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# The proapoptotic activity of C-terminal domain of apoptosis-inducing factor (AIF) is separated from its N-terminal

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**ABSTRACT**

Apoptosis-inducing factor (AIF) is a mitochondrial flavoprotein that mediates both NADH-oxidizing and caspase-independent apoptosis. Further, the proapoptotic activity of AIF is located in the C-terminus of AIF, although the precise minimum sequence responsible for apoptosis induction remains to be investigated. In the present study, we generated two truncated AIFs, AIFΔ1-480-FLAG, which is a FLAG-tagged C-terminal peptide comprising amino acids from 481 to 613, and AIF360-480 containing amino acids from 360 to 480 of AIF. We used confocal microscopy to demonstrate that both the truncated proteins are expressed and located in the cytoplasm of transfected cells. AIFΔ1-480 but not AIF360-480 induces apoptosis in transfected cells. We also found that the expression of AIFΔ1-480 could initiate the release of cytochrome *c* from the mitochondria. The suppression of caspase-9 via siRNA blocked the proapoptotic activity of AIFΔ1-480. Therefore, AIFΔ1-480 is sufficient for inducing caspase-9-dependent apoptotic signaling, probably by promoting the release of cytochrome *c*. At last, we generated a chimeric immuno-AIFΔ1-480 protein, which comprised an HER2 antibody, a *Pseudomonas* exotoxin A translocation domain and AIFΔ1-480. Human Jurkat cells transfected with the immuno-AIFΔ1-480 gene could express and secrete the chimeric protein, which selectively recognize and kill HER2-overexpressing tumor cells. Our study demonstrates the feasibility of the immuno-AIFΔ1-480 gene as a novel approach to treating HER2-overexpressing cancers.

**Key terms:** apoptosis inducing factor (AIF), apoptosis, cytochrome *c*, mitochondria, HER2.

## INTRODUCTION

The apoptosis-inducing factor (AIF) is a phylogenetically conserved mitochondrial intermembrane flavoprotein that mediates both NADH-oxidizing and caspase--independent apoptosis (Lorenzo et al., 1999, Susin et al., 2000, Cande et al., 2002). The mammalian AIF precursor consists of an N-terminal mitochondrial localization sequence (MLS, amino acid (aa) 1-100), a spacer sequence (aa101-121), an FAD-binding domain (aa122-262 and aa400-477), an NADH-binding domain (aa263-399), and a C-terminal domain (aa478-613) (Mate et al., 2002). It has been reported that the C-terminal domain is responsible for the proapoptotic activity of AIF (Ye et al., 2002). AIF is normally confined to the mitochondrial intermembrane space, where it probably acts as an NADH-oxidoreductase and catalyzes electron transfer in the mitochondrial respiratory chain (Susin et

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al., 1999). In response to the apoptosis signal, AIF is translocated into the cytosol and then, the nucleus. The AIF proteins bind to DNA and trigger peripheral chromatin condensation, large-scale DNA fragmentation (approximately 50 kb), and apoptosis in the nucleus (Zhang et al., 2002). Studies have suggested that the oxidoreductase activity of AIF is not essential for its proapoptotic activity (Delettre et al., 2006, Miramar et al., 2001). The strongly positive electrostatic potential present on the surface of the AIF protein is responsible for its DNA-binding activity, which is independent of the DNA sequence and is crucial for inducing apoptosis (Ye et al., 2002). The mechanisms of AIF-mediated chromatin condensation and DNA degradation in apoptosis have not been completely elucidated. The possible mechanisms include the following: (1) AIF may bind to DNA and increase the susceptibility of the DNA to some nucleases; (2) AIF may recruit proteases and nucleases that subsequently cause chromatin condensation and fragmentation; and (3) AIF may itself possesses some protease and nuclease activities.

Once released from the mitochondria, AIF can initiate and mediate caspase-independent apoptosis, a process that does not require the participation of cytosolic factors and that cannot be prevented by intrinsic caspase inhibitors or antiapoptotic proteins, e.g., bcl-2 (Lorenzo et al., 1999). Moreover, knockout of the AIF gene is lethal in mouse embryos, indicating the indispensable role played by critical caspase-independent apoptosis during the early stages of mammalian development (Cecconi et al., 1998). AIF-mediated apoptosis is also involved in the physiological and pathological death of most eukaryocytes (Boujrad et al., 2007). The accumulation of AIF in the extramitochondrial compartment, particularly in the nucleus, leads to caspase-independent apoptosis (Loeffler et al., 2001). Susin et al. have confirmed that AIFΔ1-120, an MLS-truncated AIF, is located in the cytoplasm and has proapoptotic activity comparable to that of wild-type AIF (Susin et al., 1999). Yu et al. generated a chimeric immuno-AIF protein, which comprised an HER2 antibody, a *Pseudomonas* exotoxin A (PEA) translocation domain, and AIFΔ1-120 and demonstrated that the antibody/AIF fusion protein efficiently triggered apoptosis of HER2-overexpressing tumor cells (Yu et al., 2006). Recently, alternatively spliced variants of AIF have been identified. AIFsh, which contains aa353-613 of AIF, is a proapoptotic isoform and another new isoform AIFsh2 possesses intact NADH oxidase activity (Delettre et al., 2006,).

