A Fas Ligand (FasL)-Fused Humanized Antibody Against Tumor-Associated Glycoprotein 72 Selectively Exhibits the Cytotoxic Effect Against Oral Cancer Cells with a Low FasL/Fas Ratio

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Abstract

Altered expression of the Fas ligand (FasL)/Fas ratio exhibits a direct impact on the prognosis of cancer patients, and its impairment in cancer cells may lead to apoptosis resistance. Thus, the development of effective therapies targeting the FasL/Fas system may play an important role in the fight against cancer. In this study, we evaluated whether a fusion protein (hcc49scFv-Fasl) comprising of the cytotoxicity domain of the Fasl fused to a humanized antibody (CC49) against tumor-associated glycoprotein 72, which is expressed on oral squamous cell carcinoma (OSCC), can selectively kill OSCC cells with different FasL/Fas ratios. In clinical samples, the significantly low FasL and high Fas transcripts were observed in tumors compared with normal tissues. A lower FasL/Fas ratio was correlated with a worse prognosis of OSCC patients and higher proliferative and invasive abilities of OSCC cells. The hcc49scFv-Fasl showed a selective cytotoxic effect on OSCC cells (Cal-27 and SAS) but not on normal oral keratinocytes cells (HOK) through apoptosis induction. Moreover, SAS cells harboring a lower FasL/Fas ratio than Cal-27 were more sensitive to the cytotoxic effect of hcc49scFv-Fasl. Unlike wild-type Fasl, hcc49scFasL was not cleaved by matrix metalloproteinases and did not induce nonapoptotic signaling in SAS cells. In vivo, we found that hcc49scFv-Fasl drastically reduced the formation of lymph node metastasis and decreased primary tumor growth in SAS orthotopic and subcutaneous xenograft tumor models. Collectively, our data indicate that a tumor-targeting antibody fused to the Fasl can be a powerful tool for OSCC treatment, especially in populations with a low FasL/Fas ratio. Mol Cancer Ther; 16(6): 1102–13. ©2017 AACR.

Introduction

Oral squamous cell carcinoma (SCC; OSCC) is the most commonly diagnosed malignancy of the oral cavity (1). Strategies used to treat OSCC include surgical extraction, radiotherapy, and chemotherapy either individually or in combination (2). Unfortunately, the prognosis is poor for relapsed and refractory disease and for patients with metastatic disease even after therapeutic interventions with chemotherapeutics, and the 5-year survival rate of patients with OSCC is around 50% which is very poor (2). It seems difficult to improve current response rates with further dose escalations to overcome drug resistance, as resistant tumor cells are able to withstand the effects of cytotoxic agents. In addition, associated cytotoxic side effects on normal tissues and organs remain a serious drawback. Therefore, searching for new and alternative therapeutic strategies such as targeted therapy has been the most essential and emergent issue.

Fas, a member of the TNF family, is a transmembrane protein with cysteine-rich extracellular domains and a death cytoplasmic domain, common to all family members and essential in the translation of the death stimulus. Immediately after receptor stimulation by the Fas ligand (Fasl), an apoptotic signal is transmitted through the adapter Fas-associated death domain (FADD), which converts caspase-8zymogen into its active form, triggering initiation of apoptosis (3). Recently, the Fasl/Fas system was reported to elicit both tumorigenic and tumor-suppressive roles in the pathogenesis of cancer (4). Fasl is expressed in two flavors, a membrane-bound form (mFasl) and a soluble form (sFasl), which is generated through cleavage of the mFasl by matrix metalloproteinases (MMP; refs. 5, 6). In vivo, the mFasl is essential for apoptosis induction, whereas the sFasl appears to promote tumorigenesis through nonapoptotic activities (7). For example, mice that only express sFasl but not mFasl suffer from large histiocytic sarcomas of the liver, and this sFasl-Fas–induced tumorigenic effect may be through inducing NFkB activation (7). More recently, sFasl was reported to trigger formation of a motility-inducing signaling complex in triple-negative breast...
cancer cells (8). To sensitize tumor cells to mFasl/Fas-mediated apoptosis, various approaches have been developed to date (9). One promising approach is the development of FasL fusion proteins which harbor a protein domain that specifically recognizes tumor cells or tumor stroma antigens, which not only achieves immobilization of the ligand to the membrane, but enables tumor-restricted action of the system through specific binding to a tumor-associated antigen. For example, the sc40-Fasl combines the extracellular domain of the Fasl and a single-chain antibody fragment (scFv) that specifically recognizes the fibroblast-activating protein (FAP), a transmembrane protein restrictedly expressed during angiogenesis and on activated fibroblasts occurring in the stroma of epithelial cancer (10), thus acting as a tumor stroma marker. This fusion protein allows the specific immobilization of the Fasl at FAP-expressing cells. Upon binding, the initially inactive protein converts into a protein with membrane-bound-Fasl-like activity, specifically inducing apoptosis in FAP-expressing tumor cells both in vitro and in vivo, without triggering systemic toxicity (11). Along the same line, a Fasl fusion protein (scFvCD7:sFasL) for CD7-restricted induction of apoptosis in T-cell leukemia was developed. As CD7 is an antigen associated with leukemia cells, it allows CD7-restricted induction of apoptosis (12).

