Overexpression of fibronectin confers cell adhesion-mediated drug resistance (CAM-DR) against 5-FU in oral squamous cell carcinoma cells

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Abstract. The tumor-associated microenvironment has been shown to protect tumor cells from treatment, and the extracellular matrix (ECM) is known to affect drug resistance as a key regulator of the tumor microenvironment. However, little is known about cell adhesion-mediated drug resistance (CAM-DR) due to cell-ECM contact in patients with oral squamous cell carcinoma (OSCC). In the present study, we evaluated the ECM molecule fibronectin (FN) using DNA microarray data obtained from parental and 5-FU-resistant OSCC cell lines. We investigated the effects of cell adhesion to FN on 5-FU resistance in OSCC cells and examined the activation of FN receptor β1 integrin-mediated survival regulators such as ILK, Akt and NF-κB. In addition, we investigated whether FNIII14, a 22-mer peptide derived from FN that potently prevents β1 integrin-mediated adhesion to FN, could overcome CAM-DR against 5-FU in OSCC cells and examined the activation of survival regulators and apoptosis-related molecules. Consequently, we obtained the following results. FN was extracellularly overexpressed in the 5-FU-resistant cells compared with that observed in the 5-FU-sensitive cells. Cell adhesion to FN enhanced 5-FU resistance and activated integrin-mediated ILK/Akt/NF-κB survival signaling in the 5-FU-resistant OSCC cells. Furthermore, the inhibition of cell adhesion to FN by FNIII14 enhanced chemosensitivity to 5-FU and apoptosis by suppressing ILK/Akt/NF-κB signaling in the 5-FU-resistant cells. These novel findings demonstrate that FN is a potentially useful biomarker and therapeutic target for improving the treatment of OSCC, particularly in the setting of 5-FU resistance.

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Abbreviations: FN, fibronectin; 5-FU, 5-fluorouracil; CAM-DR, cell adhesion-mediated drug resistance; OSCC, oral squamous cell carcinoma

Key words: fibronectin, 5-fluorouracil, cell adhesion-mediated drug resistance, oral squamous cell carcinoma

Introduction

The widely used chemotherapeutic agent, 5-fluorouracil (5-FU), is a key drug for oral cancer treatment and is known to be a potent radiosensitizer (1). Clinical studies have shown that 5-FU-based chemotherapy and chemoradiotherapy improve the survival of patients with head and neck cancer, including oral squamous cell carcinoma (OSCC) (2-4).

However, chemoresistance continues to be a major clinical obstacle to the successful treatment of OSCC. For example, patients with progressive and recurrent OSCC exhibit a poor prognosis (5,6). This is often due to treatment failure in the setting of progressive, recurrent disease that is resistant to 5-FU-based chemotherapy (7,8). On the other hand, in many cancers sensitive to 5-FU, resistance is ultimately acquired through continuous drug administration (9-11). In such cases, the drug induces alterations in the gene expression and signaling cascades that mediate resistance (12,13).

Several investigators have shown that the tumor microenvironment can modulate drug resistance (14), and it has been reported that the extracellular matrix (ECM) plays a pivotal role in cancer progression and the response to therapy (15). Integrins comprise a large family of heterodimeric ECM receptors that transmit critical signals by interacting with the ECM (16). The α subunit typically confers specificity for the ligand, whereas the β subunit couples to downstream signaling pathways (17). β1 integrin is the main receptor subunit, while α4β1 and α5β1 integrins are some of the major cellular receptors for the ECM protein fibronectin (FN) (16). β1 integrin signaling has been shown to play a significant role in mediating resistance to cytotoxic chemotherapy by enhancing cell survival in patients with hematologic

malignancies and solid tumors (18-21). Increased adhesion of β1 integrin to FN triggers the activation of the essential β1 integrin signaling mediator, integrin-linked kinase (ILK), which in turn activates Akt and NF-κB, thus contributing to cell survival (22). The form of drug resistance mediated by cell-ECM contact is called cell adhesion-mediated drug resistance (CAM-DR) (23). However, little is known about the contribution of CAM-DR in the pathogenesis of OSCC.

FNIII14, a 22-mer peptide derived from the 14th type III module of FN, has a significant inhibitory effect on β1 integrin-mediated adhesion to FN (24). Thus far, the potential of preventing cell-FN interactions using FNIII14 has been confirmed in the setting of hematologic malignancies (24-26), and it has been reported that combination therapy consisting of anticancer drugs plus FNIII14 is a promising treatment for improving the prognosis of acute myelogenous leukemia patients (25). However, the inhibitory effects of FNIII14 in solid tumor cells have not been fully elucidated.

In the present study, in order to identify novel targets implicated in CAM-DR in 5-FU-resistant OSCC, we identified ECM molecules that are commonly upregulated in two 5-FU-resistant OSCC cell lines. Based on the results, we found that the resistant cells overexpressed FN and that cell adhesion to FN confers CAM-DR against 5-FU in OSCC cells via the activation of ILK/Akt/NF-κB survival signaling. Furthermore, we demonstrated that FNIII14 sensitizes resistant cells to 5-FU with enhanced apoptosis by suppressing ILK/Akt/NF-κB signaling.