Here, we generated a truncated AIF, namely, AIFΔ1-480, which comprised aa481-613 of the wild-type protein, and demonstrated that AIFΔ1-480 effectively induced apoptosis in transfected cells a process that involves the release of cytochrome *c* from the mitochondria and possibly the activation of caspase-9. We also generated the genes of a chimeric proteins, designated immuno-AIFΔ1-480, in which AIFΔ1-480 were fused sequentially at the N-terminus to a cell translocation domain PEA, an anti-HER2 single chain antibody and a signal peptide. The chimeric proteins were expected to selectively kill HER2-overexpressing tumor cells.

## MATERIALS AND METHODS

### *DNA constructs*

The cDNAs that encode AIFΔ1-480 and AIF360-480 were amplified by polymerase chain reaction (PCR) from pcDNA3-AIFΔ1-120 with the corresponding primers (Table). The resulting fragments, which contained the coding sequence of a FLAG tag at the 3'-end, were cloned into the pcDNA3 vector (Fig 1). To inhibit the expression of caspase-9 by siRNA, a 19-nt target sequence in the coding region of the human caspase-9 gene was empirically chosen. Synthesized oligodeoxyribonucleotides encoding the corresponding siRNA were designed as follows. The 19-nt target sequence was used as the sense strand, and this was followed by a 9-nt spacer and complementary antisense strand with 5 repeat Ts as the termination signal. For the negative control, a double-

stranded oligonucleotide, which contained a 19-nt sequence that did not match any known human gene, was introduced into the pSUPER.puro vector (Table). The genes of recombinant immuno-AIFΔ1-480 was generated by sequential fusion of the coding sequences of a signal peptide (Met-Lys-His-Leu-Trp-Phe-Phe-Leu-Leu-Leu-Val-Ala-Ala-Pro-Arg-Trp-Val-Leu-Ser-), a signal-chain HER2 antibody (sFv23e), a PEA translocation domain (aa253-358) and AIFΔ1-480. The immuno-AIFΔ1-480 gene was cloned into a pCMV plasmid respectively. All constructs were confirmed by DNA sequencing.

*Cell culture and transfection*

Human cervical carcinoma cell line HeLa, human breast tumor cell line SKBR-3, human gastric cancer cell line SGC-7901, human umbilical vein endothelial cell line ECV304, human laryngeal tumor cell line Hep2 and human lymphoma cell line Jurkat were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% $CO_2$. At 24 h before transfection, the cells were seeded in 12-well plates at a density of $1 \times 10^5$ cells per well. Transfection was performed at 70-80% cell confluence by using LipofectAMINE 2000 (Invitrogen) using the standard procedure. For the transient expression of truncated AIF, the cells were harvested at the indicated time after transfection. For stable expression, transfected cells were selected using puromycin (50 μg/ml) or G418 medium (800 μg/ml) for 3-4 weeks.

*Semiquantitative RT-PCR analysis*

Total RNA was extracted, and cDNA was prepared from HeLa cells stably transfected with pSUPER.puro-siC9 (expressing shRNA targeting caspase-9) and pSUPER.puro-NC (negative control) after puromycin selection. Using C9U as the

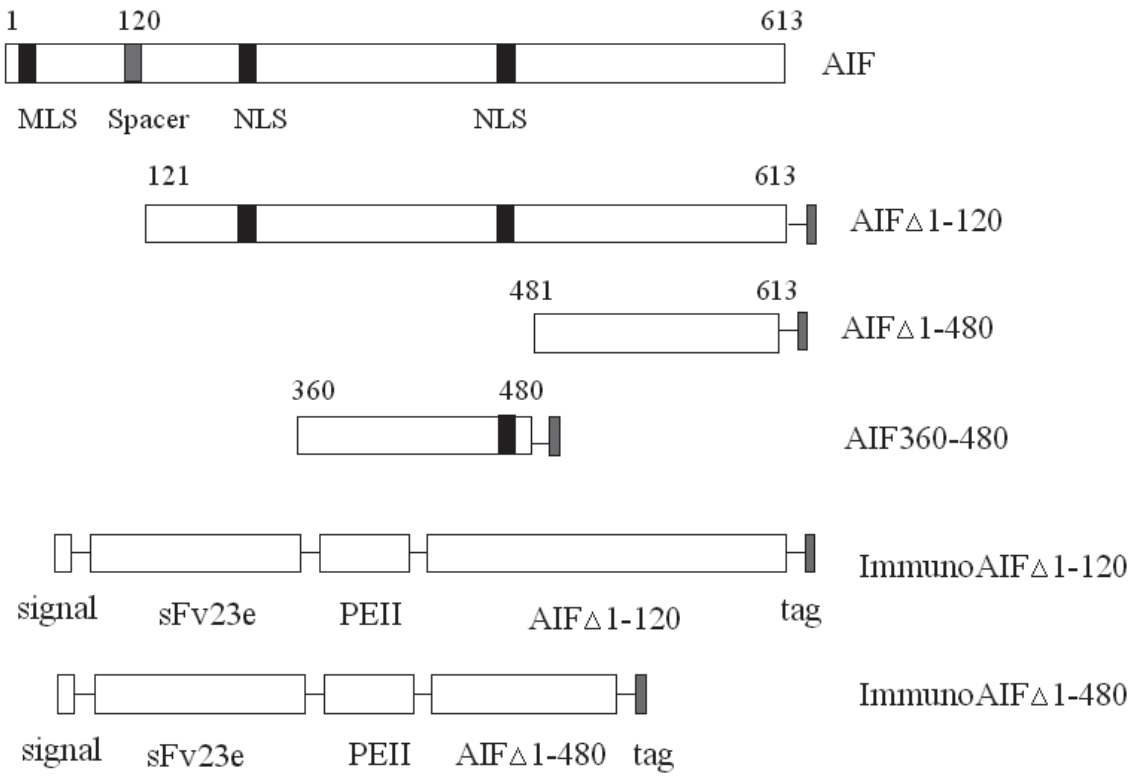


**Figure 1:** Schematic diagram of AIF-based gene constructs. MLS, mitochondrial localization sequence; NLS, nuclear localization sequence; tag, FLAG tag.

