Lin-7C/VELI3/MALS-3: An Essential Component in Metastasis of Human Squamous Cell Carcinoma

Takeshi Onda, 1 Katsuhiko Uzawa, 2,5 Dai Nakashima, 2 Kengo Saito, 2 Yasuo Iwadate, 3 Naohiko Seki, 1 Takahiko Shibahara, 1 and Hideki Tanzawa 1

1Department of Oral and Maxillofacial Surgery, Tokyo Dental College; Departments of 2Clinical Molecular Biology, 3Neurological Surgery, and 4Functional Genomics, Graduate School of Medicine, Chiba University; Division of Dentistry and Oral-Maxillofacial Surgery, Chiba University Hospital, Chiba, Japan

Abstract
Using proteomic selection, functional verification, and clinical validation, we identified specific down-regulation of Lin-7C/VELI3/MALS-3 (Lin-7C), which marks oral squamous cell carcinoma (OSCC) metastasis. Despite a rarity of sequence variations in the Lin-7C gene in both primary OSCC and OSCC-derived cells, a high prevalence of hypermethylation was detected in the CpG island region that strongly correlated with its down-regulation. Inducible Lin-7C mRNA by experimental demethylation was found in all OSCC cells tested. Overexpression of the Lin-7C gene in an OSCC cell clone does not contribute to underproliferation but results in a noninvasive phenotype with elevated b-catenin expression. Experimental metastases in multiple organs of immunodeficient mice were inhibited in cells expressing Lin-7C. Finally, the Lin-7C expression status in primary tumors afforded significantly (P < 0.001) high accuracy for predicting lymph node metastasis. These results establish Lin-7C as a novel target of early detection, prevention, and therapy for OSCC metastasis.

Introduction
Squamous cell carcinoma (SCC), which is by far the most common malignant neoplasm of the oral cavity, is one of the leading causes of death from cancer (1). The vast majority of patients who have either suspected or proven metastases in regional lymph nodes are candidates for composite resection in which the lesion, surrounding tissues, and lymph nodes of the neck are all removed. The carcinogenesis of oral SCC (OSCC) is thought to be a complex, multistep phenomenon in which a variety of genetic alterations can be segregated into early to late stages. During this process, loss of metastasis suppressor gene(s) function should be a crucial step in the progression of cancer cells from a nonmetastatic to a metastatic phenotype. Recent studies have identified the correlation between gene expression patterns and lymph node metastasis in OSCC (2, 3). Yet, there is no critical determinant that can be used to identify which primary lesions will develop into metastatic OSCC.

It has recently been recognized that proteome analysis could be a powerful analytic technology developed to enhance our study of the diagnosis, prognosis, treatment, and prevention of human diseases (4–6). In this context, two-dimensional PAGE is the principal tool in proteomics that can resolve thousands of proteins in one experiment and provide the highest resolution in protein separation. Moreover, fluorescent two-dimensional differential in-gel electrophoresis (2D-DIGE) allows the multiplex analysis of two sample proteomes on the same gel (7, 8). In a recent study, we used a model system of cell lines derived from primary normal oral tissue and oral cancer–derived cells to investigate the difference of protein expression patterns by using the 2D-DIGE combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS; ref. 9). We identified groups of proteins of which the expression differed between normal oral keratinocytes (NOK) and OSCC-derived cells. We further validated that Lin-7C/VELI3/MALS-3 (Lin-7C) mediates OSCC metastasis with b-catenin signaling and is clinically linked to the development of cervical lymph node metastasis when the Lin-7C expression level decreases in primary tumors.

Materials and Methods

Cells. All the human OSCC-derived cell lines (Ca9-22, Ho-1-N-1, Ho-1-u-1, HSC-2, HSC-3, HSC-4, OK92, Sa3, and H1) and primary cultured NOKs used as a normal control were cultured as described (10).

Tissue samples and nucleic acid isolation. Tumors with patient-matched normal oral tissues were obtained at the time of surgical resection at Chiba University Hospital after informed consent was obtained from the patients according to a protocol that was reviewed and approved by the institutional review board of Chiba University. Histopathologic diagnosis of each cancerous tissue was done according to the International Histological Classification of Tumours. Genomic DNA was extracted by a proteinase K digestion procedure and total RNA was prepared using Trizol reagent (Invitrogen) according to the manufacturer's protocol.

Proteomic analyses. We did proteomic profiling of NOKs and OSCC-derived cell lines (HSC-2 and HSC-3) using fluorescent 2D-DIGE and MALDI-TOF-MS fingerprinting in triplicate as described previously (9). Each protein extract (50 mg) was used for the 2D-DIGE analysis.