Materials and methods

Cell line and cell culture. Human OSCC cell lines derived from primary tumors, Ca9-22 (lower gingival cancer) and SAS (tongue cancer), were obtained from the RIKEN BioResource Center (Ibaraki, Japan) and cultured with DMEM supplemented with 10% FBS and maintained under humidified 5% CO_2 incubation at 37˚C. The experiments carried out to analyze the effects of cell adhesion to FN were performed using FN-coated dishes (BD Bioscience, Bedford, MA, USA).

Establishment of 5-FU-resistant OSCC cell lines. To establish 5-FU-resistant cell lines, Ca9-22 cells were continuously exposed to increasing concentrations of 5-FU over two years. The surviving cells were cloned, and one of the most 5-FU-resistant sublines, designated Ca9-22/FR2, was selected. The Ca9-22/FR2 cell line can survive exposure to 2.0 μg/ml of 5-FU. To ensure continued resistance, the cell line was maintained in a culture in DMEM containing 2.0 μg/ml of 5-FU. In order to eliminate the effects of 5-FU on the experimental outcomes, the resistant cells were cultured in a drug-free medium for at least two weeks before all experiments. Another 5-FU-resistant cell line, SAS/FR2, was previously established by us in the same way (27).

Cell proliferation assay. To assess the normal degree of proliferation, viable cells treated without 5-FU were quantified every 24 h using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).

Drug sensitivity assays. The cells ($3x10^3$/well) were seeded onto 24-well plates and incubated in DMEM with 10% FBS at 37˚C. After 24 h, DMEM containing various concentrations (0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.5, 25.0 and 50.0 μg/ml) of 5-FU was added to each well, and the cells were incubated at 37˚C for another 72 h. For the assay, WST-8 (Cell Counting Kit-8, Dojindo) was added to each well, and the plate was incubated for an additional 2 h at 37˚C. The absorbance was measured at 450 nm using a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA). Nine wells were used for each drug concentration and the experiment was performed in triplicate. The 50% inhibitory concentration (IC_{50}) was calculated from the survival curve.

Gene expression microarrays. The cRNA was amplified, labeled and hybridized to an Agilent Human GE 4x44K v2 Microarray (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. All hybridized microarrays were scanned by an Agilent scanner, and the signals of all probes were calculated using the Feature Extraction software (9.5.1.1) program (Agilent Technologies).

Data analysis and filter criteria. The raw signal intensities and flags for each probe were calculated from the hybridization intensities and spot information, according to the procedures recommended by Agilent. In addition, the raw signal intensities of two samples were log2-transformed and normalized using a quantile algorithm (28) on a Bioconductor (29,30). We selected probes that identified the 'P' flag in both the control and experimental samples. To identify up- or downregulated genes, we calculated the Z-scores (30) and ratios (non-log scaled fold change) from the normalized signal intensity of each probe. We thereafter established the criteria for the regulated genes: (upregulated genes) Z-score ≥2.0 and ratio ≥1.5-fold, (downregulated genes) Z-score ≤-2.0 and ratio ≤0.66.

Total RNA extraction and real-time qPCR (RT-qPCR). Total RNA was isolated using the RNeasy Plus mini kit (Qiagen, Hilden, Germany) according to the instructions provided by the manufacturer and reverse transcribed into cDNA using the ReverTra Ace®qPCR RT Kit (Toyobo, Osaka, Japan).

For real-time quantitative PCR (qRT-PCR), each reaction mixture was diluted 5-fold with DNase/RNase-free water (Life Technologies, Carlsbad, CA, USA), and 4 μl of each mixture was subjected to PCR. The reactions were run using the Thunderbird SYBR qPCR Mix (Toyobo) on a Light Cycler 1.5 (Roche Diagnostics, Indianapolis, IN, USA). The comparative Ct (ΔΔCt) method was used to determine the fold changes in the expressions using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each sample was run in triplicate. The following primers were used: fibronectin (forward, 5'-AGCCGCCACGTGCCAGGATTAC-3'; reverse, 5'-CTTA TGGGGGTGGCCGTTGTGG -3'); GAPDH (forward, 5'-CAA CAGCCTCAAGATCATCAGC-3'; reverse, 5'-TTCTAGACG GCAGGTCAGGTC-3'). The cycling conditions were: initial denaturation at 98˚C for 5 min followed by 45 cycles at 98˚C for 15 sec, 58˚C for 30 sec and 72˚C for 60 sec. The experiments were performed in triplicate.

Western blot analysis. Whole-cell proteins were separated using 5.0 or 10.0% SDS-PAGE, transferred onto nitrocellulose

