筑　波　大　学

博 士 （ 医 学 ） 学 位 論 文

MicroRNA-155-5p is associated with oral squamous cell carcinoma metastases and poor prognosis.

（MicroRNA-155-5p は口腔扁平上皮がんの転移と予後に関与する）

2016

筑波大学大学院博士課程人間総合科学研究科

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Contents

Abbreviations

miRNA: microRNA

OSCC: oral squamous cell carcinoma

EMT: epithelial mesenchymal transition

qRT-PCR: quantitative reverse transcriptase-polymerase chain reaction

SOCS1: suppressor of cytokine signaling 1

STAT3: signal transducer and activator of transcription 3

SEER: the surveillance, epidemiology, and end results

N-cadherin: neural cadherin

FFPE: formalin-fixed, paraffin-embedded

TNM: tumor-node-metastasis

DMEM: Dulbecco's modified eagle medium

SNORD95: small nucleolar RNA, C/D box 95

RNU6B: RNA, U6 small nuclear 2

CT: comparative threshold

E-cadherin: epithelial cadherin

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

NC: negative control

UTR: untranslated region

Abstract

Background: Abnormal miRNA expression was recently implicated in the metastasis of oral squamous cell carcinoma (OSCC) with poor prognosis. The initiation of the invasion-metastases cascade involves epithelial-mesenchymal transition (EMT). The aim of the present study was to clarify if misexpression of miRNA, particularly miR-155-5p, contributes to OSCC metastasis through EMT.

Methods: Tumor samples were collected from 73 subjects with OSCC. The transcripts were analyzed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR), and correlations between miR-155-5p levels and clinical characteristics were investigated. OSCC cell lines were analyzed by miRNA microarray and by transfection with miR-155-5p mimics or inhibitors, followed by proliferation and wound-healing migration assays. qRT-PCR analyses of EMT makers in cells transfected with miR-155-5p inhibitor were performed.

Results: High miR-155-5p expression was observed in tissue samples from subjects with OSCC that had metastasized to cervical lymph nodes. HSC-3 cells also strongly expressed miR-155-5p. The epithelial marker E-cadherin was strongly expressed in HSC-3 cells transfected with miR-155-5p inhibitor. In addition, there was a tendency of elevated SOCS1 and decreased STAT3 expression in those cells.

Conclusion: The results suggest that miR-155-5p causes OSCC to metastasize, and miR-155-5p inhibitor may function as an EMT suppressor.

Introduction

Oral cancer accounts for 1-5% of all malignant tumors in humans (1, 2), and oral squamous cell carcinoma (OSCC) comprises approximately 91% of all oral cancers (1). Despite advances in OSCC diagnosis and management, the Surveillance, Epidemiology, and End Results (SEER) data for 2011 show that the 5-year overall survival rate of patients with oral cavity and pharyngeal cancer has not significantly improved in the past decade (61.8%, 2002; 62.7%, 2011)(2). Therefore, we needed more effective methods for the diagnoses and managing OSCC. Metastases strongly decrease the likelihood of a favorable outcome for cancer. (3, 4)

MicroRNAs (miRNAs) are short non-coding RNAs consisting of 20-22 nucleotides which function in RNA silencing and post-transcriptional regulation of gene expression. (5, 6) As miRNA is involved in the normal functioning of cells, the abnormal expression of miRNAs has been implicated in numerous disease states by suppressing the translation of the target gene's mRNA (7-13), and miRNA-based therapies are under investigation. (14-17) Many miRNAs have subsequently been found to have links with various types of cancer. (18) OSCC-specific miRNAs have been identified (19-21), including miR-155. (22) Although altered miR-155 expression in OSCC cells is known to alter OSCC cell behavior (23-25), its role in OSCC is not clear.

miRNAs are initially transcribed from their own genes or introns as several hundred nucleotides long RNAs named a primary miRNAs (pri-miRNAs), which contain about 80 nucleotide RNA hairpin loop. (26, 27) A single pri-miRNA may contain from one to six hairpin loop structures. RNase III, known as Drosha, cleaves

RNA into about eleven nucleotides from the hairpin base to liberate these hairpins from pri-miRNAs. (28, 29) The resulting product is often named as a precursor-miRNA (pre-miRNA). Exportin-5, the neucleocytoplasmic shuttler, exports pre-miRNA hairpins out of the nucleus. (30) In the cytoplasm, the RNase III enzyme Dicer cleaves the pre-miRNA hairpin (31) by interacting with 5' and 3' ends of the hairpin (32) and cuts away the loop joining the 3' and 5' arms, yielding an about 22 nucleotides length miRNA duplex named a mature miRNA. (31) The mature miRNA is a part of an active RNA-induced silencing complex (RISC), where the miRNA and its mRNA target interact, containing Dicer and many associated proteins (33) including Members of the Argonaute (Ago) protein. Ago protein family is central in RISC function. Argonautes are need for miRNA-induced silencing and contain two conserved RNA binding domains. They bind the mature miRNA and orient it for interaction with a target mRNA. RISC with incorporated miRNA is referred as "miRISC." Although each strand of the duplex may potentially act as a functional miRNA, only one strand selected on the basis of its thermodynamic instability and weaker base pairing relative to the other strand is incorporated into the miRISC. (34-36) The position of the stem-loop may also influence the strand choice. (37) The other strand, named the passenger strand, is normally degraded due to its low expression level in the steady state.

