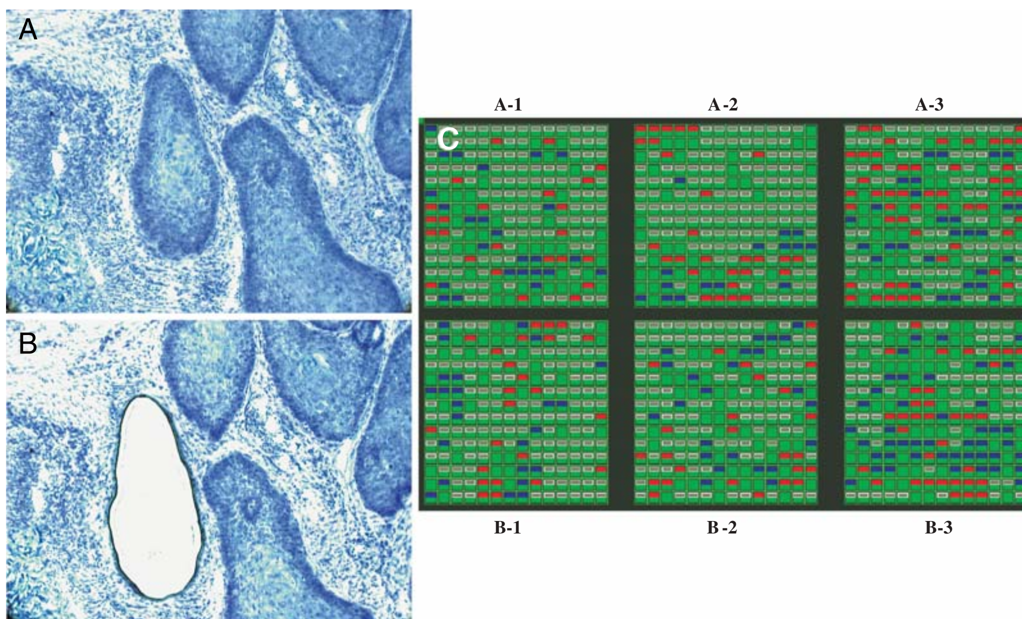


Fig. 1A–C. Gene expression profile of case 1. Specimen before (**A**) and after (**B**) laser microdissection. Result of gene expression profiling of OSCC cells compared to normal mucosal epithelium (**C**). Upregulated genes (more than 4 fold in comparison to normal mucosal epithelium) are indicated in *red*, and downregulated genes (less than 0.25 fold in comparison to normal mucosal epithelium) are indicated in *blue*. *A-1*, oncogenes; *A-2*, signal transduction proteins; *A-3*, transcription factors; *B-1*, receptors; *B-2*, growth factors, cytokines, and chemokines; *B-3*, cytoskeletal proteins, extracellular matrix proteins, and other unclassified proteins

purified using the Atlas NucleoSpin Extraction Kit, as already described. All incubations were performed using a GeneAmp 2400 PCR cycler (PE Applied Biosystems, Foster City, CA, USA).

Probe labeling and array hybridization

For array hybridization, we used nylon filters with 1176 spotted cDNA (Human Cancer 1.2 Atlas cDNA array; Clontech). The purified PCR product was labeled with α-^{32}P dATP using the Atlas SMART Probe Amplification Kit (Clontech). First, 32 μl PCR product and 1 μl CDS primer were heated at 95°C for 8 min. After 2 min at 50°C, a master mix containing 5 μl 10× labeling buffer, 5 μl dNTP (without dATP), 5 μl α-^{32}P dATP (Amersham Pharmacia Biotech, Tokyo, Japan), and 1 μl Klenow enzyme was incubated for 30 minutes at 50°C. Reaction was stopped by the addition of 2 μl 0.5 mol/l ethylenediaminetetraacetic acid. Labeled DNA was purified by the Atlas NucleoSpin Extraction Kit and eluted with 100 μl elution buffer, resulting in ~5 to 8 × 10^6 cpm. Afterward, array hybridization was performed according to the manufacturer's protocol. Filters with ^{32}P-labeled PCR product were incubated at 68°C overnight. They were washed three times in 200 ml 2 × standard saline citrate and 1% sodium dodecyl sulfate at 68°C for 30 min. Finally, they were wrapped in plastic and exposed to an imaging plate (Fuji Film, Tokyo, Japan) in lead sheathing. The imaging plate was read using a phosphorimaging system (STORM 830; Molecular Dynamic, Tokyo, Japan).

