Loss of *NKX3-1* as a potential marker for an increased risk of occult lymph node metastasis and poor prognosis in oral squamous cell carcinoma

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Abstract. The prognosis of oral squamous cell carcinoma (OSCC) is significantly dependent on the existence of cervical lymph node metastasis (LNM), with the overall survival rate being much lower in patients with LNM. Primary causes and molecular mechanisms of LNM are still largely unclear. We hypothesized that factors related with cancer progress and/or prognosis in OSCC are revealed by genome-wide investigation of DNA copy number aberrations (CNAs). In order to find biomarkers for occult LNM of OSCC, we comprehensively investigated genomic DNAs from 60 OSCC patients using Affymetrix mapping arrays and statistically analyzed correlations between CNAs of genes and the presence of occult LNM in the patients. The genome-wide CNA study indicated significant correlations between the presence of occult LNM and CNAs of certain genes. Through a literature survey, we narrowed down the candidates and focused on loss of *NKX3-1*, which is a homeodomain-containing transcription factor. *NKX3-1* is known as a tumor suppressor gene in prostate cancer but has never been reported in OSCC. Quantitative RT-PCR and immunohistochemistry (IHC) analyses also showed significantly lower expression of *NKX3-1* in the cases with occult LNM, which was further validated by IHC analysis in independent cases. The survival analyses indicated that *NKX3-1* loss is a significant risk factor to decrease the disease-free survival (DFS) and the overall survival (OS) rates. This is the first time that the significant association of *NKX3-1* loss and occult LNM was indicated in OSCC. The present results suggest that loss of *NKX3-1* may be a potential biomarker for occult LNM of OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common head and neck carcinoma, accounting for more than 260,000 cases worldwide each year (1). Although therapies for OSCC have recently been improved, only slight progress has been observed in the mortality rates over the last two decades (2). The most important prognostic factor in OSCC is the presence of lymph node metastasis (LNM). In fact, the 5-year overall survival rate in patients with LNM is approximately 25% lower than in patients without LNM (3). Especially, nodal metastasis diagnosed after surgical resection of a primary tumor site is more severe for survival and known as occult LNM even when the patient is diagnosed without cervical LNM (N0 neck) at the first visit. Therefore, the appropriate management of occult LNM is required and currently two major approaches are applied to manage the N0 neck in OSCCs; an elective neck dissection of cervical lymph node(s) and a 'wait-and-see' policy (4,5). In a case of stage N0 neck with a risk of occult LNM greater than 20%, neck dissection is a standard procedure for elective treatment (6). The risk of metastatic potential can vary according to the site, size, histo-pathological and genetic features of a primary tumor. Much controversy exists over the decision of the treatment with or without a preventive lymphadenectomy based on accurate risk stratification of potential occult LNM.

Although the associations between metastasis and particular genomic aberrations have been reported (7,8), the molecular mechanism of LNM is still not fully understood. In addition to uncovering how OSCC cells spread and migrate from the primary site to cervical lymph nodes, it is also valua-

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ble to identify genomic aberrations that are associated with LNM and are thus potential biomarkers to increase the accuracy of the prediction of occult LNM. A genome-wide copy number (CN) analysis by a high-density microarray allows us to identify numerous CNAs of genes, in which some of them may have important influence to tumor progression and poor prognosis in malignancy (9,10). We anticipate that factors related with occult LNM are also revealed by a comprehensive study of DNA copy number aberrations (CNAs) in OSCC. However, to the best of our knowledge, there is no genome-wide CNA study reporting genes associated with occult LNM of OSCC although gene- or chromosomal site-specific analyses have been reported (11,12).

The purpose of this study was to identify significant markers associated with occult LNM by a genome-wide CN analysis of the primary tumor sites. A high density SNP array was applied to screen occult LNM-related aberrations of genes in 60 OSCC patients.