upstream primer and C9D as the downstream primer (corresponding to bp 98-793 of the caspase-9 cDNA), the caspase-9 gene was amplified from the cDNA templates. PCR amplification of β-actin using primers BU and BD was used as the control. The primers used for PCR amplification are as follows. C9U 3'-agctgtt caggccccata-3'; C9D, 5'-tcacaatcttctcgaccg-3'; BU, 5'-tggcaccacaccttctacaa-3'; BD, 5'-gcagctcgtagctcttctcc-3'.

*Western blot analysis*

The lysates of the AIFΔ1-480-transfected cells were concentrated and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The proteins were transferred to polyvinylidene difluoride membranes (Amersham Biosciences) and incubated sequentially with mouse anti-FLAG primary antibody (1: 2000) and a biotin-conjugated secondary antibody. The bands were developed using 3, 3'-diaminobenzidine tetrahydrochloride (DAB) western blot detection reagents. Mouse monoclonal caspase-9 antibody (Abcam) (1: 2000) and the secondary antibodies ZB2305 anti-mouse immunoglobulin horseradish peroxidase-linked F(ab)2 fragments from goat (Zhongshan Goldenbridge Ltd.) were used for western blot analysis of caspase-9, and the bands were developed with enhanced chemiluminescence (ECL) western blotting detection reagents (Pierce Biotechnology Inc.).

*Laser confocal microscopy*

HeLa cells cultured on coverslips were fixed, permeabilized, and stained for AIF-FLAG. A polyclonal anti-FLAG antibody (Sigma) was used to detect the expression of truncated AIF, and a SABS-cy3-conjugated rabbit anti-mouse IgG was used to amplify and visualize the staining. The nuclei were stained by 4', 6-diamidino-2-phenylindole (DAPI). The expression of truncated AIF-Flag (red) in transfected cells was assessed by observation using laser confocal microscopy.

*Annexin V/PI staining*

The transfected HeLa cells were collected, washed, and stained using Annexin V-fluorescein isothiocyanate (FITC) and

TABLE

Oligonucleotides used for DNA constructs Primers for truncated AIF expression vectors

| |
|---|
| AIFΔ1-120: |
| 5'-TTTGGTACCGAATTCCACCATGGAGGAAGTTCCTCAAGACAAG-3',<br>5'-TTTTCTAGATCACTTGTCATCGTCGTCCTTGTAGTCGTCTTCATGAATGTTGAA-3', |
| AIFΔ1-480: |
| 5'-TTTGGTACCGAATTCCACCATGTTCTGGAGTGATTTGGGC-3',<br>5'-TTTTCTAGATCACTTGTCATCGTCGTCCTTGTAGTCGTCTTCATGAATGTTGAA-3', |
| AIF360-480: |
| 5'-TTTGGTACCGAATTCCACCATGGGGGTTAAGGTG ATGCCCAAT-3',<br>5'-TTTTCTAGATCACTTGTCATCGTCGTCCTTGTAGTCCATTGACTGATGCCAGTA-3', |
| shRNA espressing vector targeting caspase-9 |
| 5'-GATCCCCGGCCAGGCAGCTGATCATATTCAAGAGATATGATCAGCTGCCTGGCCTTTTTC-3',<br>5'-TCGAGAAAAAGGCCAGGCAGCTGATCATATCTCTTGAATATGATCAGCTGCCTGGCCGGG-3', |
| ShRNA espressing vector for negative control |
| 5'-GATCCCCACTACCGTTGTTATAGGTGTTCAAGAGACACCTATAACAACGGTAGTTTTTTC-3',<br>5'-TCGAGAAAAAACTACCGTTGTTATAGGTGTCTCTTGAACACCTATAACAACGGTAGTGGG-3'. |

FLAG-tag coding sequences have been underlined.

propidium iodide (PI). To quantify the apoptosis, flow-cytometric analysis was performed using an ELITE ESP Flow Cytometer (Beckman-Coulter).

*Electron microscopy*

The transfected HeLa cell pellets were fixed for 2 h at 4°C with 2.5% glutaraldehyde in 0.1 mol/l sodium phosphate buffer (pH 7.4). The pellets were washed 3 times and fixed in 1% osmic acid in phosphate buffer, followed by scraping, dehydration, and embedding. Ultrathin sections were mounted on a 200-mesh grid and examined under a GEM-2000EX electron microscope.

*Cell viability assay*

The cells transfected with truncated AIF constructs were cultured in 12-well plates for 48 h. Cells were trypsinized and resuspended in an appropriate volume of pre-warmed growth medium to give a cell density of $10^6$ cells/ml. Add 0.5 ml 0.4% (w/v) trypan blue and 0.3 ml PBS to 0.1 ml of the cell suspension. Count the stained and unstained cells using a hemocytometer. The percent viable cells was determined as follows: (number of unstained cells/total number of cells)×100%. Each assay was performed in triplicate by conducting at least 3 independent experiments.

