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Osteopontin-integrin $\alpha_v\beta_3$ axis is crucial for 5-fluorouracil resistance in oral squamous cell carcinoma

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ABSTRACT

Clinical applications of a chemotherapeutic agent, 5-fluorouracil (5-FU) in oral squamous cell carcinoma (OSCC) have been limited because of drug resistance. This study aimed to identify novel mechanisms of 5-FU resistance. Here we found increased osteopontin (OPN) gene expression in OSCC tissues with resistance to 5-FU-based chemoradiotherapy. OPN overexpression in OSCC cells led to 5-FU resistance and abrogated the prosurvival effect of the drug in a mouse xenograft model. OPN-induced 5-FU resistance required integrin $\alpha_v\beta_3$. Targeting integrin $\alpha_v\beta_3$ reversed the resistance in a 5-FU-resistant clone highly expressing OPN. Our data suggest that the OPN-integrin $\alpha_v\beta_3$ axis is crucial for 5-FU resistance in OSCC.

1. Introduction

The most common head and neck neoplasm is oral cancer, mainly oral squamous cell carcinoma (OSCC), which affects about 270 000 people throughout the world [1]. Progress in early detection, diagnosis, and treatment of head and neck squamous cell carcinoma has occurred, but the 5-year survival of patients with head and neck squamous cell carcinoma has stayed at 50% for some 30 years [2].

The widely used 5-fluorouracil (5-FU) is one of the most effective and commonly used chemotherapeutic agents for OSCC and many other solid tumors including colon, breast, and stomach tumors [3]. Inasmuch as the radiosensitizing properties of 5-FU are well established, this drug is also commonly used for chemoradiotherapy for several diseases. Despite the many advantages of 5-FU, however, its clinical applications in OSCC have been greatly limited because of intrinsic or acquired drug resistance. Therefore, new strategies for therapy and reversal of resistance are urgently required. In addition, understanding the mechanisms by which tumors gain resistance to 5-FU is essential for predicting or overcoming that resistance.

Multiple mechanisms underlying 5-FU resistance have been described for many types of cancer including OSCC. However, most preclinical and clinical studies focused on molecules associated with 5-FU metabolism including dihydropyrimidine dehydrogenase and thymidine phosphorylase, or thymidylate synthase, a well-characterized 5-FU target [4,5]. These studies so far failed to describe clinically useful predictive biomarkers and produce useful targeted agents [4]. Better understanding of the molecular signature of 5-FU-resistant populations is therefore needed. In this study, we aimed to identify novel, clinically relevant mechanisms of 5-FU resistance in OSCC patients that may be targeted for overcoming this resistance.

Abbreviations: OSCC, oral squamous cell carcinoma; OPN, osteopontin; 5-FU, 5-fluorouracil

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2. Materials and methods

2.1. Clinical characteristics of patients and patient samples

We obtained the initial biopsy specimens for gene expression microarray analysis from six patients with OSCC and specimens for real-time quantitative reverse transcription-PCR (qRT-PCR) from 49 patients with OSCC. All patients were treated at the Department of Oral and Maxillofacial Surgery, Kumamoto University Hospital, between 1999 and 2004. Tables 1 and 2 provide clinicopathological details of the patients. All patients were treated preoperatively with a total dosage of 30 Gy radiation concurrent with 5-FU before undergoing curative surgery. With regard to this chemoradiotherapy, radiation was administered at a daily dose of 2.0 Gy, five times weekly for 15 days, and an oral fluorouracil anticancer agent, S-1, was administered concurrently, at 80, 100, or 120 mg/day that depended on each patient's body surface area, for 14 days from the initiation of radiotherapy. We staged all tumors according to the TNM classification of the Union for International Cancer Control [6]. The histological response of OSCC to the preoperative chemoradiotherapy was evaluated by examining the surgically resected specimen of the primary tumor. According to the qualitative 4-stage grading system of Oboshi and Shimosato using semi-serial sections of the entire surgical specimen [7], we classified the treatment effect into two groups as follows: sensitive (grade IV), neither viable nor non-viable cancer cells are observed; resistant (grade I and IIa), cancer cell damages are noted but viable cancer cells are frequently observed. This study followed the guidelines of the Ethical Committee of Kumamoto University. We explained the nature and aims of the research to all subjects, who gave informed consent for participation in the study. All tissue samples were placed in sterile tubes, immediately frozen in liquid nitrogen, and stored at −80 °C until analysis.

2.2. Cell line and cell culture

The human OSCC cell line SAS and HSC3 were kindly provided by Dr. Shirasuna (Department of Oral and Maxillofacial Surgery, Graduate School of Dental Science, Kyushu University, Fukuoka, Japan). A 5-FU-resistant subline established from these SAS cells (SAS-FR) was kindly provided by Dr. Nagata (Department of Oral and Maxillofacial Surgery, Kumamoto University Hospital, Japan) [8]. Cells were grown in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) with 10% heat-inactivated fetal bovine serum (Gibco) in a humidified 5% CO_2 incubator at 37 °C. 5-FU was kindly provided by Nippon Kayaku (Tokyo, Japan). To establish a stable control SAS cell line and a stable SAS cell line that overexpressed osteopontin (OPN), pcDNA3 empty vector or the pcDNA3-OPN-V5 (plasmid 11617; Addgene, Cambridge, MA, USA), respectively, was transfected by using Lipofectamine 2000 (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol, after which the cells were selected by using G418 (Invitrogen, Life Technologies, 500 μg/ml).