One miRNA is complementary to a part of one or more mRNAs for the purpose of the function of miRNA in gene repression. miRNAs often have only partly the right complementary sequence of nucleotides in the 3'UTR to bond with the target mRNA. As a result, these mRNA molecules are silenced by one or more of cleavage of

the mRNA strand, leading to destabilization of the mRNA through shortening of its poly A tail, and less efficient translation of the mRNA into proteins by ribosomes. (38-40) But translational repression is accomplished through whether mRNA degradation, the translational inhibition or combination of both processes is still unclear. Partially complementary miRNAs recognize their targets. Although, 6-8 nucleotides of 5' end of the miRNA, named seed region, have to be perfectly complementary. (41-44) miRNAs that are partially complementary to a target can also increase deadenylation, causing mRNAs rapid degrading. (45)

The human genome is considered to encode over 2000 miRNAs (46-49) and appear to target about 30-60% of all human genes. (41,50) Estimates of the average number of unique mRNAs that are targets for repression by a typical miRNA vary, depending on the method used to make the estimate, (51) but a given miRNA may have hundreds of different mRNA targets, and a given target might be regulated by multiple miRNAs. (52-56) Hence, miRNA research has revealed multiple roles of miRNAs in many other biological processes.

miRNA expression can be quantified in a two-step polymerase chain reaction process of modified RT-PCR followed by quantitative PCR. miRNAs can also be hybridized to microarrays, slides or chips with probes to hundreds or thousands of miRNA targets, so that relative levels of miRNAs can be determined in different samples. (57)

miRNA mimics and inhibitors are used as analysis tools of miRNA function. (58-60) miRNA mimics and inhibitors provide means to study the function of specific

miRNAs in a range of organisms, and to validate their role in regulating targeting genes. miRNA mimics are small, chemically modified double-stranded RNA molecules that are designed to mimic endogenous mature miRNAs, resulting in artificial down-regulation of target mRNA translation and accompanied, in some cases, by reduction in transcript levels. (61) Like natural miRNAs, these mimics have two mature strands, one of which is functional and used by the Ago protein to target mRNA. miRNA inhibitors are chemically modified, single-stranded oligonucleotides designed to specifically bind to and inhibit endogenous miRNAs, resulting in artificial up-regulation of target mRNA translation. (62) The function of miRNA inhibitor is application of the antisense method using antisense nucleic acid. The antisense method is a method to inhibit or downergulate the target RNA by using antisense RNA molecules. (63, 64) Thus, it is possible to identify miRNA function in cells by specific strong inhibition of the target miRNA. (65, 66) These short RNA molecules are expected to be used as treatment agents for such human diseases as cancer.

Before cells in the primary tumor can metastasize, they must undergo invasion via epithelial-mesenchymal transition (EMT). EMT is a process by which epithelial cells in primary tumor change into mesenchymal cells; i.e., they lose cell polarity and cell-cell adhesion mediated by E-cadherin repression, and break free of neighboring cells, thereby acquiring the ability to metastasize via vascular invasion. When these circulating tumor cells exit the vessels to form micrometastases, they undergo mesenchymal-epithelial transition (MET) for outgrowth at the metastatic sites; e.g., in the cervical lymph nodes in case of OSCC. Thus, EMT, and its reverse process,

MET, form the initiation and completion of the invasion-metastasis cascade. (67, 68)

EMT and MET bring not only phenotypic but also functional changes to the cells. Epithelial cells are closely connected to each other by tight, gap and adherens junctions, have apico-basal polarity, polarization of the actin cytoskeleton and are bound to the basal membrane with their basal surface. On the other hand, mesenchymal cells lack such polarization, have a spindle-shaped morphology and interact with each other only through focal points. (69) Epithelial cells express high levels of epithelial biomarkers, E-cadherin, whereas mesenchymal cells express those of mesenchymal biomarkers, including vimentin and neural cadherin (N-cadherin), the latter is also known as cadherin-2. (70) E-cadherin is commonly found in epithelial tissues and plays a crucial role in cell-cell adhesion, forming the above-mentioned junctions to bind cells within tissues together. Vimentin is a primary cytoskeletal component of mesenchyme-derived cells or cells undergoing EMT. N-cadherin is commonly found in cancer cells; it frees cancer cells to metastasize by causing the failure of cell-cell adhesion. (71) Therefore, loss of E-cadherin is considered to be an essential event in EMT. EMT inducing transcription factors (EMT-TFs) that can repress E-cadherin directly or indirectly are previously identified. Snail, Slug, Zeb1 and Zeb2 can repress E-cadherin directly by binding E-cadherin promoter and repress its transcription, whereas factors such as Twist repress E-cadherin indirectly. (72, 73) Since EMT occurs during cancer progression, many of the EMT-TFs are thought to be involved in development of metastases. On the other hand, a recent study also showed that some miRNAs contribute to EMT. (74-76)

Suppressor of cytokine signaling 1 (SOCS1) is recently identified as a novel miR-155 target in several cancer cells. (77, 78) SOCS1 is a protein that is encoded by the SOCS1 gene in humans (79, 80). It is a tumor suppressor that functions as a negative feedback regulator of Janus activated kinase (JAK)/signal transducer and activator of transcription (STAT) signaling. SOCS genes, also known as SOCS family, encode a member of the STAT-induced STAT inhibitor (SSI). SSI family members are cytokine-inducible negative regulators of cytokine signaling. The protein encoded by this gene functions downstream of cytokine receptors, and takes part in a negative feedback loop to attenuate cytokine signaling. Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that is encoded by the STAT3 gene in humans. (81) Constitutive STAT3 activation is associated with various human cancers and commonly suggests poor prognosis. (82-85) The STAT protein regulates many aspects of growth, survival and differentiation in cells. The transcription factors of this family are activated by JAK. (86) In addition, STAT3 is described to modulate the expression of EMT transcriptional factors, including Twist, Snail, Zeb1, Zeb2, and Slug. (87, 88)