For the methyl-thiazol-tetrazolium (MTT) assay, the cells transfected with truncated AIF constructs were cultured in 96-well plates for 24-96 h. The cells were then incubated with 20 μl of 1.5 mg/ml MTT per well for 4 h, followed by the addition of 150 μl dimethyl sulfoxide (DMSO). The OD at 490 nm was determined using a Sunrise microplate reader (Tecan).

*Indirect immunofluorescence staining*

The HeLa cells cultured on coverslips were fixed, permeabilized, and stained for AIF. A polyclonal anti-Cyt C antibody (Santa Cruz) was used to detect the release of cytochrome *c*, and a rabbit anti-goat IgG conjugated to biotin and SABS-Cy3 were used to amplify and visualize the staining.

*In vitro cytotoxicity of immuno-AIFΔ1-480 proteins*

HER2-overexpressing SKBR-3 and SGC-7901 cells, as well as ECV-304 and Hep2 cells that rarely express HER2 were seeded into 12-well plates at a density of $1\times10^5$cells/well, and 0.5 ml/well supernatants prepared from $2\times10^6$ modified Jurkat cells were added every 12 h. Viable cells were counted by trypan blue exclusion at the indicated times after cocultivation. The percentages of cell killing were determined as follows: (1-the number of cells cocultured with the supernatant of Jurkat-immuno-AIFs or Jurkat-pCMV-sFv23e-PEA/the number of cells cocultured with the supernatant of untransfected Jurkat cell)×100%. Each assay was performed in triplicate by conducting at least 3 independent experiments.

*Statistical analysis*

Statistical analyses were performed with the SPSS11.5 software package for Windows. Statistical significance was based a p-value of 0.05 ($p< 0.05$).

## RESULTS

*Ectopic expression and subcellular location of truncated AIFs in HeLa cells*

HeLa cells were transfected with the pcDNA3 constructs of FLAG-tagged truncated AIF. At 24 h after transfection, the expressions of AIFΔ1-120, AIFΔ1-480, and AIF360-480 were confirmed by western blotting using a FLAG antibody (Fig. 2A). Unlike AIFΔ1-120, which was detected in both the cytoplasm and the nucleus, confocal microscopy revealed that AIFΔ1-480 and AIF360-480 were distributed exclusively in the cytoplasm (Fig. 2B).

*Expression of AIFΔ1-480 induced apoptosis in transfected HeLa cells*

The proapoptotic activity of truncated AIFs in HeLa cells was assessed. Annexin V/PI

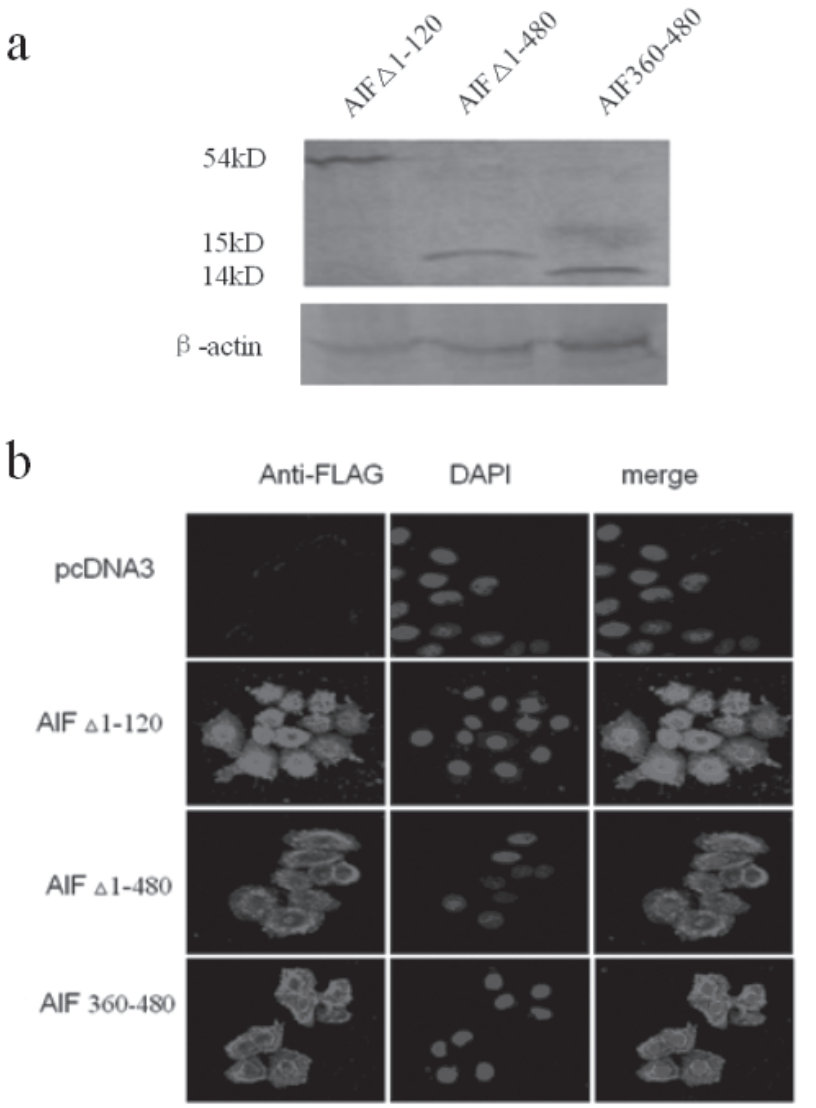


**Figure 2:** The expression of AIFΔ1--120, AIFΔ1-480, and AIF360-480 in transfected HeLa cells. (A) Western blot of the lysates of $1 \times 10^6$ transfected cells using anti-FLAG antibody at 48 h after transfection with AIFΔ1-120 (lane 1), AIFΔ1-480 (lane 2), and AIF360-480 (lane 3). (B) Laser confocal microscopy of HeLa cells transfected with the indicated gene constructs at 48 h after transfection (×400).

