

c-FLIP_{p43} induces activation of the nuclear factor- κ B signaling pathway in a dose-dependent manner in the A375 melanoma cell line

YUJUE WANG^{1*}, YATING TU^{1*}, JIEJIE LU^{2*}, JUAN TAO¹ and YAN LI¹

¹Department of Dermatology, Affiliated Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430022; ²Department of Dermatology and Venereology, Hainan Provincial Center for Skin Disease and STD Control, Haikou, Hainan 570206, P.R. China

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Abstract. In order to investigate the role of c-FLIP_{p43} in the regulation of the nuclear factor (NF)- κ B signaling pathway in melanoma cell lines, a eukaryotic expression vector for c-FLIP_{p43} was constructed with the pCMV-Tag2B plasmid. The monoclonal A375 cells with stable expression of c-FLIP_{p43} were obtained by G418 selection and were identified with western blot analysis. The protein level of NF- κ B_{p65} in the A375 cell line with stable expression of c-FLIP_{p43} was examined by western blot analysis. The translocation of NF- κ B_{p65} was examined using immunofluorescence. The A375 cell lines were transfected with the pCMV-Tag2B-cFLIP_{p43} vector at different doses and the activation of the NF- κ B signaling pathway was examined by the dual-luciferase reporter assay system. The stable expression of c-FLIP_{p43} in the A375 cell lines transfected with the pCMV-Tag2B-cFLIP_{p43} vector increased the protein level of NF- κ B_{p65} compared with in the A375 cell lines transfected with the empty vector. Transfection of the cells using the pCMV-Tag2B-cFLIP_{p43} vector increased the amount of NF- κ B_{p65} in the nucleus in a dose-dependent manner. In conclusion, the transfection of the c-FLIP_{p43} expression vector induces the protein expression of NF- κ B_{p65} and promotes the activation of the NF- κ B signaling pathway in the A375 melanoma cell line.

Introduction

Malignant melanoma (MM) is an aggressive, therapy-resistant malignancy of the melanocytes. The incidence of MM has been steadily increasing worldwide, resulting in an increasing public health problem (1). More than 95% of the MM tumors occur in the skin. MM is ranked as the third most common type of cutaneous malignant tumor, with a high malignance and high potential of hematogenous and lymphatic metastasis. During the progression and therapy of MM, melanoma cell lines demonstrate increased resistance to death receptor-mediated apoptosis and drug-induced apoptosis compared with their normal counterparts (2).

Apoptosis induced by antineoplastic drugs was not affected by the absence of Fas, indicating that cell death was in a Fas-independent manner. The insensitive manner pointed to FLIP-induced apoptosis (3), indicating the existence of another pathway responsible for preventing apoptosis.

Cellular caspase-8 (FLICE)-like inhibitory protein (c-FLIP) was originally identified as an inhibitor of death-receptor signaling through competition with caspase-8 for recruitment to FAS-associated protein with death domain (FADD). c-FLIP_L, the long isoform of c-FLIP is specifically processed by caspase 8 into N-terminal c-FLIP_{p43} and C-terminal c-FLIP_{p12}. In accordance with others studies, we previously identified that compared with the pigmented nevi lesions, the expression of c-FLIP increased and was significantly associated with the histological type and Clark's level in MM tissue samples (4-6). It was also identified that the downregulated expression of c-FLIP increases apoptosis in MM cell lines. However, the molecular mechanisms of c-FLIP in melanoma pathogenesis remain unclear.

Dohrman and Kataoka *et al* (7,8) reported that c-FLIP_L may be cleaved into c-FLIP_{p43}, promoting the activation of nuclear factor (NF)- κ B in CD8⁺ T cells, human embryonic kidney (HEK) 293 cells and 293 T cells. NF- κ B is a pro-inflammatory transcription factor and is activated by various inflammatory agents, carcinogens, tumor promoters and the tumor microenvironment. NF- κ B protein, and the proteins regulated by NF- κ B are associated with cellular transformation, proliferation, apoptosis suppression, invasion, angiogenesis and

Correspondence to: Dr Yan Li, Department of Dermatology, Affiliated Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Road, Wuhan, Hubei 430022, P.R. China
E-mail: august0907@aliyun.com

*Contributed equally

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metastasis during the genesis of tumors (9). NF- κ B is expressed and is constitutively active in the majority of tumor cell lines, including melanoma cells (10). The high level of non-canonical NF- κ B activation could be decreased by NIK depletion and thus resulted in increased apoptosis (11).

