Two Gln187 mutants of human soluble APRIL inhibit proliferation of lung carcinoma A549 cells

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Soluble APRIL (sAPRIL), the active form of a proliferation-inducing ligand (APRIL), is implicated in the proliferation of tumor cells. Suppressing APRIL function has been considered as a potential strategy for the therapy of APRIL-associated tumors. In the present study, we generated human sAPRIL and its two mutants, Gln187-D-sAPRIL (Gln187 deleted) and Gly187-sAPRIL (Gln187 replaced by Gly). *In vitro* experiments showed that the two mutants had similar specific binding capacity to lung carcinoma A549 cells compared to the wild-type sAPRIL, and both, especially Gly187-sAPRIL, exhibited significant antagonistic effect on sAPRIL-induced tumor cell proliferation in a dose-dependent manner, which might be predominantly mediated by blocking sAPRIL-induced MEK and ERK phosphorylation but not p38MAPK or JNK signaling. *In vivo* experiments with nude mice bearing A549 cell-derived xenograft tumor showed that only the Gly187-sAPRIL mutant could significantly suppress the tumor growth. These results suggest that Gln187 may be a crucial amino acid in APRIL-mediated tumor cell proliferation *via* the MEK-ERK signaling pathway and that the sAPRIL mutants may serve as novel potential antagonists of APRIL for the therapy of APRIL-associated cancers.

Keywords: APRIL, Gln187 mutant of sAPRIL, anti-tumor activity

INTRODUCTION

A proliferation-inducing ligand (APRIL), so named after its inducing effect on tumor cell proliferation, is a member of tumor necrosis factor (TNF) family (Hahne *et al.*, 1998; Medema *et al.*, 2003), which is converted in the Golgi apparatus by furin convertase, resulting in its secretion as soluble APRIL (sAPRIL, 105–250 amino acids) (López-Fraga *et al.*, 2001). sAPRIL triggers subsequent intracellular activity and acts as a homotrimer after binding its receptors including B cell maturation antigen (BCMA), transmembrane activator and calcium signal-modulating cyclophilin ligand interactor (TACI), as well as heparin sulfate proteoglycans (HSPGs) (Marsters *et al.*, 2000; Kalled *et al.*, 2005; Ingold *et al.*, 2005). The mRNA and protein levels of APRIL or sAPRIL are reported to be low in normal tissues but much higher in a variety of tumor cells and tissues, such as gastrointestinal tumors, lung carcinomas, and melanoma (Hahne *et al.*, 1998; Stein *et al.*, 2002; Roth *et al.*, 2001). Patients with certain B-cell

lymphomas, multiple myelomas, or brain glioblastomas are also reported to exhibit serum values of sAPRIL significantly higher than those detected in normal donors, and a retrospective clinical analysis also found a significant correlation between high expression of APRIL in tumor lesions and decreased overall patient survival rates (Iłzecka & Iłzecki, 2006; Planelles *et al.,* 2007; Schwaller *et al.,* 2007). Additionally, recent studies have demonstrated that APRIL promotes solid tumor development in an autocrine fashion (Rennert *et al.,* 2000; Bonci *et al.,* 2004; Roosnek *et al.,* 2009). Therefore, suppressing APRIL function may be a potential strategy for the therapy of APRIL-associated tumors.

The available researches have shown that a soluble form of APRIL's receptor BCMA (sBCMA-Fc) retards tumor growth *in vivo.* But as we know, BCMA, as well as TACI, are also receptors of another TNF family member, B-cell activation factor (BAFF) (Rennert *et al.,* 2000). So using the soluble forms of the two receptors as antagonists of APRIL to inhibit tumor cell growth may also interfere with the function of BAFF. This consideration led us to inquire into a novel strategy to develop potential antagonists of APRIL to inhibit tumor cell proliferation.

APRIL is most homologous to another TNF family ligand BAFF, which is also called TALL-1 (Yu *et al.,* 2000; Rennert *et al.,* 2000). A previous study has demonstrated that a mutant of human BAFF in which eight amino acid residues (aa217-224) are replaced with two glycines has a similar binding capacity to BAFF receptors as wild-type BAFF, but it fails to activate the receptors and to promote B lymphocyte proliferation (Liu *et al.,* 2006). Based on the BLAST alignment of the two proteins (as shown in Fig. 1) and the work of Wallweber *et al.* (2004), both indicating that aa187-188 of APRIL may functionally correspond to aa217-224 of BAFF, we presumed that mutation of aa187-188 of APRIL may block APRIL-induced tumor cell proliferation. Because residue 188 is a Gly in native APRIL, we targeted only residue 187 (Gln) by either deleting it (mutant Gln187-D-sAPRIL) or replacing it with a Gly (mutant Gly187-sAPRIL), which means aa187-188 were replaced with one or two Glys. Subsequently, their binding capaci-

ties to lung carcinoma A549 cells and effects on tumor proliferation *in vitro* and *in vivo* were analyzed.