Array analysis

Analysis was performed using the AtlasImage 2.01 software (Clontech). Global normalization was calculated by the sum method. For both arrays, differences in signal intensity minus background were added for all values over background. The normalization coefficient was determined by calculating the ratio of array 1 sum and array 2 sum. After normalization, the background was subtracted and the ratio threshold was set at 4. To average the gene expression profile results of 8 metatasis-negative cases and 4 metastasis-positive cases, the function of averaging multiple array in AtlasImage 2.01 was used. Xpression NTI (World Fusion, Tokyo, Japan) was used to sort the gene expression profile data of the 11 cases used in this study.

Results

Establishment of a gene expression profiling system using laser microdissection and cDNA microarrays from surgical materials

Typical results of gene expression profiling of OSCC cells compared to normal mucosal epithelium are shown in Fig. 1 (case 1). Microarrays consisted of nylon filters with 1176 spotted cDNA. Spotted cDNA was generally divided into six blocks. Each block chiefly consisted of genes encoding particular groups: A-1, oncogenes; A-2, signal transduction proteins; A-3, transcription factors; B-1, receptors; B-2, growth factors, cytokines, and chemokines; B-3, cytoskeletal proteins, extracellular matrix proteins, and other unclassified proteins. In Fig. 1, upregulated genes (more than 4 fold in comparison to normal mucosal epithelium) are indicated in red and downregulated genes (less than 0.25 fold in comparison to normal mucosal epithelium) are indicated in blue. Up- or downregulated genes were noted more regularly in blocks A-3 and B-3 compared with other blocks. In case 1, the gene encoding lymphokine LAG2 had the highest upregulated expression level (27

Table 2. Commonly up- or downregulated genes and their function in more than half of cases

	Function
Upregulated genes	
Vascular endothelial growth factor receptor 1 (VEGFR1)	Growth factor and chemokine receptor
rhoC (H9); small GTPase (rhoC)	G-protein
Fuse-binding protein 3 (FBP3)	Transcription activator and repressor
Replication factor C 38-kDa subunit (RFC38); activator 1 38-kDa subunit	DNA replication
Interferon gamma-induced protein precursor (gamma-IP 10)	Growth factor, cytokine, and chemokine
Downregulated genes	
CDC-like kinase 3 (CLK3)	Intracellular kinase network member
Cyclin-dependent kinase inhibitor 1 (CDKN1A)	Kinase activator and inhibitor
Leukocyte elastase inhibitor (LEI)	Inhibitor of protease
Type I cytoskeletal 10 keratin; cytokeratin 10 (K 10)	Intermediate filament protein
Neutrophil gelatinase-associated lipocalin precursor (NGAL)	Trafficking/targeting protein

fold). The gene that was downregulated the most was heparin-binding epidermal growth factor (EGF)-like growth factor (0.0296 fold). Using laser microdissection and cDNA microarray, we are able to obtain clear gene expression profiles of oral squamous cell carcinoma from small amounts of cells.

Commonly up- or downregulated genes

We examined genes that were upregulated (more than 4 fold) or downregulated (less than 0.25 fold) in cancer cells compared to normal mucosal epithelium in the majority of OSSC cases of 11 cases. Five genes were identified to be commonly upregulated and 5 genes were commonly downregulated (Table 2).

Averaged gene expression profiles in cases with or without metastasis

The results of averaged gene expression profiles of OSCC cells in cases with or without metastasis are shown in Fig. 2. Eighty-six genes were upregulated and 67 genes were downregulated in the averaged gene expression profiles of the metastasis-negative cases. In the averaged gene expression profiles of the metastasis-positive cases, 183 genes were upregulated and 96 genes were downregulated. More genes are affected in metastasis-positive cases compared with metastasis-negative cases. Blocks B-2 and B-3 showed the largest difference in averaged gene expression patterns between metastasis-positive cases and negative cases. In block B-2, 20 extra genes were upregulated and 10 extra genes were downregulated in the metastasis-positive cases compared to the negative cases. In block B-3, 15 extra genes were upregulated 12 extra were downregulated.