It remains unclear whether c-FLIP_{p43} is involved in the pathogenesis of melanoma through NF- κ B activation. The aim of the present study was to evaluate whether the regulation of c-FLIP_{p43} induces the NF- κ B signaling pathway in melanoma cell lines and subsequently promotes the progression of melanoma. The present study investigated the expression profile of c-FLIP_{p43} and NF- κ B in melanoma cell lines by immunoblotting. In addition, a eukaryotic expression vector for c-FLIP_{p43} and a monoclonal A375 cell line with the stable expression of c-FLIP_{p43} were successfully constructed. Immunofluorescent staining was also utilized to investigate the nuclear translocation of NF- κ B_{p65} with the stable expression of c-FLIP_{p43} and a dual-luciferase reporter assay system was used to investigate the NF- κ B transactivation induced by different c-FLIP_{p43} vector concentrations in the A375 cell line.

Materials and methods

Melanoma cell lines, expression vectors, antibodies and reagents. The A375 and SK-Mel-1 human malignant melanoma cell lines (Institute of Cell Biology, Shanghai Institute for Biological Science, Chinese Academy of Science, Shanghai, China) were cultured in plastic flasks in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Manheim, Germany), containing 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT, USA), 15 mm HEPES, 2 mmol/L glutamine, and 100 U/ml penicillin and streptomycin at 37°C. pCMV-Tag2B-cFLIP_{p43} and pCMV-Tag2B were constructed by the Laboratory of Molecular Biology in Hubei University (Hubei, China). pGL3-3 κ B-Luc and pRL-TK were provided by Professor Lixin Ma's experimental group of Hubei University. Antibodies against c-FLIP, NF- κ B_{p65} and β -actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The NF- κ B Activation, Nuclear Translocation Assay kit and Nuclear and Cytoplasmic Protein Extraction kit were obtained from Biyuntian Company (Beijing, China). LipofectamineTM 2000 was purchased from Invitrogen Life Technologies (San Diego, CA, USA).

Cell transfection and selection of stable G418 resistant clones. pCMV-Tag2B-cFLIP_{p43} was transfected into A375 cells with Lipofectamine 2000, and 48 h following transfection, cells were treated daily with 500 mg/ml G418 (Invitrogen Life Technologies) as a selective marker for two weeks. The surviving cells were dispersed at a density of one cell/well in 24-well multiwell plates and several stably transfected cell clones were obtained in 3-4 weeks. The stable clones were screened for pCMV-Tag2B-cFLIP_{p43} expression by western blotting.

Western blotting. A total of 1x10⁶ cells were incubated for 30 min at 4°C in 100 μ l radioimmunoprecipitation assay buffer (cell lysis buffer for western blotting and IP). A total of 30 μ g of protein per slot was separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with the gel containing 12% acrylamide. Following blotting onto a

polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA), 1 h incubation with non-fat milk was performed at 37°C using a shaker (WD-9405B; Beijing Liuyi Instrument Factory, Beijing, China) to inhibit the unspecific binding of antibodies. The blots were incubated overnight in a 4°C icebox with the primary antibody. Subsequently, the blots were washed three times each for 10 min with Tris-buffered saline containing 0.1% Tween-20 and a secondary antibody conjugated to horseradish peroxidase (dilution factor, 1:8000; Dako, Hamburg, Germany) was added. The Enhanced Chemiluminescence kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) was used to develop the blots.

Immunofluorescence assay. The cover slips were located in twenty-four-well culture plates and A375 cells, which were transfected with pCMV-Tag2B-cFLIP_{p43}, were seeded onto it. The next day, the culture media were discarded and the cells were washed once with phosphate-buffered saline (PBS). Paraformaldehyde (4%; Beyotime Institute of Biotechnology, Nantong, Jiangsu, China) was added for 10-15 min and then discarded. The cells were washed with cleaning solution three times for 3-5 min each time. Fetal Bovine Serum (Gibco-BRL, Carlsbad, CA, USA) was added for 1 h at room temperature and then discarded. The cells were incubated with antibodies against NF- κ B_{p65} overnight at 4°C. The cells were then washed with PBS (Beyotime Institute of Biotechnology) three times, for 3-5 min each time. The cells were incubated with anti-rabbit Cy3 (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. The cells were washed twice with cleaning solution for 5-10 min each time. DAPI staining solution was added for 5 min at room temperature and then removed. The cells were then washed with cleaning solution three times for 3-5 min each time. Anti-fade mounting media (Beyotime Institute of Biotechnology) were added and the cover slips were located, viewed and photographed under a laser scanning confocal microscope (Olympus, Center Valley, PA, USA). NF- κ B_{p65} staining was red and DAPI staining of the nucleus was blue.

