

## Gene expression pattern in oral cancer cervical lymph node metastasis

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**Abstract.** Treatment protocols for malignant tumors in the oral cavity differ greatly based on the presence of cervical lymph node metastasis. We applied gene expression profiling to the pathological lymph node status and used a training-test approach to evaluate the reliability of cDNA microarray-based classifications of 15 matched resected primary oral squamous cell carcinomas (OSCCs) and corresponding normal oral tissues. The clustering of all the microarray data was separated into two groups based on metastatic node positivity and node negativity. Furthermore, a 20-gene signature was identified that differentiated the testing set (n=8) with high classification accuracy (88%). Our findings support the hypothesis that the lymph node metastasis status can be predicted using the gene expression patterns of the primary OSCC, and may be a powerful tool in identifying patients at high risk of lymph node metastasis.

### Introduction

Metastasis, the dissemination of tumor cells that colonize in new areas of the body, progresses via the bloodstream or the lymphatic system. Typically, 50% of patients with oral squamous cell carcinomas (OSCCs) have detectable lymph node involvement at presentation. Patients with lymph node metastasis have a markedly worse prognosis than patients without metastasis. Only 25% to 40% of patients with lymph node metastasis at presentation survive 5 years, compared to ~90% of patients without metastasis (1).

This concept of tumor heterogeneity might indicate that studying the bulk tumor is not useful for predicting metastasis,

since a large number of poorly metastatic cells might obscure the properties of a small number of highly metastatic cells. However, gene signatures have been identified in several tumor types that correlate primary tumor gene expression with an increased risk of metastatic expression (2,3). These data are part of a growing body of studies indicating that the genetic signature of most cells in a primary tumor is extremely valuable. Identifying the differences in gene expression between metastatic and nonmetastatic primary tumors may expose the patterns of metastatic potential and serve as the basis for clinical testing for metastatic potential at biopsy.

Research in metastasis has focused largely on hematogenous metastasis, although metastasis through the lymphatic system is also common in many cancers. Certain data indicate that hematogenous metastasis and lymphatic metastasis differ markedly (4). Studies of breast, lung, colon, and pancreatic cancers have begun to address lymphatic metastasis, but these diseases are also prone to the confounding factor of distant metastasis (5,6). One study has identified a gene signature for recurrent disease in head and neck squamous cell carcinoma (HNSCC), and several studies have investigated the changes in gene expression from normal tissue to cancer tissue, but studies of lymphatic metastasis have been limited by the small number of genes assessed, the lack of a rigorous efficacy test of the metastatic signature (7-12), or both.

Oral cancer accounts for 2.6% of all cancers worldwide (13), and OSCC is the most frequently occurring malignancy in the oral cavity. The most important prognostic indicator for patients with OSCC is metastasis to the cervical lymph nodes or distant organs. Esophageal SCC is intractable, because it metastasizes to the lymph node even when the tumor is superficial. Many gene mutations are associated with metastatic potential (14). Custom cDNA arrays have been constructed (15) and used to identify new candidate genes that play an important role in esophageal carcinogenesis (16). Analysis of cDNA microarrays is a convenient method to detect differential expression (17,18), and by using this method, thousands of genes can be analyzed rapidly. By comparing gene expression profiles between tissue from oral cancer node-positive and that from oral cancer node-negative, we identified a gene that might be involved in oral cancer lymph node metastasis. We investigated whether the differences in gene expression between node-positive and node-negative

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primary tumor groups could be used to predict the nodal status of an independent patient set and whether such a discriminatory gene signature is similar to those predicting lymphatic metastasis. The goals of this study were to analyze publicly available gene expression data from OSCC samples and identify gene expression patterns that correlate with lymph node metastases and patterns of gene expression in an independent testing set.

## Materials and methods

*Tissue specimens and total RNA isolation.* Tissue samples for RNA extraction, with patient-matched normal epithelium where available, were obtained at the time of surgical resection at Chiba University Hospital after the patient's informed consent had been obtained under a protocol reviewed and approved by the institutional review board of Chiba University, Japan. Resected tissues were immediately frozen and stored in liquid nitrogen. Histopathological diagnosis of each neoplastic tissue was performed, according to the World Health Organization criteria, by the Department of Pathology, Chiba University Hospital. Clinicopathological staging was determined by the TNM classification of the International Union against Cancer. Total RNA was isolated by means of an SV Total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's protocol. The samples were stored at  $-80^{\circ}\text{C}$  until use.