Materials and methods

Patients and tissue samples. Fresh-frozen tissue samples were collected from 60 OSCC patients (39 male, 21 female) who had undergone surgical resection at the Department of Maxillofacial Surgery, Graduate School, Tokyo Medical and Dental University (TMDU; Tokyo, Japan) between 1999 and 2007. None of these patients received preoperative treatment. This study was approved by the institutional review board. Informed consent was obtained from all patients in accordance with the guidelines by the review board. The cancers were located in the tongue (34 samples), mandibular gingiva (14 samples), floor of the mouth (4 samples), buccal mucosa (6 samples), maxilla gingiva (1 sample), and pterygopalatine fossa (1 sample). Parts of the tumor specimens were collected in cryotubes, frozen just after the resection and stored at -80˚C until used in the following analyses. The remaining specimens were prepared as formalin-fixed, paraffin-embedded (FFPE) samples and used for immunohistochemistry (IHC) assay. As an independent sample set for the IHC assay, FFPE sections were prepared from 12 additional OSCC patients (6 with occult LNM, 6 without occult LNM) who underwent surgical therapy at TMDU dental hospital between 1999 and 2009.
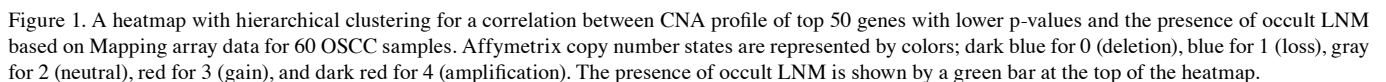
Genomic DNA extraction from primary tumors. Genomic DNAs were extracted from the frozen tumor specimens and isolated using a QIAamp DNA Mini kit (Qiagen, Valencia, CA). Contaminated RNAs were eliminated using RNase A during the purification process. The quantities and qualities of purified genomic DNAs were assessed by NanoDrop (NanoDrop Technologies, Wilmington, DE) and gel electrophoresis.

Genome-wide copy number analysis. The experiment was performed by strictly following the assay manual and using a GeneChip human mapping 250K Sty array (Affymetrix, Santa Clara, CA), which contained 238,229 SNP probe sets. The concentration of starting materials was standardized by diluting genomic DNA samples to 50 ng/μl (250 ng of genomic DNA) with reduced EDTA TE buffer. A *Sty* I

assay was performed in this study, by applying *Sty* I digestion of genomic DNA and ligation with adaptor *Sty* I prior to the PCR reaction. Amplicons were fragmented after the purification, followed by a labeling reaction. After 16 h of hybridization at 49˚C, the microarrays were transferred to a fully automated system (GeneChip Fluidic Station 450; Affymetrix) for the washing and staining steps. After the staining with streptavidin phycoerythrin (SAPE), images of the microarrays were scanned with an Affymetrix laser scanner 3000-7G. The raw intensity data of SNP probe sets were first analyzed by Affymetrix genotyping console 4.0 software to produce the genotype data. The CNA data were generated by the estimation of the copy number for each SNP probe set by comparing each normalized intensity data with Affymetrix SNP array data set of 50 normal Asians (Japanese and Chinese) from the HapMap database (http://www.hapmap.org/). The microarray data for the 60 samples used in this study are available from the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE31984.

Comprehensive correlational analysis between CNAs of genes and occult LNM. To identify an individual copy number for each gene, we first determined segmented CNA regions based on integrated chromosomal positions of SNP probes which had a unique CN profile; loss/deletion, neutral or gain/amplification. The CNA regions were annotated with relative gene information which included 22,658 gene information downloaded from the EntrezGene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene). The Fisher's exact test with the Bonferroni correction was applied on the gene CNA data from the 60 samples combined with the presence of occult LNM to determine the occult LNM-associated genes with $p < 0.05$.

Quantitative analysis by real-time RT-PCR. Using RNeasy Mini Kit (Qiagen, Valencia, CA), total RNAs were extracted from 19 OSCC tissues (9 cases without occult LNM and 10 cases with occult LNM) that were from the 60 samples used for the CNA analysis and reverse transcribed to cDNA using high-capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, CA). Real-time RT-PCR was applied to quantitatively confirm the effect of *NKX3-1* loss by comparing the samples of patients with and without occult LNM. The sequences of PCR primer sets were forward primer: 5'-CAGTCCCTACTGAGTACTCTTTCTCTC-3', reverse primer: 5'-CACAGTGAAATGTGTAATCCTTGC-3' for *NKX3-1* and forward primer: 5'-CTGGAACGGTGAAGGTGACA-3', reverse primer: 5'-AAGGGACTTCCTGTAACAACGCA-3' for *ACTB* as endogenous control. Expression level of vascular endothelial growth factor C (*VEGF-C*) was also determined to compare with the relative quantities of *NKX3-1* in the same cDNA sample set because *NKX3-1* is known to regulate expression of *VEGF-C* (13). The forward and reverse PCR primers were 5'-CACGAGCTACCTCAGCAAGA-3' and 5'-GCTGCCTGACACTGTGGTA-3', respectively. The PCR reaction was carried out with PowerSYBR Green PCR Master Mix (Applied Biosystems). The signals of the samples were detected by the 7500 Fast Real-Time PCR System (Applied Biosystems) with 7500 Fast System software v1.4.0, and the

