

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

著者別名	長谷川 正午, 内田 史彦, 山縣 憲司, 酒井 俊, 柳川 徹, 武川 寛樹
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Title

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Keywords

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Authors

Osamu Baba^a, Shogo Hasegawa^b, Hiroki Nagai^a, Fumihiko Uchida^b, Masanobu Yamatoji^b, Naomi I. Kanno^b, Kenji Yamagata^b, Satoshi Sakai^c, Toru Yanagawa^b, Hiroki Bukawa^b.

^a Oral and Maxillofacial Surgery, Clinical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan.

^b Department of Oral and Maxillofacial Surgery, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan.

° Cardiovascular division, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki,

Japan.

Corresponding author: Shogo Hasegawa

Corresponding author at: Department of Oral and Maxillofacial Surgery, Faculty of

Medicine, University of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki, Japan. Tel.: +81

029 853 3052; fax: +81 029 853 3052.

E-mail address: shogo_h@md.tsukuba.ac.jp (Shogo Hasegawa)

Abstract

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

Methods: We collected tumor samples from 73 subjects with OSCC. The samples were analyzed by 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 a miR-155-5p mimic or inhibitor, 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: We found high miR-155-5p expression 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, and we observed elevated SOCS1

and decreased STAT3 expression in these cells.

Conclusions: Our results suggest that miR-155-5p causes OSCC to metastasize, and could serve as a novel therapeutic target for OSCC.

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). Clearly, we need more effective methods for diagnosing and managing OSCC.

In general, metastasis strongly decreases the likelihood of a favorable outcome for cancer. Before cells in the primary tumor can metastasize, they must undergo epithelial-mesenchymal transition (EMT), in which epithelial cells change into mesenchymal cells; i.e., they lose cell polarity and cell-cell adhesion, and break free of neighboring cells, thereby acquiring the ability to metastasize via vascular invasion. Thus, EMT is a crucial early event in the invasion-metastasis cascade (3).

Mesenchymal biomarkers include vimentin and neural cadherin (N-cadherin), which is also known as cadherin-2 (CDH2) (4). Vimentin is the 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 (5).

MicroRNAs (miRNAs) are short, non-coding RNAs consisting of 20-22 nucleotides. A recent study showed that some miRNAs contribute to EMT induction (6). In addition, abnormal miRNA expression can lead to cancer and other diseases by suppressing the translation of the target gene's mRNA (7-11). OSCC-specific miRNAs have been identified (12), including miR-155. Although altered miR-155 expression in OSCC cells is known to alter OSCC cell behavior (13-15), its role in OSCC is not clear. Suppressor of cytokine signaling 1 (SOCS1) was recently identified as a novel miR-155 target in breast-cancer cells. SOCS1 is a tumor suppressor that functions as a negative feedback regulator of Janus activated kinase (JAK)/signal transducer and activator of transcription (STAT) signaling (16), and STAT3 modulates the expression of EMT transcriptional factors, including Twist, Snail, Zeb1, Zeb2, and Slug (17-18).

In this study, we 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. We also found that HSC-3 cells expressed increased levels of E-cadherin and decreased levels of N-cadherin and vimentin mRNAs when transfected with a 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.**

Our results showed that miR-155-5p expression correlated significantly with metastasis to the cervical lymph nodes in OSCC and with a poor prognosis. **Our 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 5 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, 7 from the cheek, 4 from the floor of the mouth, and 3 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 (UICC). 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% CO₂ 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 (19-21): 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 CATTTCG-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 comparative threshold (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). Twenty-four hours after the beginning of the transfection, we 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 h 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 (PBS), starved by incubation in serum-free medium for 24 h, and then a wound was simulated with a straight scratch using a 200- μ L pipette tip. The cells' migratory ability was measured by photographing the wound area every 3 h and quantifying it by ImageJ software. 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 (SD), 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.

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 1 a, 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 1 c). 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, we analyzed 84 miRNAs by microarray, using the Human Pathway Finder miRNA PCR Array: MIHS-102Z (Fig 2 a-c). We 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 2 b). Furthermore, the expression of miR-155-5p was 8.04-fold higher in the HSC-3 cells than in the HaCaT cells (Fig 2 c).

