IDENTIFICATION OF POTENTIAL BIOMARKERS OF LYMPH NODE METASTASIS IN ORAL SQUAMOUS CELL CARCINOMA BY cDNA MICROARRAY ANALYSIS

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We surveyed the expression of 557 cancer-related genes in 15 cases of well-differentiated OSCC by cDNA microarray analysis. To identify potential biomarkers for lymph node metastasis, all microarray data were compared by the Mann-Whitney test and the significance analysis of microarrays between OSCCs with and those without lymph node metastasis. The tissues of OSCCs with lymph node metastasis exhibited increased expression levels of MMP-1, MMP-3, uPA, integrin-α2, paxillin, tenascin C and IL-6 transcripts. All of these genes were included in common clusters on the Cluster/TreeView analysis, implying that functional gene groups of proteolytic enzymes and integrin-related molecules are involved in cervical lymph node metastasis. The results of RT-Q-PCR for differentially expressed genes were in accord with those of cDNA microarray analyses, suggesting that the data obtained by microarray gene expression analyses were valid. Consistent with cooperative expression patterns, immunohistochemical analyses demonstrated that products of MMP-1, MMP-3 and uPA were colocalized to components of the neoplastic stroma, particularly mononuclear inflammatory cells with well-developed eosinophilic cytoplasm. Our results suggest that expression levels of molecules involved in tissue remodeling and cell-ECM adhesion, especially MMP-1 and integrin-α2, can provide an accurate biomarker system for predicting the risk of cervical lymph node metastasis in OSCC.

Key words: oral squamous cell carcinoma; biomarker; lymph node metastasis; cDNA microarray; immunohistochemistry; matrix metalloproteinase-1

Cervical lymph node metastasis is a major determinant of outcome in OSCC.1,2 Although histologic evaluation of invasiveness3 provides useful information, histopathologic diagnosis provides only partial and indirect information on neoplastic changes. Consequently, biomarkers that can be used to characterize tumor behavior and predict outcome have been sought to enhance treatment planning in patients with OSCC.

Gene expression profiles of tumor tissues show accumulated genetic and epigenetic changes associated with various types of biologic and clinical behavior. Recent improvements in microarray technology have enabled extensive analysis of mRNA or protein on a scale unattainable with conventional techniques.4-7 cDNA microarray analysis can survey clinically significant molecules in various types of surgically resected tumor.8-13 We surveyed the expression of cancer-related genes in OSCC tissues by cDNA microarray analysis to predict aggressive tumor behavior, especially cervical lymph node metastasis. Many molecules have been considered potential prognostic biomarkers of SCC.14-18 However, differences among previous studies in sampling procedures, protocol design and mechanisms for tumor invasion and metastasis have led to equivocal results. To address these difficulties, we used a series of different approaches to obtain reliable, reproducible results. These approaches included selection of consistent gene expression patterns in OSCC tissues, studies comparing different patient groups to identify clinically significant genes, cluster analysis to determine functional groups of genes and validation of data from microarray expression analysis by RTQ-PCR and by immunohistochemical analysis. On the basis of these exploratory data analyses, we propose potential molecular biomarkers for OSCC that may have important prognostic implications for cervical lymph node metastasis.

MATERIAL AND METHODS

Surgical specimens

Tissue samples for mRNA extraction were obtained from 15 patients with surgically treated OSCC at the Department of Oral and Maxillofacial Surgery, Niigata University School of Dentistry, or the Division of Oral Surgery, Nagaoka Red Cross Hospital, between February 2000 and May 2001. The study protocol was approved by the ethics committees of both institutions. Tissue samples were taken after obtaining the patients’ informed consent to participate in the study and processed anonymously. All cases were diagnosed histopathologically as SCC and staged according to the TNM classification of malignant tumors. The clinical and histopathologic features of the subjects are shown in Table I.