Dual-luciferase reporter. Six-well culture plates were seeded with A375 cells. At 60-80 degrees of fusion, the cells were transiently transfected according to the manufacturer's details of the Effectene Transfection Reagent kit (Qiagen Inc., Venlo, Netherlands). The cells were collected 20 h later. The ratio of Firefly luciferase and Renilla luciferase were analyzed by Turner Bio Systems Modulus Luminometer (Reporter Microplate Luminometer; Turner BioSystems Inc., Sunnyvale, CA, USA).

Statistical analysis. Data analysis was performed using the SPSS 11.0 Statistical Software (SPSS, Inc., Chicago, IL, USA). An independent sample's t-test was used and P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of c-FLIP, c-FLIP_{p43} and NF- κ B_{p65} in A375 cells. Immunoblotting analysis of the A375 and SK-Mel-1 melanoma cell lines revealed that c-FLIP_{p43} expression in the SK-Mel-1 cells was higher than that in the A375 cells (Fig. 1A). The expression of c-FLIP was undetectable in A375 cells but high

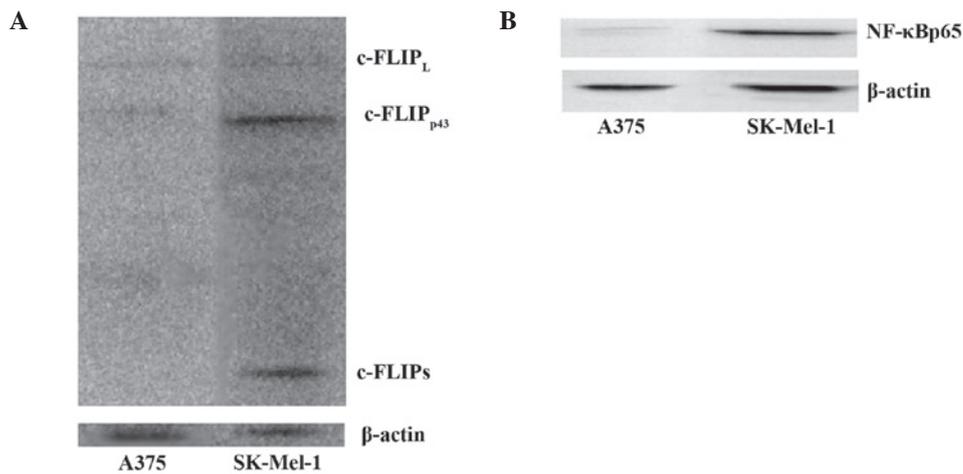


Figure 1. Expression of (A) c-FLIP and (B) NF- κ B_{p65} in A375 and SK-Mel-1 cells demonstrated using immunoblot analysis. cFLIP, cellular caspase-8 (FLICE)-like inhibitory protein; NF- κ B, nuclear factor- κ B.

expression was observed in the SK-Mel-1 cells. The expression of NF- κ B_{p65} in A375 cells was lower than that in the SK-Mel-1 cells (Fig. 1B).

Stable expression of c-FLIP_{p43} and NF- κ B_{p65} in A375 cells stably transfected with pCMV-Tag2B-cFLIP_{p43}. pCMV-Tag2B-cFLIP_{p43} was transfected into the A375 cell line and the stable clones were isolated following G418 selection. To confirm successful transfection of A375 cells with the c-FLIP_{p43} construct, immunoblotting analysis was conducted. The expression level of c-FLIP_{p43} was evidently higher in the A375 cells that were stably transfected with c-FLIP_{p43}, than in the A375 cells, which were not transfected (Fig. 2). Consistent with this, the immunoblotting results of A375 cells stably expressing c-FLIP_{p43} demonstrated increased expression of NF- κ B_{p65} (Fig. 3). These data suggest that overexpression of c-FLIP_{p43} may upregulate NF- κ B_{p65} expression at the protein level.