Table 1
Characteristics of six male patients with tongue SCC whose specimens were evaluated by means of gene expression microarray analysis.

Patient	Age (years)	TNM	Stage	Oboshi–Shimosato classification grade
Sensitive 1	71	T2N0M0	II	IV
Sensitive 2	64	T2N1M0	III	IV
Sensitive 3	64	T3N1M0	III	IV
Resistant 1	59	T2N0M0	II	IIa
Resistant 2	87	T2N2bM0	IV	I
Resistant 3	59	T4N0M0	IV	IIa

Table 2
Clinicopathological characteristics of patients with OSCC whose specimens were analyzed by means of qRT-PCR.

Characteristic	Sensitive patients (n = 30)	Resistant patients (n = 19)
Sex (male/female)	18/12	9/10
Age (years, mean ± S.E.M.)	67.77 ± 11.47	75.36 ± 8.37
Tumor location		
Gum	15	5
Tongue	8	6
Buccal area	5	5
Floor	2	3
Tumor size		
T1–T2	18	7
T3–T4	12	12
Node stage		
N0	11	9
N+	19	10
Tumor stage		
I–II	7	4
III–IV	23	15

2.3. RNA isolation and qRT-PCR

Total RNA was isolated from tissue specimens and cells by using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and was reverse transcribed to cDNA by using the ExScript RT reagent kit (Takara Bio Inc., Otsu, Japan), according to the manufacturers' instructions. The LightCycler System (Roche Diagnostics, Basel, Switzerland) with SYBR Premix DimerEraser (Takara Bio Inc.) was used to perform all PCR reactions. Primers used for qRT-PCR were as follows: OPN forward: 5′-GCCAGTTGCAGCCTTCTCA-3′, OPN reverse: 5′-AAAAGCAAATCACTGCAATTCTCA-3′; 18S rRNA forward: 5′-CGGCTACCACATCCAAGGAA-3′, 18S rRNA reverse: 5′-GCTGGA ATTACCGCGGCT-3′. Primers were purchased from Sigma (Tokyo, Japan). 18S rRNA was used as an internal control.

2.4. Gene expression microarrays

From among the tissues frozen in liquid nitrogen, we selected three sensitive and three resistant OSCC cases (Table 1). Gel electrophoresis was used to check the integration of the RNA isolated from these tissues; two bands, 18S and 28S, indicated satisfactory RNA quality. Affymetrix GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) was used for the microarray experiments according to the manufacturer's protocol. Hybridization and scanning were done according to the standard Affymetrix procedure. Signals of all probes were scanned and calculated with a Gene Array scanner (GCS3000 system; Affymetrix). Signals with at least 4.0-fold higher expression and 2.0-fold lower expression and with P values < 0.05 in Student's t test were considered to represent up-regulation and down-regulation in the resistant group, respectively. In addition, a heat map of selected genes was produced by using Cluster 3.0 and Java Treeview 1.0.12.

2.5. Protein extraction and immunoblotting

Cells were washed once in ice-cold PBS and then lysed by the addition of CelLytic M Cell Lysis/Extraction Reagent (Sigma) containing freshly added protease inhibitor cocktail (Sigma). Equal amounts of protein were fractionated via SDS–PAGE and transferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). Membranes were blocked with 5% non-fat dried milk and 0.1% Tween 20 in PBS and were then incubated overnight at 4 °C with antibodies against OPN (Immuno-Biological Laboratories Co. Ltd.,

Gunma, Japan) and β-actin (Sigma). After the membranes were washed, they were incubated for 1 h in horseradish peroxidase-conjugated secondary antibodies. After a washing, specific protein bands were detected by using ECL Prime Western Blotting Detection Reagents (Amersham Life Science, Arlington Heights, IL) according to the manufacturer's instructions.

2.6. Cell proliferation assay

The OSCC cells (9×10^4 per well) were seeded on 12-well or 24-well plates. After 24 h, various concentrations of 5-FU were added to each well, the cells were incubated at 37 °C for another 48 h, and viable cells in each well were quantified by using the Luna Automated Cell Counter (Logos Biosystems, Annandale, VA, USA). To inhibit integrin $\alpha_v\beta_3$, cells were treated with the monoclonal anti-integrin $\alpha_v\beta_3$ antibody LM609 (0.5 μg/ml; Merck Millipore, Darmstadt, Germany) or isotype-matched control antibody (R&D systems, Minneapolis, MN, USA).

2.7. Soft agar colony formation assay

The colony formation of OSCC cells was tested using CytoSelect 96-well Cell Transformation Assay Kit (Cell Biolabs, San Diego, CA, USA) following the manufacturer's instructions. Briefly, cells were plated in soft agar in a 96-well plate at 1×10^4 cells per well. After 24 h, culture medium containing 5-FU (0.5 μg/ml) or LM609 (0.5 μg/ml) was added and cells were cultured for 7 days. Colonies were photographed with a phase-contrast microscope and the absorbance was measured using Promax5 (Media Cybernetics, Silver Spring, MD, USA) with a 485/520 nm filter set.