Pathway analysis. The target gene lists were overlaid on a cellular pathway map in the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems; ref. 11). The expression levels of the mRNA of target genes were examined using quantitative reverse transcription-PCR analyses. The primer sequences of the genes related to Lin-7C are shown in the Supplementary Data.

Immunohistochemical staining. Immunohistochemical staining was done on 4-mum paraffin-embedded specimens as described with anti-Lin-7C (Santa Cruz Biotechnology) at a dilution of 1:500. All slides were finally lightly counterstained with hematoxylin and mounted. To quantitate Lin-7C protein expression, a scoring method was used as described previously (10).
Cases with a Lin-7C immunohistochemical score of <5 (minimum score of normal tissues) were considered negative.

**mRNA expression analyses.** The expression levels of Lin-7C mRNA were examined by quantitative reverse transcription-PCR with a single method using a LightCycler FastStart DNA Master SYBR Green kit (Roche) according to the manufacturer’s instructions. The primer sequences used for analysis of Lin-7C mRNA expression were shown in the Supplementary Data. The sequence of specific primers was checked.

---

**Figure 1.** Comparative analysis of OSCC-derived cells and NOKs using the 2D-DIGE system.  
A, typical 2D-DIGE gel images of OSCC-derived cells (HSC-2) and NOKs. The protein lysates are labeled with either Cy3 (green, NOKs) or Cy5 (red, HSC-2) and subjected to 2D-DIGE using Immobiline Dry IPG strips (pH 3–10), IEF, isoelectric focusing.  
B, close-ups of 2D-DIGE gel images show significant ($P < 0.001$) down-regulation in the HSC-2 cell lines compared with the NOKs.  
C, three-dimensional simulation of up-regulated and down-regulated protein spots.  
D, top, results of MALDI-TOF-MS analysis of the spot obtained from NOKs; bottom, amino acid sequences analyzed for Lin-7C by peptide mass fingerprinting analysis. Letters in red, protein sequences characterized by MALDI-TOF-MS analysis.
before use to avoid amplification of genomic DNA or pseudogenes by the Primer3 program.6

Mutational analyses. To identify sequence variations, the Lin-7C gene was analyzed based on a PCR direct sequencing method as described previously (10). Five sets of oligonucleotide primers summarized in the Supplementary Data were used to amplify the entire coding region (exons 1–5) of the Lin-7C gene.

Methylation analyses. We analyzed the methylation status of the CpG island region of the Lin-7C gene using a PCR-based methylation assay. The DNA samples were digested with mCpG-sensitive BstUI (1 μg/unit; New England Biolabs) at 60°C for 16 h. The BstUI-digested DNAs were then amplified with specific primers shown in the Supplementary Data. Peripheral blood DNA treated in vitro with SssI methylase (New England Biolabs) was used as a positive control, and the SssI-untreated DNA was used as a negative control. To assess reactivation of Lin-7C mRNA expression, a demethylating assay was done as described (12).

Functional analyses of Lin-7C. Human Lin-7C cDNA was cloned into a pME18SFL3 expression vector for transient transfection experiments (13). The nine OSCC-derived cell lines were transfected with pME18SFL3 encoding Lin-7C cDNA using the nonliposomal formulation FuGENE6 transfection reagent (Roche). All experiments using these cells were done 48 h after transfection. Mock transfection of the nine OSCC-derived cell line cultures with pME18SFL3 expression vector alone served as a control. Quantitative reverse transcription-PCR analyses and immunofluorescence were done to confirm transfection efficiency (13). To evaluate the invasive capacity of both transfected and mock cells, invasion assays were done with the QCM 96-well Cell Invasion Assay kit (Chemicon; ref. 13).

In vivo experimental metastasis assays. A full-length human Lin-7C construct was purchased from Toyobo and cloned into pcDNA4/TO vectors (Invitrogen). The vector containing Lin-7C of the empty pcDNA4/TO vector was transfected into Ca9-22 cells with the FuGENE6 kit according to the manufacturer’s protocol. Stable expression clones were selected with zeocin (Sigma) for and screened by Western blotting using monoclonal antibody for Lin-7C (Santa Cruz Biotechnology). Cells (2 × 10³) from each clone were injected directly into the tongues of the BALB/cAnNcrj-uu mice (Charles River Japan), which were sacrificed after 6 weeks. We then excised several organs, including the tongue, submandibular glands, lungs, and liver, and DNA was extracted from them for PCR amplification of the human-specific Alu sequence (15). All mice were maintained under specific