Figure 1. A heatmap with hierarchical clustering for a correlation between CNA profile of top 50 genes with lower p-values and the presence of occult LNM based on Mapping array data for 60 OSCC samples. Affymetrix copy number states are represented by colors; dark blue for 0 (deletion), blue for 1 (loss), gray for 2 (neutral), red for 3 (gain), and dark red for 4 (amplification). The presence of occult LNM is shown by a green bar at the top of the heatmap.

relative quantities of samples were calculated with an $\Delta\Delta C_t$ method with the software and DataAssist v3.0 (Applied Biosystems). Each assay was performed in triplicate.

Immunohistochemistry assay for NKX3-1. Immuno-histochemical staining was performed with a monoclonal antibody for NKX3-1 protein (1:200; abcam, Cambridge, UK) on the FFPE OSCC tissue specimens, which were 7 samples with occult LNM and 8 samples without occult LNM in the original sample set and 6 samples with occult LNM and 6 samples without occult LNM in the independent sample set. First, representative sections were stained with hematoxylin and eosin (H&E) and histologically evaluated by a pathologist. The sections were deparaffinized by xylene, transferred to graded ethanol, and rehydrated in running water. Then, after antigen retrieval by autoclave treatment (121˚C, 40 min) in citrate buffer (pH 6.0, 0.01 M), the sections were quenched for endogenous peroxidase activity and blocked with a blocking

reagent of a Histofine SAB-PO kit (Nichirei, Tokyo, Japan). Anti-NKX3-1 antibody at a dilution of 1:200 was applied on the sections and incubated at room temperature for 30 min, followed by overnight incubation at 4˚C. Subsequently, specimens were treated by the labeled streptavidin-biotinylated complex (LSAB) method using reagents of Histofine SAB-PO kit following the manufacturer protocol and stained by a 3,3'-diaminobenzidine (DAB; Merck, Darmstadt, Germany) solution containing hydrogen peroxide for 5 min. The sections were then thoroughly counterstained with hematoxylin. As negative controls, tissues were stained with PBS instead of the primary antibody solution. For each of the 27 tissue specimens in the original and independent sample sets, the numbers of positively stained carcinoma cells and non-stained carcinoma cells were separately counted in 5,000 μm^2 and the counting was repeated for nine additional areas under a light microscope. The ratio of positively stained cells over the total number of cells in a certain area and average of the ratio from the ten areas were calculated.

Table I. Clinical characteristics of 60 OSCC cases and correlation with *NKX3-1* loss.

Clinicopathological parameters	CN of *NKX3-1*		
	Normal	Loss	p-value[a]
Age			0.436
<60	21	4	
≥60	32	3	
Gender			1.000
Male	34	5	
Female	19	2	
Disease site			0.180
Tongue	31	3	
Lower gingiva	11	3	
Upper gingiva	0	1	
Buccal mucosa	6	0	
Floor of mouth	4	0	
Pterygopalatine fossa	1	0	
Growth pattern			0.741
Superficial/exophitic	25	4	
Endophitic	22	2	
Undetermined	6	1	
Cellular differentiation			0.580
Well/Moderate	44	7	
Poor	9	0	
Pathological T stage			1.000
I, II	40	6	
III, IV	13	1	
Mode of invasion			0.645
1-3	23	5	
4C, 4D	13	1	
Undetermined	17	1	
Recurrence[b]			0.012
Negative	42	2	
Positive	11	5	
Occult lymph node metastasis			<0.001
Negative	48	2	
Positive	5	5	

[a]P-values were calculated by the Fisher's exact test; [b]Recurrence includes primary recurrence, regional recurrence and distant metastasis.

Metastasis-free and overall survival analyses. To identify potential correlations between the survival rate and CNAs, we applied the Kaplan-Meier method for analyzing the disease-free survival (DFS) and the overall survival (OS) in two groups of the 60 OSCC patients: one with loss of *NKX3-1* and the other without CNA in *NKX3-1*. Follow-up periods for DFS and OS were calculated based on the interval between the first visit and the date of the patient's death or the last follow-up.