RNA extraction from oral cancer tissues

Since our aim was to identify molecules that could be used on a practical basis for cancer diagnosis and treatment, we used the simplest procedure without dissection before gene expression anal-

Abbreviations: ECM, extracellular matrix; EGF, epidermal growth factor; FDR, false discovery rate; H-E, hematoxylin–eosin; MAb, monoclonal antibody; MMP, matrix metalloproteinase; OSCC, oral squamous cell carcinoma; RAR, retinoic acid receptor; RTQ-PCR, real-time quantitative polymerase chain reaction; SAM, significance analysis of microarrays; SCC, squamous cell carcinoma; uPA, urokinase-type plasminogen activator.

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ysis. OSCC tissues samples, including tumor cells and surrounding cancer stroma, were placed in Trizol (Life Technologies, Rockville, MD) and immediately homogenized with Ultra-Turrax T8 (IKA Labortechnik, Staufen, Germany). Total RNA was isolated according to the Trizol reagent protocol. mRNA was purified with the use of oligo (dT) selection (Oligotex-dT30 Super, mRNA Purification Kit; Takara, Shiga, Japan). Samples of mRNA from 58 patients with clinically normal oral mucosa that was resected during oral surgical procedures were similarly purified and pooled to serve as control mRNA.

cDNA microarray gene expression

One microgram of poly A RNA from the OSCCs and normal oral mucosa samples was reverse-transcribed in the presence of Cy5- and Cy3-dUTP, 50 pg of λ poly A RNA as internal control, 300 pmol of oligo dT (18) primer and M-MLV reverse transcriptase (RNA Fluorescence Labeling Core Kit, M-MLV version; Takara). The cDNA microarray system was IntelliGene Human Cancer CHIP, version 2.1 (Takara), which contains 557 spots of cancer stroma, were placed in Trizol reagent (Life Technologies, Rockville, MD) and immediately homogenized with Ultra-Turrax T8 (IKA Labortechnik, Staufen, Germany). Total RNA was isolated according to the Trizol reagent protocol. mRNA was purified with the use of oligo (dT) selection (Oligotex-dT30 Super, mRNA Purification Kit; Takara, Shiga, Japan). Samples of mRNA from 58 patients with clinically normal oral mucosa that was resected during oral surgical procedures were similarly purified and pooled to serve as control mRNA.

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Extraction of valuable genes from microarray data

Fluorescence intensity differing by 2-fold or more or by half or less between OSCC tissues and normal oral mucosa control was defined as potentially different gene expression. Since our protocols involved bias associated with stage, age, sex, primary site and site of tumor sampled, we used multiple approaches to extract genes associated with metastasis of OSCC as follows.

Given that genes with consistent expression patterns have significant effects on OSCC phenotype and are potentially valuable for clinical detection, we sorted out genes whose expression levels in tumor tissue of the blocks examined were consistently increased or decreased in all cases.

Lymph node metastasis is a major indicator of aggressive behavior of OSCC. To identify potential biomarker genes that reflect the state of cervical lymph node metastasis, all gene expression data were statistically compared between groups of patients with and those without cervical lymph node metastasis. Because our ultimate objective was to divide OSCC cases into groups on the basis of lymph node metastasis, the Mann-Whitney test was considered most suitable for indicating overlapping patterns between gene expression data from 2 groups. We used the U value to evaluate the level of separate distribution patterns between the groups.

Gene expression data were further estimated by SAM, a software program specifically designed for identifying differentially expressed genes from microarray data. To control for the FDR among genes that are considered changed, SAM assigns a score to each gene on the basis of the change in gene expression relative to the SD of repeated measurements.

Then, to identify possible functional groups of cooperating or coregulated genes in OSCC tissue, hierarchical clustering was applied to the expression data, using the average-linkage clustering algorithms in Cluster and TreeView.

Validation of microarray data with RTQ-PCR

To validate the results of cDNA microarray analyses of gene expression, 7 genes suggested to be potentially valuable in the microarray analysis were subjected to RTQ-PCR. The same poly A RNAs used in microarray analyses were reverse-transcribed with oligo(dT) priming (Superscript II, Life Technologies). RTQ-PCR was carried out with specific primers for MMP-1, uPA, CD44, integrin-α3, paxillin, tenascin C and CD9. The expression of housekeeping gene β-actin was used to normalize for variances in input cDNA. A relative standard curve representing 6 10-fold dilutions of cDNA from one OSCC tissue (1:10; 100:1,000; 10,000; 100,000) was used for linear regression analyses of other unknown samples. After 30 sec at 95°C, each of 40 RTQ-PCR cycles had 5 sec at 95°C followed by 20 sec at 70°C for extension (Smart Cycler; Cepheid, Sunnyvale, CA; Takara Ex Taq, R-PCR version).