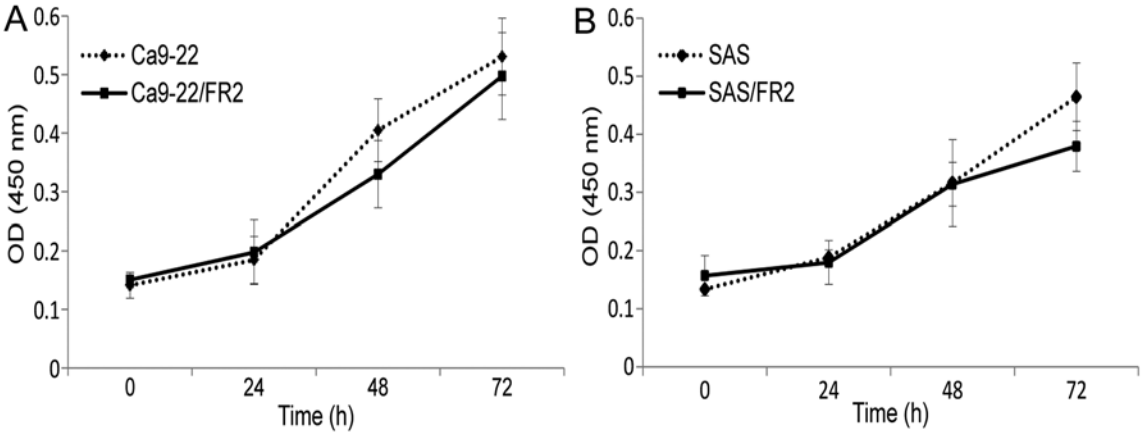

Figure 1. Cellular growth activities of the parent (Ca9-22, SAS) and 5-FU-resistant (Ca9-22/FR2, SAS/FR2) OSCC cell lines treated without 5-FU. (A) Ca9-22 and Ca9-22/FR2. (B) SAS and SAS/FR2. The degree of cell proliferation was monitored for three days using the Cell Counting Kit-8. The results represent the mean ± SD of three independent experiments.

membranes and probed with antibodies against fibronectin (1:3,000; Acris Antibodies, San Diego, CA, USA), ILK (1:1,000; Cell Signaling Technology, Danvers, MA, USA), phospho-ILK (Thr173) (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Akt (1:1,000; Cell Signaling Technology), phospho-Akt (Ser473) (1:2,000; Cell Signaling Technology), NF-κB p65 (1:1,000; Epitomics/Abcam, Burlingame, CA, USA), phospho-NF-κB p65 (Ser536) (1:1,000; Cell Signaling Technology), caspase-3 (1:1,000; Cell Signaling Technology), cleaved caspase-3 (1:1,000; Cell Signaling Technology), PARP (1:1,000; Cell Signaling Technology) and β-actin (1:10,000; Sigma, St Louis, MO, USA). Following overnight incubation, the membranes were washed and incubated wit horseradish peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark). Finally, the membranes were washed and visualized using the ECL Plus detection kit (GE Healthcare, Buckinghamshire, UK).

Enzyme-linked immunosorbent assay (ELISA) for the detection of secreted FN. The cell lines were cultured in 10% serum containing DMEM with 2.0 μg/ml of 5-FU or culture medium alone. At 24, 48, 60 and 72 h after treatment, the conditioned media were collected, and the concentration of FN was measured using the directions of the manufacturer's in the human fibronectin ELISA kit (Biomedical Technologies, Stoughton, MA, USA). The concentration of FN was calibrated from a dose response curve based on reference standards. The experiments were performed in triplicate.

Flow cytometric analysis of the cell surface integrin expression. The cell surface integrin expression was analyzed using a FACSVerse (Becton-Dickinson, Franklin Lakes, NJ, USA). All of the following antibodies used in the flow cytometric analysis were obtained from BioLegend (San Diego, CA, USA). The expression of integrin subunits was determined using fluorescein isothiocyanate (FITC)-conjugated anti-CD49d (α4 integrin), FITC-conjugated anti-CD49e (α5 integrin) and phycoerythrin (PE)-conjugated anti-CD29 (β1 integrin) antibodies. FITC-conjugated IgG1, IgG2b isotype control and non-labeled IgG2b isotype control were used as negative controls. The data were analyzed using the FlowJo software program (Treestar, Ashland, OR, USA).

FNIII14. A synthetic peptide, FNIII14, corresponding to residues 1835-1855 of FN (31) was obtained from Operon Biotechnologies (Tokyo, Japan). In the experiments performed to assess the effects of blocking cell-FN contact with FNIII14, the cells were treated with FNIII14 at a concentration of 100 μg/ml.

Statistical analysis. The differences in the mean values between the two groups were statistically analyzed using Student's t-test. All p-values were based on two-tailed statistical analyses, and a p-value of <0.05 was considered to be statistically significant ($^{*}p<0.05$; $^{**}p<0.01$). All statistical analyses were performed using the JMP 9 software program (SAS Institute Inc., Cary, NC).

Results

Growth of the 5-FU-resistant OSCC cell lines and the cytotoxic effects of 5-FU in the cells. The cellular growth activities of the two 5-FU-resistant cell lines treated without 5-FU were evaluated for 72 h. No significant differences were found between the cellular growth of the 5-FU-sensitive (SAS, Ca9-22) and -resistant (SAS/FR2, Ca9-22/FR2) cell lines (Fig. 1A and B), thus suggesting that the 5-FU resistance of OSCC cells is not due to increased cell proliferation. We next examined the cytotoxic effects of 5-FU in the 5-FU-sensitive and -resistant cells. Fig. 2A and B show the drug sensitivity curves for the two sets of cell lines after 72 h of incubation with various concentrations of 5-FU. After 72 h of incubation with 2.0 μg/ml of 5-FU, increased apoptotic cell changes (shrinkage and rounding-up of the cells) were noted in the 5-FU-sensitive cells compared with that observed in the 5-FU-resistant cells under phase-contrast microscopy (Fig. 2C and D). A comparison of the IC_{50} value for 5-FU