The tumor-associated glycoprotein (TAG)-72 is a membrane protein complex of approximately 220 kDa (13). It was demonstrated that TAG-72 is overexpressed in a number of human tumors such as colon cancer, ductal breast cancer, lung cancer, epithelial ovarian cancer, hepatocellular carcinoma, and oral cancer, but is not expressed by most normal tissues (14–18). An anti-TAG-72 mAb (CC49) was studied in preclinical animal models and in humans for cancer detection based on its high specificity against cancer antigens in various solid cancers (17, 19). Herein, we investigated the anticancer effects on oral cancer in vitro and in tumor xenografts using our synthesized fusion protein, hcc49scFv-Fasl (20), which is comprised of the extracellular cytotoxic domain of the Fasl fused to a humanized TAG-72 antibody, CC49.

We found that the Fasl/Fas ratio in OSCC cells was inversely correlated with the in vitro proliferative and invasive abilities and in vivo tumor growth rate, and positively correlated with the clinical prognosis of OSCC patients. We demonstrate that the recombinant hcc49scFv-Fasl selectively induces apoptosis in OSCC cells (Cal-27 and SAS), but not normal oral keratinocytes. Interestingly, cells harboring a lower Fasl/Fas ratio, such as SAS, presented higher sensitivity to hcc49scFv-Fasl treatment compared with cells harboring a higher Fasl/Fas ratio, such as Cal-27. In vivo, the hcc49scFv-Fasl dramatically inhibited tumor growth through apoptosis induction in SAS subcutaneous and orthotopic graft models through intratumoral and intraperitoneal (i.p.) administration, respectively. In addition, intraperitoneal administration of the hcc49scFv-Fasl strongly limited the formation of metastasis to neck lymph nodes and prolonged survival times in the orthotopic metastatic mouse model.

Materials and Methods

Reagents

A recombinant hcc49scFv-Fasl was generated according to our previous report (20). The recombinant Fasl, MMP7, and proform SPPI (Osteopontin) proteins were purchased from (R&D Systems). Antibodies against phospho-p38 (9211), p38 (9212), phospho-extracellular signal-regulated kinase (ERK)1/2 (9101), Erk1/2 (9102), phospho-NFκB-p65 (3066), NFκB-p65 (3034), phospho-IκBα (9246), IκBα (4818), pro-caspase-3 (9662), cleaved caspase-3 (9661), and Snail (3895) were obtained from Cell Signaling Technology. Antibodies against Fas (GTX116044) and Fasl (GTIX116044) were purchased from Genetex. An antibody specific for N-cadherin (610921) was purchased from BD Biosciences. An antibody against α-tubulin (T5168) was purchased from Sigma. An antibody neutralized Fas activity (05-338) was purchased from Merck Millipore. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) powder was purchased from Sigma.

Processing of microarray data from GEO database

Microarray datasets with accession number GSE42743 and GSE13601 were obtained from Gene Expression Omnibus (GEO) database and normalized by GeneSpring (Agilent Technologies) software as log2 values.

Cell culture

The OSCC lines Cal-27, Detroit-562, and RPMI-2650 were obtained from ATCC and cultured in modified Eagle medium (MEM) containing 10% FBS, 2 mmol/L 1-glutamine and 100 U/ml penicillin. HSC-4 and SAS cell lines were obtained from Japanese Collection of Research Bioresources (JCRB) Cell Bank and cultured in MEM and DMEM, respectively, supplemented with the same additives. Human lung epithelial cell line WI-38 was obtained from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) and cultured in MEM with 10% FBS, 2 mmol/L 1-glutamine, 0.1 mmol/L nonessential amino acids, 1.0 mmol/L sodium pyruvate, and 100 U/mL penicillin. Human oral keratinocytes (HOK) cells were purchased from ScienCell and cultured in oral keratinocyte medium (catalog no. 2611, ScienCell). Cells were incubated in 5% CO2 at 37°C. All components used for cell cultures were purchased from Gibco (Invitrogen). All cells were routinely authenticated on the basis of morphologic and growth characteristics as well as by STR analysis and confirmed to be free of mycoplasma.

Establishment of red fluorescence protein (DsRed2)-expressing SAS cells

The DsRed2 gene was cloned into the pDsRed2-C1 vector (Clontech) with EcoRI and NotI and then subcloned into the pCI-Neo expression vector with the same restriction enzymes. SAS cells were transfected with pCI-DsRed2-Neo by calcium phosphate transfection reagent (Invitrogen). DsRed2-expressing SAS cells were further selected in the presence of 800 μg/mL neomycin. SAS-DsRed2 cells were maintained with the condition medium in the presence of 200 μg/mL neomycin.