Real-time monitoring of PCR products was done with fluorescence of SYBR green I (Takara). Expression levels of specific genes were represented as ratios to that of β-actin from the same master reaction. PCR primer pairs (5′ to 3′) used for each gene were as follows: MMP-1, CCAGAAACCCCCAAGGCGTGG and CTGGTACATCTGTTCCCTGGAAC; uPA, CCACACGACATTGCTTGC and TGGTGACTTTGAGCCGTAGTATG; CD44, GCATTGGTGAACAGAGGAGTGC and CCAGGGTTTGGCCATATGAAG; integrin-α3, TAAAGGCAGAGCCGCGAGATG and CTTGGGACCTGCTAGGCGTAACA; paxillin, TGCTCCCTCGGATTTTC and GTCAAGGGCTGTCACCACTT; tenascin C, GAGGATGACGGCCGTTCTGAG and GGTGACCATCCAGGAGATTTG; and CD9, CATTGGAC-

1T and N categories of the UICC TNM classification of malignant tumors for lip and oral cavity.–2TNM stage grouping.–3Major width of tumor.–4Histopathologic classification of OSCC based on the mode of invasion used by Yamamoto et al.19–5Delayed lymph node metastasis detected after primary surgery.–6Metastasis in multiple distant organs accompanied by bilateral cervical lymph node metastasis.

| Case | Primary site | Age (years) | Sex | TN1 | TNM stage2 | Size (mm)3 | Mode of invasion4 |
|------|--------------|-------------|-----|-----|------------|------------|------------------|
| 1    | Lower gingiva | 50          | F   | T4N1 | IVA        | 25         | 3                |
| 2    | Lower gingiva | 68          | F   | T4N2a | IVA       | 30         | 4d               |
| 3    | Lower gingiva | 72          | M   | T4N0 | IVA        | 31         | 4e               |
| 4    | Lower gingiva | 70          | M   | T4N0 | IVA        | 34         | 4e               |
| 5    | Upper gingiva | 72          | M   | T4N06 | IVA    | 25         | 4e               |
| 6    | Upper gingiva | 84          | M   | T2N0 | II         | 30         | 3                |
| 7    | Tongue        | 79          | F   | T1N05 | I       | 12         | 4e               |
| 8    | Tongue        | 64          | M   | T2N1 | III        | 40         | 4d               |
| 9    | Tongue        | 53          | F   | T1N0 | I         | 19         | 3                |
| 10   | Tongue        | 81          | F   | T2N06 | II       | 22         | 4d               |
| 11   | Tongue        | 65          | M   | T4N2b | IVA      | 35         | 4d               |
| 12   | Tongue        | 76          | M   | T1N0 | I         | 15         | 3                |
| 13   | Oral floor    | 53          | F   | T2N0 | II        | 22         | 3                |
| 14   | Oral floor    | 50          | F   | T4N1 | IVA        | 40         | 4e               |
| 15   | Buccal mucosa | 77          | M   | T2N0 | II        | 24         | 4e               |

The expression of MMP-1, uPA, CD44, integrin-α3, paxillin, tenascin C and CD9. The expression of housekeeping gene β-actin was used to normalize for variances in input cDNA. A relative standard curve representing 6 10-fold dilutions of cDNA from one OSCC tissue (1:10; 100:1,000; 10,000; 100,000) was used for linear regression analyses of other unknown samples. After 30 sec at 95°C, each of 40 RTQ-PCR cycles had 5 sec at 95°C followed by 20 sec at 70°C for extension (Smart Cycler; Cepheid, Sunnyvale, CA; Takara Ex Taq, R-PCR version). Real-time monitoring of PCR products was done with fluorescence of SYBR green I (Takara). Expression levels of specific genes were represented as ratios to that of β-actin from the same master reaction. PCR primer pairs (5′ to 3′) used for each gene were as follows: MMP-1, CCAGAAACCCCCAAGGCGTGG and CTGGTACATCTGTTCCCTGGAAC; uPA, CCACACGACATTGCTTGC and TGGTGACTTTGAGCCGTAGTATG; CD44, GCATTGGTGAACAGAGGAGTGC and CCAGGGTTTGGCCATATGAAG; integrin-α3, TAAAGGCAGAGCCGCGAGATG and CTTGGGACCTGCTAGGCGTAACA; paxillin, TGCTCCCTCGGATTTTC and GTCAAGGGCTGTCACCACTT; tenascin C, GAGGATGACGGCCGTTCTGAG and GGTGACCATCCAGGAGATTTG; and CD9, CATTGGAC-