staining revealed that the percentages of apoptotic cells in the transfectants of AIFΔ1-120 and AIFΔ1-480 were 25.1% and 30.4%, respectively, which was in contrast to 2.0% and 2.4% apoptotic cells observed in the parental HeLa cells and AIF360-480-transfected cells, respectively (Fig. 3A). These data suggested that AIFΔ1-480 could efficiently trigger apoptosis and that cytoplasmic AIF play a role in mediating apoptotic signals.

The typical features of apoptotic cells as detected by electron microscopy were plasma membrane blebbing, peripheral chromatin condensation, and nuclear shrinkage (Fig. 3B). Cell viability assay showed decreased viable cells in AIFΔ1-120 and AIFΔ1-480 transfectants when compared with AIF360-480-transfected and the parental HeLa cells (Fig. 3C). Thus, the apoptosis-inducing ability of the N-terminal-truncated AIF, AIFΔ1-480, which

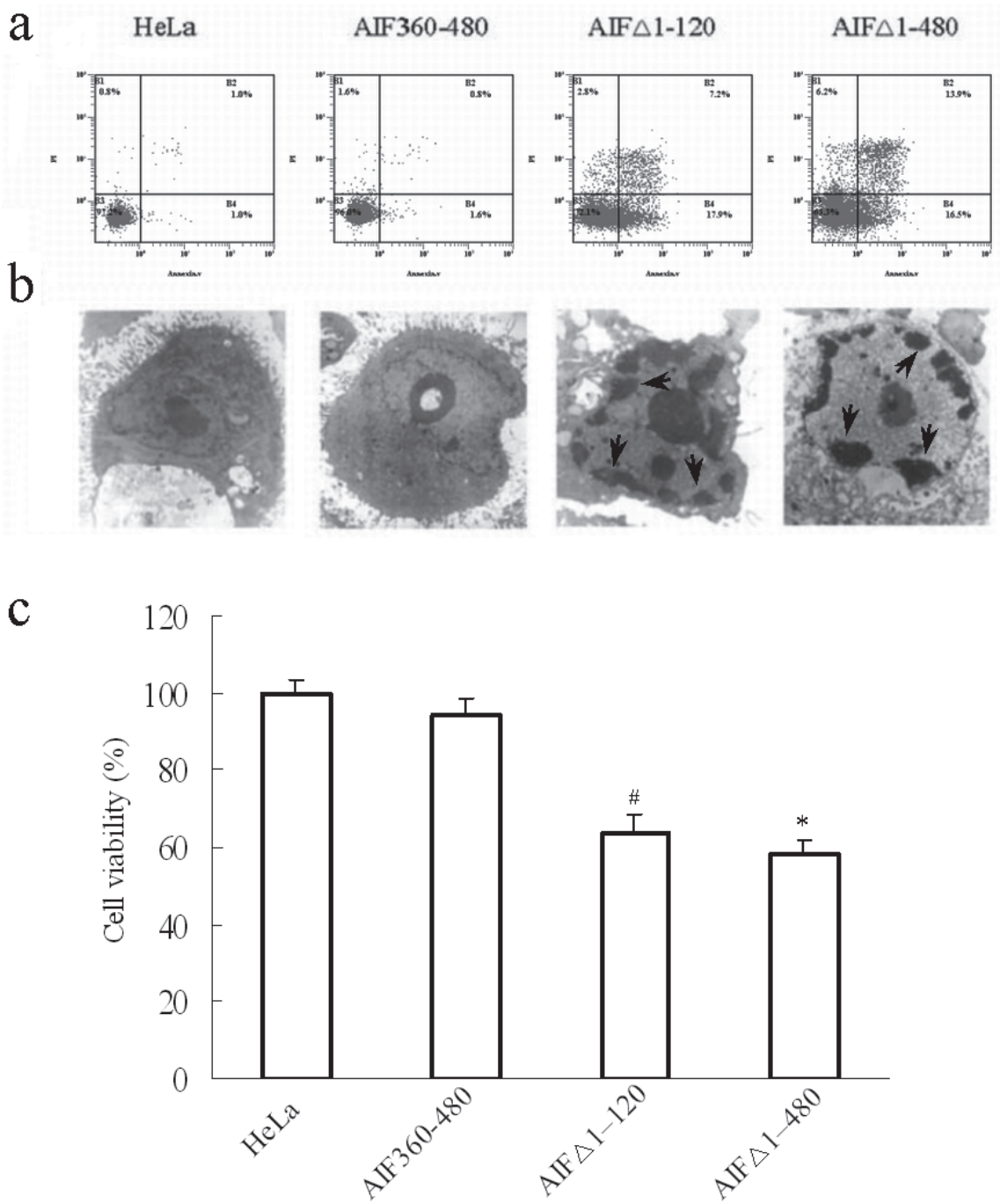


**Figure 3:** Proapoptosis of AIFΔ1-480 in HeLa cells. (A) Apoptosis induced by the overexpression of transfected, recombinant AIF genes. HeLa cells were transiently transfected with the indicated genes. After 48 h in culture, annexin V/PI staining was performed and the cells were analyzed by flow cytometry. (B) Electron microscopy was used for observing ultramicrostructural alterations 48 h post-transfection. Arrows show nuclear condensation (×4000). (C) Cell viability assays of HeLa cells performed 48 h post-transfection with different gene constructs. The data represent the mean values of 2 independent experiments. Each experiment was performed in triplicate. *$p<0.05$, #$p<0.05$.