Crispr/Cas9–mediated knockout of Fas and Fasl in OSCC cells

All-in-one Cas9-sgRNA lentiviral expression Crispr nuclease vector were obtained from RNAiCore facility (Academia Sinica, Taipei, Taiwan). The FAS (GGAGTTGATGTCAGTCACTT) and FASL (AAATGTCATGCAATCACTCC) sgRNA sequences were designed by using E-Crispr design algorithm and synthesized as a double-strand DNA prior to clone into Cas9-sgRNA expression vector according the manufacturer’s guidelines. All of the constructs were verified via double-strand plasmid sequencing. The control (no-sgRNA) and the Crispr knockout pseudovirus particles were done by following the standard protocol from RNAiCore.
facility. Cells were infected with designated pseudovirus particles with the incubation for 48 hours and then selected with 10 μg/mL puromycin for another 48 hours. The single-cell dilution was performed to obtain the successful Crispr knockout cells and verified by Sanger DNA sequencing against the specific sgRNA targeting genomic DNA regions.

Real-time PCR assay

mRNA was isolated and amplified as described previously (20). The real-time PCR experiments for FAS and FASLG were performed by using paired primers (for FAS: forward primer_5'-TCTGGTTCTAAGGTTGTGC-3', reverse primer_5'-CTTGTCAGTCCCTAGCTTCC-3'; for FASLG: forward primer_5'-CTCCGAGTGCTACAGCCA-3', reverse primer_5'-TGGACCTGCTGT-TAAATGG-3').

Western blot analysis

Protein lysates were prepared as described previously (20). A Western blot analysis was performed with primary antibodies for phospho-p38, p38, phospho-ERK1/2, ERK1/2, phospho-JNK, JNK, phospho-NFκB-p65, NFκB-p65, pro-caspase-3, cleaved caspase-3, N-cadherin, Snail, or α-tubulin.

Cytotoxicity assay

Cells (2 × 10^5) were seeded in wells of a 96-well plate. After 24 hours, medium was replaced with 200 μL of fresh medium containing various dosages of the hcc49scFv-Fasl recombinant protein at designated concentrations was applied by an intratumoral injection into a subcutaneous tumor twice daily or by an intraperitoneal injection in the orthotopic experiment. Tumor growth was measured with a caliper or fluorescent in vivo imaging system (Kodak). The length (L) and width (W) of the tumor were measured with a caliper every week, and the tumor volume (TV) was calculated as TV (mm^3) = (L × W^2)/2. Tumor metastasis was observed by fluorescent in vivo imaging system. After the mice were sacrificed, organs and primary tumors were recorded with fluorescent stereomicroscope (Olympus). Tissues were fixed with fixation solution (10% formalin, 5% glacial acetate, and 72% ethanol), dehydrated, and embedded in paraffin blocks. All animal procedures were approved by the

TUNEL assay

Cells were grown on glass cover slides in 6-well plates. Cells were fixed with 4% paraformaldehyde for 1 hour and further incubated with permeabilization solution (sodium citrate buffer containing 0.1% of Triton X-100) for 2 minutes. For formalin-fixed, paraffin-embedded (FFPE) cases, samples were deparaffinized using xylene and rehydrated in a graded series of ethanol with a final wash in tap water. Antigen retrieval was treated with Target Retrieval Solution (DAKO). After a PBS wash, terminal deoxynucleotidyl transferase deoxyuridinyl triphosphate nick end labeling (TUNEL) reaction mixtures were applied to whole samples in the dark at 37°C for 1 hour. A DAPl solution (Vector Laboratories) was used to stain nuclei for 5 minutes before sealing on a slide for observation by fluorescence microscopy.

Transwell invasion assay

The invasion assay was performed using Transwell inserts for a 24-well plate that contained 8-μm pores (Corning Costar). OSCC cells plated in a Matrigel (BD Biosciences) coated top chamber and incubated for 24 hours. The numbers of cells that invaded were normalized to the growth rate for each cell line.

Animal model

SAS-DsRed2 cells were collected and diluted to 5 × 10^5 cells/mL in PBS. Each mouse was injected subcutaneously with 100 μL of the cell suspension on both rear sides or orthotopically with 20 μL of a cell suspension into buccal tissues under anesthesia. The hcc49scFv-Fasl recombinant protein at designated concentrations was applied by an intratumoral injection into a subcutaneous tumor twice daily or by an intraperitoneal injection in the orthotopic experiment. Tumor growth was measured with a caliper or fluorescent in vivo imaging system (Kodak). The length (L) and width (W) of the tumor were measured with a caliper every week, and the tumor volume (TV) was calculated as TV (mm^3) = (L × W^2)/2. Tumor metastasis was observed by fluorescent in vivo imaging system. After the mice were sacrificed, organs and primary tumors were recorded with fluorescent stereomicroscope (Olympus). Tissues were fixed with fixation solution (10% formalin, 5% glacial acetate, and 72% ethanol), dehydrated, and embedded in paraffin blocks. All animal procedures were approved by the

Figure 1.