NF- κ B_{p65} nuclear translocation. Immunofluorescent staining of NF- κ B_{p65} was performed in the A375 cell line stably expressing c-FLIP_{p43}. An overlay of merged images (NF- κ B_{p65} in red and DAPI in blue) demonstrates that NF- κ B_{p65} was predominantly localized in the nuclei and plasma in A375 cells transfected with pCMV-Tag2B-cFLIP_{p43}, while there was weak plasma and nuclei staining in the untreated cells (Fig. 4). c-FLIP_{p43} transfection increased the NF- κ B_{p65} fluorodensitometric value ($P < 0.01$) compared with the transfection with empty vector (Table I). Collectively, these results indicate that NF- κ B_{p65} nuclear translocation was increased in the A375 cells following transfection with pCMV-Tag2B-cFLIP_{p43}.

NF- κ B_{p65} activation examined by the dual-luciferase reporter assay. To determine whether c-FLIP_{p43} was able to induce NF- κ B transactivation, the luciferase activity was measured using a dual-luciferase reporter assay system. The A375 cells, which were transfected with pCMV-Tag2B-cFLIP_{p43}, induced a 1.5-fold increase in promoter activity compared with the control cells (Fig. 5). This implies that effective c-FLIP_{p43} expression is required for NF- κ B activation and this increase was in a dose-dependent manner.

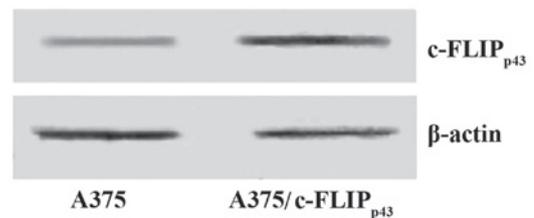


Figure 2. Expression of c-FLIP_{p43} in A375 cells stably transfected with pCMV-Tag2B-cFLIP_{p43} by immunoblot analysis. cFLIP, cellular caspase-8 (FLICE)-like inhibitory protein.

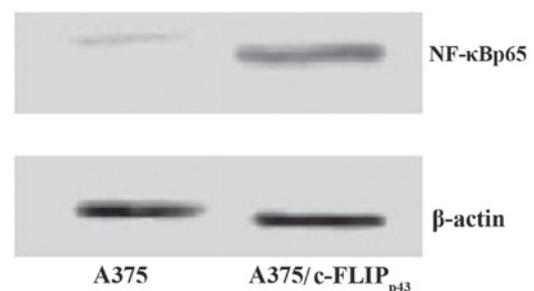


Figure 3. Expression of NF- κ B_{p65} in A375 cells stably transfected with pCMV-Tag2B-cFLIP_{p43} by immunoblot analysis. NF- κ B, nuclear factor- κ B; FLIP, cellular caspase-8 (FLICE)-like inhibitory protein.

Discussion

c-FLIP has been observed at increased levels in a number of cell lines or cancerous tissues, including melanoma (5,6,12), gastric (13-15), lung (5,16), colorectal (16), hepatocellular (17), pancreatic (18), ovarian (19) and prostate carcinoma (20), and B-cell chronic lymphocytic leukemia (21,22). Elevated levels of c-FLIP have been demonstrated to correlate with more aggressive tumors and a poor prognosis (23-28).

Zeise *et al* (29) found that different melanoma cells demonstrated sensitivity or resistance towards TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis, which may have been due to different expression and the pre-apoptotic processing status of FLIP. The authors also found that