Genes that shared common behavioral patterns between metastasis-positive cases and metastasis-negative cases

Figure 3 illustrates genes that tended to be downregulated in metastasis-negative cases and upregulated in metastasis-positive cases. Columns represent individual genes and rows represent individual cases; upregulated genes are indicated in red and downregulated genes in green. Areas marked within Fig. 3 have been enlarged and are shown in Fig. 4. Area A shows the top 10 genes that tended to be downregulated in metastasis-positive cases and upregulated in metastasis-negative cases; area B shows the top 10 genes which tended to be upregulated in metastasis-positive cases and downregulated in metastasis-negative cases.

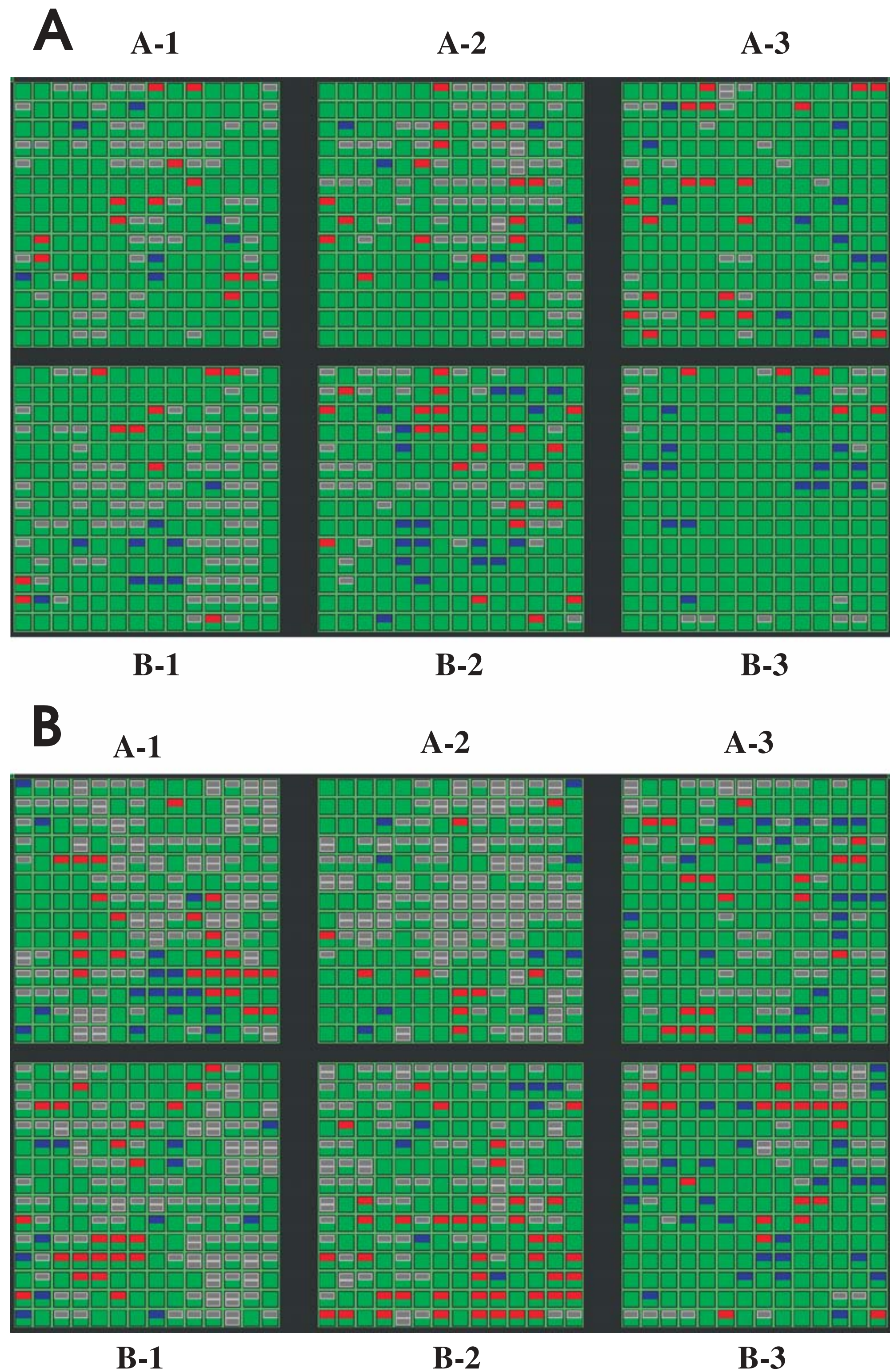
Discussion

Necessity of the microdissection method in microarray analysis of cancer and the risk of microarray analysis without using microdissection