Clinical relevance of the Fas ligand (Fasl)/Fas ratio in OSCC. A and B, Gene expression levels of the Fas ligand (FASLG) and Fas (FAS) in unpaired (GSE42743; A) and paired (GSE13601; B) normal and tumor tissues derived from OSCC patients. Statistical significance was analyzed by a t-test in A and a paired t-test in B. C and D, Kaplan–Meier analysis of FASLG, FAS (C), and combined FASLG/FAS (D) gene expressions in OSCC tissues (GSE42743).

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Institutional Animal Care and Use Committee at Academia Sinica (Taipei, Taiwan).

Hematoxylin and eosin staining

Tissue samples were set in a 60°C oven for 1 hour, and then sequentially soaked in xylene, 100% EtOH, 95% EtOH, 75% EtOH, 50% EtOH, and 30% EtOH prior to performing hematoxylin staining for 5 minutes. After a wash with tap water for 5 minutes, samples were incubated with an eosin solution for 1 minute and then sequentially dipped into 75% EtOH, 95% EtOH, 100% EtOH, and xylene. Finally, samples were mounted with mounting solution (DAKO).

Statistical analysis

Each experiment was repeated three times. The values are presented as the means ± SE. The statistical analysis was performed using Statistical Package for Social Science software, version 16 (SPSS). When two groups were compared, the data were analyzed using Student’s t test. A one-way ANOVA followed by Tukey post hoc test was used to analyze three or more groups.

Results

A low FasL/Fas ratio is associated with tumorigenesis and predicts a poorer prognosis in patients with OSCC

To examine FasL and Fas expressions in patients with OSCC, 74 OSCC cases were analyzed from the Gene Expression Omnibus (GEO) database (GSE42743). Significantly low FasL and high Fas transcripts were observed in tumors compared with normal tissues (Fig. 1A). Moreover, an analysis of 40 matched tumor tissues and their corresponding normal tissues (GSE13601) revealed lower FasL and higher Fas expressions in the tumors (Fig. 1B). The Kaplan–Meier plot revealed a favorable overall survival of patients with high FasL expression (P = 0.037; Fig. 1C).

Relationships between the level of FasL/Fas expression and the prognosis of OSCC patients are shown in Fig. 1D. Most...
importantly, patients who had FasL \textsubscript{low}/Fas \textsubscript{high} tumors had shorter survival times compared with those who had Fas \textsubscript{high}/Fas \textsubscript{low} tumors. Taken together, the above clinical data indicate that downregulation of the FasL/Fas ratio is a critical event in promoting OSCC progression.

**Effect of the FasL/Fas ratio on the proliferative abilities of OSCC cells in vitro and in vivo**

The clinical findings suggested that the FasL/Fas ratio may affect the proliferative ability of oral cancer cells. We next evaluated the correlation between the FasL/Fas ratio and the cell proliferative ability, because OSCC is the most common malignant tumor of the head and neck. First, we used a set of head and neck SCC cell lines including Cal-27, HSC-4, SAS, Detroit-562, and RPMI-2650 to investigate expression levels of the FasL (Fig. 2A) and Fas (Fig. 2B) using a quantitative PCR. Next, proliferation rates of these cell lines were monitored in real time with the IncuCyte Life-Cell Imaging System (Fig. 2C). We observed that SAS cells expressed the lowest and highest levels of the FasL and Fas, respectively, in these cell lines (Fig. 2A and B) and also exhibited the highest

![Diagram](https://example.com/diagram.png)

**Figure 3.** The recombinant hcc49scFv-Fas ligand (FasL) effectively killed oral cancer cells with a low FasL/Fas ratio in vitro. A, The putative strategy of using recombinant hcc49scFv-FasL to kill OSCC. B, The killing efficacy of recombinant hcc49scFv-FasL at various concentrations on normal cell lines, HOK and WI-38, and the OSCC lines, Cal-27 and SAS. Data from three independent experiments were shown as mean ± SE. Statistical differences were analyzed by one-way ANOVA with Tukey post hoc tests and the symbol "∗" represents P < 0.001. C, The killing efficacy of recombinant FasL at various concentrations HOK and SAS cells. Data from three independent experiments were shown as mean ± SE. D, TUNEL assay for SAS cells without (control, CTL) or with hcc49scFv-FasL (50 μg/mL) treatment. E, The cleaved caspase-3 (top) and phosphorylation levels of ERK1/2, p38, and p65 (bottom) were assessed by a Western blot analysis after treatment of SAS cells with 50 μg/mL hcc49scFv-FasL for the indicated time points. Quantitative results of p-ERK, p-p38, and p-p65 protein levels, which were respectively adjusted to their total protein levels.
proliferative index among these cell lines (Fig. 2C). To further compare the correlation between the FasL/Fas ratio and the proliferative index among these cell lines, we found a nonsignificant trend toward an inverse correlation (Spearman rank correlation coefficient: \( \rho = -0.863, P = 0.078 \)) between the FasL/Fas ratio and proliferative abilities of these cell lines (Fig. 2D). We next examined the in vivo effects of the FasL/Fas ratio on tumor growth. Five head and neck SCC cells were subcutaneously injected into BALB/c nude mice, and we found that SAS-injected mice formed larger tumors than those in mice injected with other SCC cells after 3 weeks (Fig. 2E). These data suggest that altered expression of the FasL/Fas ratio in OSCC might be important for influencing tumor growth in vitro and in vivo.