*Preparation of labeled probes and hybridization on oligonucleotide arrays.* The AceGene Human oligo chip (30K) (Hitachi Software Engineering Co., Ltd., Kanagawa, Japan) containing 10,800 probes was used for mRNA expression profiling. We obtained information for each gene on the chips from the National Center for Biotechnology information database, and the experiments for the oligonucleotide microarray were performed according to the manufacturer's instructions (<http://dnasis.hskbio.hitachi-sk.co.jp/acegene/>). Briefly, cDNA probes labeled with aminoallyl-dUTP (Ambion Inc., Austin, TX, USA) were synthesized using Oligo(dT) primer and SuperScript II (Invitrogen, Carlsbad, CA, USA), and were purified using the QIAquick PCR purification kit (Qiagen K K., Tokyo, Japan).

The labeled test and reference cDNA probes were coupled with Cy3- and Cy5-monoreactive dye (Amersham Biosciences Inc., Tokyo, Japan), respectively, and purified using microbio-spin columns (Bio-Rad Laboratories Inc., Tokyo, Japan).

*Extraction of candidate genes from microarray data.* To identify the potential biomarker genes that reflect the state of cervical lymph node metastasis, all gene expression data sets were statistically compared between the patients with node-positive and node-negative disease. Because our ultimate objective was to divide OSCC cases into groups on the basis of node positivity and negativity, a permutation test was most suitable for indicating overlapping patterns between the gene expression data from the two groups. We used an absolute value of a difference of the mean to evaluate the level of separate distribution patterns between the groups. The gene

expression data set was further estimated by Significance Analysis of Microarrays (SAM), a software program specifically designed to identify differentially expressed genes from microarray data (19). To control for the false discovery rate 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 discrimination score 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.

## Results

*Identification of candidate OSCC with cervical lymph node metastasis target genes.* The characteristics of the patients and tumors are shown in Table I. The clustering of all the microarray data was used to classify the 15 OSCCs into two meaningful groups based on pathological node positivity ( $n=5$ ) and node negativity ( $n=10$ ). We identified a 20-gene signature for node positivity (Fig. 1) by regression analysis. The distribution of the total expression of 20 candidate genes was identified between the node-positive and node-negative groups within the training data set (Fig. 1).

*Prediction of lymph node status in the training set.* Analysis of the training data set using microarray analysis identified 20 genes that were correlated with the pathological lymph node status (Table II). The 20-gene classification algorithm generated on the training set was applied to the data as a testing set. The expression patterns of the 20 genes in the eight independent OSCC sets were similar to those in the 15 cases of the training set.

*Functional annotation of 20 genes identified from OSCC-derived training set.* Functional annotation analysis of the 20 genes was performed, and they were grouped and named according to the Gene Ontology convention (20). Information on the four functional categories, i.e., biological process, cellular component, molecular function, and cancer-related gene, was collected from the 20 genes and shown in Table II.

*Prediction of lymph node status in the testing set.* The 20-gene classification algorithm generated by the training set was then applied to the data from the eight independent OSCCs as a validation test. In this data set, the classification accuracy for the training set in node-positive cases remained high (100%; 3/3) but accuracy in the node-negative group was 80% (4/5). Overall, classification accuracy for microarray in the testing set was 87% (7/8) when compared with pathology. The classification probabilities for individual samples were similar to those in the testing set with 100% probability for 0 samples, 80% to 99% for three samples, and 45% to 80% for five samples (Fig. 2).