2.8. Animal experiments

BALB/c-nu/nu female mice (nude mice), 4-6 weeks old, were purchased from Charles River Japan (Yokohama, Japan) and maintained at the Center for Animal Resources and Development of Kumamoto University. The mice were handled in accordance with the animal care policy of Kumamoto University. SAS and SAS-OPN cells were harvested and resuspended in PBS, after which 5×10^6 cells were injected subcutaneously into the left axilla of each mouse. After the mice developed palpable tumors, they were placed in the 5-FU or PBS treatment group according to their closely matched tumor volumes. The mice then received intraperitoneal injections of 5-FU (100 mg/kg) or PBS once weekly. The health of the mice and evidence of tumor growth were evaluated every 3–4 days. Tumor development was followed in individual animals by sequential caliper measurements of length (L) and width (W). Tumor volume was calculated by means of the formula $LW^2\pi/6$.

2.9. Statistical analysis

Student's t test was used to evaluate differences between two groups. All analyses were performed with JMP software Version 5.1 for Windows (SAS Institute Japan, Tokyo, Japan). Statistical significance was defined as $P < 0.05$.

3. Results

3.1. OPN gene expression was up-regulated in OSCC tissues with resistance to 5-FU-based preoperative therapy

To clarify the drug resistance mechanism in OSCC tissue, we first evaluated clinical biopsy tissues by means of microarray gene expression analysis. Patients with OSCC who received one preoperative S-1 chemoradiation treatment were assigned to one of two groups on the basis of a pathological evaluation of the response to treatment of the surgical specimens. We compared, by means of microarray analysis, the expression of numerous genes in the specimens from sensitive and resistant groups of patients, whose information is provided in Table 1. This analysis indicated 187 genes with more than 4-fold higher expression and 2309 genes with more than 2-fold lower expression in the resistant group compared with the sensitive group. Fig. 1A provides a heat map representing the gene expression levels. Our gene ontology (GO) analysis of the microarray data with DAVID, which is an annotation and integration tool, identified possible indicators in pathways that may be associated with drug resistance ($P < 0.01$, Fig. 1B). With our therapeutic strategy in mind, we narrowed our search, via our GO molecular function analysis, for up-regulated genes that encode secretory proteins, extracellular matrix, and receptors. We discovered several significantly up-regulated genes including trichohyalin (TCHH; 34.78-fold, $P = 0.0199$), OPN (13.96-fold, $P = 0.0296$), and plasminogen activator inhibitor-1 (PAI-1; 9.06-fold, $P = 0.0236$) in the resistant group (Supplementary Table S1).

Our qRT-PCR analyses of OSCC biopsy samples obtained from 30 sensitive and 19 resistant cases revealed OPN as the only significantly up-regulated gene ($P < 0.05$, Table 2, Fig. 1C and data not shown). OPN protein, also called SPP1, is a multifunctional binding glycophosphoprotein that is involved in several pathological processes including inflammation and cancer [9]. We confirmed the involvement of OPN in the main GO terms such as cell adhesion, biological adhesion, cell–cell adhesion, regulation of response to external stimulus, and positive regulation of homeostatic process (Fig. 1B).

3.2. OPN overexpression led to 5-FU resistance in OSCC cells

S-1 is an oral fluorouracil antitumor drug that combines three pharmacological agents: tegafur, a pro-drug of FU; gimeracil, which is designed to enhance potency by inhibiting the catabolism of 5-FU; and oteracil, which is designed to decrease gastrointestinal tract toxicities by inhibiting the activity of 5-FU selectively within the intestinal lumen. Because antitumor activity of S-1 is originated from 5-FU, we focused on 5-FU resistance in OSCC cells. To determine whether OPN is involved in 5-FU resistance in OSCC cells, we established SAS-OPN, an OPN-overexpressing subclone of the OSCC cell line SAS (Fig. 2A). No apparent difference in cell proliferation was observed between SAS-OPN and controls cells (SAS-vector) (Fig. 2B). As Fig. 2C shows, sensitivity to 5-FU was significantly decreased in SAS-OPN cells compared with SAS-vector cells in vitro, which indicates that OPN overexpression contributed to 5-FU resistance in OSCC cells.

To further investigate the significance of OPN overexpression for 5-FU resistance, we injected nude mice subcutaneously with SAS-OPN and SAS-vector cells. Consistent with our in vitro data, OPN overexpression by itself did not apparently affect tumor growth (Fig. 2D). However, although treatment with 5-FU strongly suppressed the growth of control tumors, as expected (PBS, 1245 mm^3 vs. 5-FU, 288 mm^3, at day 24; $P < 0.01$), 5-FU did not inhibit the growth of OPN-overexpressing tumors (PBS, 1314 mm^3 vs. 5-FU, 1080 mm^3, at day 24; $P = 0.89$). Furthermore, 5-FU treatment significantly prolonged survival of mice bearing control tumors (median survival 5.14 weeks vs. 7.57 weeks $P < 0.05$, log-rank test, Fig. 2E), whereas such a prosurvival effect of 5-FU was abolished in the SAS-OPN group (PBS-treated OPN group vs. 5-FU-treated OPN group, $P > 0.1$, log-rank test, Fig. 2E). OPN overexpression by itself did not affect survival of mice (Fig. 2E). These data indicate that OPN overexpression in OSCC cells led to poor survival by inducing 5-FU resistance without apparent tumor-promoting effects.