MATERIALS AND METHODS

Cell culture and animals. The human lung carcinoma cell line A549 was obtained from American Type Culture Collection and cultured in DMEM supplemented with 10% FBS (Hyclone, USA), streptomycin (100 μg/mL), penicillin (100 U/mL), and 2 mM glutamine. Male nude mice, 5–6 weeks old and weighing 18–23 g, were purchased from Vital River Experimental Animals Co. Ltd. (Beijing, China) and housed in the Experimental Animals Center of the Third Military Medical University (Chongqing, China).

Synthesis of proteins for sAPRIL and its mutants. Using the total RNA extracted from human tonsil tissue as the template, human sAPRIL cDNA (encoding aa 105–250) was amplified by RT-PCR with the oligonucleotides 5'-GCGGGTACCGCAGT-GCTCACCCAAAAACA-3' (forward primer) and 5'-GCGAAGCTTTCACAGTTTCACAAACCCCA-3' (reverse primer), and cloned into vector pUC19 after being digested with *Kpn*I and *Hin*dIII. Using this recombinant plasmid as the template, the two kinds of mutants of sAPRIL targeting Gln187 were constructed by a one-step opposite-direction PCR with TaKaRa MutanBest Kit (TaKaRa, Japan) according to the manufacturer's protocol. The primers for the mutant Gln187-D-sAPRILwere 5'-GAAGGCGGAAGGCAG-GAGACT-3' (forward primer) and 5'-TCGAGACAC-CACCTGACCCAT-3' (reverse primer), and the primers for the mutant Gly187-sAPRIL were 5'-GAAG-GCGGAGGAAGGCAGGAG-3' (forward primer) and 5'-TCGAGACACCACCTGACCCAT-3' (reverse primer). The DNA fragments of wild-type sAPRIL and its two mutants digested with *Kpn*I and *Hin*dIII were inserted into pQE-80L with 6×His-tag and then expressed in *Escherichia coli* DH5α. The His-tagged sAPRIL, Gln187-D-sAPRIL and Gly187-sAPRIL were purified from the bacterial lysate by binding to Ni^{2+}-NTA agarose (Qiagen, USA) and eluted with 200 mM imidazole (pH 5.9). After refolding by dialysis as previously described (Wingfield *et al.,* 1995), homotrimers (functional forms) of sAPRIL and its two mutants were isolated using Sephacryl S-200 gel filtration chromatography separately. The purity of the proteins was further confirmed by 15% SDS/PAGE with Coomassie blue staining and Western blotting using mouse anti-His-tag antibody (Qiagen, USA) as described (Gao

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                              187           188
hAPRIL   172   QDVTFTMGQVVSREGQ ....... GRQETLFRCIRSMP   201

hBAFF    202   TDKTYAMQHLIQRKKVHVFGDELSLVTLFRCIQNMP   237
                              217           224
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Figure 1. Sequence alignment of human APRIL and human BAFF.
Amino-acids sequence of APRIL (GenBank BAE16556.1) and BAFF (GenBank AAQ89240.1) were alined and the targets of mutation in APRIL were chosen by protein-protein BLAST as previously described (Wallweber *et al.,* 2004). APRIL aa 187–188 and corresponding BAFF aa 217–224 are marked in red. Residues forming the receptor binding-site are marked in green. The mutation site is outside the receptor binding region.

et al., 2006). Briefly, after separation by SDS/PAGE, the proteins were transferred to a nitrocellulose membrane and incubated with the primary and secondary antibodies. The immunoreactive bands were visualized using the ECL system.

Flow cytometry analysis. The binding activities of sAPRIL and its mutants to A549 cells were measured with mouse anti-His-tag antibody (Qiagen, USA) and FITC-conjugated goat anti-mouse IgG (Promega, USA) using a FACS Calibur analyzer with corrected mean fluorescence intensity and the binding affinity was scored by Fluorescence index (FI): high (FI>1.5), intermediate (1.0<FI<1.5), or weak (FI>0.5) (Nijman *et al.*, 1993).

In vitro cell proliferation assay. A549 cells were arrested in G0/G1 phase by serum deprivation for 24 h and then administered with increasing doses (0, 0.01, 0.1 or 1 μg/mL) of the three recombinant proteins alone or mixture of 1 μg/mL recombinant sAPRIL and various concentrations (0, 0.01, 0.1 or 1 μg/mL) of the mutants in DMEM supplemented with 2% FBS. After 48 h, cells were pulsed with 1 μCi of [^{3}H]thymidine for a further 8 h at 37°C. The cells were incubated overnight with 10% trichloroacetic acid (TCA) at 4°C, and incorporated [^{3}H]thymidine was then extracted in NaOH and measured in a liquid scintillation counter (LS 6500, Beckman Instruments; Fullerton, CA, USA). To facilitate comparison between different conditions, absolute counts were converted to a percentage of control (untreated) counts. All values are means ±S.E.M. of three independent experiments done in triplicate.