## Discussion

Clustering analysis of tumor samples on the basis of gene expression might facilitate a detailed diagnosis of individual cancers. Recently, a cDNA microarray system was used to

Clinical classification	Training set			No. of patients (%)	Testing set			No. of patients (%)
	Total	pN <sup>+</sup>	pN <sup>-</sup>	P-value	Total	pN <sup>+</sup>	pN <sup>-</sup>	P-value
Age at surgery				0.5338				1
<60	8	4 (50)	4 (50)		3	1 (33)	2 (67)	
≥60, <70	1	0	1 (100)		2	1 (50)	1 (50)	
≥70	6	1 (17)	5 (83)		3	1 (33)	2 (67)	
Gender				0.714393				0.465209
Male	8	3 (37)	5 (63)		4	2 (50)	2 (50)	
Female	7	2 (28)	5 (72)		4	1 (25)	3 (75)	
T-primary tumor				1				1
T1	0	0	0		0	0	0	
T2	1	0	1 (100)		0	0	0	
T3	6	2 (33)	4 (67)		4	1 (25)	3 (75)	
T4	8	3 (37)	5 (63)		4	2 (50)	2 (50)	
N-regional lymph node				0.000333				0.00467773
N <sup>-</sup>	10	0	10 (100)		5	0	5 (100)	
N <sup>+</sup>	5	5 (100)	0		3	3 (100)	0	
Stage				0.230769				1
I	0	0	0		0	0	0	
II	1	0	1 (100)		0	0	0	
III	4	0	4 (100)		4	1 (25)	3 (75)	
IV	10	5 (50)	5 (50)		4	2 (50)	2 (50)	
Histopathological type				0.56044				1
Well differentiated	11	3 (27)	8 (73)		7	3 (43)	4 (57)	
Moderately differentiated	4	2 (50)	2 (50)		1	0	1 (100)	
Poorly differentiated	0	0	0		0	0	0	
Tumor site				0.246753				0.678571
Gingiva	1	1 (100)	0		0	0	0	
Tongue	10	2 (20)	8 (80)		4	1 (25)	3 (75)	
Buccal mucosa	0	0	0		1	0	1 (100)	
Oral floor	2	1 (50)	1 (50)		3	2 (67)	1 (33)	
Orpharyngeal isthmus	2	1 (50)	1 (50)		0	0	0	
Lower lip	0	0	0		0	0	0	
Soft palate	0	0	0		0	0	0	

identify the genes involved in many types of malignant tumors, such as malignant lymphoma (21), hepatocellular carcinoma (22), breast cancer (23), prostate cancer (24), colorectal cancer (25), lung carcinoma (26), esophageal cancer (27), gastric cancer (28), and ovarian carcinoma (29). Although investigators have reported possible regulators related to the lymph node metastasis of cancer cells, the available information is still very limited. We can now recognize a novel type of classification based on gene expression by exploring a large number of cancerous tissues, and current

methods of cancer therapy might be improved if this novel classification could be implemented.

The cluster analysis undertaken in this study identified candidate genes associated with lymph node metastasis in 15 OSCCs. These results demonstrate that measurable differences in gene expression exist between node-positive and node-negative primary tumors and that these differences are sufficient to predict the nodal status of an independent set of patients using a leave-one-out cross-validation approach (9,10). Despite the relatively small sample size, significance