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

We 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 a miR-155-5p mimic or inhibitor. We 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 3 a). 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 3 b). Together, our 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, we 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 4 a). In contrast, N-cadherin and vimentin tended to be downregulated in HSC-3 cells transfected with the miR-155-5p inhibitor (Fig 4 a).

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, we used qRT-PCR to analyze SOCS1 and STAT3 in HSC-3 cells transfected with miR-155-5p inhibitor. We found trends for SOCS1 to be upregulated and STAT3 to be downregulated (Fig 4 b).

Discussion

OSCC's most dangerous characteristic 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. Our 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 (22-23). We 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, we conducted a series of loss- and gain-of-function assays with a miR-155-5p mimic or inhibitor. Our 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 (24-26). 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 (24). 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 markers are progressively lost as mesenchymal markers increase, and the cells develop a mesenchymal phenotype (4). 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, we 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 metastasis (21). **In head and neck squamous cell carcinoma, previous study reported SOCS1 regulates STAT3 activation in cell line and tissue samples (27) and STAT3 alternation correlates poor prognosis (28).** Our results suggest that miR-155-5p inhibitor may suppress EMT by downregulating STAT3

via SOCS1 upregulation. Both increased SOCS1 and decreased STAT3 were observed in HSC-3 cells transfected with miR-155-5p inhibitor, suggesting that inhibited miR-155-5p function downregulates STAT3 by activating SOCS1, as previously described (16). STAT3 activation has been associated with EMT in several carcinomas (17). Thus, miR-155-5p inhibitor may function as an EMT suppressor that increases SOCS1 expression and suppresses STAT3 signaling. (Fig 5)

Taken together, our 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 our 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, we 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 5 different sites and 5 controls from gingiva were included to this study. Although there was no statistically significant difference of miR-155-5p expression between each biopsy site, some factors may affect our 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.**

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Conflict of interest statement

Conflicts of interest: none

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Figure legends

Table 1: Clinical characteristics of patients with OSCC

Figure 1: High expression of miR-155-5p is associated with 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 (* $P < 0.05$). (b) miR-155-5p levels in 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 ($P=0.04$). (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 OSCC patients

Figure 2: miR-155-5p expression in HSC-3 cells. miRNA microarray analysis of 84

miRNAs that are expressed differently in HSC-3 versus 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 (* $P < 0.05$).

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 a 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 a 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 EMT related mRNA. (a) E-cadherin expression is significantly increased in HSC-3 cells transfected with an miR-155-5p inhibitor (*P<0.05). HSC-3 cells transfected with an miR-155-5p inhibitor shows trends toward decreased N-cadherin and vimentin expression. (b) SOCS1 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: SOCS1 is potential target of miR-155-5p. miR-155-5p is associated with EMT through STAT3-signaling modulation via SOCS1. (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.

Table 1

Patient characteristics	Number of patients (total n=73)	miR-155-5p expression Cancer / normal		P value
		High expression n=30	Low expression 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.

Table 2

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.