In microarray analysis of cancer, the use of normal tissue as a normal control in the same patient is needed. Therefore, in the analysis of gene expression profiles in carcinoma cells, the use of laser microdissection is required for the procurement of carcinoma cells and normal epithelial cells. The advantages of using laser microdissection in microarray analysis of cancer in clinical application are as follows: (1) it permits microarray analysis from biopsied samples, (2) it permits the procurement of normal epithelial cells from biopsied samples of the same patient, and (3) it is unnecessary to collect any other tissue from patients, thus avoiding unnecessary surgical stress to cancer cells.

Alternately, there are serious problems in microarray analysis without microdissection. When using bulk normal tissue as a normal control in microarray analysis, various stromal cells are included. The gene expression profile result of cancer cells is then influenced by the difference in the amount of stromal cells in bulk normal tissue. It should be considered that the sort and amount of stromal cells are not constant, even if in the interstitium of normal tissue. Moreover, in spite of the diversity of cancer itself, the sort and amount of stromal cells in cancer tissue are more variable than that in normal tissue. Analysis of prostate carcinoma using microdissection, mRNA amplification, and microarray compared with microarray analysis using prostate carcinoma in bulk prostate tissue without microdissection

Fig. 2A,B. Averaged gene expression profiles in cases with or without metastasis. **A** Averaged gene expression profiles in metastasis-negative cases. **B** Averaged gene expression profiles in metastasis-positive cases



showed remarkable differences.[17] This study reported over- and underexpressed genes in bulk prostate tissue using microarray and in microdissected tumor epithelia using microarray and mRNA amplification. A total of 19 genes were statistically significant. When comparing these two results (with or without microdissection), only 6 genes were commonly noted as significantly over- or underexpressed in both bulk prostate tissue and microdissected tumor epithelia. This result indicated that the difference in microarray analysis with or without microdissection was remarkable. Consequently, it is important to recognize that microarray analysis without microdissection results in irreproducible data because of slight differences in the site of collection and the amount of sample. The disadvantages of microarray

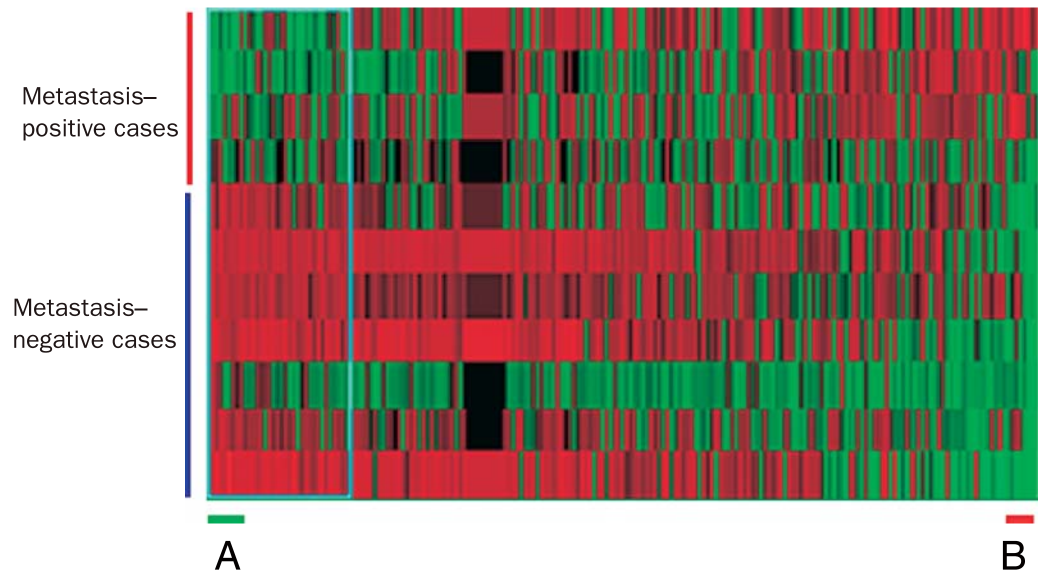


Fig. 3. Result view of sorting of all data to indicate genes showing common behavior between metastasis-positive cases and metastasis-negative cases. Each gene was arranged in order of tendency to be downregulated in metastasis-negative cases and upregulated in metastasis-positive cases. *Columns* represent individual cases; *rows* represent individual genes. Genes that are upregulated appear in *red*; those that are downregulated appear in *green*; *black* indicates approximately the same gene expression as the median

