Effects of let-7a microRNA and C–C chemokine receptor type 7 expression on cellular function and prognosis in esophageal squamous cell carcinoma

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Abstract

Background: C–C chemokine receptor type 7 (CCR7) participates in chemotactic and metastatic responses in various cancers, including in esophageal squamous cell carcinoma (ESCC). The microRNA (miRNA) let-7a suppresses migration and invasion of various types of cancer cells by downregulating CCR7 expression.

Methods: The expression levels of CCR7 and let-7a were measured in the cell lines, tumor, and peritumoral tissues of ESCC patients. KYSE cell lines were transfected with synthetic let-7a miRNA and a let-7a miRNA inhibitor, and their CCR7 expression levels as well as invasive ability were evaluated. A highly invasive cell line was established via an invasion assay, and CCR7 expression level along with let-7a level was subsequently evaluated. Cancer cells overexpressing CCR7 were injected subcutaneously into mice, and the animals were monitored for tumor growth along with lymph node metastasis.

Results: A negative correlation between CCR7 and let-7a expression was observed in the ESCC cell lines as well as in tissue samples from patients. Synthetic let-7a decreased CCR7 expression level, while the let-7a inhibitor increased it. In vitro, the established highly invasive cancer cells with high and low levels of CCR7 and let-7a expression, respectively, exhibited a greater invasive ability than the wild-type cell line. The cells were associated with tumor growth and lymph node metastasis in mice. Patients in the high-CCR7/low-let-7a group had the worst prognosis, with a five-year recurrence free survival (5-RFS) rate of 37.5%, followed by the high-CCR7/high-let-7a (5-RFS: 60.0%) and low-CCR7 (5-RFS: 85.7%; p = 0.038) groups.

Conclusions: The expression of CCR7 was downregulated by let-7a miRNA in esophageal cancer cells. The decrease in let-7a expression level led to the increased expression level of CCR7 in ESCC cells, consequently increasing their invasive ability and malignancy and resulting in a worse prognosis for ESCC patients.

Trial registration. Retrospectively registered.

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Background

Cancer incidence and mortality are rapidly increasing worldwide. In 2018, esophageal cancer ranked seventh and sixth in terms of incidence and overall mortality, respectively [1]. Although the survival rate of esophageal cancer has improved due to the development of multidisciplinary treatment, the five-year post-esophagectomy survival rate is approximately 50% [2, 3]. Therefore, there is an urgent need to investigate the mechanisms of cancer progression and metastasis to improve the prognosis of patients with esophageal cancer.

Chemokines regulate tumor cell proliferation, infiltration, and metastasis [4, 5]. In a previous study, we demonstrated the significant clinicopathological relationship and functional causality between C–C chemokine receptor type 7 (CCR7) expression and lymph node metastasis in patients with esophageal squamous cell carcinoma (ESCC) [6].

MicroRNAs (miRNAs) are non-coding small RNA molecules that can control the translation of mRNAs and regulate several cancer-related genes [7–9]. The miRNA let-7a suppresses various types of cancers [10–13]. For example, in breast cancer, let-7a suppresses the expression of CCR7 and reduces the ability of cancer cells to migrate and invade. Furthermore, a recent prospective study has reported that high let-7a expression level can be a predictive factor for favorable response to chemotherapy [14]. For gastric cancer, a lack of let-7a expression increases CCR7 expression level and is associated with metastasis, contributing to a poor prognosis [12]. Similarly, in esophageal cancer, plasma levels of let-7a miRNA are significantly lower in cancer patients than in healthy participants [15].

However, the effect of let-7a on molecular expression is still unclear, and it has not been confirmed whether the downregulation of let-7a miRNA expression is responsible for increased CCR7 expression levels in ESCC tissues. Here, we investigated the relationship between CCR7 and let-7a miRNA expression as well as the underlying regulatory mechanism, in esophageal cancer cell lines, tumor tissues, and peritumor tissues of patients with ESCC.

Methods

Tissue samples

Tissue samples were obtained during a biopsy from 17 ESCC patients at the Keio University School of Medicine in Japan. Immediately after the procedure, the samples were frozen in liquid nitrogen and stored at -70 °C until use.