lacks FAD- or NADH-binding activity and nuclear translocation ability, was almost unaffected.

*The expression of AIFΔ1-480 induced cytochrome c release from the mitochondria*

Since the AIFΔ1-480 protein was located in the cytoplasm of transfected cells, the direct binding of truncated AIF to chromosomal DNA was prevented, implying that the cytoplasmic targets and signaling of truncated AIF are responsible for the initiation of apoptosis. Therefore, we examined the release of cytochrome *c* in the transfected cells. In fact, indirect immunofluorescence staining revealed that the expression of AIFΔ1-480 in HeLa cells induced cytochrome *c* release from the mitochondria, which is a process also observed in AIFΔ1-120-transfected cells but not in the parent cells or cells transfected with AIF360-480 constructs (Fig. 4).

*Caspase-9 is required for the proapoptotic activity of AIFΔ1-480*

In cells undergoing typical apoptosis, the release of cytochrome *c* from the mitochondria leads to the activation of caspase-9, and then, caspase-3 (Yang et al., 1997). This involves the formation of an apoptosome, which is a multiprotein complex comprising cytochrome *c*, Apaf-1, pro-caspase-9, and ATP (Lorenzo et al., 2007). To test whether caspase-9 participates in the apoptotic pathway mediated by AIFΔ1-480, we knocked down the expression of the caspase-9 gene in HeLa cells by using RNAi. The stable expression of caspase-9-targeted siRNA from a pSUPER.puro vector resulted in a decrease of approximately 70% in caspase-9 expression (Fig. 5A and B). The cell viability assay demonstrated that the proapoptotic activity of AIFΔ1-480 was inhibited by caspase-9 knockdown (Fig. 5C). These results suggest that the proapoptotic activity of AIFΔ1-480 is mediated by caspase-9-dependent cytoplasmic signaling events.

*Secreted immuno-AIFΔ1-480 from modified Jurkat cells selectively kill HER2-overexpressing tumor cells*

To further probe whether the AIF C-terminal domain is structurally and functionally independent and whether it maintains a proapoptotic activity when covalently incorporated into heterogeneous peptides, we generated the genes of a chimeric proteins, designated immunoAIFΔ1-480. Human lymphoma Jurkat cells stably transfected with pCMV-immuno-AIFΔ1-480 or previously reported pCMV-immuno-AIFΔ1-120 were modified to express and secrete the corresponding chimeric proteins. Western blot analysis confirmed the expression and the secretion of immuno-AIFΔ1-480 and immuno-AIFΔ1-120 proteins, as shown in Figure 6A.

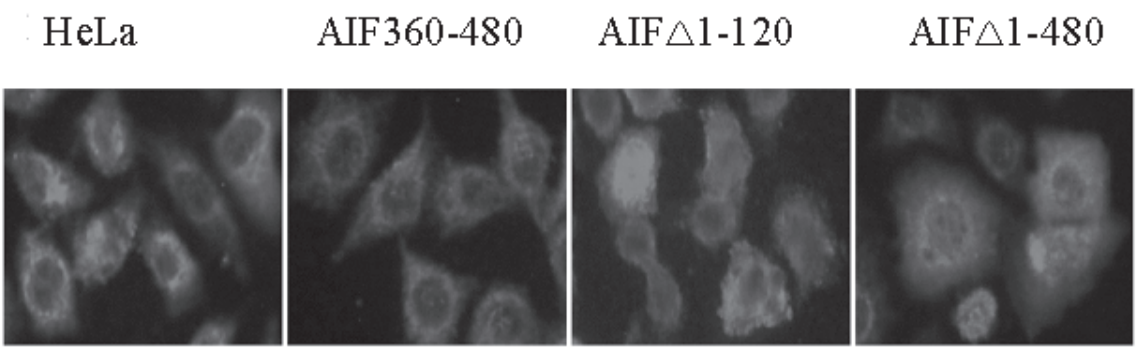


**Figure 4:** Expression of AIFΔ1-480 initiates the release of cytochrome *c* from the mitochondria Indirect immunofluorescence staining of cytochrome *c* in HeLa cells transfected with the indicated gene constructs 48 h after transfection.