Significant and selective antiproliferative effects of the hcc49scFv-Fasl fusion protein in OSCC cells

In our previous study, we developed the hcc49scFv-Fasl fusion protein which can specifically target the tumor antigen, TAG-72, and showed it to be 30,000-fold more cytotoxic against TAG-72\(^+\) Jurkat lymphoma cells compared with sFasL treatment (20). TAG-72 was also found to be expressed by oral cancer cells (18, 21), indicating that hcc49scFv-Fasl, possibly through binding to TAG-72, induced Fasl-mediated cell death (Fig. 3A). To determine the in vitro efficacy of the hcc49scFv-Fasl in OSCC cells, the cytotoxic effects of hcc49scFv-Fasl were examined in two OSCC cell lines (SAS and Cal-27) which represent different FasL/Fas ratios. As shown in Fig. 3B, the cytotoxicity of hcc49scFv-Fasl toward SAS, Cal-27, and normal cell lines, NHOK (normal human oral keratinocytes) and WI38 cells (human lung fibroblast cells) at various concentrations (0–50 \( \mu \)g/mL) for 72 hours were determined by an MTT assay. The results showed that hcc49scFv-Fasl exhibited much more potent cytotoxicity against the OSCC cell lines than normal cells especially cells (SAS) with a lower FasL/Fas ratio (Fig. 3B). Compared with hcc49scFv-Fasl, wild-type Fasl did not show different cytotoxic effects on NHOK and SAS cells (Fig. 3C). To investigate the mode of cell death induced by hcc49scFv-Fasl, SAS cells were treated with hcc49scFv-Fasl (50 \( \mu \)g/mL) for 72 hours and then subjected to a TUNEL assay. Nuclei of hcc49scFv-Fasl–treated cells were small, condensed, and TUNEL positive, suggesting apoptosis was induced by hcc49scFv-Fasl (Fig. 3D). The NFkB and MAPK signaling pathways were shown to control the cell proliferation and apoptosis of a great variety of cancers (22, 23). Moreover, sFasL stimulation in cancer cells can induce nonapoptotic events such as motility and invasiveness promotion through activating ERK and NFkB signaling pathways (24, 25). We therefore examined the effect of hcc49scFv-Fasl on the MAPKs and NFkB pathways in OSCC cells. Our results showed that treatment of SAS cells with hcc49scFv-Fasl (50 \( \mu \)g/mL) for different time points (0, 1, 6, and 24 hours) induced activation of caspase-3 and suppressed activation of ERK1/2 and NFkB (Fig. 3E), suggesting that hcc49scFv-Fasl did not turn on the nonapoptotic signaling and suppression of NFkB and ERK pathways might be involved in hcc49scFv-Fasl–mediated cell apoptosis in OSCC cells.

Significant antitumorigenic effect of hcc49scFv-Fasl in SAS subcutaneous and orthotopic graft models

We next examined the in vivo effects of hcc49scFv-Fasl on tumor growth. Previous reports showed that transduction and expression of fluorescent proteins in cancer cells can be used for real-time tracking of cells in mouse internal organs and does not affect cell behaviors such as metastasis (26–28). Here, DsRed2-expressing SAS cells were established, subcutaneously injected into BALB/c nude mice, and allowed to become stable for 3 days before initiating treatment. SAS-DsRed2 graft mice were directly intratumorally injected with hcc49scFv-Fasl (1 mg) or vehicle control twice daily, and tumor growth was monitored by fluorescent light imaging. Figure 4A shows the dominant inhibitory effect of hcc49scFv-Fasl on tumor growth after 10 days of treatment by in vivo fluorescent detection. Next, we examined the effects of hcc49scFv-Fasl on cell apoptosis.
within SAS tumors after 10 days of treatment. The number of TUNEL-positive cells had dramatically increased in hcc49scFv–FasL–injected tumors compared with vehicle-injected tumors (Fig. 4B). Moreover, we also established SAS-DsRed2 orthotopic graft mice, and 3 days after cells were injected, we treated mice with the hcc49scFv–FasL (1 mg) or the vehicle control every day by intraperitoneal administration for 5 or 10 days. Tumor growth inhibition was also observed after injecting tumor cells for 2 and 4 weeks (Fig. 4C).