Esophageal cancer cell lines

For this study, we used six established ESCC cell lines from the KYSE series (KYSE-350, 510, 590, 1260, 1440, and 2400), purchased from the Japanese Collection of Research Bioresources Cell Bank of the National Institutes of Biomedical Innovation, Health, and Nutrition.

Cell culture

Cells were maintained and cultured in a Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37 °C and 5% CO2 atmospheric content. For the experiments, cells were collected and prepared as single-cell suspensions in phosphate-buffered saline.

Transfection

The sequence of double-stranded RNA (dsRNA) used in transfection experiments as scrambled small interfering RNA was 5′-UCA CAA CCU CCU AGA AAG AGU AGA-3′, that of synthetic let-7a-5p miRNA was 5′-UGAGGU AGUAGGUUGUAUGUU-3′, while inhibitor let-7a-5p miRNA was 5′-UGAGGUAGGUUGUAUGUU-3′. These RNAs were synthesized by Applied Biosystems (Tokyo, Japan). The cell lines were transfected with dsRNA using the reagents Lipofectamine RNAiMAX and Lipofectamine 2000 (Invitrogen, Tokyo, Japan), according to the reagent manufacturer’s instructions. The cell lines were harvested 2 d after transfection and subjected to various analyses.

Isolation and quantitative determination of expression level of let-7a

Isolation of let-7a miRNA from the cell lines was conducted using the mirVana™ miRNA Isolation Kit. The RNA concentration was quantified via the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Tokyo, Japan). The complementary DNA (cDNA) was synthesized by the reverse transcription of let-7a miRNA. A quantitative reverse transcription polymerase chain reaction (RT-PCR) was conducted using Viia™ 7 (Applied Biosystems); the thermal cycling consisted of an initial cycle of 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles for 15 s at 95 °C and 60 s at 60 °C.
Relative let-7a expression level was quantified using the $2^{-\Delta\Delta Ct}$ method.

Isolation of RNA and synthesis of single-stranded cDNA
Isolation of total RNA from cell lines was conducted using an RNeasy® Micro Kit (QIAGEN, Tokyo, Japan). The RNA concentration was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). Then, synthesis of cDNA from total RNA was performed using an RNA-to-cDNA kit (Applied Biosystems). The quality and quantity of the cDNA samples were evaluated via standard electrophoresis and a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific).

Real-time PCR
The quantitative RT-PCR analysis was performed via ViiA™ 7 (Applied Biosystems) and the Fast SYBR® Green Master Mix (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and CCR7 expression levels were evaluated using PCR primers. The thermal cycling consisted of an initial 20 s at 95 °C, followed by 40 cycles for 1 s at 95 °C and 20 s at 60 °C, using GAPDH as an internal control. The relative amount of CCR7 expression in KYSE cell lines was calculated using the $2^{-\Delta\Delta Ct}$ method, wherein the KYSE-350 expression level was defined as 1.

Let-7a transfection
A synthetic let-7a and a let-7a inhibitor were used for transfection. We diluted 15 μL of the Lipofectamine® RNAiMAX reagent (Life Technologies) in 2 mL of OptiMEM® (Life Technologies), then added 2 mL of diluted let-7a miRNA (300 pmol) to diluted Lipofectamine® RNAiMAX and incubated the mixture for 5 min at room temperature. Next, we plated the transfection reagent onto a 10 cm plate, which was incubated for 10–20 min in a CO$_2$ incubator at 37 °C. A suspension of 5 × 10$^5$ cells in an antibiotic-free medium was added to the plate and incubated for 3 days in a CO$_2$ incubator at 37 °C.

Invasion assay
To create highly invasive cell lines, we used Corning Bio-Coat Matrigel Invasion Chambers containing a polyester membrane with 8 μm pores and a thin layer of a Matrigel Basement Membrane Matrix. We prepared a suspension of 2 × 10$^6$ cells/mL in culture medium for 24-well chambers and incubated it in an invasion chamber for 72–96 h in a humidified tissue culture incubator at 37 °C in 5% CO$_2$ atmosphere. After incubation, the non-invading cells were removed from the upper surface of the membrane while the cells in the lower surface were collected and reseeded to the chamber. After repeating this process six times, a highly invasive cell population was obtained and isolated using a cell dissociation solution, solubilized, and stained using a staining solution. The number of infiltrated cells were quantified by measuring the absorbance of the solution.