Fig1

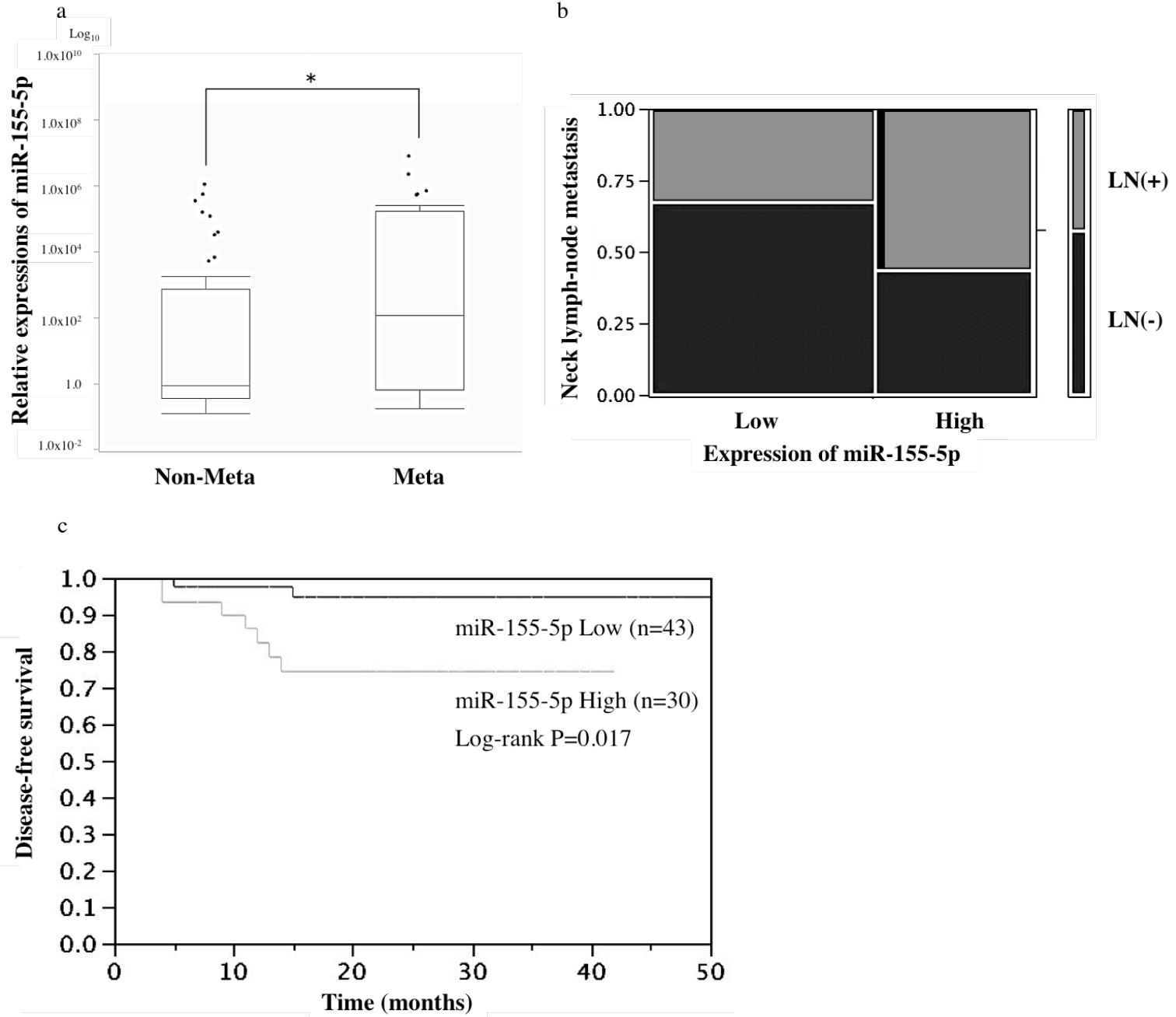


Fig2 a

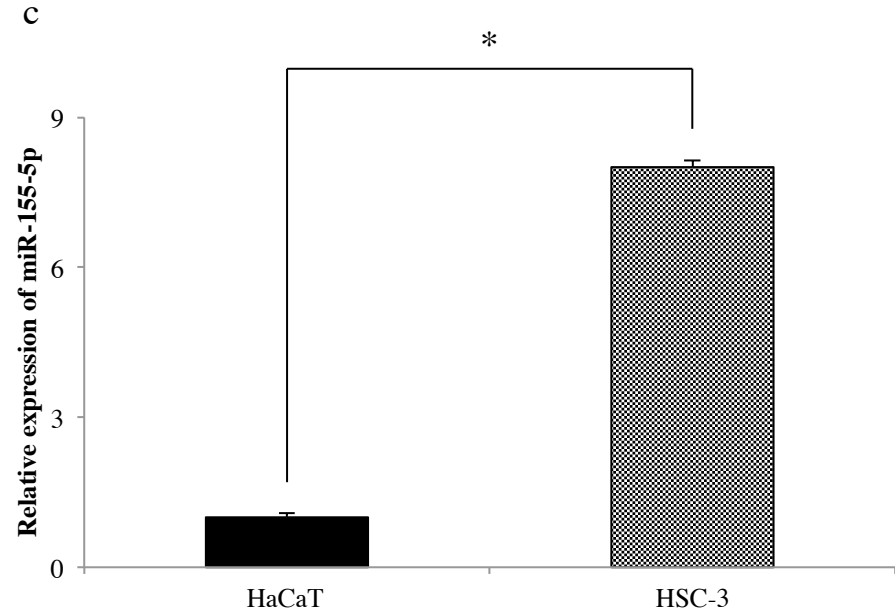