Signaling pathway analysis. Using Western blotting, we assayed phosphorylated p38 MAPK, JNK, MEK and ERK (p-p38, p-JNK, p-MEK, and p-ERK). Total MEK and ERK were also assayed to identify p-MEK and p-ERK expressions. Briefly, after treatment with 1 μg/mL sAPRIL in the presence of various concentrations (0.01, 0.1 or 1 μg/mL) of the two mutants, total cellular extracts of A549 cells were separated on a 10% SDS/PAGE gel and transferred to nitrocellulose membranes. Following blocking in TBS and Tween 20 (TBST; 10 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 10% nonfat instant milk powder, the nitrocellulose membranes were incubated overnight at 4°C with primary antibodies (1:1000 dilution of anti-phospho-p38, anti-phospho-JNK, anti-

phospho-MEK, anti-phospho-ERK, anti-total MEK or anti-total ERK antibodies; Cell Signaling, USA) in TBS containing 10% milk. After washing with TBST and incubation with the secondary antibody, the immunoreactive bands were visualized employing an ECL detection system (GE, USA).

In vivo xenograft tumor growth assay. A549 cells were harvested, washed twice and resuspended in pyrogen-free PBS at $5×10^6$ cells/mL. Mice were anesthetized using an i.p. injection of 100 mg/kg ketamine and 5 mg/kg xylazine. Then 200 μL of A549 cells was carefully implanted subcutaneously in the flank abdominal area of the mice. Purified proteins were injected i.p. into the mice just before tumor cell implantation and every three days thereafter. The injected dose for both Gln187-D-sAPRIL and Gly187-sAPRIL was 1 mg/kg, and the control group received PBS. Xenograft tumor diameter was measured on day 30 after implantation using a caliper, and tumor volume was calculated using the formula $v=4/3πR^3$.

Statistical analysis. Graphic data were expressed as mean ±S.E.M. Statistical analyses of the data were performed by Student's *t*-test and one-way ANOVA. $P<0.05$ was considered as a significant difference.

RESULTS

Recombinant sAPRIL and its two mutants. Recombinant human sAPRIL and its two mutants targeting Gln187 expressed in *E. coli* were purified by Ni^{2+}-NTA agarose. After dialysis for refolding, all proteins were over 90% pure, and the molecular mass of each monomer was about 17 kDa, as revealed by Coomassie blue staining of SDS/PAGE (Fig. 2A). The proteins were further analyzed by Western blotting (Fig. 2B).

The two sAPRIL mutants had a similar binding capacity to A549 cells with the wild-type sAPRIL. Following preparation of the recombinant sAPRIL and its two mutants, we used flow cytometry analysis to detect their binding capacity to lung carcinoma A549 cells, with PBS as negative control. As shown in Fig. 3, all three recombinant proteins could bind to A549 cells with similar affinities at the middle level. In addition, no significant difference of FI was observed in them. These results indicated that the two kinds of mutations targeting Gln187 in sAPRIL do not substantially affect its binding capacity.
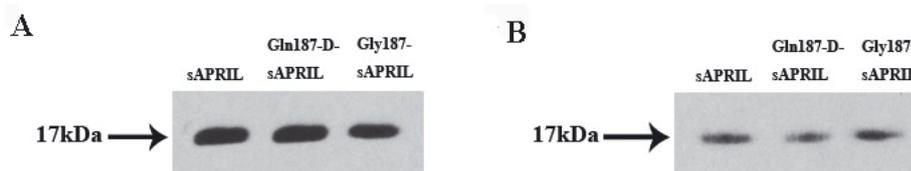