Development of lymph node metastasis model
A lymph node metastasis model was developed through the following steps: 1) a xenograft tumor was created subcutaneously by injecting 1 × 10$^7$ wild-type and invasive KYSE-510 cells into five-week-old mice, visualized with Green Fluorescent Protein (GFP) transfection; 2) after growth (4–6 weeks post injection), the resulting tumors were resected and cut into small pieces (approximately 2 to 8 mm$^3$), then transplanted into the elbows of different five-week-old mice; 3) the accessory axillary lymph nodes were removed and examined 8 weeks after transplantation.

Statistical analysis
Statistical analysis was done using the IBM SPSS statistics 26.0 software. The continuous variables were expressed as mean ± SD and compared with the Student’s t-test. Recurrence-free survival (RFS) was measured from the time of surgery until the recurrence of tumor or death, whichever came first. The RFS curves were estimated with the Kaplan–Meier method and compared by the log-rank test.

Univariate analysis was then performed using Cox’s proportional hazard model, and a p-value of 0.05 was considered as statistically significant.

Results
Expression of CCR7 and let-7a in esophageal cancer cell lines
Using quantitative RT-PCR analysis, CCR7 mRNA expression was confirmed in all esophageal cancer cell lines (Fig. 1A), and the level of let-7a miRNA expression was also determined (Fig. 1B). KYSE-510 and 1440 exhibited significantly high CCR7 and low let-7a expression levels, whereas KYSE-590 had significantly low CCR7 and high let-7a expression levels. The expression levels of the CCR7 and let-7a were normalized based on the expression levels in KYSE-350 cell, which were defined as 1 (Table S1).

Relationship between let-7a and CCR7 expression in ESCC cells
As determined via quantitative RT-PCR analysis, the expression of CCR7 was downregulated by the transfection of let-7a in five of the six KYSE cell lines (Fig. 2A). The decrease in CCR7 expression level was most
prominent in KYSE-1440; CCR7 expression level was the highest in the wild-type.

**Downregulation of let-7a increases CCR7 expression level and promotes invasion ability in KYSE-590 cells**

The inhibitory effect of let-7a was determined by using synthetic anti-let-7a oligonucleotides in KYSE-590 esophageal cancer cells, which expressed a low level of CCR7 and a high level of let-7a in the wild-type. After transfection with synthetic anti-let-7a oligonucleotides, the level of CCR7 expression in KYSE-590 cells increased ($p < 0.001$; Fig. 2B), along with their invasive ability ($p = 0.061$; Fig. 2C).
Development of highly invasive cell lines and lymph node metastasis model

After six courses of the invasion assay, the invasive ability of the highly invasive lines increased (KYSE-510: \( p = 0.004 \), KYSE-590: \( p = 0.001 \), Fig. 3A–B). Compared to that in the wild-type, CCR7 expression level increased (KYSE-510: \( p = 0.04 \), KYSE-590: \( p = 0.203 \), Fig. 3C) in the invasive type, but let-7a expression level decreased (KYSE-510: \( p < 0.001 \), KYSE-590: \( p = 0.001 \), Fig. 3D).

Since the KYSE-510 cell line alone could be injected into mice for the in vivo experiment, we used it for the mouse model. The implanted primary tumor of invasive KYSE-510 was both larger (6.5 ± 3.51 vs. 13.0 ± 3.31 mm, \( p = 0.025 \)) and heavier (0.158 ± 0.161 vs. 0.481 ± 0.263 g, \( p = 0.070 \)) than that of wild-type cells (Fig. 4A–D). Similarly, the percentage of positive lymph node metastasis of the invasive-type cells, labeled with GFP, was higher than that of the wild-type — 25% (1/4) and 0% (0/5), respectively (Fig. 4E–F).