In this study, I, with colleagues found that miR-155-5p was significantly upregulated both in OSCC cell lines and in formalin-fixed, paraffin-embedded (FFPE) tissue samples from patients with OSCC metastases to cervical lymph nodes, which is associated with a poor prognosis. I, with colleagues also found that HSC-3 cells expressed increased levels of E-cadherin and decreased levels of N-cadherin and vimentin mRNAs when transfected with an miR-155-5p inhibitor. Furthermore, there

was trend that SOCS1 was upregulated and STAT3 was downregulated in HSC-3 cells transfected with the miR-155-5p inhibitor.

My results showed that miR-155-5p expression correlated significantly with metastasis to the cervical lymph nodes in OSCC and with a poor prognosis. My findings also suggest that miR-155-5p would be a potential novel target for the prevention of OSCC metastasis.

Materials and methods

Clinical samples

The study included 73 patients with OSCC and five patients who did not have cancer. All subjects visited the University of Tsukuba Hospital for the first time between February 2008 and November 2010, and tissue samples were collected on each subject's first visit, before administering treatment. Patients with oral cancers other than OSCC were excluded from this study. Samples of oral tumors were collected as part of a biopsy procedure. Thirty-four samples were from the tongue, 25 from the gingiva, seven from the cheek, four from the floor of the mouth, and three from the soft palate. Five patients without OSCC who had an impacted wisdom tooth volunteered to provide an oral biopsy sample for this study. All control samples were from the gingiva around the wisdom tooth. The samples were prepared for FFPE histology using standard procedures. OSCC was diagnosed and classified based on the Tumor-Node-Metastasis (TNM) system of Unio Internationalis Contra Cancrum. All cases were diagnosed histologically and clinically, as confirmed by pathologists. The median follow-up period was 24 months (range, 3–50 months).

Table 1 shows the clinical characteristics of subjects with OSCC. Follow-up data were obtained from each patient's medical chart. Disease-free survival time was calculated from the date of the patient's first visit to a final time point of 60 months, when the overall survival rate was poor.

This study was reviewed and approved by the Ethics Committee University of Tsukuba Hospital (No.215). All patients gave informed written consent prior to

enrollment.

Cell lines, reagents, and cultures

HaCaT and HSC-3 cell lines were obtained from the Japanese Collection of Research Bioresources. HSC-3 is a human oral squamous carcinoma cell line with high metastatic potential. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Nichirei Bioscience, Tokyo, Japan) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. HaCaT cells, an immortalized human keratinocyte line, were used as a control.

TaqMan-based quantitative reverse-transcription polymerase chain reaction (qRT-PCR) assays of miRNA expression

Mature miRNA expression levels were analyzed by TaqMan miRNA assay. Total RNA was extracted with the miRNeasy Mini Kit (Qiagen, Venlo, Limburg, Netherlands) for cell lines and the miRNeasy FFPE Kit (Qiagen). Total RNA was then reverse-transcribed into complementary DNA using a TaqMan MicroRNA Reverse-Transcription Kit (Applied Biosystems, Foster city, CA). The miR-155-5p level in the HSC-3 cell line was compared with the level in HaCaT cells. PCR reactions were first incubated at 16°C for 30 min and then at 42°C for 30 min, followed by inactivation at 85°C for 5 min. For the miRNA microarray analysis, reactions were then incubated in an miRNA PCR array platform (Human Cancer Pathway Finder miScript miRNA PCR array, MIHS-102Z, Qiagen), and other samples were incubated in a

384-well plate at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, using the 7500 Fast Real-Time PCR System (Applied Biosystems) for MIHS-102Z, and the CFX384 Real-Time System (Bio-Rad Laboratories, Pleasanton, CA, USA) for 384-well plates. Relative miRNA expression was normalized against SNORD95 in the miRNA microarrays, and other samples were normalized against RNU-6B. Relative expression was calculated by the comparative threshold (CT) method. All experiments were performed at least in quintuplicate.

TaqMan-based qRT-PCR assays of mRNA expression

The qRT-PCR primers for E-cadherin, N-cadherin, and vimentin mRNA were as described previously (89-91): E-cadherin, 5'-TGCCCAGAAAATGAAAAAGG-3' (forward) and 5'-GTGTATGTGGCAATGCGTTC-3' (reverse); N-cadherin, 5'-ACAGTGGCCACCTACAAAGG-3' (forward) and 5'-CCGAGATGGGGTTGATAATG-3' (reverse); vimentin, 5'-GAGTCCACTGAGTACCGGAGAC-3' (forward) and 5'-TGTAGGTGGCAATCTCAATGTC-3' (reverse); SOCS1, 5′-GAGGGAGC GGATGGGTGTA-3′ (forward) and 5′-GAGGTAGGAGGT GCGAGTTCAG-3′ (reverse); and STAT3, 5′-CCAAGGAGGAGG CATTCG-3′ (forward) and 5′-ACATCGGCAGGTCAATGG-3′ (reverse). Total RNA was extracted with the RNeasy Mini Kit (Qiagen) and then reverse-transcribed into complementary DNA using a PrimeScript RT Reagent Kit (TaKaRa, Shiga, Japan). PCR reactions were first incubated at 16°C for 30 min and then at 42°C for 30 min followed by inactivation at

85°C for 5 min. Reactions were then incubated in a 384-well plate at 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s using the CFX384 Real-Time system (Bio-Rad Laboratories). Relative mRNA expression was normalized against GAPDH. Relative expression was calculated by the CT method. All experiments were performed at least in quintuplicate.