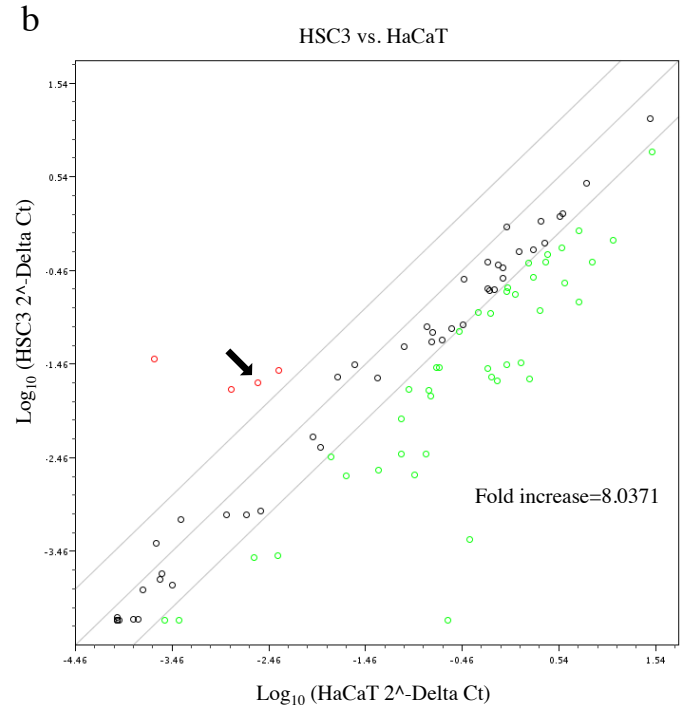
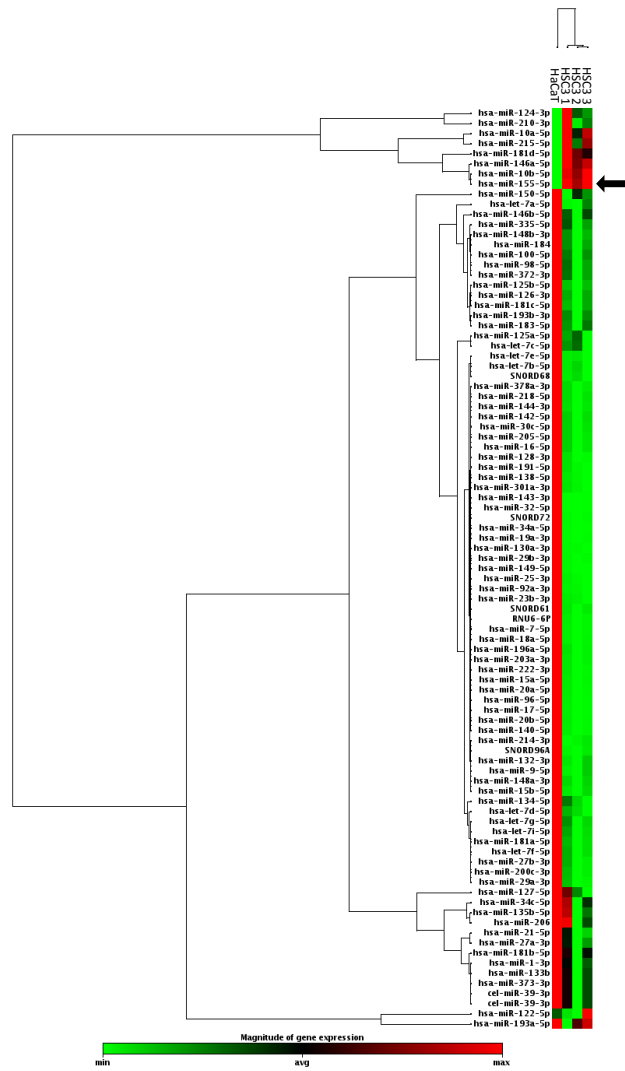