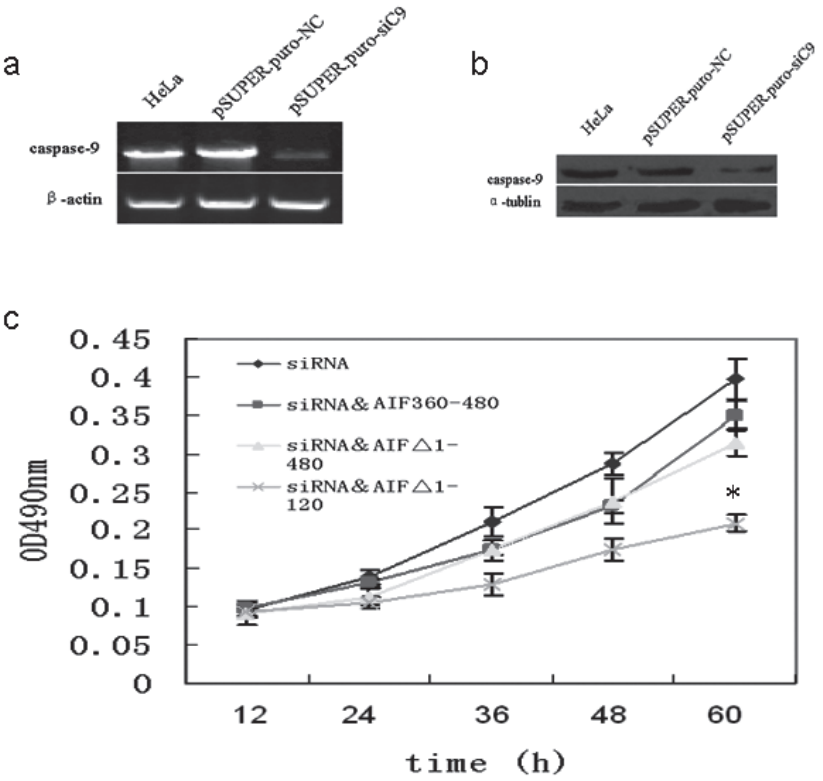


**Figure 5:** Caspase-9 knockdown abrogated the proapoptotic activity of AIFΔ1-480 in HeLa cells (A) RT-PCR and (B) western blot demonstrated a significant knockdown of caspase-9 by a vector-based siRNA assay. (C) Caspase-9-knockdown HeLa cells were further transiently transfected with truncated AIF constructs or a control vector. The cells were then subjected to an MTT assay at the indicated time. *p<0.05.

The secreted immuno-AIFΔ1-480 and immuno-AIFΔ1-120 proteins show significant cytotoxic effects on both HER2-overexpressing cells lines, but not on HER2-negative cells (Fig. 6B). These data indicate that immuno-AIFΔ1-480 molecules secreted from lymphocytes specifically recognize and kill HER2-overexpressing tumor cells, but not normal or tumor cells lacking HER2 expression *in vitro*.

## DISCUSSION

AIF is a multifunctional mitochondrial protein, which mediates both oxidoreductase activity (via its FAD- and NADH-binding domains) and caspase-independent apoptosis (partially by its DNA-binding activity) (Mate et al., 2002, Miramar et al., 2001). Studies on the crystal structures of mouse and human AIF have provided substantial information regarding its role in the cell (Mate et al., 2002), which have extended our understanding of molecular mechanisms underlying the proapoptotic activity of AIF; thus, making it a potential candidate for cancer gene therapy (Lorenzo et al., 2007). However, a previous study has shown that the C-terminal domain, consisting of 136 amino acid residues, is responsible for the proapoptotic activity of AIF protein, and Susin et al. demonstrated that AIFΔ1-120 could induce cell apoptosis and that further truncation impaired the proapoptotic activity of AIF (Susin et al., 1999). Ce´ cile Delettre et al. found a novel AIF proapoptotic isoform AIFsh in many cancer cells. AIFsh contains the C-terminal amino acids from 353-613, and a study has demonstrated that AIFsh triggers chromatin condensation and DNA