Transfection with miR-155-5p mimic or inhibitor

Cells were transfected with miR-155-5p mimic or inhibitor or with scrambled negative control (NC; Ambion, Austin, TX, USA) using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA). Twenty-four hours after the beginning of the transfection, I, with colleagues isolated RNA and assayed cell proliferation and wound-healing migration.

Cell-proliferation assay

HSC-3 cells were seeded in triplicate 6-well plates with 3 ml of cell suspension per well (total 2.5×10^5 cells/well) and incubated for 24 h to allow attachment. The cells were transfected with miR-155-5p mimic or inhibitor or with NC, and were counted 24 and 48 h after transfection using a TC10 (Bio-Rad) automated cell counter.

Wound-healing migration assay

HSC-3 cells were seeded in 6-well plates with 3 ml of cell suspension per well (total 2.5×10^5 cells/well), cultured until approximately 80% confluent, and then transfected with miR-155-5p mimic or inhibitor or with NC. Twenty-four hours later, the cells were

washed twice with phosphate-buffered saline, starved by incubation in serum-free medium for 24 h, and then a wound was simulated with a straight scratch using a 200-μl pipette tip. The cells' migratory ability was measured by photographing the wound area every 3 h and quantifying it by ImageJ software (U. S. National Institutes of Health). (92, 93) All experiments were performed at least in duplicate.

Statistical analysis

Data from the OSCC cell line were compared by the Student's *t*-test. Data from the FFPE tissue samples were compared by the Mann-Whitney *U*-test. Data from the Student's *t*-test are presented as the mean + standard deviation, and data from the Mann-Whitney *U*-test are presented as boxplots. Data from cervical lymph-node metastasis and disease-free survival data were analyzed by the chi-square test. Survival data were analyzed by Kaplan-Meier survival analysis; Kaplan-Meier curves were compared using the log-rank test. In chi-square tests and the Kaplan-Meier survival analysis, the miR-155-5p expression level was classified as high or low by normalizing miR-155-5p expression to that of normal tissue (expression of miR-155-5p in normal tissue = 1, high > 1, low < 1). Data from FFPE tissue samples and patients' clinical characteristics, including treatments for OSCC, were used for multivariate analysis of prognostic variables by Cox regression analysis. Metastasis to cervical lymph nodes was excluded from the multivariate analysis because of its obvious contribution to a poor prognosis. A two-tailed P value <0.05 was considered to be statistically significant. All statistical analyses were performed using JMP for Macintosh version 11 (SAS

institute).

Results

High miR-155-5p expression was associated with poor overall survival in OSCC patients

To determine the clinical significance of miR-155-5p expression, tissue samples from patients with OSCC and those without cancer were collected, prepared for FFPE, and analyzed by qRT-PCR. The miR-155-5p levels were then compared with patients' clinical characteristics, to look for significant correlations (n=73; Table 1) after confirmation that there were no statistically significant differences in expression of miR-155-5p between each site. Mann-Whitney *U* and chi-square tests showed that high miR-155-5p expression was positively correlated with cervical lymph-node metastasis in OSCC patients (Fig. 1a, b). Kaplan-Meier survival analysis of miR-155-5p expression and OSCC recurrence or metastasis showed a significant correlation between high miR-155-5p expression and a poor disease-free survival rate (P = 0.017, Fig. 1c). In addition, multivariate analysis of prognostic variables in OSCC patients identified miR-155-5p expression as the specific factor leading to a poor OSCC prognosis (P = 0.023, Table 2).

miR-155-5p was upregulated in OSCC cells

To determine which miRNAs are differentially expressed in HSC-3 cells, I, with colleagues analyzed 84 miRNAs by microarray, using the Human Pathway Finder miRNA PCR Array: MIHS-102Z (Fig. 2a-c). I, with colleagues found that miR-146a-5p, miR-10b, miR-155-5p, and miR10a-5p were upregulated more than 4-fold in HSC-3

cells. Conversely, 38 miRNAs were downregulated, albeit less than 4-fold (Fig. 2b). Furthermore, the expression of miR-155-5p was 8.04-fold higher in the HSC-3 cells than in the HaCaT cells (Fig. 2c).

Effect of miR-155-5p mimic or inhibitor on OSCC-cell proliferation and migration

I, with colleagues investigated correlations between miR-155-5p and the ability of HSC-3 cells to proliferate and migrate by assaying cell proliferation and wound-healing migration in cells transfected with an miR-155-5p mimic or inhibitor. I, with colleagues first confirmed that transfection was successful by performing qRT-PCR for miR-155-5p. Proliferation did not differ markedly between HSC-3 cells transfected with the miR-155-5p mimic or the inhibitor (Fig. 3a). Furthermore, although there was no statistically significant difference, the migratory ability of HSC-3 cells tended to increase by the miR-155-5p mimic and to decrease by the miR-155-5p inhibitor (Fig. 3b). Together, my data suggest that miR-155-5p may affect the ability of HSC-3 cells to migrate rather than their ability to proliferate.