Figure 2. Gene expression profiling of Lin-7C. A, immunohistochemical staining of Lin-7C in normal and cancerous oral tissues. Top, typical examples of Lin-7C–positive staining; bottom, examples of the negative cases. Normal oral tissues show strong Lin-7C protein expression limited to the cell membrane. A typical example of primary OSCC without a metastatic lesion is positive for Lin-7C. In contrast, primary cases with a metastatic lesion and the metastatic OSCC itself are negative for Lin-7C. Original magnification, ×400. B, state of Lin-7C protein expression in primary OSCCs with node-positive (pN+) or node-negative (pN−) metastatic lesions. The Lin-7C immunohistochemical (IHC) scores were calculated as follows: immunohistochemical scores = the percentage of positive tumor cells × staining intensity. Both the test set (n = 80) and the validation set (n = 30) indicated that node-positive cases have significantly (P < 0.001) decreased Lin-7C protein expression. C, bottom, typical results of direct DNA sequencing of PCR products from a primary case of OSCC (case 20) show one-base nucleotide substitution in exon 1 (codon 6) of the gene compared with the DNA from normal tissues. D, typical results of the methylation assay for OSCC-derived cells and OSCC cases. Lin-7C methylation is seen in all OSCC cell lines (top), which show reduced Lin-7C mRNA expression compared with the NOKs. Bottom, in addition, whereas no normal tissue has methylated Lin-7C, most primary tumors do. DNA from NOK treated in vitro with SssI methylase is used as a positive control (lane PC) for methylated alleles. SssI-untreated DNA is used as a negative control (lane NC) for methylated genes. M, molecular marker.

6 http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi
pathogen-free conditions. All animal experiments were conducted in accordance with the guidelines of the Chiba University laboratory animal center.

**Results**

**Proteomic analyses.** An average of 2,500 protein spots was detected across all five 2D-DIGE gels (Fig. 1A). A 2-fold or greater change in protein expression was considered significant. In OSCC-derived cell lines (HSC-2 and HSC-3), we found 226 differentially expressed protein spots compared with the NOKs. Among them, 92 were up-regulated and 134 were down-regulated. Among the protein spots detected, 9 proteins were commonly up-regulated and 15 were commonly down-regulated in both cell lines studied (Supplementary Table S1). MALDI-TOF-MS measurement and database matching identified most of the matched proteins with high sequence coverage and mass accuracy (Fig. 1B–D).

**Gene networks of Lin-7C gene.** Using IPA, we found that one down-regulated protein, Lin-7C, may link to β-catenin, and because of that subsequent analysis focused on Lin-7C. This analysis validated that 21 known genes could generate a network (Fig. 3A). Down-regulation of the Lin-7C gene seemed to be one of the central events affecting the Lin-2-β-catenin signal. Transiently forced expression of the Lin-7C gene in three independent OSCC-derived cells (HSC-2, HSC-3, and HSC-4) significantly enhanced the mRNA expression levels of the identified genes and, in particular, led to a significantly (P = 0.01) elevated level of the Lin-2-β-catenin signal even at protein expression levels (Fig. 3A–C).

**Lin-7C status in human OSCCs.** We analyzed a large cohort (80 samples) of OSCCs by semiquantitative immunohistochemical analysis. Thirty samples (38%) showed down-regulation of Lin-7C, defined as 59, which is below the lowest immunohistochemical score of expression in corresponding normal tissues. Interestingly, whereas most primary OSCCs (20 of 25 Lin-7C–negative cases...
with Lin-7C down-regulation had regional lymph node metastasis, tumors without metastatic lesions were positive for Lin-7C (45 of 55 Lin-7C–positive cases; P < 0.001; Fig. 2A). In addition, metastatic OSCC lacked Lin-7C protein expression (n = 25). Representative micrographs of normal tissue, Lin-7C–positive OSCC, Lin-7C–negative OSCC, and metastatic OSCC are presented in Fig. 2B. We next tested the sequence variations of the entire coding region of the gene. Four novel nucleotide substitutions of the gene were identified at Glu 6 (Fig. 2C), Arg27, Arg40, and Lys168 in tumor tissues from three patients with OSCC (15%), resulting in three amino acid replacements.

Quantitative reverse transcription-PCR analysis of OSCC-derived cell lines and NOKs confirmed that significant (P = 0.005) down-regulation of Lin-7C mRNA expression was evident in the cancer cells. In addition, a high prevalence of tumors showed significant (P < 0.001) down-regulation of Lin-7C, whereas corresponding normal oral tissues exhibited steady-state levels of the mRNA. This down-regulation also is closely associated with lymph node status seen in the protein expression status analyzed by immunohistochemistry (Supplementary Figs. S1 and S2). Moreover, methylation analysis showed that all DNA samples from both tumors (n = 20) and OSCC-derived cells (n = 9) were methylated in the promoter region of the Lin-7C gene and there was decreased mRNA expression in both tumors and OSCC-derived cells (Fig. 2D). Treatment using the DNA demethylating agent 5-aza-2'-deoxycytidine resulted in induced reactivation of Lin-7C mRNA expression in OSCC-derived cells (Supplementary Fig. S2).