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Immunohistochemical analysis

Immunohistochemical analyses of gene products that were suggested to be differentially expressed were performed on 10% formaldehyde-fixed, paraffin-embedded sections of the OSCCs. The antibodies used were as follows: goat antimouse MMP-1 antibodies (Fuji, Takaoka, Japan); goat antihuman MMP-3 and uPA antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit antihuman tenasin C antibody (Chemicon, Temecula, CA); rabbit polyclonal antihuman collagen type IV antibody for the detection of microvessels (ICN Pharmaceuticals, Costa Mesa, CA) and mouse antihuman macrophage CD68 clone PG-M1 MAb as a marker for macrophage lineage cells (Dako, Kyoto, Japan). Primary antibodies were incubated overnight at 4°C. For MMP and uPA staining, sections were treated with biotin-conjugated rabbit antibodies were incubated overnight at 4°C. For MMP and uPA antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); goat antimouse MMP-1 formaldehyde-fixed, paraffin-embedded sections of the OSCCs.

RESULTS

Gene expression patterns in OSCC tissues

Consistently higher expression levels of 14 genes and lower expression levels of 19 genes compared to those in normal oral mucosa were demonstrated in 15 OSCC tissues (Table II). These changes manifest altered summation of gene expression in neoplastic cells and all cells involved in the reaction of neoplastic stroma.

Genes showing higher expression levels in OSCC tissues included 6 proteolytic enzymes (5 MMP family genes and uPA) and 2 ECM molecule genes (fibronectin-1 and tenascin C). Among the MMP family, the average expression level of MMP-1 in the OSCCs was 35 times higher than that in normal mucosa. Consistent increases in gene expression levels were also shown by some signal-transduction or transcription molecules known to be inducible by growth factors, e.g., MIP, IP-10, BIGH3 and STAT1.

Consistently decreased expression levels were observed in series of genes that are involved in differentiation and formation of epithelial structures. Keratin family genes constituted a major group. Genes that serve as components of desmosome plaques or keratin-binding proteins, i.e., function plakoglobin, desmoplakin and envoplakin, also showed decreased expression levels. In addition, slightly decreased expression of RAR-γ and v-erb-b2 was consistently found in OSCC tissue.

Differentially expressed genes associated with cervical lymph node metastasis

Mann-Whitney tests were carried out to estimate the separation patterns of gene expression levels between the 8 OSCCs with lymph node metastasis and the 7 without lymph node metastasis. Six ECM-degrading enzymes, 5 cell adhesion molecules and 4 other molecules were genes that showed higher expression levels with lymph node metastasis (Table III). Table IV shows 4 genes that showed decreased levels of expression with lymph node metastasis. All genes in Tables III and IV were selected on the