Effect of miR-155-5p on E-cadherin, N-cadherin, and vimentin mRNA expression

To investigate correlations between miR-155-5p and the mRNA of genes related to epithelial or mesenchymal properties, I, with colleagues analyzed E-cadherin, N-cadherin, and vimentin in HSC-3 cells transfected with miR-155-5p inhibitor. E-cadherin was significantly upregulated in HSC-3 cells transfected with the miR-155-5p inhibitor (Fig. 4a). In contrast, N-cadherin and vimentin tended to be

downregulated in HSC-3 cells transfected with the miR-155-5p inhibitor (Fig. 4a).

miR-155-5p inhibitor suppressed the STAT3 signaling pathway through SOCS1

To identify the biological mechanism by which miR-155-5p inhibitor mediates the upregulation of E-cadherin and the downregulation of N-cadherin and vimentin, I, with colleagues used qRT-PCR to analyze SOCS1 and STAT3 in HSC-3 cells transfected with miR-155-5p inhibitor. I, with colleagues found trends for SOCS1 to be upregulated and STAT3 to be downregulated (Fig. 4b).

Discussion

The most fatal characteristics of oral squamous cell carcinoma is its potential for metastasis. When removing a primary OSCC tumor, surgeons may also remove nearby lymph tissue—especially cervical lymph nodes, as these are frequently the first metastasis sites. Once OSCC metastasizes to the lymph system, the likelihood of a favorable outcome decreases significantly. (3, 4) My study suggests that miR-155-5p induces metastasis to the lymph nodes, which leads to a poor prognosis. Thus, miR-155-5p might be a useful prognostic biomarker and an important therapeutic target for OSCC.

High levels of miR-155-5p were significantly associated with a poor prognosis, metastasis to cervical lymph nodes, and poor overall survival. Multivariate analysis confirmed that this association of miR-155-5p with a poor prognosis for OSCC was not influenced by other prognostic variables such as treatment, pTNM stage, or age. Therefore, miR-155-5p merits consideration as a potential prognostic biomarker.

Analysis by miRNA microarray confirmed that the miR-155-5p levels were high in OSCC cell lines compared with HaCaT cells. The roles of miR-155 upregulation and EMT in metastasis have been investigated in several cancers. (94, 95) I, with colleagues hypothesized that miR-155-5p also plays a crucial role in OSCC metastasis through EMT and could serve as a novel target for OSCC treatment. To investigate miR-155-5p's effect on OSCC cell proliferation, migration, and expression of EMT-related mRNAs, I, with colleagues conducted a series of loss- and gain-of-function assays with an miR-155-5p mimic or inhibitor. My data showed that

increased miR-155-5p function caused trends toward enhanced OSCC-cell migration rather than enhanced proliferation.

Recently, miRNAs have been found in serum, plasma, saliva, and other body fluids. In addition, circulating extracellular vesicles, such as exosomes, containing miRNA are significantly associated with treatment resistance, metastatic properties, and a poor prognosis. (96-98) These findings suggest that the effect of miRNAs is not limited to the cancer cell itself, but that miRNAs can influence the behavior of both neighboring and distant cells. In other words, miRNAs exert not only autocrine effects, but also paracrine or endocrine effects. The miRNAs contained in exosomes influence the microenvironment of cells in the stroma of the neoplasm, including endothelial cells and fibroblasts, permitting them to begin migrating and invading other tissues (96). This mechanism may explain why the changes in the HSC-3 cells' migration were not statistically significant, even though a clear relationship between metastasis and the expression of miR-155-5p was shown in clinical samples.

In HSC-3 cells transfected with miR-155-5p inhibitor, the epithelial marker E-cadherin was upregulated while the mesenchymal markers N-cadherin and vimentin decreased. During EMT, epithelial makers are progressively lost as mesenchymal markers increase, and the cells develop a mesenchymal phenotype. (70) However, one fundamental function of miRNA is to silence mRNAs by cleaving their target mRNA strand or by decreasing the efficiency of its translation into protein. Thus, I, with colleagues hypothesized that miR-155-5p induced EMT by inhibiting an unknown biological mechanism. This would explain the high levels of epithelial-related mRNAs

in HSC-3 cells transfected with miR-155-5p inhibitor and the high miR-155-5p levels in both the HSC-3 cell line and the FFPE tissue samples. This would also support the potential for developing a novel OSCC therapeutic agent based on miR-155-5p function.

A recent study reported a STAT3-mediated association between miR-155 and EMT that leads to invasion and metastases. (91) In head and neck squamous cell carcinoma, previous study reported SOCS1 regulates STAT3 activation in cell line and tissue samples (99) and STAT3 alternation correlates with poor prognosis (100, 101) and is considered to be a novel therapeutic target. (102)

Expression of E-cadherin mRNA was upregulated in HSC-3 cells transfected with miR-155-5p inhibitor (Fig. 4a), and significant contribution of miR-155-5p inhibitor to EMT inhibition was confirmed. Abundant expression of SOCS1 and STAT3 mRNAs was also confirmed in HSC-3 cells. The SOCS1 and STAT3 levels showed tendencies to be up- and down- regulated, respectively, by the transfection with miR-155-5p inhibitor, although the changes were not statistically significant (Fig. 5). miR-155-5p inhibitor may function as an EMT suppressor that increases SOCS1 expression and suppresses STAT3 signaling as previously described (77, 87). Thus, it is strongly suggested that miR-155-5p inhibitor causes inhibition of EMT, which could be mediated by the SOCS1-STAT3 signaling cascade. Further studies are needed to clarify the mechanisms how miR-155-5p inhibitor leads to EMT inhibition.