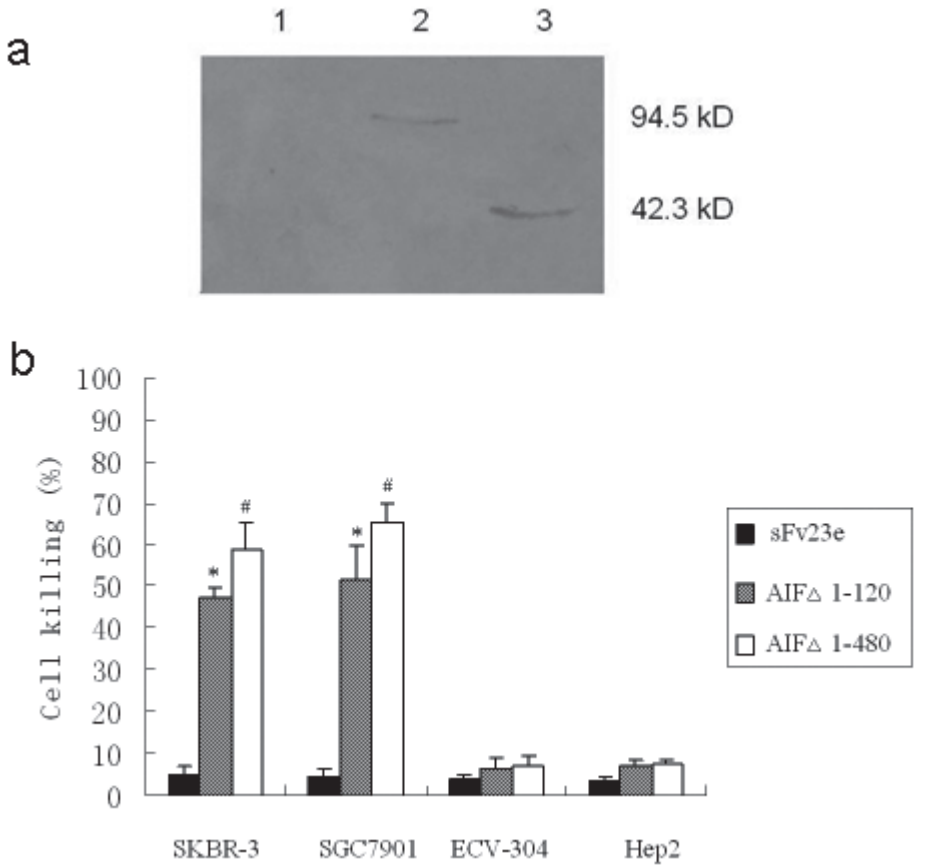


**Figure 6:** Selective cytotoxicity of immuno-AIF gene-modified Jurkat cells to HER2-overexpressing tumor cells in vitro. (A) Jurkat cells were transfected with the pCMV construct of immuno-AIFΔ1-120 (lane 2) and immuno-AIFΔ1-480 (lane 3) respectively, or mock-transfected with pCMV-sFv23e-PEA (lane 1). The G418-resistant cells were amplified. The serum-free medium of immuno-AIFΔ1-480 and immuno-AIFΔ1-120 gene-modified Jurkat cells were condensed after 48 h culture and subjected to western blot analysis using anti-FLAG antibody. (B) tumor cells were cultured $1\times10^5$/well in 12-well plates, and 0.5 ml/well supernatants prepare from $2\times10^6$ modified Jurkat cells were added every 12 h. The cells were then subjected to a cell viability assay at day 4. *$p<0.05$, #$p<0.05$.

fragmentation of isolated nuclei (Delettre et al., 2006). In our study, we deleted the N-terminal 1-480aa of the AIF protein and demonstrated that this truncated variant, despite lacking NADH-binding and nuclear translocation abilities, induces remarkable apoptosis of HeLa cells in cytochrome *c*- and caspase-9-dependent manners.

The cell viability assay showed that the proliferation of AIFΔ1-480- and AIFΔ1-120-transfected HeLa cells was significantly inhibited. We also detected the cell cycle of transfected cells. Compared to the parent or negative cells, the cell cycle of AIFΔ1-480-transfected cells was not obviously affected (data not shown). Together, these results demonstrate the effectiveness of AIFΔ1-480 in inhibiting cell survival and growth via the triggering of apoptotic signal pathways.

A previous study has shown that AIF induces apoptosis in a caspase-independent manner as this process is not inhibited by the pan-caspase inhibitor z-VAD-fmk (Daugas et al., 2000, Pérez-Galán et al., 2002). By introducing serially truncated AIF into HeLa cells, we observed significantly inhibited cell growth and increased apoptosis

following the expression of AIFΔ1-120 or AIFΔ1-480. Unlike AIFΔ1-120, the ectopic expression of AIFΔ1-480 did not affect cell cycle, suggesting a diversity of mechanisms through which AIF induces apoptotic cell death. Cytochrome *c* is a key regulator of apoptosis (Zou et al., 1999, Yoshida et al., 1998, Li et al., 2000). The release of cytochrome *c* from the mitochondria leads to the activation of caspase-9 and subsequently of caspase-3. This involves the formation of an apoptosome, which is a multiprotein complex comprising cytochrome *c*, Apaf-1, pro-caspase-9, and ATP (Genini et al., 2000, Jiang et al., 2000). In our study, we observed that cytochrome *c* was released from the mitochondria in AIFΔ1-480- and AIFΔ1-120-transfected cells. These data suggest a crosstalk between the classical AIF apoptotic signaling and caspase pathways. Although the mechanism by which cytoplasmic AIF causes the release of cytochrome *c* from the mitochondria remains to be elucidated, the caspase-9-dependent signaling may represent an alternative apoptotic pathway of AIF and a conserved approach through which AIF joins the mainstream cellular apoptotic machinery.

Chimeric proteins comprising antibodies and cytotoxic molecules hold out great promise in targeted elimination of cells undergoing pathological alterations, e.g. carcinogenesis (Yu et al., 2006, Jia et al., 2003, Wang et al., 2007). Yu et al. generated a fusion protein, immuno-AIFΔ1-120, which consisted of a HER2 antibody, a PEA translocation domain, and AIFΔ1-120. The chimeric protein could be internalized via endocytosis and undergoes a furin-mediated cleavage between Arg279 and Gly280 of the original PEA peptide, releasing its C-terminal peptide into the cytosol. This peptide, which comprises a PE sequence and AIF1-120, then induces apoptosis (Yu et al., 2006). However, only a minority of the truncated AIF could be released into the cytosol probably due to a large molecular weight of AIFΔ1-120. Given that we have demonstrated a similar proapoptotic activity of AIFΔ1-480 with AIFΔ1-120, we developed a smaller immuno-AIF variant, immuno-AIFΔ1-480. Indeed, immuno-AIFΔ1-480 exhibited a significantly higher efficiency than immuno-AIFΔ1-120 in specifically killing HER2-overexpressing tumor cells. Our finding that AIF exerts a proapoptotic effect independent of its large FAD- and NADH-binding domains has implications in the development of tumor-targeted therapeutics with simplified constituents but enhanced cytotoxicity.

## ACKNOWLEDGMENTS

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