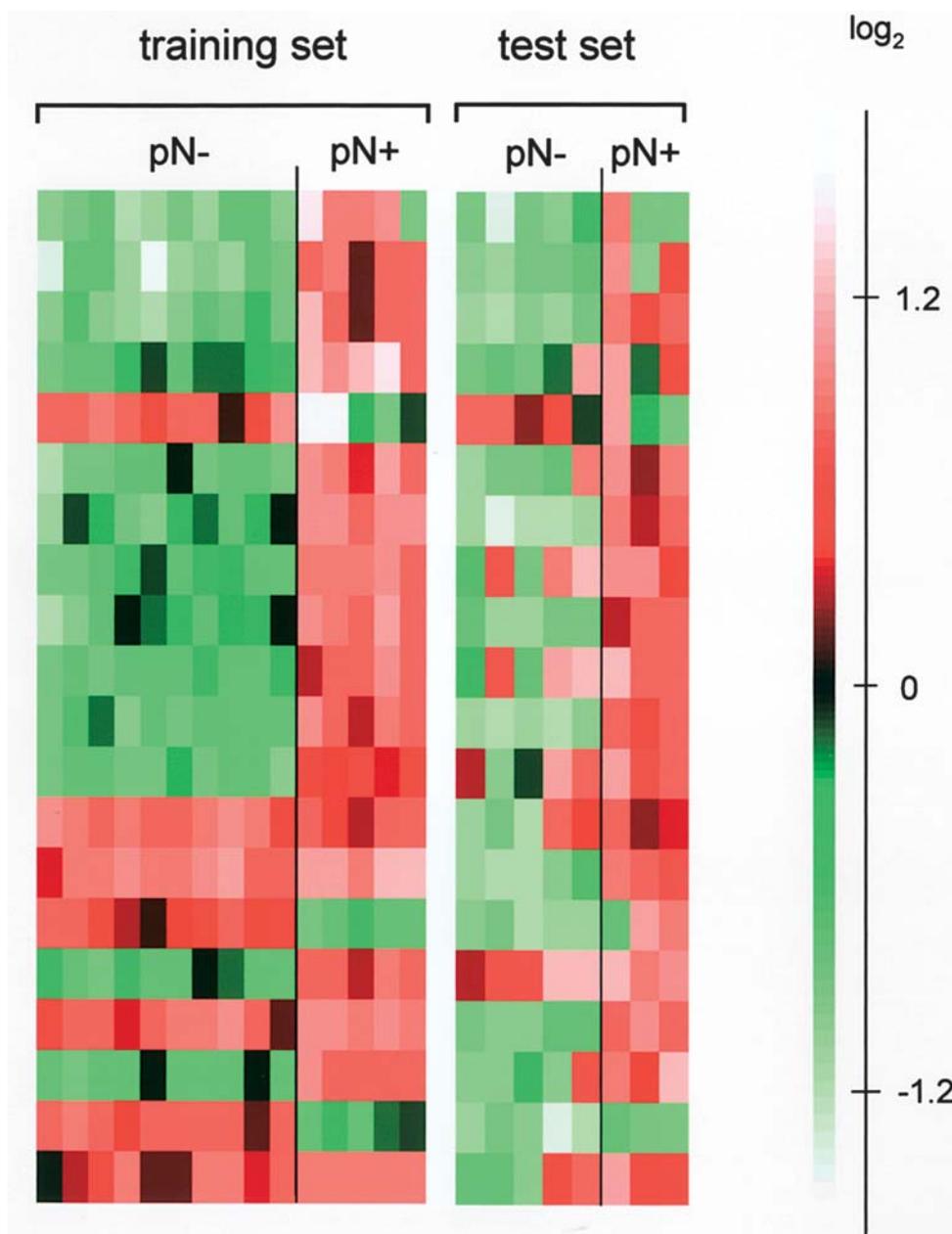


Figure 1. Selection procedures for lymph node metastasis. pN- indicates node negativity and pN+ node positivity.

was high. In the current study, we sought to determine whether the analysis of gene expression patterns in primary OSCCs could predict lymph node status and, if so, predict outcome in individual patients, many of whom are presumed to have lymph node metastases. For this, we used pathological lymph node status as the training classifier and SAM to identify gene expression patterns that specifically correlate with pathological nodal status. Twenty significant genes were associated with metastasis to the lymph nodes.

One limitation of the current study is that the patients with node-negative disease may actually have occult nodal involvement and, therefore, our gold standard for the training classifier is not necessarily accurate in pathologically node-negative cases. However, it should be 100% accurate in the case of node-positive tumors. Indeed, in both the training and testing data sets, we found extremely high (88%) concor-

dance between microarray analysis and pathological node status for tumors with pathologically positive lymph nodes. In node-negative tumors, however, microarray analysis of the training set data predicted that one of five (20%) node-negative tumors was actually node-positive. We believe that the poor outcome in the patients who tested as node-negative reflects understaging (possibly occult nodal disease) in a higher than normal percentage of patients, and to some degree this is responsible for the high number of patients considered node-positive in microarray analysis. We hypothesize that this inability to attain a good classification accuracy (even within the training set itself) may be the result of a high frequency of occult lymph node disease in this patient set. This may be a result of differences between the two microarray data sets or may reflect the need to refine the analysis tools used for prediction. Another possibility is that