Since, high correlation of miRNA expression and carcinogenesis is observed, the development of nucleic acid medicine for variety of human diseases like cancer

have been carried out. Recently, reducing expression level of the oncomiR has attracted attention as one of the developing therapeutic agents of human diseases including cancer. (103-106) In addition, use of the disease signature miRNAs has been increasingly investigated in clinical trials in several countries. (107)

Taken together, my findings indicate that the induction of metastasis and the poor prognosis associated with high miR-155-5p expression may be a direct result of EMT. Although my results indicate that miR-155-5p merits consideration as a prognostic biomarker and a treatment target, the details of the mechanism by which miR-155-5p exerts its effects remain obscure. Furthermore, I, with colleagues have evaluated the function of miRNA only at the miRNA and mRNA levels, and since the no significant differences for N-cadherin, Vimentin, STAT3 and SOCS1 mRNA, it is only speculations that miR155-5p contributes to EMT through STAT3 signaling pathway via SOCS1 in HSC-3 cell. Further studies of OSCC, specifically focusing on mRNA translation to protein, will be necessary to determine the mechanism underlying miR-155-5p's association with poor prognosis in more detail. Additionally, Only 73 OSCC specimens from five different sites were included to this study. Although there was no statistically significant difference in miR-155-5p expression between each biopsy site, some factors may affect my results depending on which site in the oral cavity the biopsy is taken. Larger number of patients and comparison of each biopsy site may improve the study quality.

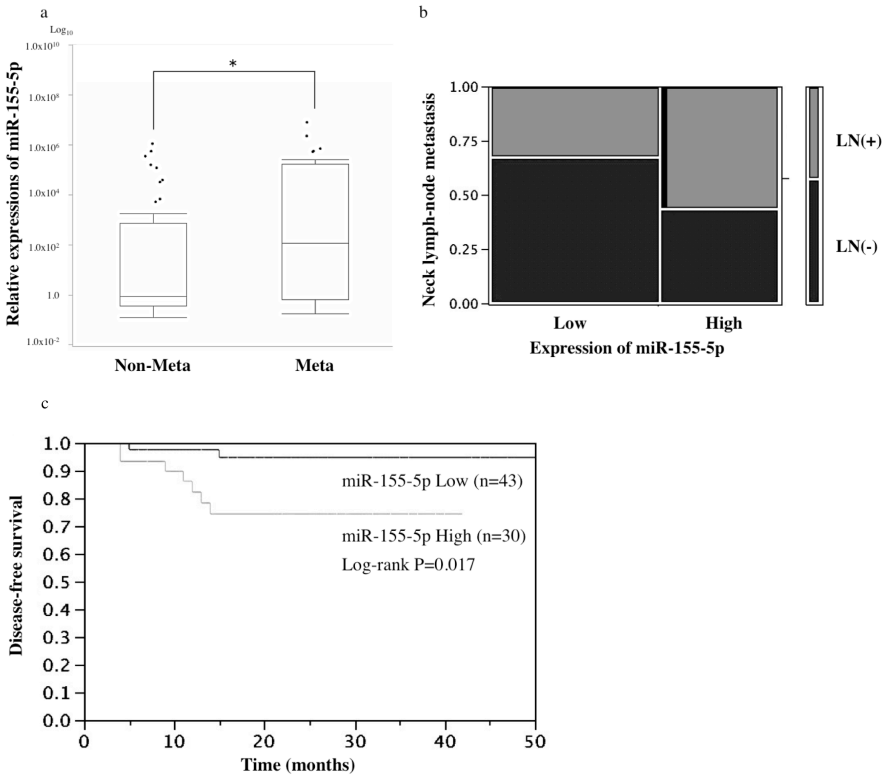
Figures and tables

Table 1 Clinical characteristics of patients with oral squamous cell carcinoma

Patient characteristics	Number of patients (total n=73)	miR-155-5p expression Cancer / normal		P value
		High expression	Low expression	
		n=30	n=43	
Age				
＜60	18	7	11	0.48
≧60	55	23	32	
Gender				
Male	49	21	28	0.15
Female	24	9	15	
T-primary tumor				
T1, 2	37	14	23	0.60
T3, 4	36	16	20	
N-regional lymph node				
N(−)	42	13	29	0.045*
N(+)	31	17	14	
pTNM stage				
I-II	29	11	18	0.60
III-IV	44	19	25	
Treatment				
Surgery only	24	9	15	0.57
Surgery + preoperative therapy	41	17	24	0.94
Radiotherapy only	3	2	1	0.28
Chemotherapy + radiotherapy	3	1	2	0.96
Unknown	2	1	1	0.87

*P<0.05.