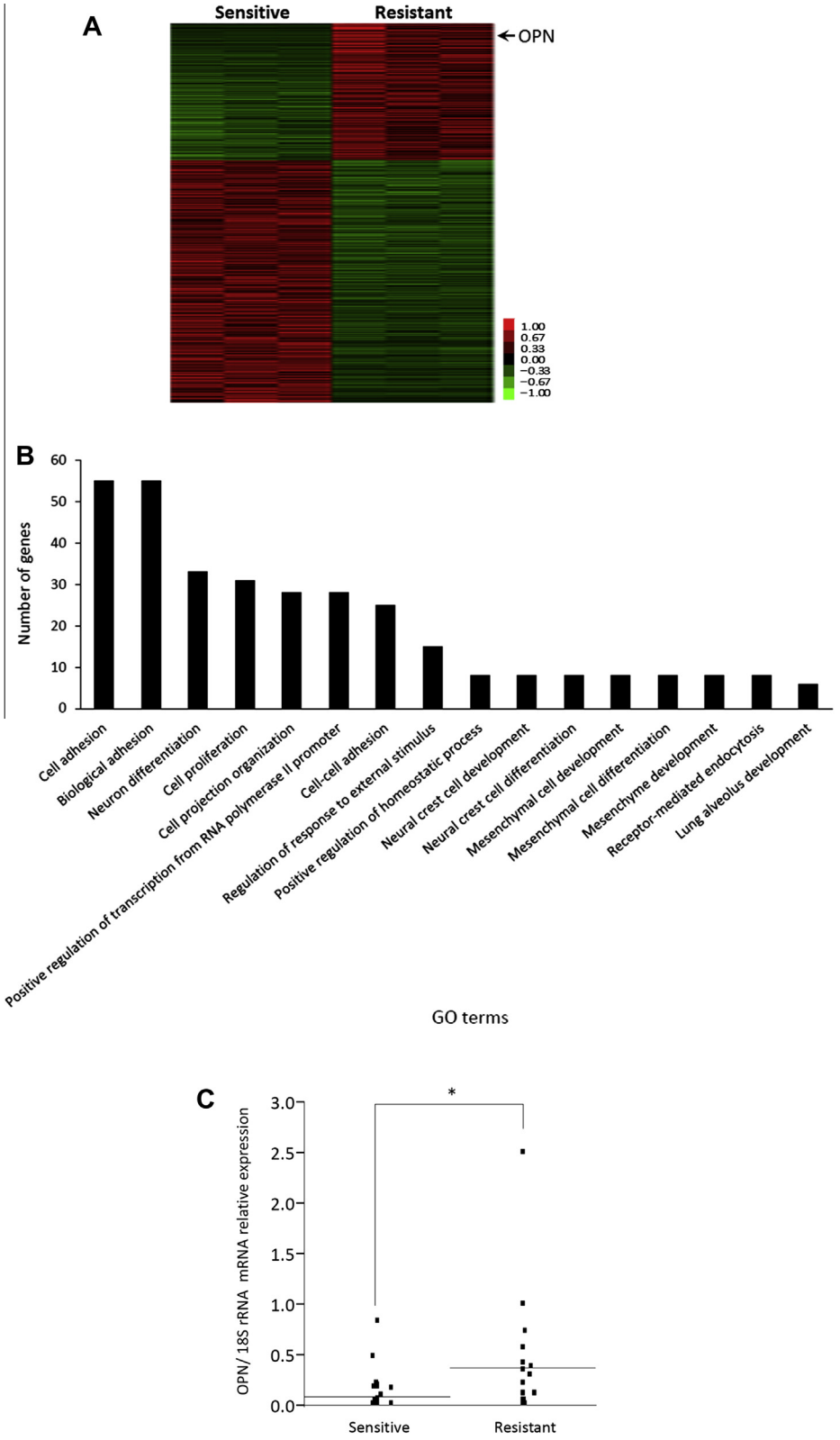

Fig. 1. OPN gene expression was up-regulated in OSCC tissues with resistance to 5-FU-based preoperative therapy. (A) Heat map, obtained by means of a microarray analysis, depicting differential gene expression in 5-FU-sensitive and 5-FU-resistant OSCC tissues ($P < 0.05$, Student's t test). Red indicates up-regulated genes; green, down-regulated genes (see color bar). (B) The number of genes expressed in different cellular mechanisms and pathways as revealed by GO analysis of microarray data with DAVID ($P < 0.01$). (C) OPN mRNA expression in 5-FU sensitive ($n = 30$) and resistant ($n = 19$) OSCC tissues. OPN mRNA expression in OSCC tissues was determined via qRT-PCR. $^{*}P < 0.05$. Values are means ± S.D.