The FasL/Fas ratio is inversely correlated with the invasive abilities of OSCC cells and significant antimetastatic effects of the hcc49scFv–FasL in a SAS orthotopic graft model

In addition to the effects of the FasL/Fas ratio on the proliferation of OSCC cells, we further investigated the correlation between the FasL/Fas ratio and cell invasive ability. We first evaluated the invasive abilities of five head and neck SCC cell lines (Cal-27, HSC-4, SAS, Detroit-562, and RPMI-2650) by a transwell invasion assay and found a variety of invasive abilities.

Figure 5.
The FasL/Fas ratio is inversely correlated with the invasive abilities of OSCC cells and significant antimetastatic effects of the hcc49scFv–FasL in a SAS orthotopic graft model. A, In vitro invasive abilities of OSCC cell lines. Values are presented as the mean ± SE of three independent experiments. Data were analyzed using a one-way ANOVA with Tukey post hoc tests at 95% confidence intervals; different letters represent different levels of significance. B, Correlation between the FasL/Fas ratio and the invasiveness of the OSCC cell lines. Spearman correlation coefficient = 0.9; P = 0.037. C, SAS cells were treated by the 50 µg/mL hcc49scFv–FasL for the Matrigel invasion assays. Values are presented as the mean ± SE of three independent experiments. * P < 0.05 compared with the vehicle groups. D, Mesenchymal markers, N-cadherin, and Snail were expressed in hcc49scFv–FasL–treated SAS cells. Lysates were collected from cells cultured with or without hcc49scFv–FasL (50 µg/mL) for 24 hours and subjected to a Western blot analysis.

E, Administration of the hcc49scFv–FasL through an intraperitoneal injection prevented the neck lymph node metastasis of DsRed-labeled SAS cells. F, Neck lymph node tissues were histologic analyzed by H&E staining. Histopathology of the lymph nodes are shown at ×100 (top) and ×400 (bottom). T, tumor part; NT, nontumor part. G, An overall survival curve was produced for tumor-bearing mice after treatment without or with the hcc49scFv–FasL using the Kaplan–Meier method. The P values were determined using a log-rank test.
among these cell lines, with SAS cells exhibiting the highest invasive ability (Fig. 5A). We combined this result with the results from Fig. 2A and B and found a significant inverse correlation (Spearman rank correlation coefficient \( \rho = -0.9, P = 0.037 \)) between the Fasl/Fas ratio and the invasive abilities of these cell lines (Fig. 5B). A previous report indicated that sFasL can induce epithelial–mesenchymal transition (EMT) to promote motility of gastrointestinal cancer (24). We next evaluated the \textit{in vitro} effects of the hcc49scFv-Fasl on tumor-invasive ability. In contrast to sFasL, hcc49scFv-Fasl can suppress invasion of SAS cells by inhibiting expression of mesenchymal markers, N-cadherin and Snail (Fig. 5C and D). Furthermore, we examined the \textit{in vivo} effects of the hcc49scFv-Fasl on tumor metastasis. SAS-DsRed2 cells were orthotopically injected into BALB/c nude mice for 3 days, and then mice were treated with the hcc49scFv-Fasl (1 mg) or the vehicle control every day by intraperitoneal administration for 5 days. Figure 5E shows that the formation of spontaneous cervical lymph nodes metastasis was dramatically prevented by the hcc49scFv-Fasl according to evidence from \textit{in vivo} and \textit{ex vivo} fluorescent imaging 6 weeks after injecting tumor cells. In addition, hematoxylin and eosin (H&E) staining revealed a prevention of cervical lymph node tumor metastasis in response to hcc49scFv-Fasl treatment (Fig. 5F). Most importantly, mice with tumors and hcc49scFv-Fasl treatment had significantly longer survival times compared with mice with tumors and vehicle treatment (Fig. 5G). Together, these results revealed that hcc49scFv-Fasl treatment suppressed invasion and lymph node metastasis of OSCC \textit{in vitro} and \textit{in vivo}, respectively.

The \textit{in vitro} and \textit{in vivo} antitumor effects of hcc49scFv-Fasl rely on endogenous Fas receptor in OSCC cells

To prove the tumour effects of hcc49scDv-Fasl, we were relying on endogenous Fas receptor, we preformed the Crispr knockout system on endogenous Fas of SAS cells. The deletion mutants with early translational termination codon were identified in two alleles of Fas Crispr knockout SAS cells (Fig. 6A; Supplementary Fig. S1A). The Fas Crispr knockout appeared to completely deplete the endogenous Fas expression (Fig. 6B), abolish the tumouricidal effects of hcc49scDv-Fasl \textit{in vitro} and \textit{in vivo} (Fig. 6C–F), prevent the proteolytic activation of caspase-3, and restore the phosphorylation of ERK1/2, p38, IxB, and NFkB (Supplementary Fig. S2C) in SAS cells. Accordingly, the neutralization of Fas with its specifically blocking antibody was also capable of releasing cell death (Supplementary Fig. S2A), preventing caspase-3 cleavage (Supplementary Fig. S2B) and rescuing the phosphorylation of ERK1/2, p38, IxB, and NFkB (Fig. 6G) in SAS cells. Together, these results showed that the antitumor function of hcc49scDv-Fasl heavily relies on endogenous Fas receptor in OSCC cells and the Fasl/Fas ratio determines the antitumor effectiveness of hcc49scDv-Fasl.