Figure 1 Association of high miR-155-p expression with oral squamous cell carcinoma (OSCC) metastasis to neck lymph nodes and poor prognosis for OSCC



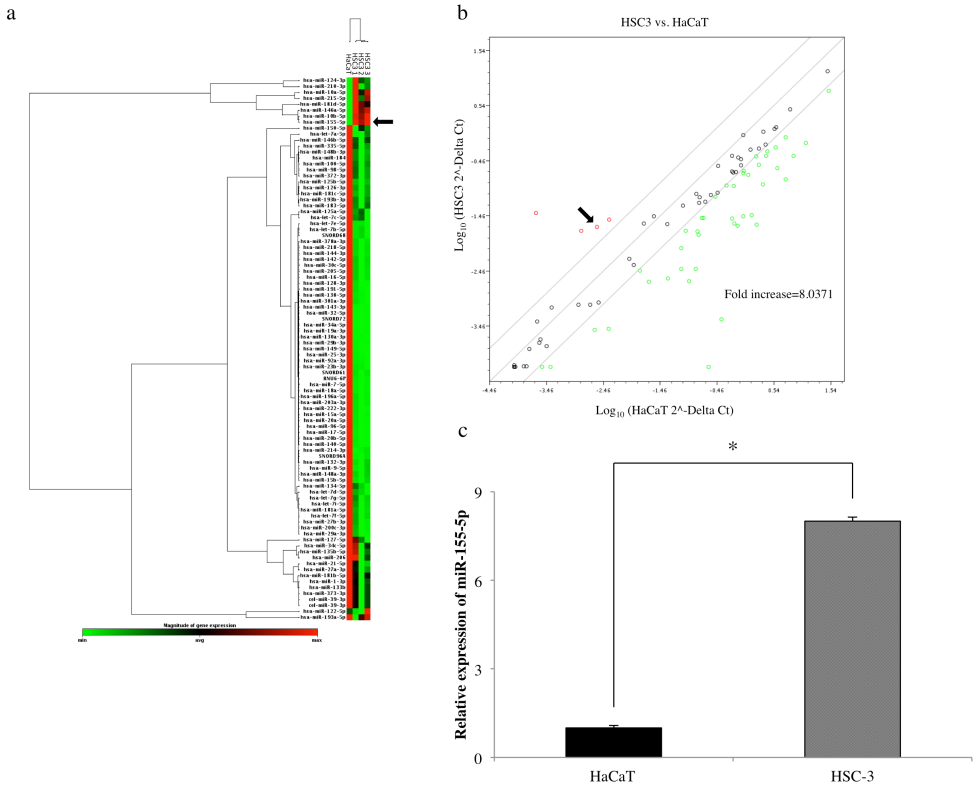
(a) High miR-155-5p levels are found in tissue samples from OSCC patients with metastasis to neck lymph nodes (*P = 0.045). (b) miR-155-5p levels in formalin-fixed, paraffin-embedded (FFPE) tissue samples from patients with OSCC correlate with metastasis to neck lymph nodes. Significance of correlation between miR-155-5p expression in FFPE tissue samples and the clinical index (presence or absence of metastasis) are determined by chi-square test (P = 0.040). (c) Kaplan-Meier survival analysis support the correlation between a poor disease-free survival rate for OSCC patients and high miR-155-5p expression (P = 0.017).

Table 2 Cox-regression multivariate analysis of prognostic variables in oral squamous cell carcinoma patients

Patient characteristics		Number of patients (total n=73)	Hazard ratio	P value
Expression of miR-155-5p				
	High	30	5.156	0.023*
	Low	43		
Age				
			0.845	0.358
pTNM stage				
	I	13	1.097	0.778
	II	16		
	III	10		
	IV	34		
Treatment				
	Surgery only	24	2.480	0.115
	Surgery + preoperative therapy	41	1.363	0.243

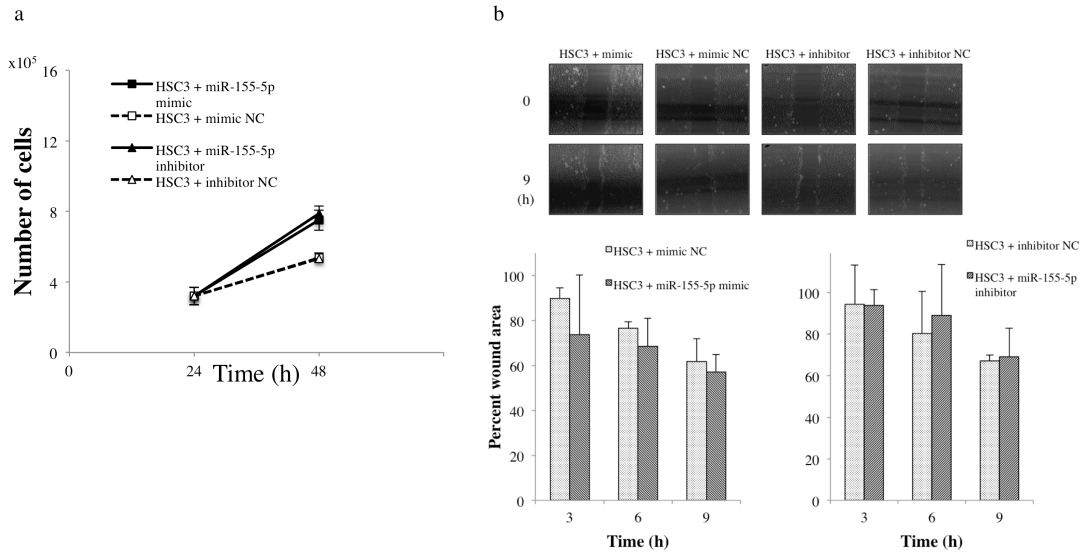
*P<0.05.