Relationship between CCR7 and let-7a expression in ESCC patients

Tissue samples were obtained from 17 ESCC patients during a biopsy, and their characteristics are listed in Table 1. Figure 5A shows the expression levels of CCR7 and let-7a in cancer tissue relative to those in normal tissue, defined as 1 based on quantitative RT-PCR. The graph uses a logarithmic notation, thus presenting negative values when the CCR7 and let-7a expression level ratio is less than 1. In 11 ESCC patients, CCR7 expression levels were higher in malignant tissues than in normal tissues (positive value). In contrast, in a different group of 11 ESCC patients, let-7a expression was downregulated more in malignant tissues than in normal tissues (negative value). Around nine ESCC patients exhibited a negative correlation between CCR7 and let-7a expression.

The impact of CCR7 and let-7a expression on prognosis

Recurrence of tumor was observed in six patients, and the most predominant sites were the lymph nodes. Using the median value, patients were split into two
respective groups with low and high CCR7 expression levels; patients with high CCR7 expression levels were further divided into two sub-groups with low and high let-7a expression levels. The high-CCR7/low-let-7a group (5-RFS: 37.5%) had the worst prognosis, followed by the high-CCR7/high-let-7a (5-RFS: 60.0%), and then the low-CCR7 group (5-RFS: 85.7%; \( p = 0.038 \), Fig. 5B).

Cox proportional hazards analysis identified the high-CCR7/low-let-7a group as a significant prognostic factor (Table 2).

### Discussion

This study showed that synthetic let-7a and the let-7a inhibitor respectively decreased and increased CCR7 expression levels in KYSE cells, demonstrating a negative correlation between CCR7 and let-7a expression. Therefore, the down-regulation of let-7a miRNA is a factor contributing to increased CCR7 expression levels in esophageal cancer cells and the development of malignant tumors.

High CCR7 expression level is related to lymphatic tumor metastasis and prognosis in gastric as well as colorectal cancer [16, 17]. Crucially, the elevated CCR7 expression level caused by a deficiency in let-7a expression level is intricately linked to the metastasis and progression of cancer [12]. Furthermore, CCR7 is correlated

| Variables                  | esophageal cancer patients |
|----------------------------|----------------------------|
| Gender                     | n = 17                     |
| Male                       | 14                         |
| Female                     | 3                          |
| Age (median, range)        | 69 (35–75)                 |
| UICC TNM 8th               |                            |
| Pathological T factor      |                            |
| T1                         | 3                          |
| T2                         | 1                          |
| T3                         | 13                         |
| Pathological N factor      |                            |
| N0                         | 6                          |
| N1                         | 4                          |
| N2                         | 7                          |
| Pathological Stage         |                            |
| Stage I                    | 1                          |
| Stage II                   | 7                          |
| Stage III                  | 9                          |

*UICC Union for international cancer control tnM classification of malignant tumors (8th edition)*
with lymphatic metastasis and poor prognosis in different types of breast cancer, while let-7a has been found to suppress breast cancer cell migration and invasion through the downregulation of CCR7 expression [10, 18]. Thus, our study confirmed the negative correlation between CCR7 and let-7a expression in ESCC, which strongly suggests that let-7a suppresses the expression of CCR7. Regarding the direct interaction between CCR7 and let-7a, Kim et al. used the luciferase assay to show that let-7a directly regulates CCR7 expression by binding with its 3′-UTR [10]. In addition, we can check what type of gene sequence can be regulated by specific miRNA at Target Scan Human 8.0 [19]; it showed that CCR7 is regulated by let-7a binding its 3′-UTR.

In normal human cells, CCR7 is mainly expressed in differentiated lymphocytes as well as the surface of dendritic cells, and it is thought to mediate lymphocyte migration [20]. The invasive ability of cancer cells is affected by the expression of CCR7 [10, 18]. In this study, the invasive ability of ESCC increased after transfection with synthetic anti-let-7a because of the increased and decreased CCR7 and let-7a expression levels, respectively. After the invasion assay, the expression level of CCR7 in the invasive-type cells increased while that of let-7a decreased, in comparison with those in the wild-type. Therefore, the respective increase and decrease in CCR7 and let-7a expression levels are important factors affecting the invasive ability of ESCC. We also used the highly invasive cell line for in vivo experiments on mice. The implanted primary tumor of the invasive-type was larger in size and mass than that of the wild-type, and the percentage of positive lymph node metastasis was higher as well. Therefore, the highly invasive cell line was more likely to cause and accelerate lymph node metastasis in vivo.