Figure 2. SDS/PAGE and Western blot analysis of purified sAPRIL and the two mutants.
(**A**) SDS/PAGE analysis. (**B**) Western blot analysis. The purified sAPRIL and its two mutants were about 17-kDa (monomers).
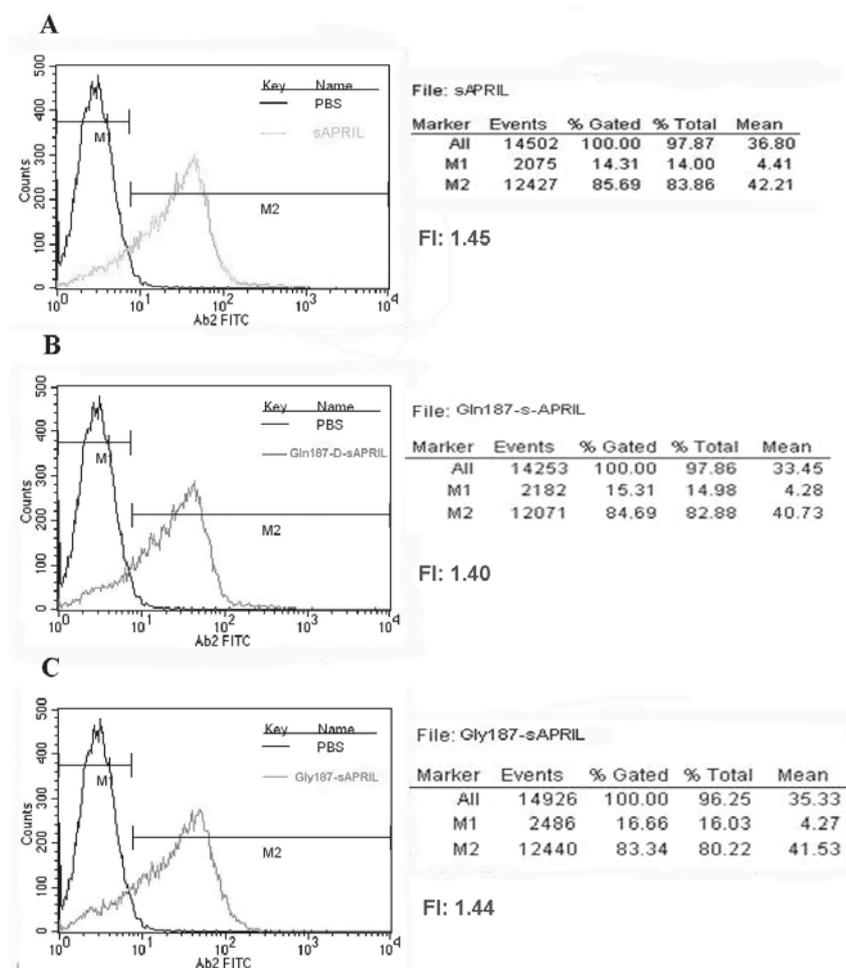
Figure 3. Binding of sAPRIL and its two mutants to A549 cells.
The binding activities of sAPRIL and its mutants to A549 cells were measured by flow cytometry analysis. (**A**) sAPRIL binding to A549 cells. (**B**) Mutant Gln187-D-sAPRIL binding to A549 cells. (**C**) Mutant Gly187-sAPRIL binding to A549 cells. PBS served as control.

Both mutants inhibit sAPRIL-induced A549 cell proliferation *in vitro*. To investigate whether the recombinant proteins affected tumor cell growth, we measured proliferation of A549 cells treated with either of the three proteins by the [³H]thymidine incorporation assay. As shown in Fig. 4A, sAPRIL alone promoted proliferation, but two mutants, especially Gly187-sAPRIL, inhibited proliferation of A549 cells in a dose-dependent manner.

In competitive experiments, A549 cells were challenged with 1 µg/mL sAPRIL in the presence of various concentrations of Gln187-D-sAPRIL or Gly187-sAPRIL. Gly187-sAPRIL could completely block the sAPRIL-induced cell proliferation (Fig. 4C), while Gln187-D-sAPRIL had only a partial blocking effect (Fig. 4B).

Inhibition of MEK/ERK signaling was involved in the mutant-mediated blocking of sAPRIL-induced A549 cell proliferation. To investigate the potential MAPK signaling pathway involved in the antagonism of sAPRIL by the mutants, p-p38, p-JNK, p-MEK, and p-ERK were assayed using Western blotting. As shown in Fig. 5, there were no significant changes of p-p38 or p-JNK in cells treated with sAPRIL alone or with 1 µg/mL sAPRIL in the presence of different concentra-

tions of the mutants. But as shown in Fig. 6A and Fig. 6B, sAPRIL treatment alone clearly increased p-MEK level, which could be dramatically blocked by Gly187-sAPRIL in a dose-dependent manner (Fig. 6B) and partially blocked by Gln187-D-sAPRIL (Fig. 6A). MEK operates upstream of ERK1/2, so p-ERK was also determined at the same time points. The results showed that changes of p-ERK1/2 paralleled those of p-MEK in cells treated with sAPRIL or its mutants (Fig. 6C and Fig. 6D). This result suggested that inhibition of APRIL-induced activation of MEK/ERK signaling might be a mechanism of the mutant-mediated blocking of sAPRIL-induced proliferation of A549 cancer cells.