Discussion

The extrinsic apoptosis pathway can be triggered by enzymes of the TNF family, including Fas and the Fasl. Fasl-positive T cells can eliminate Fas-positive tumoral cells by inducing apoptosis (29). Fas is expressed by almost all human tumor cells including oral cancers (30, 31); however, given the fact that almost all established cancers express Fas, most cancer cells are resistant to apoptosis induction (4). This is because increased amounts of the sFasL are produced by MMP-mediated cleavage of extracellular domains of the mFasL and are released by these apoptosis-resistant cancer cells (5). The sFasL enables a powerful “counterattack” against antitumor immune effector cells, such as cytotoxic T cells, resulting in a repressed tumor attack by the immune system, and this phenomenon was also observed in head and neck cancers (32–34). Actually, higher concentrations of sFasL in the serum of many cancer patients (4) may be interpreted as the context of sFasL counterattack theory. Moreover, previous reports also indicated that altered Fasl and Fas expressions by peripheral blood T cells as well as Fasl- and Fas-mediated apoptosis of the major T-cell subsets are possible mechanisms for the down-regulation of immune functions and reduced apoptosis of tumor cells in patients with tobacco-related OSCC (35, 36). According to these results, the major cause of cancer cell resistance to Fas-mediated apoptosis is the Fasl aimed at the wrong target.

An alternative strategy to overcome these limitations is to develop a novel Fas agonist with high sensitivity and specificity for cancer cells. In this respect, we generated a Fasl fusion protein (20) and selected the anti-TAG-72 antibody as a fusion partner due to its specific binding ability to the TAG-72 antigen in tumors, including oral cancer (18). Anti-TAG-72 mAbs were studied in preclinical animal models and in humans for cancer detection based on their high specificity against cancer antigens in various solid cancers. For example, the anti-TAG-72 antibody was used as a radioimmunotherapeutic agent to treat ovarian cancer (37). Moreover, several nanoparticles conjugated with the anti-TAG-72 antibody were also used as targeting agents for radioimmunotherapy and tumor imaging (13, 38). The CC49 mAb recognizes the sialyl-Tn and sialyl-T epitopes, which are disaccharide carbohydrate antigens present on TAG-72. This antibody showed better immunoreactivity compared with other TAG-72–reactive antibodies in tumors (19). Hence, we investigated the anticancer effects of the fusion protein hcc49scFv-Fasl of the extracellular cytototoxic domain of the Fasl fused to a humanized antibody, CC49, on oral cancer. We found that the hcc49scFv-Fasl, but not recombinant Fasl, exhibited higher cytotoxicity and a greater apoptosis-inducing effect on OSCC cells compared with normal oral keratinocytes and lung fibroblasts, suggesting the problem of wrong targeting by the Fasl can be improved. Although we did not test the cytotoxic effect of the hcc49scFv-Fasl on primary T cells, it was demonstrated that TAG-72 is not expressed by T cells (39). We assumed that the hcc49scFv-Fasl might also exhibit lower sensitivity to cytotoxic T cells than cancer cells. The wild-type Fasl can be cleaved from the cell surface by MMPs and accumulates as an sFasL (40). It was observed that sFasL can have proapoptotic and antiapoptotic functions depending on the local microenvironment (5, 40). The sFasL can induce apoptosis following association/aggregation with ECM proteins or after spontaneous aggregation (40, 41). However, the antiapoptotic function is mediated by sFasL that competes with mFasL for Fas binding. The sFasL exists as a
Fas signaling plays a critical role in chemoresistance to doxorubicin and oxaliplatin by interacting with MMP-7 (45, 46). The chemotherapeutic drug-induced upregulation of MMP-7 can generate tumor-derived sFasL by cleavage of its membrane form and further counteract the host immune system by eliminating Fas-sensitive cytotoxic T cells (34, 45, 46). Actually, according to immunohistochemical staining results of esophageal squamous cell carcinoma (ESCC). FasL expression was diffusely observed in stromal cells around ESCC cells following chemoradiotherapy (CRT), supporting the CRT-induced sFasL counterattack theory (47). In the current study, we incubated recombinant hcc49scFv-Fasl or osteopontin (SPP1) with purified active MMP-7 in vivo. SPP1 was used as a positive control for MMP-7 cleavage (48). We found that only the SPP1, but not hcc49scFv-Fasl, was cleaved by MMP-7 (Supplementary Materials and Methods and Supplementary Fig. S4B), suggesting that a combination of chemotherapy and hcc49scFv-Fasl (specifically targeting Fas on cancer cells) may be beneficial for OSCC treatment.