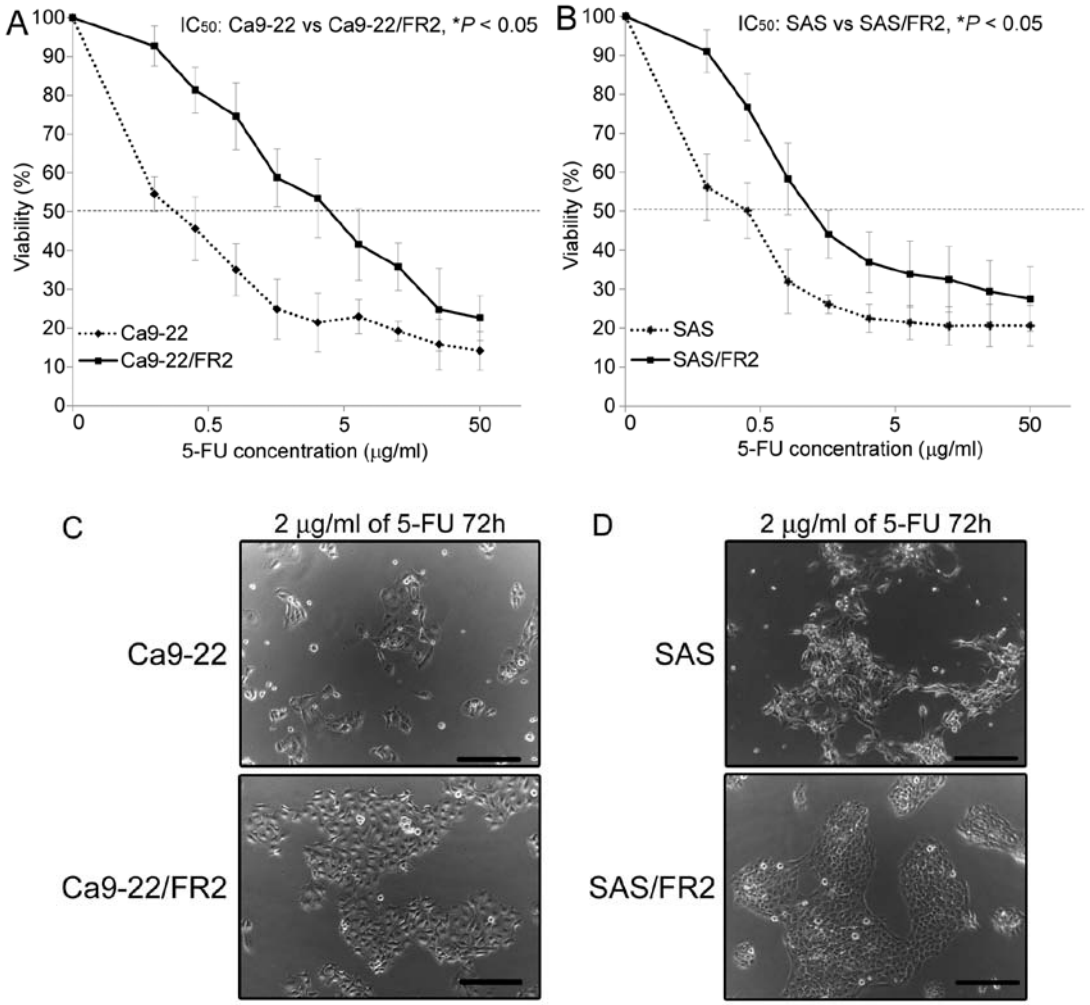

Figure 2. Cytotoxic effects of 5-FU against the 5-FU-sensitive and -resistant OSCC cell lines. (A and B) The cell survival of the parental (Ca9-22, SAS) and resistant (Ca9-22/FR2, SAS/FR2) cells was monitored 72 h after incubation with various concentrations of 5-FU using the Cell Counting Kit-8. The results represent the mean ± SD of three independent experiments. (C and D) Morphological differences under phase-contrast microscopy between the parental and resistant cells 72 h after treatment with 2.0 μg/ml of 5-FU. Bar, 100 μm.

revealed that Ca9-22/FR2 and SAS/FR2 showed significantly higher resistance (13.7- and 3.0-fold, respectively) to 5-FU than the parent cells.

DNA microarray analysis and the upregulation of FN in the 5-FU-resistant OSCC cell lines. To identify genes that are differentially expressed between 5-FU-sensitive and -resistant cell lines, a DNA microarray analysis that contains 34,127 oligonucleotide-based probe sets was performed. The results of the analysis showed that the expression levels of 546 genes were elevated, while those of 442 genes were decreased, in the Ca9-22/FR2 cells compared with those observed in the parental Ca9-22 cells. On the other hand, the expression levels of 598 genes were elevated and the expression levels of 447 genes were decreased in the SAS/FR2 cells compared with those observed in the parental SAS cells. Among these genes, we narrowed our search to ECM molecules and found that the expression level of the FN gene was remarkably increased in the Ca9-22/FR2 cells (ratio, 27.9-fold; Z-score, 5.7) and that an increased expression of the FN gene was further confirmed in the SAS/FR2 cells (ratio, 7.2-fold; Z-score, 2.5) (Table I). In addition, FN was the only ECM molecule that was significantly upregulated in both the resistant cell lines. It has been reported that cell adhesion to ECM proteins, such as FN, regulates apoptosis and cell survival in a wide variety of cell types (18,32-35). Therefore, we focused on the analysis of FN in the present study. We first confirmed the expression levels of FN in the 5-FU-sensitive and -resistant cells at both the gene and protein levels (Fig. 3A-D). Consistent with the data obtained

Table I. Comparison of FN genes between the 5-FU-sensitive and -resistant OSCC cell lines using a DNA microarray analysis.