Mutant Gly187-sAPRIL but not Gln187-D-sAPRIL suppressed tumor growth *in vivo*. The demonstration that the two mutants can inhibit the sAPRIL-induced A549 cell proliferation *in vitro* prompted us to investigate whether they have an anti-tumor effect *in vivo*. The lung carcinoma cell line A549 was grown as subcutaneous tumors in nude mice and subjected to Gln187-D-sAPRIL or Gly187-sAPRIL treatment. As shown in Fig. 7, 30 days after tumor cell implantation, the xenograft tumor size for control group mice was 0.09–0.12 cm³, while treatment with Gly187-sAPRIL yielded a sig-
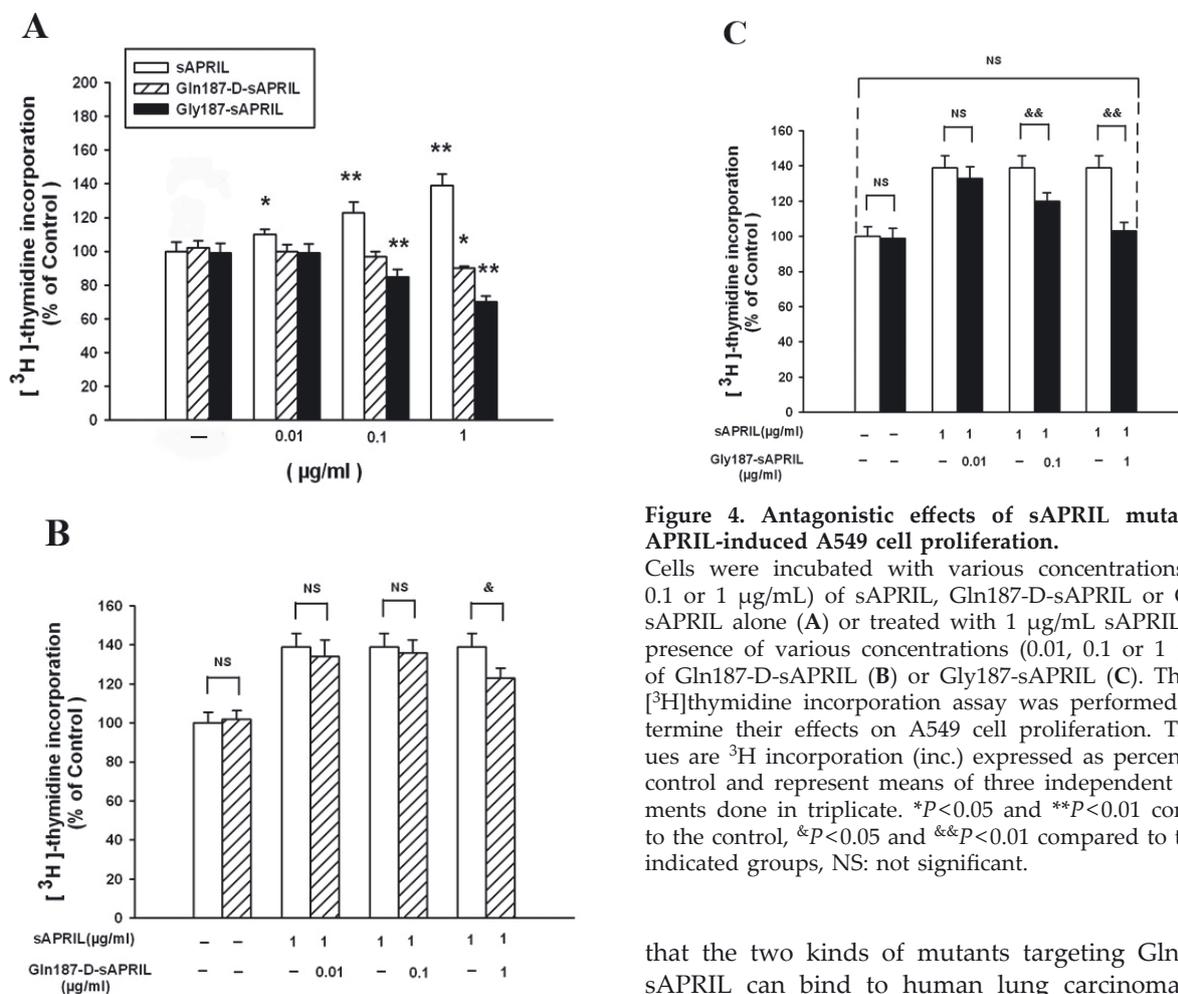
A

B

C

Figure 4. Antagonistic effects of sAPRIL mutants on APRIL-induced A549 cell proliferation.
Cells were incubated with various concentrations (0.01, 0.1 or 1 μg/mL) of sAPRIL, Gln187-D-sAPRIL or Gly187-sAPRIL alone (**A**) or treated with 1 μg/mL sAPRIL in the presence of various concentrations (0.01, 0.1 or 1 μg/mL) of Gln187-D-sAPRIL (**B**) or Gly187-sAPRIL (**C**). Then, the [^{3}H]thymidine incorporation assay was performed to determine their effects on A549 cell proliferation. The values are ^{3}H incorporation (inc.) expressed as percentage of control and represent means of three independent experiments done in triplicate. *$P<0.05$ and **$P<0.01$ compared to the control, &$P<0.05$ and &&$P<0.01$ compared to the two indicated groups, NS: not significant.

nificant reduction in tumor size to about 0.02–0.05 cm^3 ($P<0.01$). However, no such tumor growth retardation was observed in the nude mice with the administration of Gln187-D-sAPRIL (tumor size 0.08–0.13 cm^3).