Statistical analysis. The statistical analyses were performed with the statistical software R 2.11.1 (http://www.r-project.
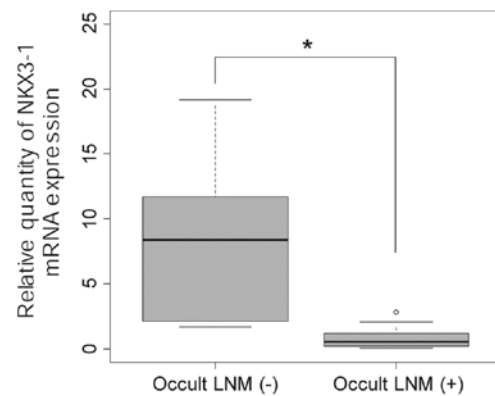

Figure 2. A box-plot for quantitative analysis of *NKX3-1* gene expression between 2 groups (9 cases without occult LNM, 10 cases with occult LNM). p-value was calculated by the Wilcoxon rank sum test. *p<0.001.

org/). The Wilcoxon rank sum test was applied to determine the difference in the relative quantities of gene expression between the occult LNM positive and negative cases. In the IHC assay, the difference of the positive staining rates between the occult LNM positive and negative cases was statistically analyzed by the Wilcoxon rank sum test. The survival curves were estimated by the Kaplan-Meier method and the significances of differences between the survival curves were calculated by the log-rank test. Risk factors for DFS and OS were examined by the Cox proportional hazards analysis. A probability level <0.05 was used for a statistical significance.

Results

Correlation analysis between CNAs and occult LNM. We analyzed the correlation between CNA data for genes and the presence of occult LNM using the Fisher's exact test. The heatmap in Fig. 1 shows that several gene clusters of loss/deletion or gain/amplification of genes were significantly associated with occult LNM (p<0.05). Through a literature survey using PubMed (http://www.ncbi.nlm.nih.gov/pubmed/), we narrowed down the significant genes detected by the Fisher's exact test. We then focused on *NKX3-1*, which is one of the homeodomain-containing transcription factors, encoded on 8p21.2 and known as a putative prostate tumor suppressor gene (14-17). Therefore, we further investigated its validity as a marker in OSCC through the qRT-PCR and IHC assays. The clinical characteristics of the 60 OSCC patients and correlation with *NKX3-1* loss are summarized in Table I. Recurrence (p=0.012) and occult LNM (p<0.001) were significantly associated with loss of *NKX3-1* and none of the other factors was significant.