Cell line	Ratio	Z-score
Ca9-22/FR2	27.9	5.7
SAS/FR2	7.2	2.5

Ratio represents the gene expression ratio of resistant/sensitive with fold change.

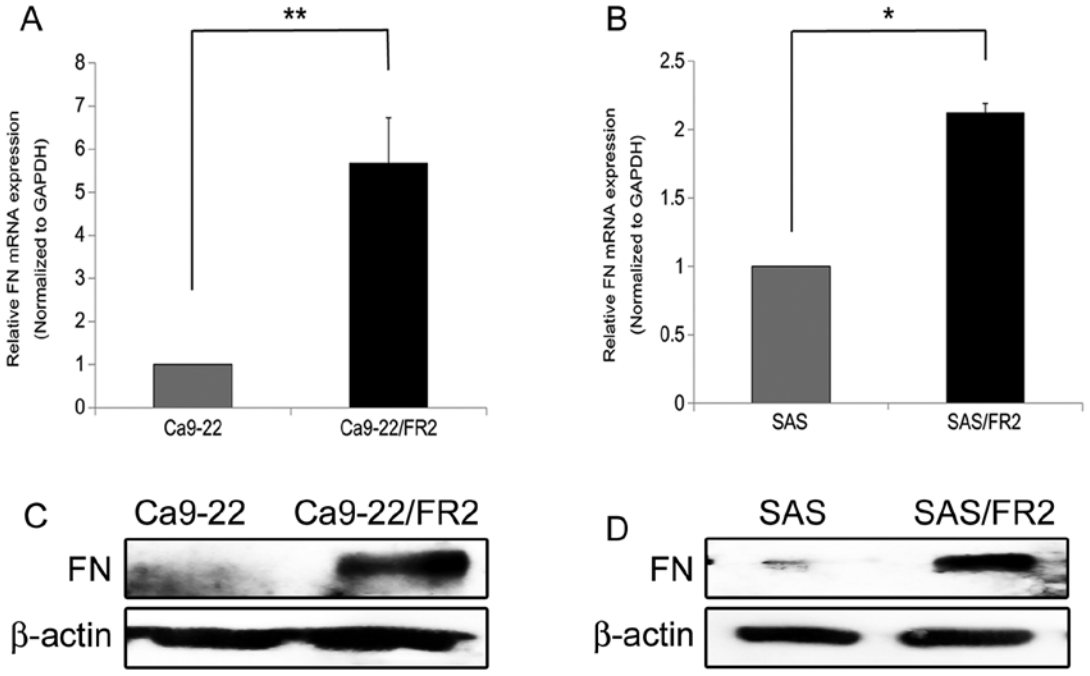

Figure 3. Overexpression of FN in the 5-FU-resistant OSCC cells treated without 5-FU. (A and B) The mRNA levels of FN in the 5-FU-sensitive (Ca9-22, SAS) and -resistant (Ca9-22/FR2, SAS/FR2) cells. Total RNA was extracted, and the expression levels of FN mRNA were analyzed using RT-qPCR. The results represent the mean ± SD of three independent experiments. *P<0.05; **P<0.01. (C and D) The protein levels of FN in the 5-FU-sensitive and -resistant cells. Whole-cell lysates were prepared, and the expression levels of FN proteins were examined using a western blot analysis.

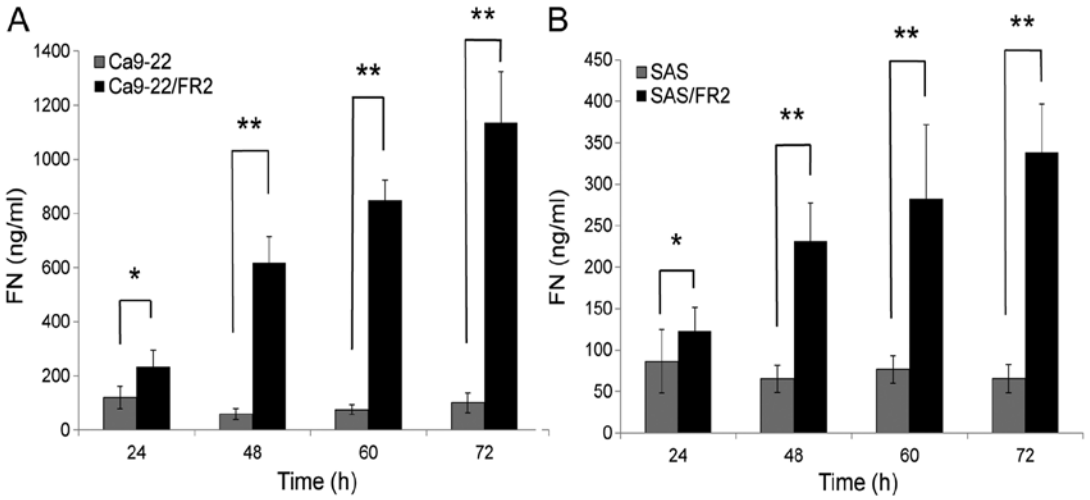

Figure 4. The release of FN in the 5-FU-resistant OSCC cells was enhanced compared with that observed in the 5-FU-sensitive OSCC cells. (A and B) The amount of FN in the conditioned media of the 5-FU-sensitive and -resistant cells cultured on non-coated dishes was measured using ELISA kits after 24, 48, 60 and 72 h of incubation in DMEM containing 10% FBS. The results represent the mean ± SD of three independent experiments. *P<0.05; **P<0.01.

from the DNA microarray analysis, the 5-FU-resistant cells clearly expressed higher levels of FN than the parental 5-FU-sensitive cells.