DISCUSSION

In the present study, we successfully generated two sAPRIL mutants targeting residue Gln187 with a similar specific binding capacity to lung carcinoma A549 cells as that of wild-type sAPRIL. Both mutants, especially Gly187-sAPRIL, inhibited A549 cell proliferation and exhibited antagonistic effects on sAPRIL-induced tumor cell proliferation *in vitro*, which might be mediated by blocking sAPRIL-induced MEK/ERK signaling. However, *in vivo* experiments indicated that only the Gly187-sAPRIL mutant significantly suppressed tumor growth in mice transplanted with A549 cells.

It has been reported that sAPRIL exerts its effect by binding to three receptors–BCMA, TACI and HSPG (Marsters *et al.*, 2000; Kalled *et al.*, 2005; Ingold *et al.*, 2005). Our FACS analysis showed

that the two kinds of mutants targeting Gln187 of sAPRIL can bind to human lung carcinoma A549 cells as well as does wild-type sAPRIL. Since BCMA and TACI are heavily absent on A549 cells (Rennert *et al.*, 2000) and previous research has found that HSPG binding promotes APRIL-induced tumor cell proliferation (Hendriks *et al.*, 2005), it seems likely that HSPG expressed on A549 cells is responsible for the binding of recombinant sAPRIL and its mutants. Whether HSPG is the only receptor mediating the sAPRIL-induced tumor cell proliferation or some other receptor of APRIL take part in the response is not clear and requires further investigation. However, the deletion of Gln187 or its replacement with a Gly in sAPRIL had no effect on their specific binding to the receptors, indicating that Gln187 of sAPRIL is not necessary for its binding.

A549 cells express endogenous sAPRIL which can stimulate cell proliferation, a process called autocrine regulation (Rennert *et al.*, 2000). In the cell proliferation assay, we found that the two mutants alone can retard cell proliferation in a dose-dependent manner. Because sAPRIL and its two mutants bind to A549 cells with similar efficiency, it seems likely that the two mutants may compete with endogenous sAPRIL for binding to the same receptors on A549 cells and antagonize sAPRIL-induced tumor cell proliferation. To confirm this hypothesis, we challenged
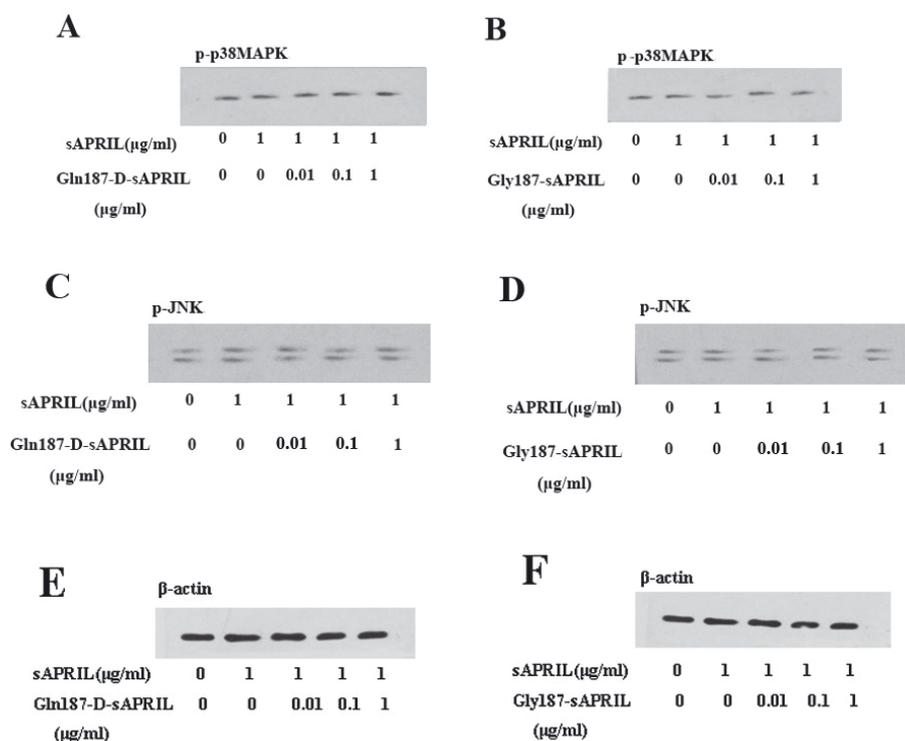
Figure 5. p-p38 MAPK and p-JNK in the signaling of mutant-mediated blocking of sAPRIL-induced proliferation of A549 cells.