We also investigated the expression of CCR7 and let-7a miRNA in ESCC patient tissues and demonstrated that they tend to have a higher CCR7 and a lower let-7a miRNA expression levels than normal tissues. Furthermore, we found that the expression levels of CCR7 and let-7a miRNA can stratify the prognosis of the ESCC patients. In the two high-CCR7 groups had a worse prognosis than those in the low-CCR7 group, with the low-let-7a group exhibiting a worse prognosis than the high-let-7a group. These results suggest that during carcinogenesis, the suppression of CCR7 expression diminishes owing to the decreased expression level

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**Table 2** Univariate Cox proportional hazard analysis

| Variables                  | HR  | 95% CI          | p-value |
|----------------------------|-----|-----------------|---------|
| Age                        |     |                 | 0.547   |
| < 70                       | 1.00|                 |         |
| ≥ 70                       | 1.644| 0.326–8.306     |         |
| Pathological T factor      | 1.00|                 | 0.381   |
| T1-2                       |     |                 |         |
| T3-4                       | 2.639| 0.302–23.083    |         |
| Pathological N factor      |     |                 | 0.831   |
| negative                   | 1.00|                 |         |
| positive                   | 0.831| 0.152–4.552     |         |
| CCR7 high / Let-7a low     |     |                 | 0.028   |
| None                       | 1.00|                 |         |
| Yes                        | 6.476| 1.222–34.309    |         |

**Fig. 5** Relationship between CCR7 and let-7a expression and the prognostic implication. A CCR7 and let-7a expression levels in ESCC patients. B Recurrence-free survival curve for ESCC patients in the low-CCR7, high-CCR7/high-let-7a, and high-CCR7/low-let-7a groups (p = 0.038)
of let-7a, and that these changes may affect the prognosis of ESCC patients. Supporting this finding, He et al. [15] found that the plasma levels of let-7a miRNA were significantly lower in ESCC patients than in healthy participants.

However, our study has three main limitations. First, while we confirmed the negative correlation between CCR7 and let-7a, we did not account for the potential influence of other oncogenes controlled by let-7a. Second, in the mouse experiment, we demonstrated the in vivo malignancy of the synthesized highly invasive cell line but did not measure CCR7 or let-7a expression levels in metastatic lymph nodes. Nevertheless, the relationship between CCR7 expression level and lymph node metastasis in ESCC cells has been examined in a previous study [6]. Third, the number of cell lines and patient tissue samples used in this study was relatively small; therefore, additional studies are required to validate these results.

Conclusions
The expression of CCR7 was found to be downregulated by let-7a miRNA in ESCC cells. Thus, a decrease in let-7a expression level led to an increase in the expression level of CCR7 in ESCC cells, which consequently acquired increased invasive ability and malignancy, resulting in a worse prognosis for patients with ESCC.

Abbreviations
3′-UTR: 3′-untranslated region; CCR7: C–C chemokine receptor type 7; cDNA: Complementary DNA; dsRNA: Double-stranded RNA; ESCC: Esophageal squamous cell carcinoma; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GFP: Green fluorescent protein; miRNA: MicroRNA; RFS: Recurrence-free survival; RT-PCR: Reverse-transcription polymerase chain reaction.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12885-022-10178-2.

Additional file 1: Table S1. Quantitative RT-PCR analysis.

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Authors’ contributions
MY and KF designed the study. MY is the first author, while KF checked the manuscript and advised the first author on its preparation. MS and IT contributed to the collection of data and approved the final version to be submitted. RN and HK conceived the study and supervised all the experiments performed. HT and YK supervised the study as principal investigators. All participants provided their commentary, corrected, and approved the final manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials
The datasets KYSE-350, 510, 590, 1260, 1440, and 2400 for this study can be found and are available in the JCRB cell bank (https://cellbank.nibiohn.go.jp/english/).

Declarations
Ethics approval and consent to participate
This study was approved by the Institutional Review Board of Keio University (approval number: 20120340).

Consent for publication
All participants provided informed, written consent for their participation in the study. All procedures were performed in accordance with the Helsinki Declaration.

Competing interests
The authors declare that they have no competing interests.

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