The release of FN in the 5-FU-resistant OSCC cells was enhanced compared with that observed in the 5-FU-sensitive OSCC cells. To determine whether the upregulated FN in the resistant cells is extracellularly released, we measured the amount of FN in the conditioned media of the 5-FU-sensitive and -resistant cells using ELISA kits after 24, 48, 60 and 72 h of incubation without 5-FU treatment (Fig. 4A and B). Considering that there were no differences in cell proliferation between the 5-FU-sensitive and 5-FU-resistant cells, it was confirmed that the release of FN in the resistant cells was significantly increased compared with that observed in the sensitive cells. This result suggests that resistant cells possess the capacity to create the tumor-associated microenvironment that protects tumor cells from therapy.

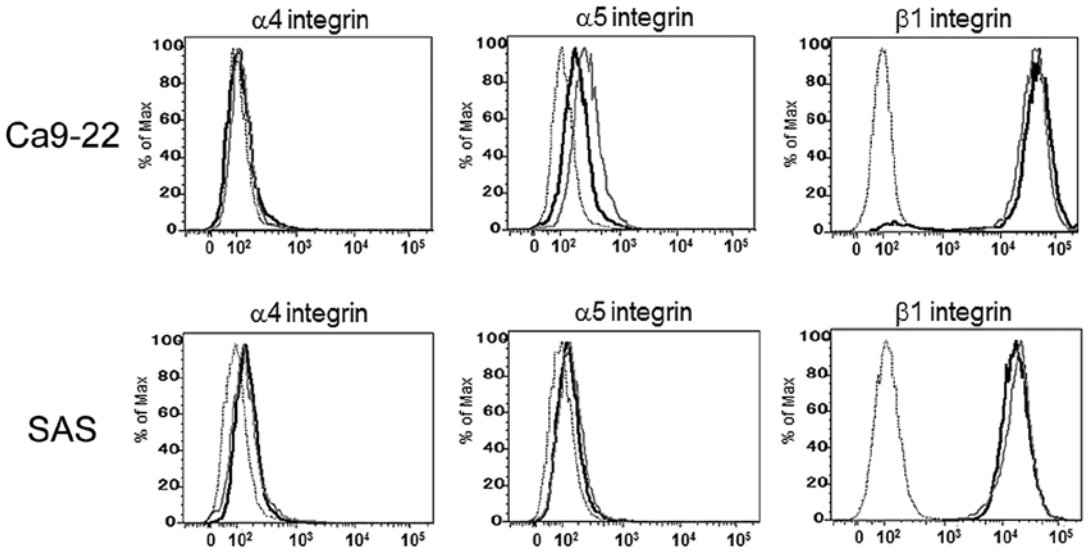

Figure 5. Expression levels of cell surface integrins in the 5-FU-sensitive and -resistant OSCC cells. The expression of integrin subunits in each cell line were determined using a flow cytometric analysis with monoclonal antibodies for α4, α5 and β1 integrin. Each cell line was incubated with an integrin-specific antibody or an irrelevant control antibody, followed by incubation with the secondary antibody. The black lines represent the resistant cell lines (Ca9-22/FR2 or SAS/FR2), and the grey lines represent the sensitive cell lines (Ca9-22 or SAS). The histograms are representative of three independent experiments.

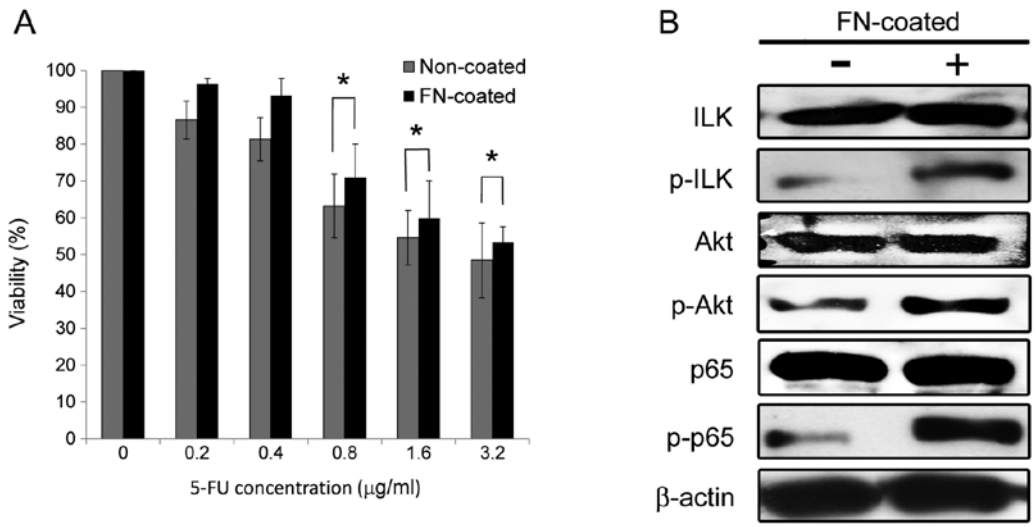

Figure 6. Culture on FN-coated dishes enhanced 5-FU resistance and activated integrin-mediated ILK/Akt/NF-κB survival signaling in the 5-FU-resistant OSCC cells. Ca9-22/FR2 cells were seeded on non-coated or FN-coated 24-well dishes and incubated in DMEM containing 10% FBS for 24 h. (A) Following an additional 72 h of 5-FU treatment, the degree of cell survival in the 5-FU-resistant cells was monitored using the Cell Counting Kit-8. The results represent the mean ± SD of three independent experiments. *P<0.05. (B) Following an additional 72 h of 5-FU treatment, whole-cell lysates were prepared, and the expression level of each protein was examined using a western blot analysis.