In conclusion, our study shows that increased FasL/Fas expression ratio correlated with favorable prognoses in OSCC patients and worse proliferative and invasive abilities of OSCC cells. We clearly demonstrated that fusing the CC49 antibody to the extracellular cytotoxic domain of the FasL is a promising approach for suppressing tumor growth and metastasis of OSCC cells, especially cells harboring a low FasL/Fas expression ratio. This fusion protein exhibited powerful tumor-targeting and tumor-binding abilities because of the anti-TAG-72 antibody and no MMP cleavage sites within the extracellular domain of the FasL. Moreover, the tumor-promoting signals of Fas cannot be turned on by this fusion protein. Previous reports indicated that labeling of nucleus and cytoplasm of cancer cells with different fluorescent proteins or tumor cells expressing DsRed2 are implanted in mice transgenic for green fluorescence are suitable models to, respectively, observe the nuclear–cytoplasmic dynamics and tumor–host interactions in vivo (49, 50).

Our future works will try to use these models to further investigate the diverse anticancer effects of hcc49scFv-Fasl in vivo. Moreover, a detailed preclinical drug safety analysis will be required to further assess the promise of this fusion protein. Taken together, the hcc49scFv-Fasl fusion protein provides a novel approach to developing death receptor–targeting anticancer therapeutics, which merit additional investigation.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Figure 6.**

Fas Crispr knockout relieves the tumoricidal efficacy of hcc49scFv-Fasl on SAS cells in vitro and in vivo. A. Genomic DNA sequencing results of SAS cells without (no sgRNA) or with Fas (Fas sgRNA) Crispr knockout. The protospacer adjacent motif (PAM) sequence was marked in red and the red arrow indicated the cutting site of Crispr nuclease. The oligonucleotides underlined in green, blue, and black denote the original DNA sequences of Fas. B, Western blot analysis for Fas and β-actin protein expression in Fas-sgRNA SAS cells. C, The killing efficacy of hcc49scFv-Fasl at various concentrations on parental (no-sgRNA) and Fas Crispr knockout (Fas-sgRNA) SAS cells. Data from three independent experiments were shown in mean ± SE. Statistical differences were analyzed by one-way ANOVA with Tukey post hoc tests and the symbol “***” represents P < 0.001. D, TUNEL assay for SAS cells without (no-sgRNA) or with Fas (Fas-sgRNA) Crispr knockout in the absence (PBS) or presence of hcc49scFv-Fasl (50 μg/mL) treatment for 24 hours. E, Tumor appearance (top) and tumor volume (bottom) of parental (no-sgRNA) and Fas Crispr knockout SAS cells in the absence or presence of the treatment with hcc49scFv-Fasl (1 mg) twice daily in a subcutaneous implantation model. F, The distribution (top) and fluorescent intensity (bottom) of DsRed2-expressing SAS cells without (no-sgRNA) or with Fas Crispr knockout after the treatment with hcc49scFv-Fasl for 10 days in an orthotopic mouse model. Statistical differences were analyzed by Student t test. The symbol “*” and “**” represent P < 0.05 and 0.001, respectively. G, Western blot analysis for the protein expression of pro/cleaved caspase-3 and β-actin (top), as well as the phosphorylated and total ERK1/2, p38, Jnk, and p65 (bottom), in whole-cell lysates derived from parental and Fas Crispr knockout SAS cells posttreatment with hcc49scFv-Fasl at 50 μg/mL for 24 hours. In B and G, β-actin was used as an internal control of protein loading.
Mol Cancer Ther; 16(6) June 2017

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Grant Support

This study was supported by a grants [NSC102-2320-B-038-038-MY3] from the National Science Council of Taiwan (to M.H. Chien) and [MOST104-2320-B-038-010- MY2 and MOST 104-2320-B-038-061-MY3] from the Ministry of Science and Technology (to Y.F. Lin). This study was supported by Academia Sinica and Ministry of Science and Technology grants MOST 105-20110-01-09-MOST 105-02110-11-01-01, and MOST-406-0210-01-15-02 to M. Hsiao. This study was also supported by grants [1R01DK105833 and 2R01AI16725] to R. Sharma from National Institutes of Health.

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Received May 17, 2016; revised June 27, 2016; accepted March 7, 2017; published OnlineFirst March 14, 2017.

Acknowledgments

We would like to thank Dr. Shy-Te Ju for help and advice on hcc49/FcV-FasL fusion protein.

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A Fas Ligand (FasL)-Fused Humanized Antibody Against Tumor-Associated Glycoprotein 72 Selectively Exhibits the Cytotoxic Effect Against Oral Cancer Cells with a Low FasL/Fas Ratio

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