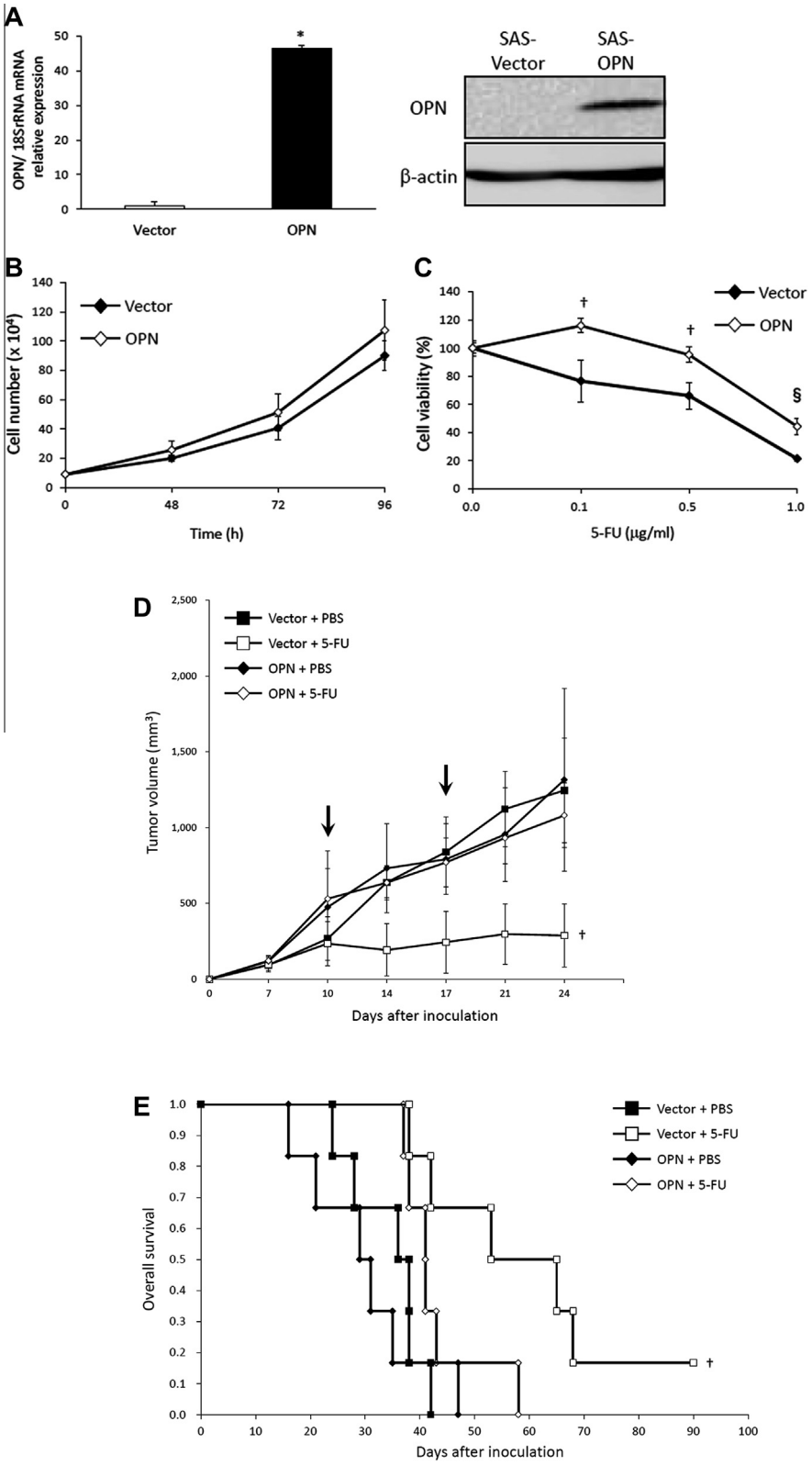

Fig. 2. OPN overexpression led to 5-FU resistance in OSCC cells. (A) OPN mRNA (left panel) and protein expression (right panels) in SAS-vector (Vector) and SAS-OPN (OPN) cells was determined via qRT-PCR and western blotting, respectively. $^{*}P < 0.001$. (B) The number of SAS-vector and SAS-OPN cells was measured. (C) SAS-vector and SAS-OPN cells were treated with 5-FU at the indicated concentrations for 48 h, after which cell numbers were determined. $^{\dagger}P < 0.05$; $^{\S}P < 0.01$. Values are means ± S.E.M. of triplicate samples. (D) SAS-vector and SAS-OPN cells were injected subcutaneously into mice. After 10 days, the tumor-inoculated mice received intraperitoneal injections of 100 mg/kg 5-FU or PBS every 7 days for 24 days. The graph indicates the mean tumor growth rates ± S.D. of six animals per experimental condition. Arrows indicate days of administration of 5-FU or PBS. $^{\dagger}P < 0.05$ compared with the other groups. (E) Kaplan–Meier plots of overall survival of each experimental group. $^{\dagger}P < 0.05$ compared with PBS-treated vector group (log-rank test).