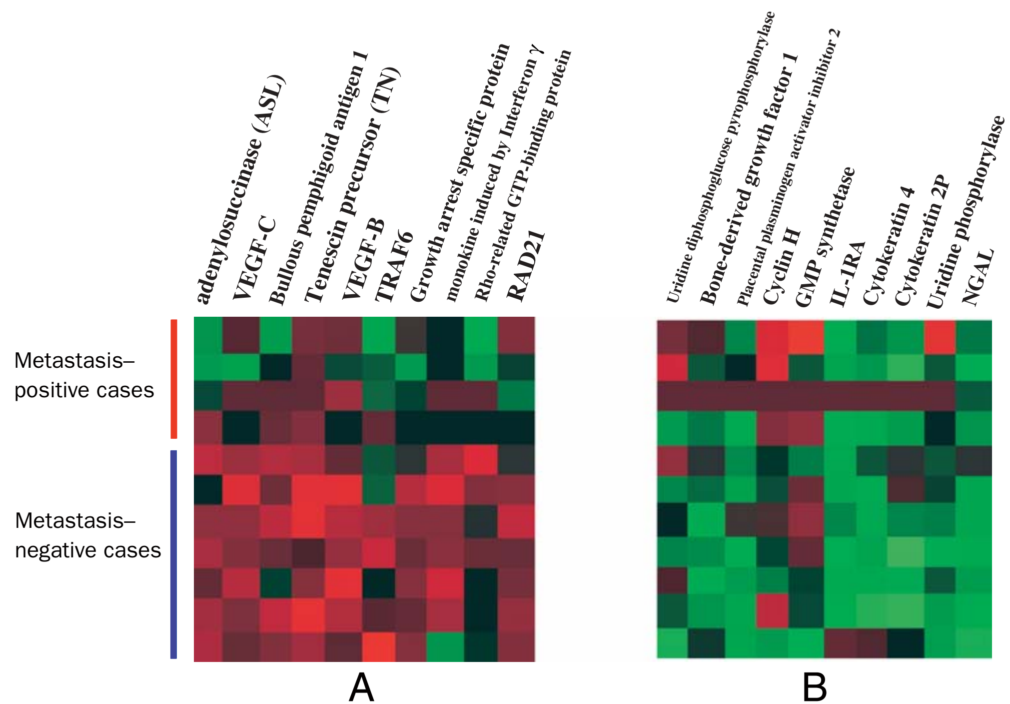


Fig. 4A,B. Enlarged view of areas indicated in **A** and **B** of Fig. 3, including gene description

analysis without microdissection in clinical application are as follows: (1) necessity of sampling of normal tissue and cancer tissue from the patients other than biopsy in preoperative examination, (2) unnecessary surgical stress to cancer, and (3) severe patient suffering.

Commonly up- or downregulated genes in more than half the cases

It was speculated that commonly up- or downregulated genes were an important factor in the histogenesis and acquisition of invasion and proliferation capability of carcinoma cells. It may also be possible to use these genes as tumor markers and prediction of cellular state. The results of our analysis are shown in Table 2. It was previously reported that overexpression of vascular endothelial growth factor receptor 1 (VEGFR-1) was related to poor prognosis of patients with endometrial carcinomas.[18] VEGFR-1 and other angiogenic factors and receptors contribute to the development of colon cancer.[19] VEFGR-1 might participate in histogenesis and acquisition of malignancy in OSCC. Overexpression of RhoC GTPase was identified in inflammatory breast cancer, which is the most lethal form of locally advanced breast cancer with remarkable vessel permeation.[20] RhoC GTPase might be involved in acqui-

sition of invasion capability and malignant potential in OSCC.

It was demonstrated that cyclin-dependent kinase inhibitor 1 (CDKN1A) downregulated replication by inhibiting proliferating-cell nuclear antigen (PCNA) and could retard progression of cells through S phase in vivo.[21] CDKN1A might lead OSCC cells to acquire proliferation capability. Leukocyte elastase inhibitor (LEI) and cytokeratin 10 proteins were reported to be expressed in well-differentiated squamous cell carcinomas in the oral cavity[22] and esophagus.[23] It may be possible to evaluate OSSC malignancy using the expression profiles of these genes.