Functional analyses of Lin-7C gene in OSCC cells. Although two clones from the Ca9-22 cells (Lin7 a-1 and Lin7 5-5) that stably expressed the Lin-7C gene showed no significant differences in their phenotypic changes and the proliferation rate, they showed significant inhibition of motility and invasiveness as determined by a wound-healing assay and invasive assay when compared with the cells with empty vector controls (mock experiment, Fig. 4A–C). To determine whether down-regulation of Lin-7C induces metastasis in vivo, we then injected the Lin-7C–expressing cells (Lin7 a-1 and Lin7 5-5) into the tongue of nude mice. Although no visible metastatic lesions were observed in distant organs, such as the lung, liver, kidney, and colon, the human-specific Alu sequence was detected only in the organs of the mice injected with the Ca9-22 cells with empty vector controls (Fig. 4D).
Discussion

In this report, we showed that Lin-7C plays an important role in the metastatic capacity of OSCC cells via the Lin-7C-CASK (Lin-2)-CTNNB1 (β-catenin) network (Fig. 3A–C) and that the Lin-7C status in the primary tumor may be a potential predictor of regional metastasis.

Although Lin-7C is on 11p14, a region that is deleted in 38% of human head and neck cancers (16), the identity of crucial gene(s) for oral cancer, where Lin-7C is located, is still unclear. Lin-7C contains one PDZ domain that is known to mediate protein-protein interactions with COOH-terminal tails of transmembrane protein. Recent studies have reported that although the Lin-7C PDZ protein is a component of the mature cadherin-based junctional domain in glioma cells, the immature junctions of migrating cells fail to accumulate Lin-7C, suggesting that its presence could be a marker of junctional maturity and invasiveness. An interesting study has shown that microtubule disruption of the cadherin-catenin complex resulted in relocalization of the E-cadherin-catenin-actin complex in normal human epidermal keratinocytes but not in squamous cancer cell lines (17). Thus, the loss of cell-cell adhesion via the cadherin-catenin complex in SCC cells is likely due to this irreversible modification. Lin-2 is a cell adhesion molecule and also has a PDZ domain. The central PDZ domain of Lin-2 binds to the cytoplasmic tails of several cell surface proteins. However, no relationship between human carcinogenesis and Lin-2 status has been confirmed thus far. No reports have been published about an association between Lin-2 and human carcinogenesis, and further studies should evaluate whether down-regulation of Lin-2 may play a role in oral tumorigenesis. β-catenin, whose function is cellular adhesion through the Wnt signaling pathway (18), is down-regulated in the metastatic lesions of OSCC (19), with decreased expression of the Wnt pathway or due to structural abnormalities of the gene. In the current study, overexpression of Lin-7C results in repression of Lin-2 and β-catenin and reduction of invasive/metastatic phenotypes in OSCC cells in vitro (Fig. 3B and C) and in vivo (Fig. 4A–D), suggesting that Lin-7C expression in OSCC is part of the β-catenin signaling pathway that regulates β-catenin and that this pathway may be one of the most important factors in metastatic suppression.

Whereas inactivation of tumor suppressor genes caused by deletion or mutation enhances tumor growth, the loss of Lin-7C function in OSCC induced tumor cell invasion without affecting the growth of the primary tumor. Therefore, we have hypothesized that Lin-7C might suppress metastasis in oral carcinogenesis and that Lin-7C up-regulation, possibly by demethylation (Supplementary Fig. S2), in primary OSCC and probably other types of human cancers may suppress tumor invasion and metastasis. The crucial role of the significant down-regulation of Lin-7C could determine the type of primary tumor that arises in metastatic phenotype. It will be important to design clinical studies to assess the predictive value of Lin-7C down-regulation in patients with OSCC because our test set consisting of newly diagnosed OSCC (n = 30) has been divided into two groups (i.e., those with and without regional metastasis) based on the status of Lin-7C expression in primary tumors.

Acknowledgments


Grant support: The 21st Century Center of Excellence Programs Grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (H. Tanzawa).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Takaki Hiwasa (Department of Biochemistry and Genetics, Chiba University Graduate School of Medicine) for invaluable advice in proteomic analysis and Lynda C. Charters for editing this manuscript.

References

Lin-7C/VELI3/MALS-3: An Essential Component in Metastasis of Human Squamous Cell Carcinoma

Takeshi Onda, Katsuhiro Uzawa, Dai Nakashima, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/67/20/9643

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2007/10/23/67.20.9643.DC1

Cited articles
This article cites 20 articles, 6 of which you can access for free at:
http://cancerres.aacrjournals.org/content/67/20/9643.full.html#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
/content/67/20/9643.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.