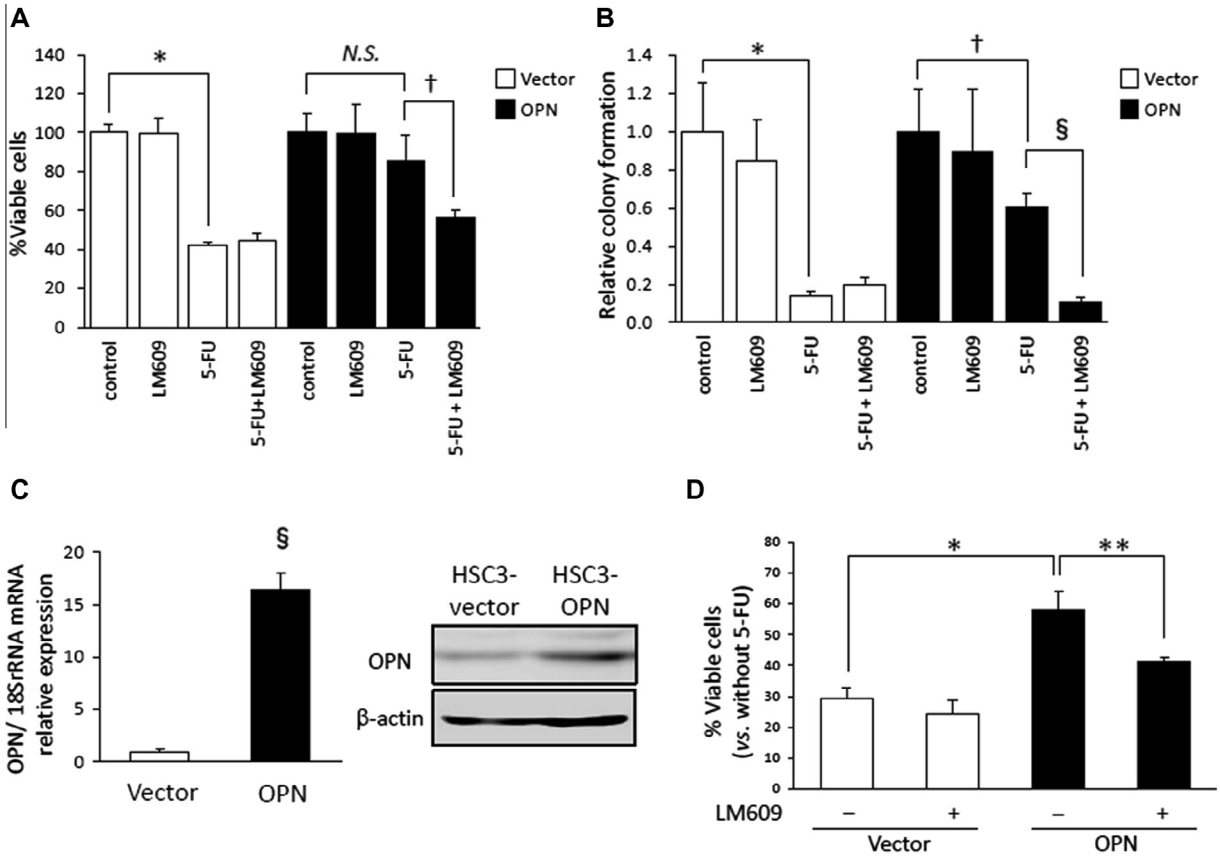

Fig. 3. Integrin $\alpha_v\beta_3$ was required for 5-FU resistance induced by OPN overexpression in OSCC cells. (A) SAS-vector (Vector) and SAS-OPN (OPN) cells were treated with 5-FU (0.5 μg/ml) and/or LM609 (0.5 μg/ml) for 48 h, after which cell numbers were determined. $^{*}P < 0.005$; $^{\dagger}P < 0.05$. *N.S.*, not significant. (B) Cells were plated in soft agar in a 96-well plate at 1×10^4 cells per well. After 24 h, culture medium containing 5-FU (0.5 μg/ml) and/or LM609 (0.5 μg/ml) was added and cells were cultured for 7 days. Absorbance of colonies in each well was measured using a 485/520 nm filter set. $^{*}P < 0.005$; $^{\dagger}P < 0.05$; $^{\S}P < 0.0005$. (C) mRNA (left panel) and protein expression (right panels) of OPN in HSC3 cells transfected with empty vector (Vector) or OPN expression vector (OPN) were determined via qRT-PCR and western blotting, respectively. $^{\S}P < 0.0005$. (D) HSC3-vector and HSC3-OPN cells were treated with 5-FU (0.5 μg/ml) and/or LM609 (0.5 μg/ml) for 48 h, after which cell numbers were determined. Results are expressed as a percentage relative to cells without 5-FU in each experimental group. $^{*}P < 0.005$; $^{**}P < 0.01$. Values are means ± S.E.M. of triplicate samples.

3.3. Integrin $\alpha_v\beta_3$ was required for 5-FU resistance induced by OPN in OSCC cells

We next sought to determine the precise mechanisms underlying OPN-induced 5-FU resistance. OPN was shown to interact with multiple integrins, which led to activation of various downstream pathways. In view of the data showing that, in particular, tumor cell expression of integrin $\alpha_v\beta_3$ correlated with poor prognosis of various tumor types [10] and our microarray data demonstrated elevated expression of integrin β_3 (Supplementary Table S1), we investigated the involvement of integrin $\alpha_v\beta_3$ in OPN-induced 5-FU resistance by using the anti-integrin $\alpha_v\beta_3$ antibody LM609. LM609 almost completely blocked OPN-induced 5-FU resistance but did not affect the basal cytotoxic effects of 5-FU (Fig. 3A). In addition, we confirmed these results by soft agar colony formation assay (Fig. 3B). Furthermore, in another OSCC cell line HSC3, transient OPN overexpression (Fig. 3C) induced 5-FU resistance, and this resistance was strongly reversed by LM609 (Fig. 3D). These data showed that integrin $\alpha_v\beta_3$ is critically contributing to OPN-triggered 5-FU resistance.