Quantitative RT-PCR verifies gene expression for NKX3-1 and correlation with VEGF-C. The gene expression analysis for *NKX3-1* was performed by quantitative real-time RT-PCR in order to verify whether the DNA copy number of *NKX3-1* affected the gene expression in the 19 OSCC cases (9 cases without occult LNM, 10 cases with occult LNM) in the original sample set. The result of quantitative analysis
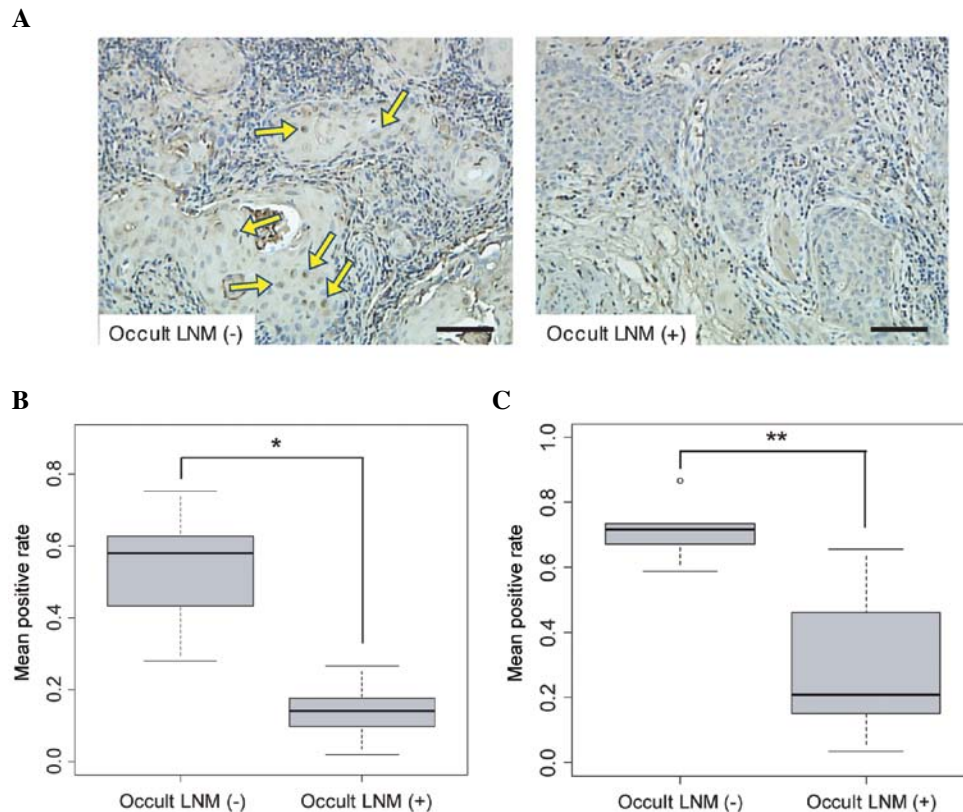
Figure 3. Immunohistochemistry for FFPE OSCC tissues with monoclonal anti-NKX3-1 antibody (1:200). (A) Positive staining (pointed by yellow arrows) for NKX3-1 was more frequently observed in the group without occult LNM than the group with occult LNM. Scale bar: 100 μm. (B) the Wilcoxon rank sum test was applied for the IHC results to test the difference of average positive rates between OSCC cases with and without occult LNM. *p<0.001. (C) the Wilcoxon rank sum test was applied for the IHC results to test the difference of average positive rates between the independent OSCC cases with and without occult LNM. **p=0.004.
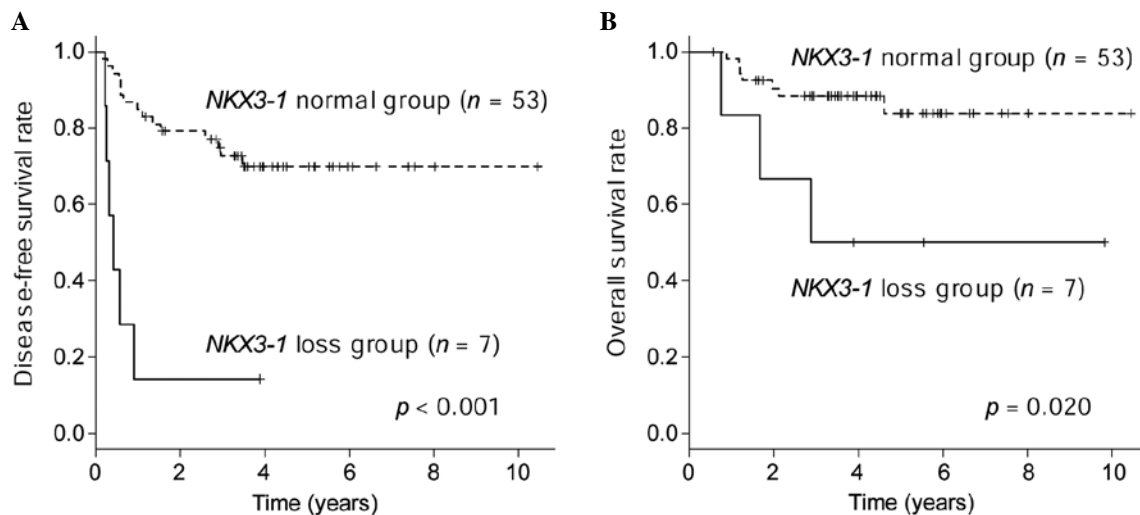

Figure 4. Disease-free and overall survival curves analyzed for the 60 OSCC cases with the Kaplan-Meier method. (A) The disease-free survival rate of patients with *NKX3-1* loss was significantly lower than that of patients with normal copy number of *NKX3-1* (log-rank test, p<0.001). (B) The overall survival rate of patients with *NKX3-1* loss was significantly lower than that of patients with normal copy number of *NKX3-1* (log-rank test, p=0.020).

showed a significant decrease (p<0.001) in the mean relative gene expression of *NKX3-1* in the OSCC cases with occult LNM (Fig. 2). To investigate the transcriptional influence of *NKX3-1* expression on *VEGF-C*, the quantitative results from qRT-PCR were compared. A weak negative correlation of

r=-0.256 was observed between the two genes although it did not reach a statistical significance.