### Table II - Genes showing consistently different expression levels in OSCC tissues

| Name                  | GenBank1 | Fold change ± std | Functional description of molecules                                                                 |
|-----------------------|----------|-------------------|-------------------------------------------------------------------------------------------------------|
| Higher level          |          |                   |-------------------------------------------------------------------------------------------------------|
| MMP-1                 | M13509   | 35.62 ± 24.86     | Interstitial collagenase                                                                             |
| MMP-3                 | X05232   | 15.00 ± 9.73      | Stromelysin 1, progelatinase                                                                        |
| MIF                   | X7255    | 14.94 ± 18.57     | Chemokine, inducible by IFN                                                                         |
| MMP-10                | X07820   | 14.72 ± 12.49     | Stromelysin 2                                                                                        |
| IP-10                 | X02530   | 12.67 ± 11.31     | Chemokine, inducible by IFN                                                                         |
| Fibronectin 1         | X02761   | 10.19 ± 10.33     | ECM                                                                                                 |
| BIGH3                 | M7349    | 10.10 ± 7.55      | Cell adhesion protein                                                                                |
| MMP-13                | X75308   | 9.78 ± 9.79       | Collagenase 3                                                                                        |
| MMP-7                 | Z11887   | 9.39 ± 10.32      | Matrixlysin                                                                                         |
| Tenascin C            | X75665   | 6.84 ± 4.09       | ECM                                                                                                 |
| uPA                   | X02419   | 6.28 ± 2.79       | Membrane protein, proteolytic enzyme                                                                |
| STAT1                 | M97935   | 4.67 ± 3.02       | Transcription factor                                                                                |
| P-cadherin            | X63629   | 3.62 ± 1.50       | Cell adhesion molecule                                                                              |
| Superoxide dismutase 2| AL050388 | 3.46 ± 1.44       | Intramitochondrial free radical scavenging enzyme                                                    |
| Lower level           |          |                   |-------------------------------------------------------------------------------------------------------|
| v-erb-b2              | M11730   | 0.47 ± 0.24       | Growth factor receptor                                                                              |
| MAPK 3                | X60188   | 0.40 ± 0.14       | Serine/threonine kinase; cell cycle, thymocyte development                                          |
| RAR-γ                 | M38258   | 0.40 ± 0.13       | Nuclear receptor; cell growth, differentiation and apoptosis                                        |
| Macrophage-stimulating 1 | U28054 | 0.38 ± 0.11       | Macrophage activation                                                                               |
| Junction plakoglobin  | Z68228   | 0.37 ± 0.21       | Component of desmosomes and adhering junctions                                                      |
| Bone morphogenetic protein 7 | X51801 | 0.37 ± 0.27       | A member of the TGF-β superfamily                                                                  |
| Protocadherin 1       | L11370   | 0.36 ± 0.13       | Cadherin homolog; cell aggregation                                                                  |
| Keratin 8             | X74929   | 0.36 ± 0.14       | Cytokeratin: cytoskeletal element                                                                  |
| Keratin 7             | A1238246 | 0.34 ± 0.20       | Cytokeratin: cytoskeletal element                                                                  |
| IL1RN                 | U65590   | 0.29 ± 0.27       | Receptor antagonist; neutralization of immune responses                                             |
| Keratin 19            | Y00503   | 0.25 ± 0.14       | Cytokeratin: cytoskeletal element                                                                  |
| Desmoplakin           | AL031058 | 0.24 ± 0.12       | Component of desmosomal plaque                                                                     |
| Envoplakin            | U53786   | 0.24 ± 0.11       | Desmosome component; keratin binding protein                                                       |
| Deoxyribonuclease 1-like 3 | U75744 | 0.23 ± 0.15       | DNAse; primarily expressed in spleen and liver cells                                               |
| Human G0S3            | L49169   | 0.21 ± 0.54       | Transcription factor, G1/G0, switch                                                                 |
| Keratin 13            | X14640   | 0.14 ± 0.25       | Cytokeratin: cytoskeletal element                                                                  |
| Keratin 10            | X14487   | 0.11 ± 0.12       | Cytokeratin: cytoskeletal element                                                                  |
| Keratin 4             | X07695   | 0.11 ± 0.18       | Cytokeratin: cytoskeletal element                                                                  |
| Keratin 1             | AF237621 | 0.08 ± 0.11       | Cytokeratin: cytoskeletal element                                                                  |

1GenBank, GenBank accession number. 2Fold change represents the mean signal ratio of described gene in OSCC tissue relative to that in normal oral mucosa.
Hierarchical clustering analysis

Cluster analyses of the gene expression data demonstrated gene clusters, one designated as a among decreased genes and another designated as b among increased genes (Fig. 1). Cluster a included CD9, IGFBP2 and genes for major elements of epithelial structure or differentiation. The increased gene cluster b included 2 subclusters: (i) tenascin C, uPA, MMP-1 and MMP-3 and (ii) paxillin, moesin and integrin-α3. Interestingly, all of these flanking genes were differentially expressed between the group with and that without lymph node metastasis (Tables III, IV). This finding implied coregulated or cooperative mechanisms of these genes in the metastatic behavior of OSCC.