3.4. The OPN-integrin $\alpha_v\beta_3$ axis may be important for acquired 5-FU resistance in OSCC

Even in cancers that are primarily sensitive to 5-FU, resistance may ultimately be acquired through continuous drug administration, which may lead to alterations in gene expression and signaling cascades [11–15]. Thus, we utilized a 5-FU-resistant SAS clone (SAS-FR), which was previously established via continuous exposure to increasing concentrations of 5-FU for 2 years, to investigate whether the OPN-integrin $\alpha_v\beta_3$ axis was also involved in acquired 5-FU resistance. SAS-FR has the same proliferative activity as the parental line [8]. We first confirmed a lower sensitivity of SAS-FR cells to 5-FU compared with the parental SAS cells (SAS-parental) (Fig. 4A). Our qRT-PCR and western blot analyses revealed significantly increased OPN expression in SAS-FR cells compared with SAS-parental cells (Fig. 4B). Furthermore, neutralizing integrin $\alpha_v\beta_3$ activity by LM609 significantly increased the sensitivity of SAS-FR cells to 5-FU without an apparent effect on the cell proliferation and the sensitivity of SAS-parental cells (Fig. 4C). Furthermore, the colony formation assay revealed that LM609 abolished the 5-FU-resistance phenotype of SAS-FR cells (Fig. 4D). These data suggest that the OPN-integrin $\alpha_v\beta_3$ axis is also important for an acquired 5-FU resistance and that integrin $\alpha_v\beta_3$ was a useful target for overcoming 5-FU resistance in OSCC.

4. Discussion

5-FU-based chemoradiotherapy improves the survival of patients with OSCC. However, despite the many advantages of this therapy, development of resistance to 5-FU, whether intrinsic or acquired, is a major obstacle to its successful clinical application in OSCC. We demonstrate here, for the first time, that OPN overexpression contributed to 5-FU resistance in OSCC via integrin $\alpha_v\beta_3$ and