Protein expression level of NKX3-1 in occult LNM cases. Following the verification of DNA copy number loss and tran-

Table II. Cox proportional hazard analysis for clinicopathological factors with DFS and OS.

Variables	DFS			OS		
	HR	95% CI	p-value	HR	95% CI	p-value
Age <60 ≥60	0.975	(0.411-2.314)	0.954	1.848	(0.477-7.152)	0.374
Gender Male Female	0.797	(0.336-1.893)	0.608	0.949	(0.266-3.383)	0.936
Alcohol habit Negative Positive	0.614	(0.238-1.585)	0.313	0.634	(0.151-2.658)	0.533
Smoking habit Negative Positive	1.797	(0.707-4.569)	0.218	1.284	(0.321-5.140)	0.724
Growth pattern Superficial/exophitic Endophitic	0.884	(0.355-2.200)	0.790	1.587	(0.425-5.919)	0.492
Cellular differentiation Well/Moderate Poor	1.004	(0.296-3.414)	0.994	2.694	(0.694-10.460)	0.152
Pathological T stage I, II III, IV	1.014	(0.371-2.769)	0.978	1.628	(0.420-6.309)	0.481
pN Negative Positive	1.861	(0.783-4.426)	0.160	2.560	(0.738-8.874)	0.138
Mode of invasion 1-3 4C, 4D Undetermined	1.260	(0.464-3.418)	0.650	2.222	(0.555-8.903)	0.259
CN of *NKX3-1* Normal Loss	7.574	(2.835-20.240)	<0.001	4.351	(1.124-16.840)	0.033

DFS, disease-free survival; OS, overall survival; HR, hazard ratio.

scriptional down-regulation of *NKX3-1*, the protein expression of NKX3-1 was also determined by the IHC assay. NKX3-1, a transcription factor, was stained in a nucleus of oral squamous malignant cell and less stained in the occult LNM-positive cases (Fig. 3A). The average positive rates of NKX3-1 staining were significantly lower (p<0.001) in the group with occult LNM (Fig. 3B). The significant difference was validated by the additional IHC assay in the independent sample set and the average positive rates of NKX3-1 was significantly lower in occult LNM-positive cases (p=0.004; Fig. 3C).

Loss of NKX3-1 is correlated with low survival rates. To determine the correlation between loss of *NKX3-1* and survival rates in the OSCC patients (n=60), the Kaplan-Meier curves were constructed for both DFS and OS. Among the OSCC

patients, the cases with loss in *NKX3-1* had a significantly lower DFS rate (log-rank test, p<0.001; Fig. 4A). The OS analysis also demonstrated a significant difference between the patients with and without loss of *NKX3-1* (log-rank test, p=0.020; Fig. 4B). Among the various clinicopathological parameters, the Cox proportional hazards analysis showed that loss of *NKX3-1* was the only factor significantly associated with poor prognosis (Table II).

Discussion

Precedent studies demonstrated that genome-wide CNA analyses could identify CNA-based markers associated with metastasis in breast cancer, colorectal cancer and endometrioid carcinoma (18-20). Therefore, a comprehensive CNA

analysis with microarray can be valuable in an investigation of causative genomic changes for occult LNM of OSCC. To the best of our knowledge, this is the first study of genome-wide aberrations of DNA copy number using Mapping 250K array on primary tumor tissues as metastatic profiles after surgical treatment of primary OSCC sites.

The present study identified a significant correlation between loss of *NKX3-1* and occult LNM of OSCC based on the genome-wide CNA analysis on the 60 OSCC patients. The results of quantitative RT-PCR and IHC revealed significantly lower expression of NKX3-1 in the primary tumor of the occult LNM cases. The Kaplan-Meier analysis showed that both DFS and OS rates significantly decreased in the patients with *NKX3-1* loss. The expression pattern of NKX3-1 protein was reproduced in the independent patient group, i.e., the expression in the patients with occult LNM was significantly lower than those without occult LNM. All these findings indicated that loss of *NKX3-1* is involved in occult LNM in OSCC. This is the first report that loss of *NKX3-1* is significantly associated with the presence of occult LNM in OSCC although there are several papers reporting involvement of NKX3-1 mostly in prostate cancer (14-17,21-23) but a few in acute lymphoblastic leukemia (24), adrenal gland tumor (25), breast cancer (26,27), kidney cancer (25,28) and testis cancer (25,29). In the following, we will discuss possible roles of NKX3-1 in occult LNM of OSCC and its validity as a marker.