Fig3

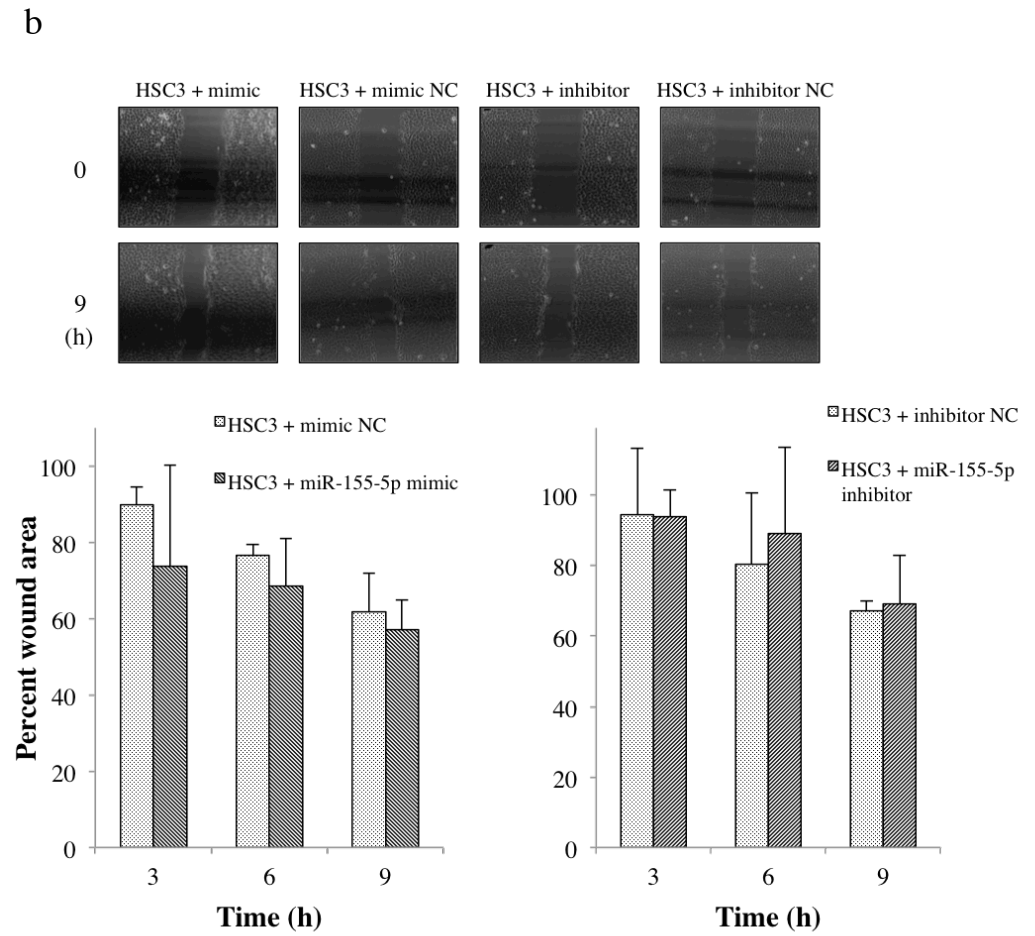