A549 cells were treated with various concentrations (0.01, 0.1 or 1 μg/mL) of Gln187-D-sAPRIL or Gly187-sAPRIL in the presence of 1 μg/mL sAPRIL. Thirty minutes later, whole cell proteins were extracted for Western blot. The blots were probed with phospho-specific antibody for p-p38 or p-JNK to determine their phosphorylation. (**A**) and (**B**): p-p38 level after challenge with Gln187-D-sAPRIL or Gly187-sAPRIL. (**C**) and (**D**) p-JNK level after challenge with Gln187-D-sAPRIL or Gly187-sAPRIL. (**E**) and (**F**) β-actin was the control.
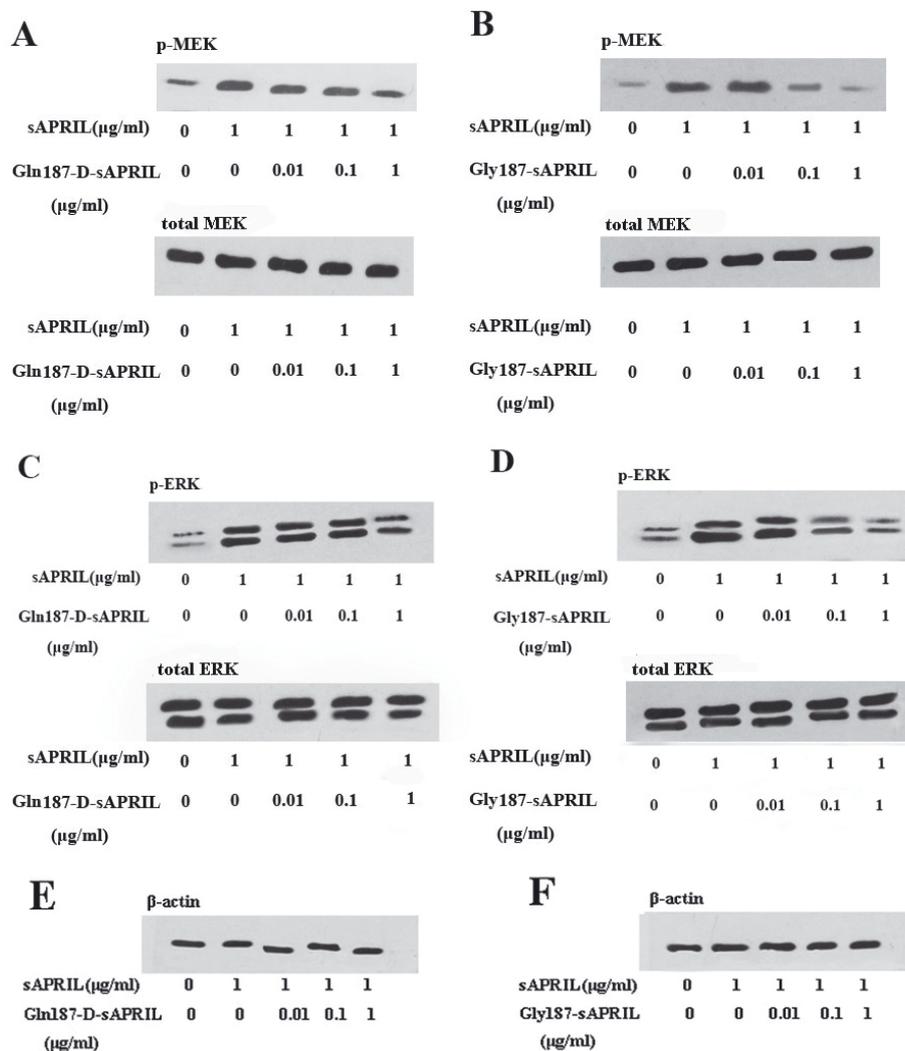

Figure 6. p-MEK and p-ERK in the signaling of mutant-mediated blocking of sAPRIL-induced proliferation of A549 cells.

