Short Communication

The SQUU-B cell line spreads its metastatic properties to nonmetastatic clone SQUU-A from the same patient through exosomes

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Article info

Article history:
Received 29 June 2015
Received in revised form 28 August 2015
Accepted 29 September 2015

Keywords:
Oral squamous carcinoma cells
invasion
exosome

Emerging evidence indicates that cancer-derived exosomes increase the tumorigenic potential of tumor cells by reprogramming the cells associated with the tumor microenvironment. Our aim was to examine the cross talk via exosomes between two oral squamous cell carcinoma (OSCC) clones from the same patient. Our data showed that exosomes derived from highly metastatic SQUU-B cells conferred metastatic ability to nonmetastatic SQUU-A cells and subsequently reduced mRNA expression of cytokeratin 13, which is strongly linked to malignant transformation of OSCCs. The results suggest that multiple cell clones secrete their unique exosomes within the malignant tumor mass, which mediate paracrine interactions with other cell clones and affect clinical prognosis.

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1. Introduction

Local invasion is a central process of the metastatic cascade [1]. Many reports have shown that there are extracellular modulators in the tumor microenvironment that can influence the invasive potential [2]. Among them, exosomes recently emerged as crucial components of cell-to-cell and cell-to-matrix communication related to invasive and metastatic processes [3,4]. Exosomes are small membrane vesicles of endocytic origin that are released from various types of cells into the extracellular environment after fusion of multivesicular bodies with the plasma membrane [5]. Recent evidence highlights the ability of tumor-released exosomes to stimulate invasion and migration in various cancer models [4–7]. Nevertheless, the majority of these studies have been focused on the activity of tumor-related exosomes toward stromal cells or malignant cells from another patient.

Oral squamous cell carcinoma (OSCC) is a type of invasive epithelial cancer that carries a high risk of lymph node metastasis and shows poor prognosis [8]. Our aim was to examine the cross talk via exosomes between two OSCC clones from the same patient. We demonstrated that a highly metastatic OSCC clone (SQUU-B) conferred metastatic potential to a nonmetastatic OSCC clone (SQUU-A) derived from the same tumor microenvironment. Our findings provide new insights into the function of tumor-derived exosomes: the cross talk among different malignant cell clones in the same tumor microenvironment.

2. Materials and methods

2.1. Cell culture

The cell lines SQUU-A and SQUU-B (established from local recurrence events of a tongue cancer [9]) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Nacalai Tesque Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum (BioWest, Nuaillé, France) at 37 °C in a humidified atmosphere containing 5% of CO2.

2.2. The cell invasion assay

The hardware for the assay was assembled from 8-μm-pore Transwell inserts (Corning Inc., Corning, NY) as upper chambers and 24-well plates as lower chambers. The cell culture inserts were coated with 100 μL of Matrigel (diluted 1:20 with DMEM). After removal of the medium, 5 × 10⁴ SQUU-A or SQUU-B cells were plated in the upper chamber and incubated for 21 h.

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The inserts were then stained with Giemsa. The invading cells were detected and counted under a microscope (EVOS Cell Imaging System, Life technologies Corporation, Carlsbad, CA).

2.3. Real-time PCR

Total RNA extraction, reverse transcription, and real-time PCR analysis were performed by means of the High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany), Verso cDNA Synthesis Kit (Life Technologies), and LightCycler 480 System (Roche Diagnostics), respectively.

2.4. Statistical analysis

The Mann-Whitney U test was used to analyze differences in relative mRNA expression (data from real-time PCR) and in the numbers of invasive cells. Differences with a \( P \) value less than 0.05 were considered statistically significant.

![Graph showing mRNA abundance for CK13 and CK17 in SQUU-A and SQUU-B cells.](image)

**Fig. 1.** Characterization of the SQUU-A and SQUU-B cell lines. (A) Relative expression levels of KRT13 (cytokeratin 13) and KRT17 mRNA in SQUU-A and SQUU-B cells. Analysis of GAPDH was used as an internal control. Gene expression that was normalized to ACTB (β-actin) data manifested the same trends (data not shown). The cycling conditions were as follows: 95 °C for 10 min (hot-start PCR), followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 s (for an acquisition of the cDNA amplification step). The PCR primer sequences (forward and reverse, respectively) and amplicon sizes were as follows: GAPDH (5'-AGC CAC ATC GCT CAG ACA C-3' and 5'-GCC CAA TAC GAC CAA ATC C-3', 66 bp), ACTB (5'-CCA ACC GCG AGA AGA TGA-3' and 5'-CCA GAG GCG TAC AGG AG-3', 97 bp), KRT13 (5'-GAA GAG CTC CGG GAC AAG A-3' and 5'-TGA GCC TGA AGT CTA G-3', 100 bp), and KRT17 (5'-AAT TGA GGA GCT GCA GAA CAA-3' and 5'-AAA CTT GGT GCG GAA GTC AT-3', 109 bp). TaqMan probes specific for each sequence were selected from the LightCycler Universal Probe Library (Roche Diagnostics): probes # 60 (for GAPDH), # 64 (for ACTB and KRT13), and # 71 (for KRT17). The experiments were performed three times in duplicate. The error bars represent SEM. **P < 0.001.** (B) The Matrigel invasion assay and Giemsa staining of SQUU-A and SQUU-B cells cultured in serum-free Dulbecco’s modified Eagle’s medium (DMEM). The upper panels and the lower panels are representative images acquired at weaker (50 x) and stronger magnification (200 x), respectively. Scale bars: 100 μm. Quantification was performed by counting the number of invasive cells in a visual field at 200 x magnification. The analysis was performed three times in triplicate on four random visual fields per insert.