Chromosomal site 8p, where NKX3-1 gene is encoded, is frequently reported as one of the major alteration sites having genomic loss and LOH which are associated with tumorigenesis, metastasis or poor prognosis of cancer patients. It has been discussed that some of the genes encoded on the region might be tumor suppressor genes and potential markers for tumorigenesis, metastasis or poor prognosis (30-33). NKX3-1, which is one of the homeodomain-containing transcriptional factors, was first reported for an association with prostate cancer in 1996 (15). From that time on, NKX3-1 has been studied for the involvement in the carcinogenesis and progression of prostate cancer as a putative prostate tumor suppressor gene that shows prostate-specific gene expression (16,17,22). Some reports showed that in prostate cancer the DNA copy number loss or down-regulation of *NKX3-1* had a significant correlation with progression, LNM and poor prognosis (14,21,23). In breast cancer, *NKX3-1* was considered as a potential transcriptional inhibitor of estrogen receptor response and tumor suppressor gene (26,27). NKX3-1 was also identified in prostate and kidney cancer cell lines as an enhancer of topoisomerase I, which is an enzyme regulating the overwinding or underwinding of DNA and involved in DNA replication, transcription, or DNA repair (28). On the other hand NKX3-1 could have an activity of either tumor suppressor or oncogene in T cell acute lymphoblastic leukemia depending on tissue expression (24).

Interestingly, NKX3-1 has a binding domain to the promoter region of *VEGF-C*, which is a lymphangiogenesis factor, and NKX3-1 normally regulates the expression of *VEGF-C* (13). Lymphangiogenesis is the formation of novel lymphatic vessels from existing lymphatic vessels. Significant involvement of VEGF family in vasculogenesis, angiogenesis and lymphangiogenesis has been reported (34). Tumor cells require angiogenesis to obtain oxygen and nutrients from a regular blood stream, and overexpressions of VEGF proteins are observed in several malignancies (35,36). In addition, progression and metastasis of tumor cells and poor prognosis are also correlated with overexpressions of VEGFs (37-40). VEGF-C is classified into lymphatic markers, which are desmoplakin, LYVE-1, PROX-1, VEGF-C, VEGF-D and VEGFR-3, and generally studied in the correlation with nodal metastasis of cancers (36,41). Therefore, up-regulation of VEGF-C is induced by reduction of NKX3-1 expression, resulting in driving lymphangiogenesis (13).

Based on these facts, we hypothesize that lymphangiogenesis induced by VEGF-C is suppressed under the normal condition of cells because NKX3-1 negatively regulates transcription of *VEGF-C*. By contrast, novel lymphatic vessels may tend to be produced around tumor cells while up-regulation of VEGF-C is induced by reduction of NKX3-1 expression. In consequence, metastatic tumor cells can spread to regional lymph nodes through the newly produced and existing lymphatic vessels, followed by extravasation and proliferation in lymph nodes, resulting in the metastasis in regional lymph nodes. Since status of occult LNM is one of the critical risk factors to lower the OS rate, the significant decrease in OS rate observed in the OSCC patients with loss of *NKX3-1* can be explained in part by *NKX3-1* loss being a risk factor to increase an incidence of occult LNM. Amplifications of *CCND1* and *CCNL1* have been reported as CNA-based promising biomarkers for occult LNM (11,12). Using the new marker, loss of *NXK3-1*, in addition could improve the accuracy of prediction for occult LNM. In this study, although we focused only on NKX3-1, we will continue to investigate the other genes significantly associated with occult LMN as potential biomarkers.

In conclusion, the genome-wide CNA analyses on 60 OSCC patients identified several genes having significant correlation with occult LNM. We then focused on the significant correlation between loss of *NKX3-1* and occult LNM based on the literature. Our findings suggest that DNA copy number loss and low expression of *NKX3-1* may be one of the responsible factors for driving the lymphangiogenesis to accomplish metastasis at the secondary site, implying that NKX3-1 becomes a novel prognostic factor and a promising biomarker to improve a molecular-based prediction of occult LNM in patients with OSCC. Together with the conventional biomarkers, loss of *NKX3-1* would provide insight into selecting an appropriate management approach.

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