Validation of cDNA microarray data by RTQ-PCR

Figure 2 shows the results of RTQ-PCR with specific primers for 7 genes that showed different expression levels between the group with and that without cervical lymph node metastasis.
Mann-Whitney test with relative quantification of data yielded $U_{11005} = 4.0$ for MMP-1, $U_{11005} = 5.0$ for uPA and $U = 0$ for integrin-$\alpha$-3. The results of RTQ-PCR were in accord with those of cDNA microarray analyses, suggesting that the data obtained by microarray gene expression analyses were valid and that these genes could potentially serve as biomarkers.

**Immunohistochemical studies**

Antigens of MMP-1, MMP-3, MMP-9 (data not shown), uPA and uPAR (data not shown) were commonly localized in particular cell types and ECM (Fig. 3a–h), while carcinoma cell nests showed only faint or no positive findings on immunostaining for ECM-degrading enzymes (Fig. 3a–c, asterisks). Immunoreactivity for ECM-degrading enzymes was colocalized in neoplastic stroma associated with advanced ECM degradation (Fig. 3a–c). ECM-degrading enzyme$^+$ cells were also observed at the interface between the neoplastic stroma and surrounding host tissues, where degradation of interstitial fibers was found (Fig. 3e–h). ECM-degrading enzyme$^+$ mononuclear cells contained well-developed eosinophilic cytoplasm with a one-sided nucleus, indicating active protein production (Fig. 3f). The distribution pattern of CD68$^+$ cells suggested that ECM-degrading enzyme$^+$ cells were a specifically differentiated population among the macrophage lineage cells (Fig. 3g,i). Staining for collagen type IV revealed angiogenesis with MMP-1$^+$, mononuclear cell invasion (Fig. 3h,j). Tenascin C was immunoreactive in the neoplastic stroma near tumor cell nests and weakly positive on the basal side of tumor cells (Fig. 3d). The localization of tenascin C differed from that of ECM-degrading enzymes, suggesting distinct roles in metastasis.

**DISCUSSION**

The higher expression level of ECM-degrading enzyme genes in OSCC tissues suggests that activated ECM degradation plays a fundamental role in the progression of OSCC. On cluster analysis, we found that MMP-1, MMP-3, uPA, MMP-10 and uPAR belonged to the same cluster, implying that a common mechanism coregulates these genes. Promoters for MMP genes show a similar general arrangement of binding sites for Ets and AP-1. These transcription factors are required to induce ECM-degrading enzymes by growth factors, and their expression levels correlate with...
invasive or angiogenic phenotypes. Soluble factors from SCC cells activated expression of MMP-1 in stromal fibroblasts through AP-1 and Ets association. Consistent with in vitro data, topographic correlations among MMP-1, MMP-9 and Ets in breast cancers and between uPA and Ets in meningiomas have been demonstrated in vascular endothelial cells, stromal fibroblasts and tumor cells. Our immunohistochemical data also indicated colocalization of 5 ECM-degrading enzymes in inflammatory monocellular cells of the neoplastic stroma. These findings alternatively support the existence of coregulated and synergistic functions among ECM-degrading enzymes in tumor tissues.

Our study showed that increased expression levels of ECM-degrading enzyme genes were associated with lymph node metastasis. MMP-1 consistently showed high expression levels in the 15 OSCC tissues but was only slightly detected in normal oral mucosa on RTQ-PCR (data not shown). Strikingly, among the genes implicated in lymph node metastasis, MMP-1 had by far the highest expression ratio in the 8 cases of OSCC with lymph node metastasis. MMP-1 expression ratios <20 were associated with nonaggressive metastatic behavior, whereas aggressive tumors with cervical lymph node metastasis had higher ratios, ranging from 50 to 70. The U value for MMP-1 on the Mann-Whitney test was 0, indicating no overlap in the distribution of MMP-1 expression between cases with and those without lymph node metastasis. Previous studies have proposed that MMP-1 is a prognostic factor related to poor outcomes in esophageal, gastric, pancreatic and colorectal cancers, as well as cutaneous malignant melanomas.