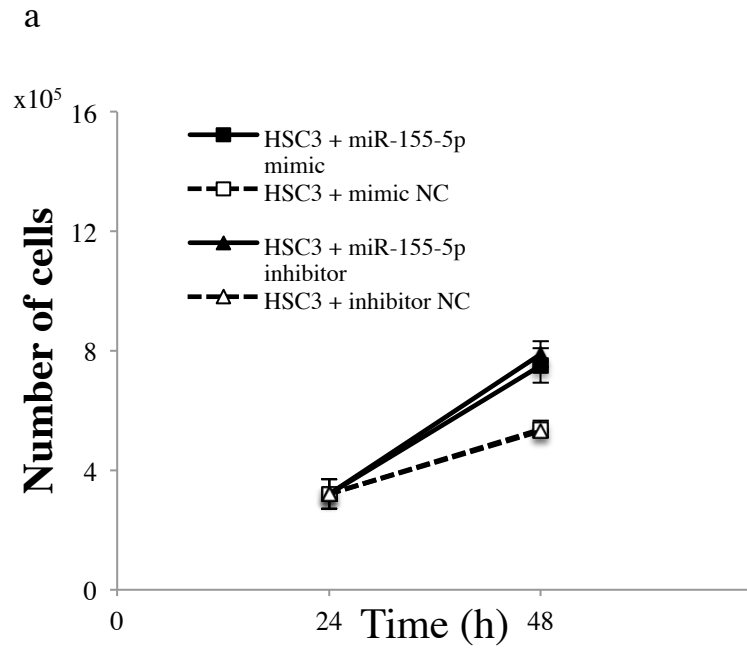
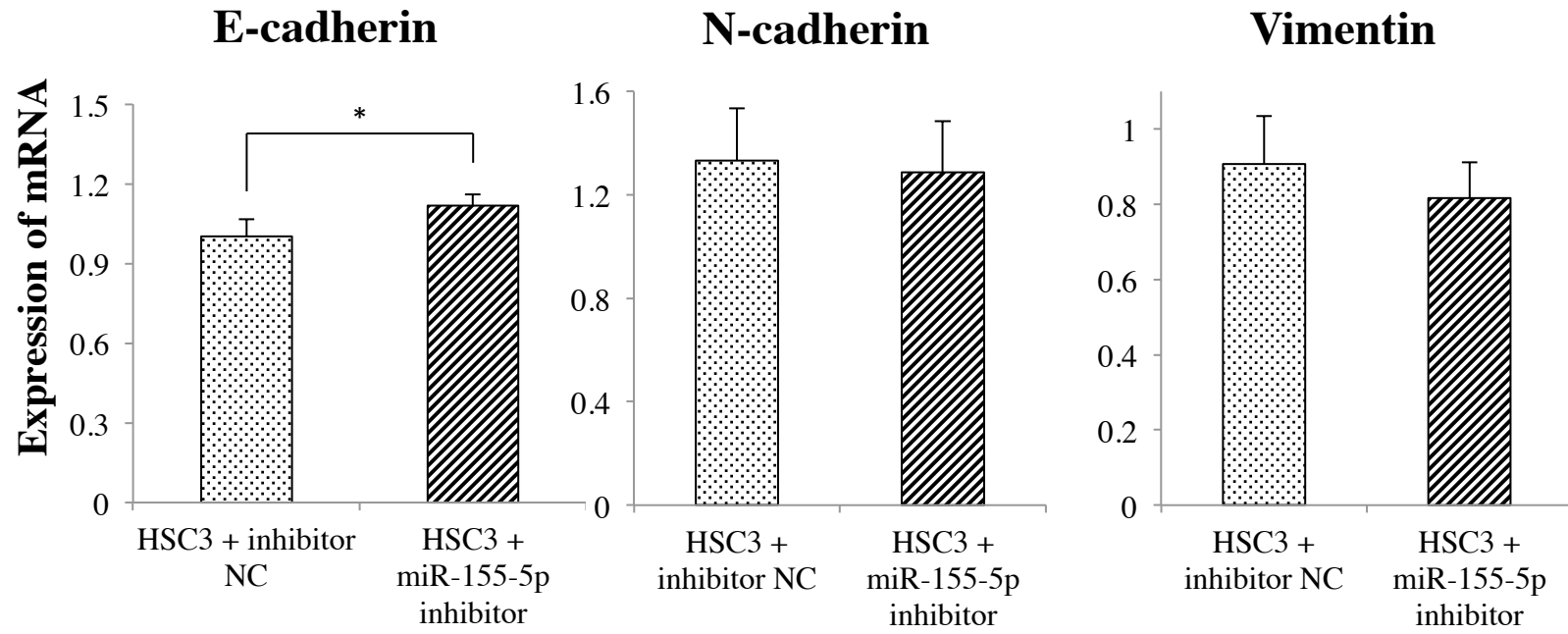