Expression levels of cell surface FN receptors in the 5-FU-sensitive and -resistant OSCC cells. To elucidate the influence of the cell surface FN receptor expression on 5-FU resistance, we next examined the expression levels of the α4, α5 and β1 integrin subunits using a flow cytometric analysis. As shown in Fig. 5, there were no differences in the expression levels of these FN receptor components between the 5-FU-sensitive and -resistant cells.

Culture on FN-coated dishes enhances 5-FU resistance and activates integrin-mediated ILK/Akt/NF-κB survival signaling in the 5-FU-resistant OSCC cells. The Ca9-22/FR2 cells exhibited much higher elevation of 5-FU resistance and FN production than the SAS/FR2 cells compared with each parental cell line, as shown in Figs. 2-4, suggesting that cell adhesion to FN exerts protective effects against 5-FU more potently in Ca9-22/FR2 cells than in SAS/FR2 cells. Therefore, we conducted further experiments using Ca9-22/FR2 cells. To investigate the effects of cell adhesion to FN on 5-FU resistance in OSCC cells, we performed drug sensitivity assays using the cells cultured on non-coated or FN-coated dishes. As shown in Fig. 6A, cell adhesion to FN

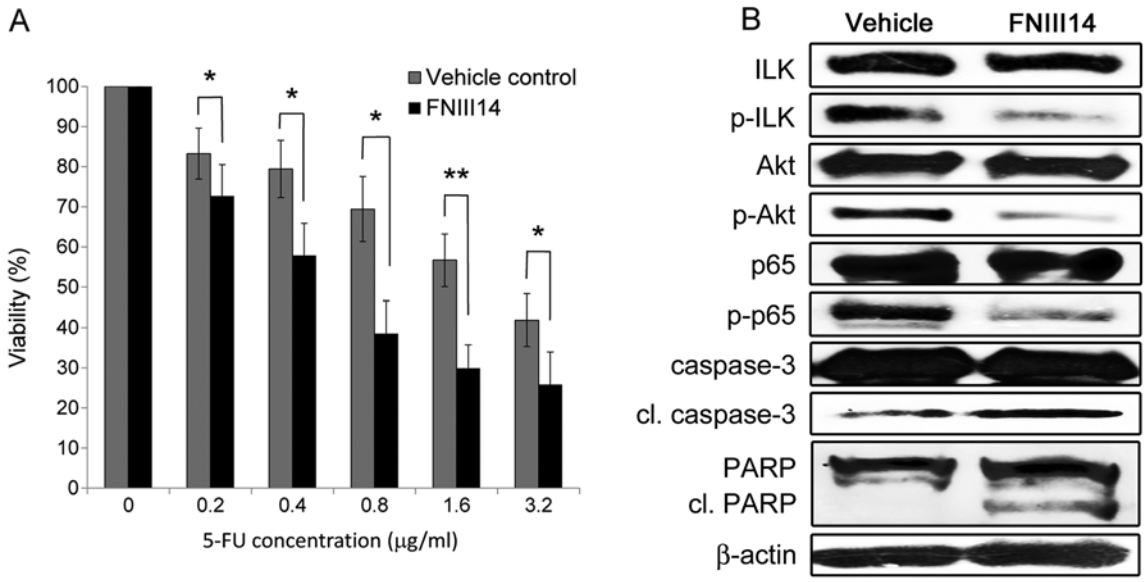

Figure 7. Inhibition of cell adhesion to FN by FNIII14 enhances chemosensitivity and apoptosis by suppressing integrin-mediated ILK/Akt/NF-κB signaling in the 5-FU-resistant OSCC cells. Following suspension with DMEM containing 10% FBS plus vehicleor FNIII14, Ca9-22/FR2 cells were seeded on FN-coated 24-well dishes and incubated in the culture medium for 24 h. (A) Following an additional 72 h of 5-FU treatment with vehicle or FNIII14, the degree of cell survival was monitored using the Cell Counting Kit-8. The results represent the mean ± SD of three independent experiments. *P<0.05; **P<0.01. (B) Following an additional 72 h of 0.8 mg/ml of 5-FU treatment with vehicle or FNIII14, whole-cell lysates were prepared, and the expression level of each protein was examined using western blot analysis.

significantly enhanced resistance to 5-FU under treatment with 0.8, 1.6 and 3.2 μg/ml of 5-FU in the 5-FU-resistant cells. In addition, a western blot analysis revealed that ILK, Akt and NF-κB (p65) were activated in the cells cultured on FN-coated dishes (Fig. 6B). These results suggest that enhanced 5-FU resistance by cell adhesion to FN is implicated in the activation of integrin-mediated ILK/Akt/NF-κB survival signaling.