An experimental study reports that, although activated forms of MMP-2 and MMP-9 are present in highly invasive OSCC cells, only induction of MMP-1 by EGF stimulation can result in complete degradation of collagen type I matrix. To our knowledge, however, no previous clinical study has focused on the relation between MMP-1 and aggressive behavior of OSCCs. Our results suggest that MMP-1, in concert with other proteases, plays a key role in OSCC associated with lymph node metastasis. MMP-1 may thus be a useful biomarker of cervical lymph node metastasis in OSCC.

Our immunohistochemical studies showed prominent staining of ECM-degrading enzymes in mononuclear cells with eosinophilic cytoplasm adjacent to degraded collagen fibers within newly formed neoplastic stroma. Production of MMP-1 by eosinophils in neoplastic stroma and its roles in tumor invasion and metastasis have been suggested in colon and gastric, and colorectal cancers and SCCs. In our study, the distribution of ECM-degrading enzyme cells only partly overlapped that of CD68 cells. Although ECM-degrading enzyme cells showed well-developed eosinophilic cytoplasm, they lacked bilobed nuclei. These findings suggest that the ECM-degrading enzyme mononuclear cell is not eosinophilic but a differentiated population of macrophage lineage cells. Results of our immunohistochemical analysis and prior studies provide evidence that MMP-1 and other ECM-degrading enzymes play crucial roles in providing a pathway for tumor invasion.

Increased expression ratios of the integrin-α3 and paxillin genes were also associated with lymph node metastasis. Integrin-α3β1 is a major receptor for laminin-5 in basement membrane. Integrin-α3β1 is also expressed at high levels in the majority of adherent transformed cells and in most tumors, and it could have similar roles in ECM remodeling during tumorigenesis and cell invasion. Paxillin is localized to focal adhesions and permits the regulated recruitment of downstream effectors of the integrin signaling pathway in cytoplasm. The flanking position of integrin-α3 and paxillin in the cluster analysis reflected coregulation and a close relation in function between these genes. Among genes that had decreased expression levels in the tissue associated with lymph node metastasis, CD9 is thought to modulate integrin signaling to inhibit cancer cell invasion. Although several previous studies have reported that integrin-α3–ECM interactions play an important role in tumor invasion and metastasis, no clear evidence has been demonstrated in vivo. In our quantitative measurement of integrin-α3 expression by RTQ-PCR, the Mann-Whitney test indicated a completely separate distribution between the groups with and without cervical lymph node metastasis (U = 0). All of these findings may provide clinical evidence for the involvement of cell–ECM interactions through integrin-α3 signaling in the development of lymph node metastasis. Integrin-α3 expression level is one of the important candidate biomarkers for lymph node metastasis in OSCC.

Among the genes expressed differentially in previous microarray analyses of OSCC, we could find only a few identical to those identified in this study. Although analysis was performed with laser-dissected epithelial components, Alevizos et al. reported that MMP-1, uPA and fibronectin-1 were commonly upregulated in carcinoma cells. However, the inconsistency with the results of previous studies is difficult to explain. Such inconsistency may have been caused by differences in microarrays, sample sites or conditions, experimental procedures, types of control RNA, unexpectedly wide heterogeneity of tumors within a small sample size or different methods for biostatistical analysis and interpretation of data. Increased emphasis on analytic planning and the use of standardized procedures is necessary to produce comparable gene expression data.

Early identification of cervical lymph node metastasis associated with OSCC is likely to improve treatment planning. The results of our cDNA microarray analysis revealed gene sets with distinct expression patterns in terms of epithelial structure, intercellular and intracellular signaling and ECM degradation. Some of these gene groups were clearly shown to reflect the clinical status of cervical lymph node metastasis. However, further clinical trials are needed before routine clinical application. We are examining the association between candidate gene expression and lymph node metastasis in larger numbers of OSCC cases, using RTQ-PCR methods for quantification. Our results will hopefully contribute to the development of improved molecular diagnostic techniques.

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