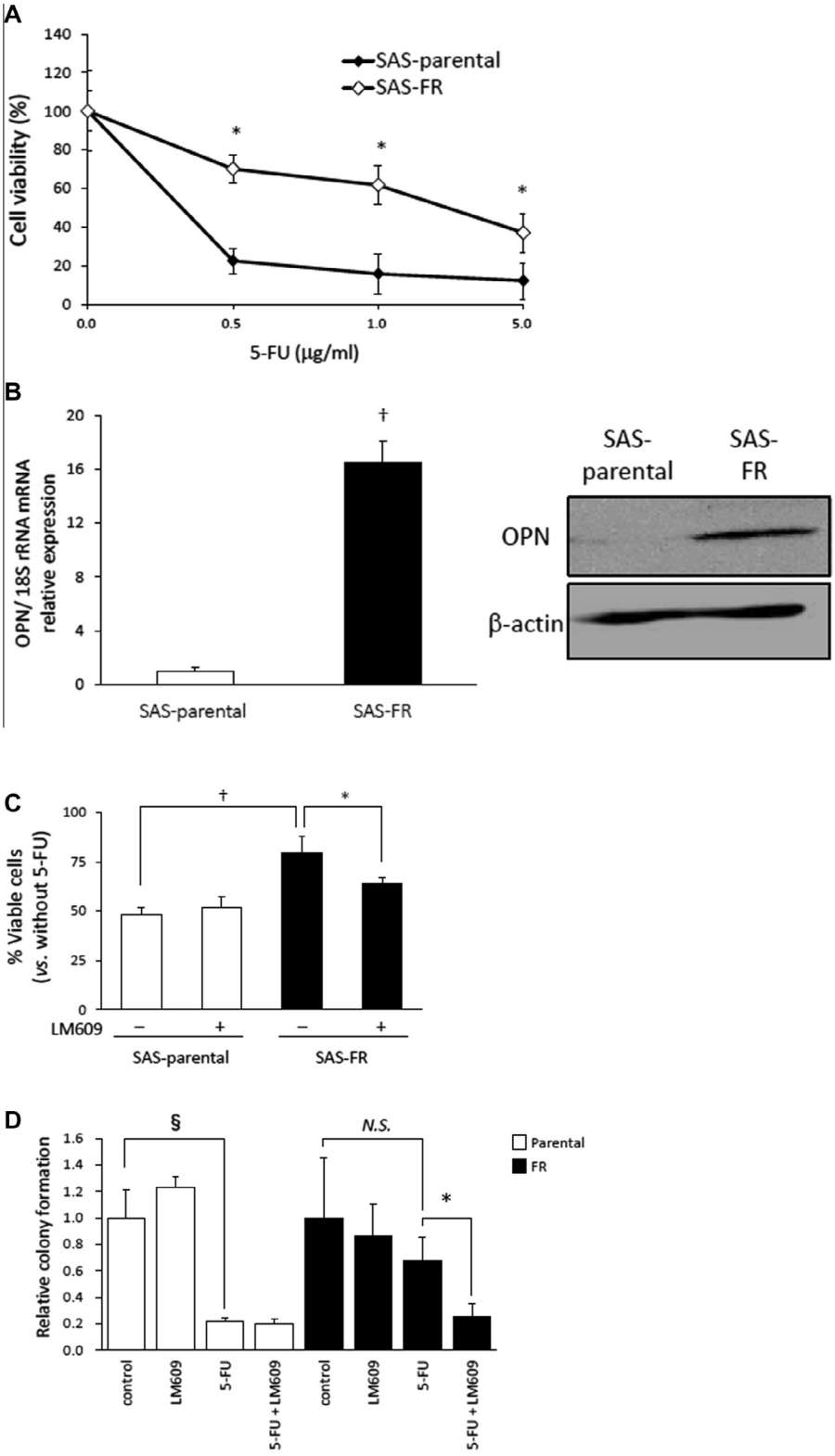

Fig. 4. A 5-FU-resistant OSCC cell clone highly expressed OPN, and inhibition of integrin $\alpha_v\beta_3$ reversed the resistance. (A) SAS-parental and SAS-FR cells were treated with 5-FU at the indicated concentrations for 48 h, after which cell numbers were measured. $^{*}P < 0.05$ compared with SAS-parental cells. (B) OPN mRNA (left panel) and protein expression (right panels) in SAS-parental and SAS-FR cells was determined via qRT-PCR and western blotting, respectively. $^{\dagger}P < 0.001$. (C) SAS-parental and SAS-FR cells were treated with 5-FU (0.5 μg/ml) and/or LM609 (0.5 μg/ml) for 48 h, after which cell numbers were measured. Results are expressed as a percentage relative to cells without 5-FU in each experimental group. $^{*}P < 0.05$; $^{\dagger}P < 0.001$. (D) Cells were plated in soft agar in a 96-well plate at 1×10^4 cells per well. After 24 h, culture medium containing 5-FU (0.5 μg/ml) and/or LM609 (0.5 μg/ml) was added and cells were cultured for 7 days. Absorbance of colonies in each well was measured using a 485/520 nm filter set. $^{*}P < 0.05$; $^{\S}P < 0.005$. *N.S.*, not significant. Values are means ± S.E.M. of triplicate samples.

that pharmacological inhibition of integrin $\alpha_v\beta_3$ may be useful for overcoming resistance to 5-FU-based therapy.

OPN belongs to the small integrin-binding ligand, N-linked glycoprotein family, with all members having a functional Arg-Gly-Asp (RGD) domain that interacts with multiple integrins. OPN also interacts with the CD44 receptor in an RGD-independent manner [16–18]. Through these receptors, OPN triggers cell signaling that leads to promotion of cell adhesion, survival, migration, and immune regulation [18,19]. Aberrant OPN expression was said to be involved in many pathophysiological processes including cancer [20–25]. In many types of cancer, an increased OPN level was found in the blood and tumor tissues, which was correlated with a poor prognosis [26,27]. In addition, several lines of evidence suggest that OPN is important for invasion, metastasis, and angiogenesis [28]. Plasma OPN is an independent prognostic marker for OSCC [29–32]. In particular, OPN was recently proposed as a surrogate marker for hypoxia, the most important predictor for a response to radiation, and thus for radiotherapy outcome in head and neck cancer [30,33–38]. Our present study using microarray and qRT-PCR analyses showed that OPN gene expression was significantly higher in OSCC tissues in patients with pretreatment resistance to 5-FU-based chemoradiotherapy compared with patients who had sensitivity to this chemoradiotherapy. In fact, OPN overexpression in OSCC cells clearly led to 5-FU resistance in a cell-autonomous fashion, whereas, unexpectedly, OPN overexpression by itself did not affect tumor growth both in vitro and in vivo. Survival data for our mice suggested that 5-FU resistance induced by OPN did not depend on the tumor-promoting effect. Further investigation may clarify molecular mechanisms underlying OPN overexpression and whether OPN expression is useful for prediction of response to not only radiotherapy but also 5-FU treatment.

With regard to mechanisms of 5-FU resistance, we identified integrin $\alpha_v\beta_3$ as a critical mediator of OPN-induced 5-FU resistance. Our finding that inhibition of integrin $\alpha_v\beta_3$ alone neither affected cell proliferation nor improved the cytotoxic efficacy of 5-FU indicates the importance of OPN as a trigger for 5-FU resistance. We also found that pharmacological inhibition of integrin $\alpha_v\beta_3$ reversed the acquired 5-FU resistance in OPN-overexpressing SAS-FR cells without apparent effects on basal cell proliferation and colony formation. Together, OPN-integrin $\alpha_v\beta_3$ axis might be important for not only intrinsic but also acquired resistance to 5-FU. A recent study showed that the binding of OPN to integrin $\alpha_v\beta_3$ induced expression of P-glycoprotein, a subfamily of the ATP-binding cassette transporter in prostate cancer cells [39]. In addition, OPN-integrin $\alpha_v\beta_3$ signaling has been shown to promote cell survival and prevents apoptosis [40,41]. Importantly, as OPN-induced 5-FU resistance was more clearly observed in mouse xenografts than in vitro, synergy between tumor microenvironment and OPN autocrine/paracrine signals also should be considered [42,43].

Recent clinical trials for several types of cancer demonstrated the safety and promising feasibility of etaracizumab, a humanized anti-integrin $\alpha_v\beta_3$ antibody developed from LM609, and cilengitide, an RGD-containing cyclic peptide [44–48]. Further in vivo studies using these drugs combined with 5-FU would contribute to develop novel therapeutic strategies for OSCC.

In conclusion, we demonstrated here, for the first time, the OPN-integrin $\alpha_v\beta_3$ axis as a crucial signal for 5-FU resistance in OSCC. Our findings emphasize the need to evaluate OPN expression levels in tumor tissues in OSCC patients before and during 5-FU-based therapy in relation to outcome. Pharmacological targeting of integrin $\alpha_v\beta_3$ combined with 5-FU-based therapy might improve the prognosis of OSCC patients.

5. Conflict of interest

The authors disclosed no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.12.004.

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