Treatment of A549 cells and Western blot were done as described in Fig. 4 using total MEK- or total ERK- and p-MEK- or p-ERK1/2-specific antibodies. (**A**) and (**B**): p-MEK and total MEK after challenge with Gln187-D-sAPRIL or Gly187-sAPRIL. (**C**) and (**D**): p-ERK and total ERK after challenge with Gln187-D-sAPRIL or Gly187-sAPRIL. (**E**) and (**F**) β-actin was the control.
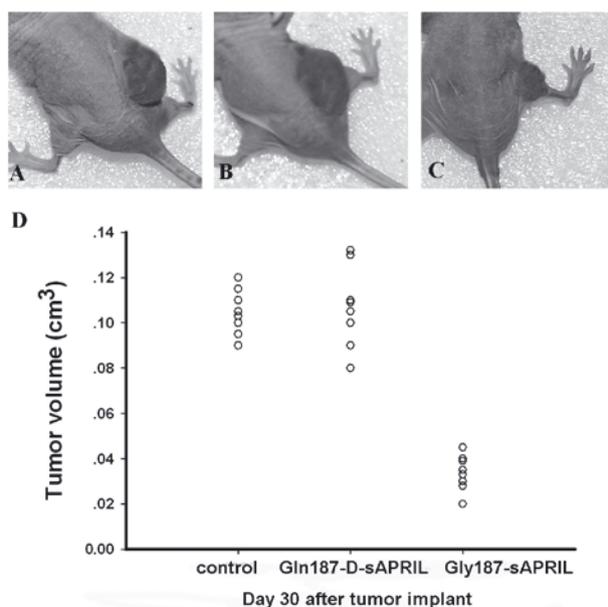
Figure 7. *In vivo* anti-tumor effect of two sAPRIL mutants.
A549 cells were implanted into nude mice subcutaneously. The animals were injected i.p. with 1 mg/kg Gly187-D-sAPRIL (**B**) or Gly187-sAPRIL (**C**) just before tumor cell implantation and every three days thereafter. Mice treated with vehicle served as control (**A**). Xenograft tumor volumes were measured at 30 days after A549 cell implantation (**D**) (n=8 mice/group).

A549 cells with 1 μg/mL sAPRIL in the presence of various concentrations of Gln187-D-sAPRIL or Gly187-sAPRIL. As expected, Gly187-sAPRIL completely blocked and Gln187-D-sAPRIL partially blocked the sAPRIL-induced proliferation of A549 cells. These results confirmed that both mutants of Gln187 have an antagonistic or competitive activity (whether strong or weak) against sAPRIL *in vitro*. This finding also suggests that Gln187 is not required for the binding of sAPRIL to A549 cells but is necessary for its function in inducing tumor cell proliferation.

So far, the signaling pathway responsible for the tumor growth-inducing function of sAPRIL is poorly known. Previous reports have shown that the growth-promoting effects of APRIL on some tumor cells are associated with activation of p38 MAPK signaling (Trabach *et al.*, 2002; Mackay *et al.*, 2003). It is also possible that the other two MAPK signaling pathways, JNK and MEK/ERK, are also involved in the activity of APRIL. To identify the signaling pathway involved in the APRIL-antagonizing effects of its two mutants, we investigated three crucial component of the MAPK pathways: p38, JNK, and MEK/ERK. The results showed that sAPRIL strongly increased the phosphorylation of MEK and, to a lesser extent, of p-ERK but not of p38 and JNK. This stimulating effect could be suppressed by Gln187-D-sAPRIL partially and by Gly187-sAPRIL completely.

ERK is the best-studied molecule of the MAPK pathway and is activated by many growth factors and cytokines that are important in driving proliferation and preventing apoptosis in cancers, such as hepatocellular carcinoma, lung cancer, breast cancer, and pancreatic cancer (Ito *et al.*, 1998; Dhillon *et al.*, 2007). In the present study, we found that activation of the MEK/ERK pathway might be involved in sAPRIL-induced A549 cell proliferation and that Gln187 might be a key amino-acid residue for sAPRIL in triggering this signaling. In addition, inhibition of APRIL-induced activation of MEK/ERK (but not p38 and JNK) signaling might be one of the mechanisms for the mutant-mediated blocking of sAPRIL-induced proliferation of A549 cancer cells. Previous studies have demonstrated the elevated ERK activation mediated by HSPGs contributes to the overexpression of fibrotic proteins (Rabenstein, 2002; Chen *et al.*, 2008), which to some extent supports our speculation that HSPG expressed on A549 cells might mediate the binding and activity of sAPRIL and its mutants. But to confirm this signal pathway, further investigation is needed.

The results of the *in vitro* experiments showed that the beneficial anti-tumor effect of the Gly187-sAPRIL mutant was much stronger than that of Gln187-D-sAPRIL, and the *in vivo* experiments confirmed that Gly187-sAPRIL significantly suppressed xenograft tumor growth in nude mice implanted with A549 cells, while Gln187-D-sAPRIL had little effect. We speculate that replacement of Gln187 with Gly can make the recombinant mutant protein more flexible, better refolded for exerting a stronger effect compared with the mutant with deletion of Gln187. More studies are needed to show whether it is true.

Taken together, we successfully generated two Gln187 mutants of sAPRIL and using *in vitro* and *in vivo* assays showed that it is a promising way to developing a novel anti-cancer strategy for the treatment of APRIL-associated tumors.

Acknowledgements

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