Inhibition of cell adhesion to FN by FNIII14 enhances chemosensitivity and apoptosis by suppressing integrin-mediated ILK/Akt/NF-κB signaling in the 5-FU-resistant OSCC cells. We next examined the effects of blocking cell adhesion to FN on 5-FU resistance using FNIII14, which has been shown to have a strong inhibitory effect on the β1 integrin-mediated adhesion to FN. Consequently, FNIII14 treatment significantly increased the chemosensitivity of the 5-FU-resistant cells compared with that observed following treatment with the vehicle control (Fig. 7A). We also examined alterations in the expressions of integrin-mediated signaling molecules and apoptosis-related molecules using a western blot analysis (Fig. 7B). The inhibition of cell adhesion to FN by FNIII14 decreased the phosphorylation of ILK, Akt and NF-κB and increased the expression levels of cleaved caspase-3 and cleaved PARP, thus leading to enhanced apoptosis by suppressing integrin-mediated ILK/Akt/NF-κB signaling in the 5-FU-resistant cells.

Discussion

5-FU is a key drug in the treatment of many solid tumors, including OSCC, although some tumors exhibit 5-FU resistance. Therefore, it is extremely important to understand the molecular mechanisms of resistance in order to develop better treatment options. Hence, establishing 5-FU-resistant cell lines is absolutely essential for obtaining novel insights into resistance mechanisms. We recently reported that we had established an 5-FU-resistant OSCC cell line, SAS/FR2, for the first time, over a two-year period (27). In the present study, we used two 5-FU-resistant cell lines, including the newly established Ca9-22/FR2 line, that demonstrate higher resistance to 5-FU than SAS/FR2. We believe that the establishment of another cell line, Ca9-22/FR2, largely contributed to obtaining novel findings in this study.

Among the data obtained in the DNA microarray analysis, we narrowed our search to ECM molecules produced by cancer cells in order to identify targets implicated in CAM-DR using two sets of cell lines. As a result, we focused on FN as a key regulator exerting CAM-DR in 5-FU-resistant OSCC. Therefore, selecting novel targets based on data found commonly in two 5-FU-resistant cell lines appears to be a rational approach.

Consistent with our results, previous reports based on microarray analyses of human cancers have shown a link between ECM overexpression and chemoresistance (36,37). Furthermore, ECM overexpression has been demonstrated to not only enhance chemoresistance, but also act as a negative prognostic factor (21). These findings indicate that drug-resistant cells can alter the composition of the ECM in order to accelerate the acquisition of CAM-DR, thus resulting in a more favorable microenvironment for tumor cells. This phenomenon represents an autocrine-like survival enhancement response, as reported by Morin (38).

To date, the importance of CAM-DR achieved via cell adhesion to FN has been reported in various malignancies, including small cell lung cancer (21), myeloma (32), breast

cancer (18), colon cancer (33), acute myelogenous leukemia (35) and pancreatic cancer (39). Therefore, it is conceivable that the overexpression of FN in cancer cells confers CAM-DR in patients with OSCC. To the best of our knowledge, no other report has demonstrated the contribution of FN overexpression to the development of CAM-DR against 5-FU.

CAM-DR functions as a powerful stimulus that triggers several signal transduction pathways, leading to a decreased sensitivity to apoptosis. Cell adhesion to FN via integrin receptors has been demonstrated to protect both hematological and solid tumor cells from a number of apoptotic stimuli (23). FN associates with the major FN receptors α4β1 and α5β1 integrins and has been demonstrated to mediate prosurvival effects in several cell systems (40). However, as shown in Fig. 5, the expressions of these FN receptor components were not elevated in the 5-FU-resistant cells. These data suggest that the upregulation of FN receptors is not essential for exerting CAM-DR in OSCC cells.

ILK, an important β1 integrin signaling mediator, promotes the phosphorylation of Akt (41) and its consequent activation of downstream anti-apoptotic pathways mediated through NF-κB activation (42,43). In the present study, as expected based on the results of previous studies, our data demonstrated that the activation of ILK, Akt and NF-κB in 5-FU-resistant cells was enhanced by culture on FN-coated dishes. In addition, a previous report indicates that ILK is overexpressed in SCC of the head and neck (SCCHN) tumor specimens and that targeting ILK induced apoptosis in the SCCHN cell lines (44). Collectively, the ILK/Akt/NF-κB signaling pathway appears to play an important role in apoptosis resistance, thus contributing to the development of CAM-DR in the setting of OSCC.

We investigated whether FNIII14, which potently impairs the interaction of FN with β1 integrin, is able to overcome CAM-DR in 5-FU-resistant cells. An impairment of cell adhesion to FN by FNIII14 enhanced the chemosensitivity of the 5-FU-resistant cells cultured on FN-coated dishes, and this effect was accompanied by the suppression of ILK/Akt/NF-κB signaling. These results demonstrate that combination therapy consisting of 5-FU and FNIII14 can be used to effectively overcome CAM-DR in 5-FU-resistant cells. However, the major limitation of our study is that these novel findings were obtained based on *in vitro* data only. Therefore, further studies are required to confirm the effects of combination therapy with FNIII14 and 5-FU on 5-FU-resistant OSCC cells using *in vivo* models.

In conclusion, we herein highlighted the potential importance of CAM-DR achieved via cell adhesion to FN in 5-FU-resistant OSCC cells. Our data indicate that FN is a potentially useful biomarker and therapeutic target for improving the treatment of OSCC, particularly in the setting of 5-FU resistance.

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