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3. Results and discussion

3.1. Verification and characterization of SQUU-A and SQUU-B cells

First, we successfully confirmed that cytokeratin (CK) 13 and CK17 expression levels in SQUU-A cells were significantly higher than those in SQUU-B cells (Fig. 1A), in line with other studies [9,10]. We next performed cell invasion assays based on the migratory ability of invasive and metastatic cancer cells [11]. As shown in Fig. 1B, the invasive ability of SQUU-B cells was markedly greater than that of SQUU-A cells. Given that the migratory and invasive abilities of cancer cells are strongly related to the metastatic process physiologically [12], these data confirmed that SQUU-A and SQUU-B cells retained their nonmetastatic and metastatic state, respectively, in vitro [9].

3.2. The SQUU-B cell culture supernatant can induce invasive properties

We next tested whether soluble factors can mediate cancer cell invasiveness in the two cell culture supernatants. A 17.5-fold-concentrated SQUU-A cell culture supernatant had no effect on the SQUU-B cell culture supernatant (supA) or SQUU-B cells (supB) contained serum-free Dulbecco's modified Eagle's medium (DMEM). After 17.5-fold concentration by means of Amicon Ultra-15 (molecular weight cutoff: 100,000 Da; Merck Millipore, Darmstadt, Germany; in panels A and B) and further concentration with Amicon Ultra-15 (molecular weight cutoff: 30,000; Merck Millipore; panel B), the concentrated supernatant was filtered through a membrane (pore size 0.45 μm) and then applied to culture inserts for invasion assays. The upper panels and the lower panels are representative images acquired at weaker (50 ×) and stronger magnification (200 ×), respectively. Scale bars: 100 μm. Quantification involved counting the number of invasive cells. All the experiments were performed twice in triplicate.

Fig. 2. SQUU-B-derived culture supernatant induced invasiveness in SQUU-A cells. Matrigel invasion assays and Giemsa staining of SQUU-A and SQUU-B cells. The culture supernatant from SQUU-A cells (supA) or SQUU-B cells (supB) contained serum-free Dulbecco's modified Eagle's medium (DMEM). After 17.5-fold concentration by means of Amicon Ultra-15 (molecular weight cutoff: 100,000 Da; Merck Millipore, Darmstadt, Germany; in panels A and B) and further concentration with Amicon Ultra-15 (molecular weight cutoff: 30,000; Merck Millipore; panel B), the concentrated supernatant was filtered through a membrane (pore size 0.45 μm) and then applied to culture inserts for invasion assays. The upper panels and the lower panels are representative images acquired at weaker (50 ×) and stronger magnification (200 ×), respectively. Scale bars: 100 μm. Quantification involved counting the number of invasive cells. All the experiments were performed twice in triplicate.

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invasive potential of SQUU-A and SQUU-B cells, whereas a 17.5-fold-concentrated SQUU-B supernatant significantly enhanced the invasiveness of SQUU-A cells (Fig. 2A). We then performed another invasion assay with SQUU-A cells using the supernatants fractionated by molecular size: < 30 kDa, 30–100 kDa, and > 100 kDa (Fig. 2B). As a result, only the fraction > 100 kDa from SQUU-B cells induced an invasive activity in SQUU-A cells; the size of the effect was similar to that in Fig. 2A. This result suggests that the factors of invasiveness were estimated to be molecules > 100 kDa or nanoparticles (e.g., exosomes) with a diameter > 10 nm [13].

3.3. Exosomes from SQUU-B cells confer the invasive potential on SQUU-A cells

We next isolated exosomes from cultured SQUU-A cells (exosomes exoA) and SQUU-B cells (exosomes exoB) and added them to the culture media of each cell line in the invasion assays (these exosome supplements were estimated to be equivalent to a 17.5-fold concentrated supernatant). After purifying the exosomes, we confirmed the presence of common exosomal markers, CD9 and CD81 [14]; this result was consistent with successful purification of the exosome samples (Fig. 3A). During the invasion assays, exoB induced an invasive activity in SQUU-A cells (Fig. 3B), and the effect was stronger than that of the concentrated supernatant (Fig. 3B compared to Fig. 2A). This result means that exoB spread the invasive potential to SQUU-A cells. Because it was previously reported that the change in expression patterns of CKs is closely related to the invasive and metastatic properties of cancer [9,10,15], we analyzed mRNA expression of CK13 (KRT13) and CK17 (KRT17) 21 h after introduction of exosomes into the SQUU-A cell culture. The mRNA levels of KRT13, whose down regulation is strongly linked to malignant transformation [9,15], but not KRT17 mRNA levels, decreased in SQUU-A cells in a time-dependent manner as a result of addition of exoB to the culture medium (data not shown and Fig. 3C, respectively). Therefore, it appears that cytoskeletal system can enhance cancer cell invasiveness and the metastatic potential via exosomes.

4. Conclusions

Our study shows the spread of the metastatic potential between OSCC cell clones (derived from the same tumor mass) via exosomes. Our findings suggest that there is an exosome-mediated system of interaction among tumor cell clones, and this system may affect clinical prognosis.

Ethical approval

The study did not require any ethical approval.

Fig. 2. (continued)
Conflict of interest

The authors have no conflicts of interest to disclose.

References


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