Gene name	Accession no.	Symbol	Description
similar to 40s s3A	XM_517871	RPS3A	similar to 40s ribosomal protein s3a ; loc137437
ribosomal protein L32	NM_000994_1	RPL32	ribosomal protein l32; rpl32
ribosomal protein S28	NM_001031_1	RPS28	ribosomal protein s28; rps28
ornithine aminotransferase (gyrate atrophy)	NM_000274_1	OAT	ornithine aminotransferase precursor; oat
alpha-2-hs-glycoprotein; ahsg	NM_001622_1	AHSG	alpha-2-hs-glycoprotein; ahsg
ribosomal protein S8	NM_001012_1	RPS8	ribosomal protein s8; rps8
ribosomal protein S11	NM_001015_1	RPS11	ribosomal protein s11; rps11
aldehyde dehydrogenase 3 family, member A2	NM_000382_1	ALDH3A2	aldehyde dehydrogenase 3a2; aldh3a2
cadherin 1, type 1, E-cadherin (epithelial)	NM_004360_1	CDH1	cadherin 1, type 1 preproprotein; cdh1
src homology three (SH3) and cysteine rich domain	NM_003149_1	STAC	src homology three (sh3) and cysteine rich domain; stac
ribosomal protein S24	NM_001026_1	RPS24	ribosomal protein s24, isoform c; rps24
flj22184	NM_025094_1	FLJ22184	hypothetical protein flj22184; flj22184
tenascin C (hexabrachion)	NM_002160_1	TNC	hexabrachion (tenascin c, cytotactin); hxb
pro1316	NM_018580_1	PRO1316	hypothetical protein pro1316; pro1316
absent in melanoma 1-like	NM_017977_1	AIM1L	absent in melanoma 1-like
Ras-related GTP binding A	NM_006570_1	RRAGA	ras-related gtp-binding protein; raga
prostate androgen-regulated transcript 1	NM_016590	PART1	prostate androgen-regulated transcript 1 protein; part1
ribosomal protein L3	NM_000967_1	RPL3	ribosomal protein l3; rpl3
small EDRK-rich factor 1A (telomeric)	NM_021967_1	SERF1A	small edrk-rich factor 1a, telomeric; serf1a
immediate early response 2	NM_004907_1	IER2	immediate early protein; etr101

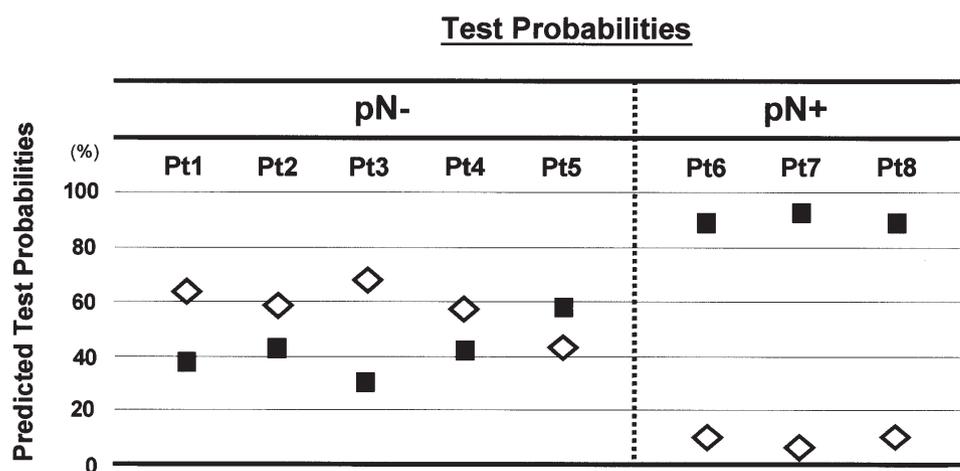


Figure 2. Classification probability for the test set. Original sample names and pathological lymph node status are labeled on the top of each graph. Classified nodal status is indicated by ◊, indicating node negativity and ■, indicating node positivity, with the Y axis indicating the probability associated with the classification of each patient.

some of the misclassified tumors actually have the potential to metastasize (2,5,30) but have not yet done so.

In conclusion, our analysis of gene expression in primary tumors correlated very highly in pathologically node-positive

patients. This analysis may identify patients whose tumors have already metastasized or have the potential to do so. However, a problem with this data interpretation is that we do not have actual data on occult lymph node disease in

patients who are node-negative. This problem may be resolved in a situation in which tumor gene expression, accurate clinical and surgical staging, and the presence of occult nodal disease are determined in the same set of patients.

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