Averaged gene expression profiles in cases with or without metastasis

We averaged gene expression profiles in cases with or without metastasis to objectively evaluate the biological characteristics of a cancer from a pattern of gene expression profiles by means of procurement of cancer cells from biopsied samples using microdissection. Information on the expression of a large number of genes enables proper evaluation of the biological characteristics of cancer. Therefore, microarray analysis can contribute to cancer diagnosis and therapy by clarifying the pattern of gene expression profiles. Our results need further analysis, but if the increase in up- or downregulated genes between normal and cancer cells is reproducible, clinicians must be made aware of the need for intensive care of regional lymph node and distant metastasis.

Genes sharing common behavior between metastasis-positive cases and metastasis-negative cases

The aim of clarifying genes sharing common behavior between metastasis-positive cases and metastasis-negative cases is to identify genes that play a large role in cancer metastasis. In our results (see Fig. 4), genes that were downregulated in metastasis-negative cases and upregulated in metastasis-negative cases may be inhibitory in cancer metastasis (Fig. 4A). Genes indicated in Fig. 4B may promote cancer metastasis. In Fig. 4A, it was reported that adenylosuccinase (ASL) was downregulated during neoplastic transformation of colon carcinoma cells.[24] Downregulation of bullous pemphigoid antigen 1 is frequently observed in nasopharyngeal carcinoma cells in contrast to nonmalignant primary cultured nasopharyngeal epithelial cells.[25] It was hypothesized that monokine, induced by interferon gamma (MIG), might be an endogeneous inhibitor of non-small cell lung carcinoma (NSCLC) growth in vivo. In support of this hypothesis, it was demonstrated that overexpression of MIG resulted in inhibition of NSCLC tumor growth by attenuation of tumor-derived angiogenesis.[26] The human rad21 gene is downregulated by hypoxia in human tumor cells.[27] Of relevance to this finding is that a growing body of evidence from clinical and experimental studies points to a fundamental role for hypoxia in metastatic progression.[28] Therefore, downregulation of human rad21 gene in metastasis-positive cases and upregulation in metastasis-negative cases in our study correlates with these reports. As shown in Fig. 4B, neutrophil gelatinase-associated lipocalin (NGAL) modulated matrix metalloproteinase 9 (MMP-9) activity by forming a complex of NGAL and MMP-9, thus protecting it from degradation.[29] Moreover, MMP-9 is involved in invasion and intravasation of OSCC.[30] It is speculated that NGAL may promote cancer metastasis. It was demonstrated that uridine phosphorylase (Upase) was a potential prognostic factor in patients with OSCC.[31] A high rate of Upase staining in primary tumors is associated with the presence of metastasis to lymph nodes and with lower overall survival rates.

Previous studies have shown that cyclin H expression was, on average, threefold higher in mammary gland carcinomas compared to control mammary gland tissues: this correlated with proliferation, as measured by PCNA staining.[32] The level of mRNA for guanine monophosphate (GMP) synthetase was substantially higher in proliferating, transformed cells compared to nontransformed cells.[33] In transformed cell lines, treatment with phorbol ester inhibited proliferation and resulted in a dramatic downregulation in the level of GMP synthetase mRNA and protein. It has not been clarified whether cyclin H and GMP synthetase are directly concerned with cancer metastasis, but such a possibility cannot be denied. Plasminogen activator inhibitor 2 (PAI-2), in stage II–IV, was increased in invasive carcinoma of cervix, and it was suggested that PAI-2 played an important role in invasion and metastasis in the advanced stages of this tumor.[34] It is speculated that PAI-2 may also promote cancer metastasis in OSCC.

As described here, the results from several genes that we have clarified are supported by results from other research groups. Further studies on OSCC are needed to confirm our findings and to establish their clinical significance using more sophisticated bioinformatic tools.

Conclusion

Our results indicated that application of laser microdissection and cDNA microarrays has large potential in cancer diagnosis and therapy. Objective evaluation of the biological characteristics of cancer, based on the pattern of gene expression profiles from biopsied samples, is expected to be established by further studies, and in doing so, we hope to contribute to the improvement of quality of life for cancer patients.

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