Figure 2 miR-155-5p expression in HSC-3 cells



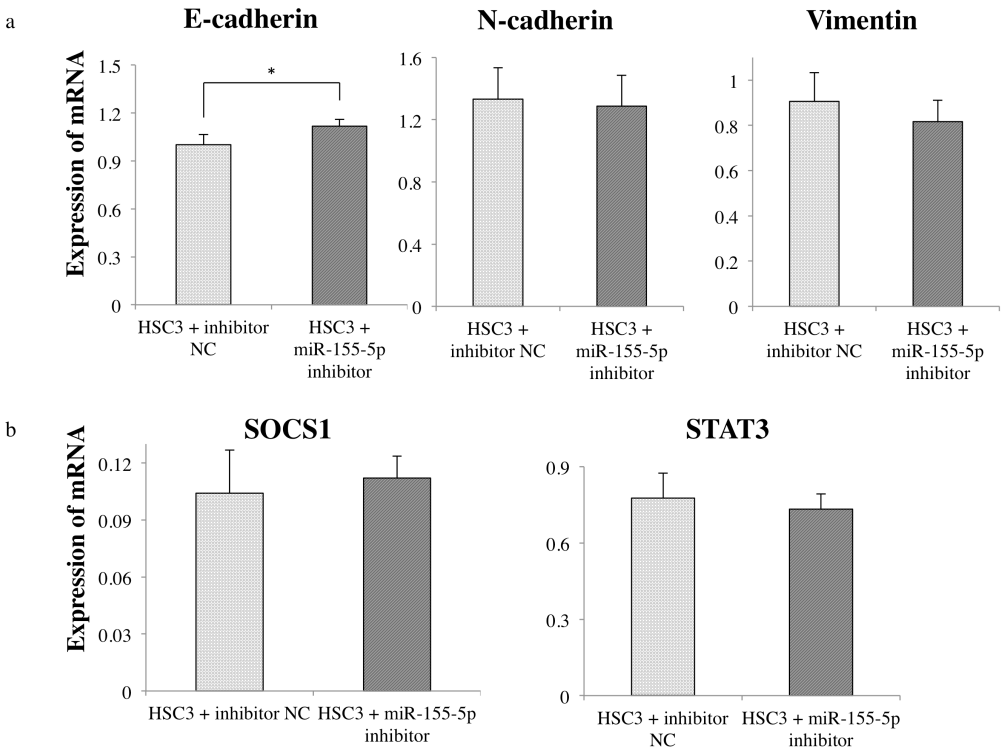
miRNA microarray analysis of 84 miRNAs that are expressed differently in HSC-3 vs. HaCaT cells. (a) miR-155-5p express more strongly in HSC-3 cells than HaCaT cells (black arrow). Clustergram generated from miRNA microarray data of HaCaT and HSC-3 cells. Red: high expression; green: low expression; levels are continuously mapped on the color scale at the bottom of the figure. (b) miR-155-5p expression is 8.04-fold higher in HSC-3 cells than HaCaT cells. Scatterplot generated from miRNA microarray data from HaCaT and HSC-3 cells. (c) miR-155-5p expression is significantly higher in HSC-3 cells than HaCaT cells (*$P = 0.026 \times 10^{-4}$).

Figure 3 Effect of miR-155-5p regulation on the proliferation and migration of HSC-3 cells



(a) Proliferation assay of HSC-3 cells transfected with an miR-155-5p mimic or inhibitor. There are no significant differences in HSC-3 cell proliferation in either condition. (b) Wound-healing migration assay of HSC-3 cells. HSC-3 cells' migration is increased by transfection with an miR-155-5p mimic and decreased by transfection with an miR-155-5p inhibitor, but the difference is not statistically significant.

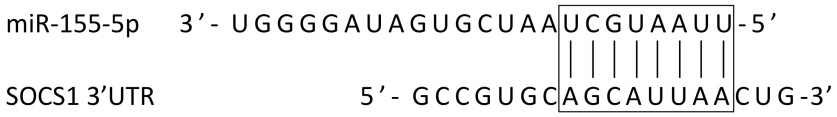
Figure 4 Effect of miR-155-5p inhibitor on the expression of epithelial-mesenchymal transition related mRNA



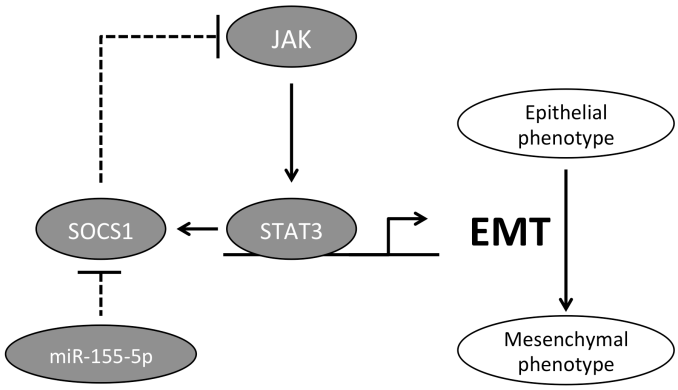
(a) E-cadherin expression is significantly increased in HSC-3 cells transfected with an miR-155-5p inhibitor (*P = 0.041). HSC-3 cells transfected with an miR-155-5p inhibitor shows trends toward decreased N-cadherin and vimentin expression. (b) Suppressor of cytokine signaling 1 expression tend to be upregulated in HSC-3 cells after transfection with the miR-155-5p inhibitor, while STAT3 expression tend to be downregulated.

Figure 5 Suppressor of cytokine signaling 1 (SOCS1) is a potential target of miR-155-5p. miR-155-5p is associated with epithelial-mesenchymal transition (EMT) through STAT3-signaling modulation via SOCS1

a



b



(a) The seed region of miR-155-5p shows good complementarity to the SOCS1 3'UTR.

(b) miR-155-5p induces EMT by upregulating STAT3 via SOCS1 downregulation.

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