Fig4

a



b

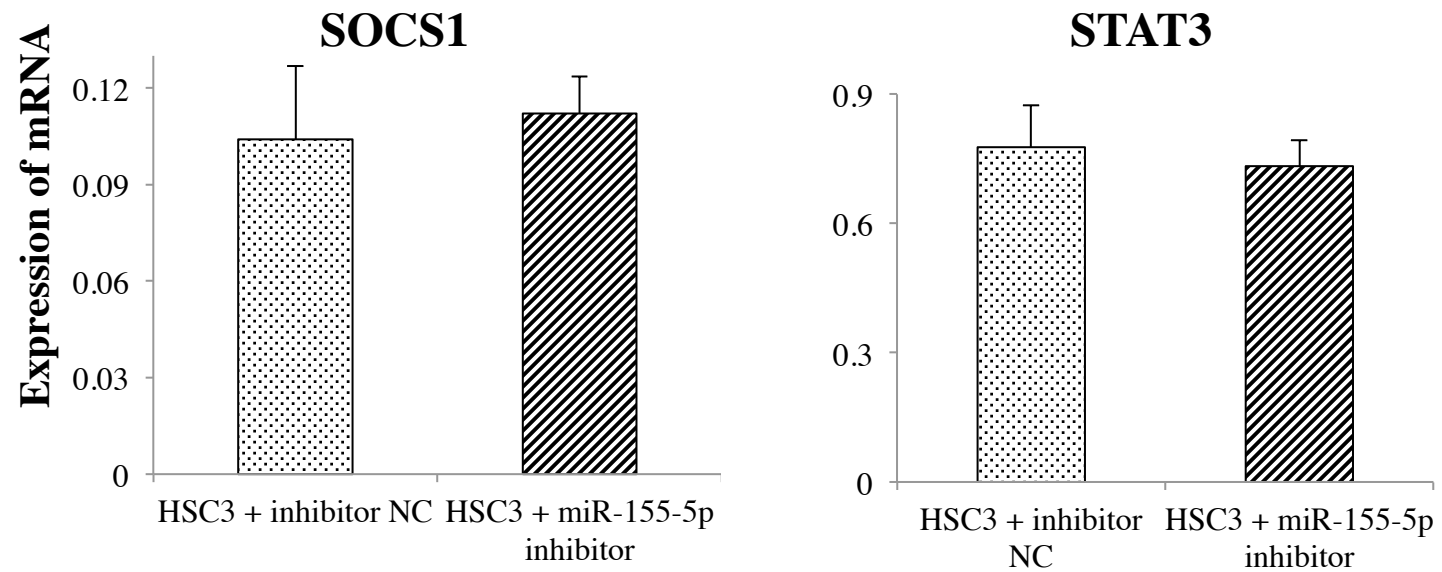
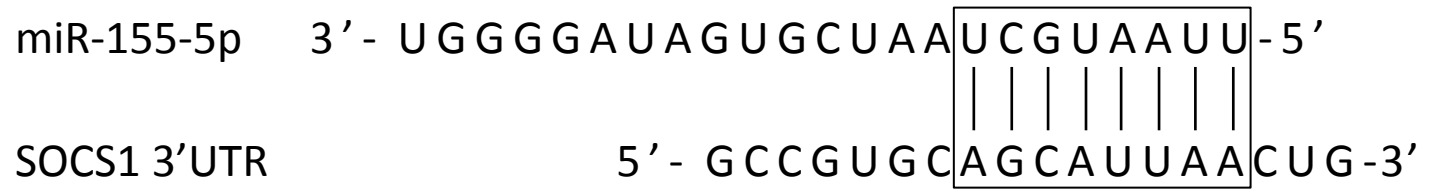


Fig5

a



b