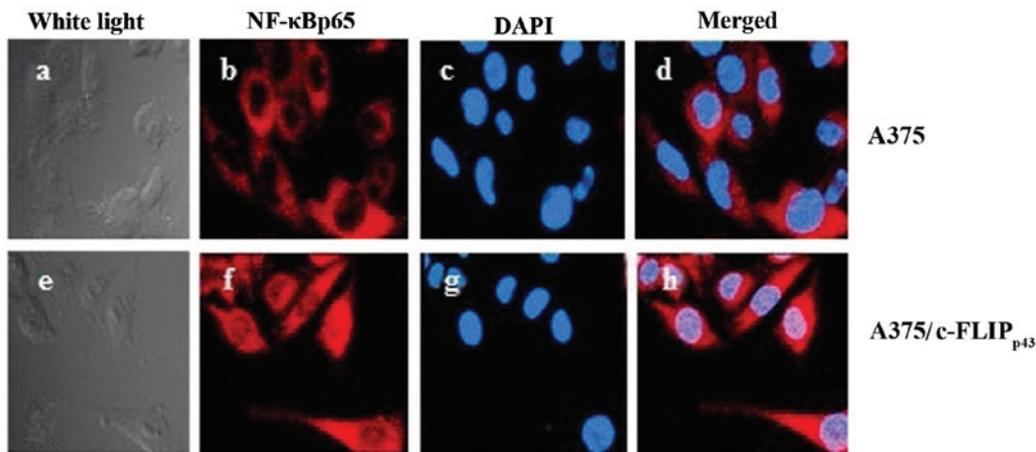


Figure 4. Immunofluorescence images of untreated A375 cells (upper panel) and A375 cells stably expressing c-FLIP_{p43} (lower panel) stained with the indicated antibodies. (A and E) Represent white light images in each cell line; (B and F) demonstrate the expression of NF- κ B_{p65} (in red) and DAPI (in blue). (C and D) NF- κ B_{p65} was predominantly localized in the nuclei and plasma in A375 cells stably transfected with pCMV-Tag2B-cFLIP_{p43}, while there was weak nuclei staining in the untreated A375 cell line. (D and H) The merged images of NF- κ B_{p65} and DAPI staining. cFLIP, cellular caspase-8 (FLICE)-like inhibitory protein; NF- κ B, nuclear factor- κ B.

Table I. Fluorodensitometric value of NF- κ B_{p65} in the nuclei.

	Fluorodensitometric value	P-value
A375	17.34±8.22	
A375/cFLIP _{p43}	60.71±18.32	<0.01

cFLIP, cellular caspase-8 (FLICE)-like inhibitory protein.

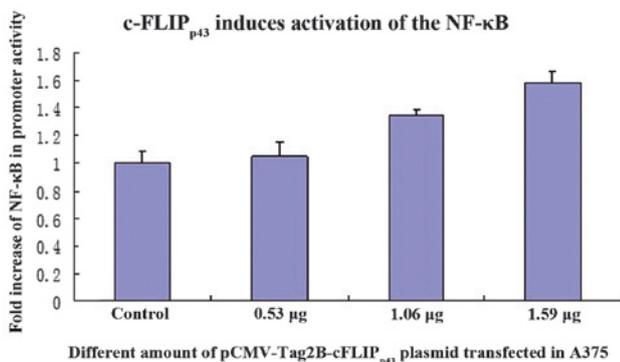


Figure 5. Fold increase of promoter luciferase activity of NF- κ B in the control A375 cell line and A375 cells transfected with pCMV-Tag2B-cFLIP_{p43} with different amounts of plasmid was measured using a Dual-Luciferase Reporter Assay system. cFLIP, cellular caspase-8 (FLICE)-like inhibitory protein; NF- κ B, nuclear factor- κ B.

A375 cells appeared sensitive to TRAIL-induced apoptosis and a number others, including SK-Mel-30, were resistant to TRAIL-induced apoptosis. The present study demonstrated that the expression of c-FLIP_{p43} was low in A375 cells but high in the SK-Mel-1 cells. High c-FLIP expression has been found to inhibit TRAIL-induced apoptosis by preventing activation of the caspase cascade (30,31). Differential sensitivity of cancer cells to TRAIL-induced apoptosis may be determined by intracellular c-FLIP levels. Though TRAIL is regarded as a promising anticancer drug due to its high selectivity, the

regulation of c-FLIP may be considered as a potential strategy in certain TRAIL-resistant cancer cells.

It was noted that the expression of NF- κ B_{p65} in A375 cells was lower than that in the SK-Mel-1 cells. It has been reported that besides the crucial role of c-FLIP in death receptor (DR)-induced apoptosis, the c-FLIP cleavage product c-FLIP_{p43} may also regulate DR-induced NF- κ B activity (8,32-34). Others studies have reported that the regulation of c-FLIP_L in CD95-mediated NF- κ B activation may be concentration- or cell line-dependent and different levels of c-FLIP expression may promote or inhibit the pathway (35).

To improve the understanding of the role of c-FLIP_{p43} in the pathogenesis of melanoma, a eukaryotic expression vector for c-FLIP_{p43} with the plasmid pCMV-Tag2B was constructed and the stable expression of c-FLIP_{p43} monoclonal A375 cells was obtained.

It was identified that NF- κ B activity was elevated following transfection with pCMV-Tag2B-cFLIP_{p43} in the A375 cells. As the expression of c-FLIP_{p43} increased, the NF- κ B_{p65} activity was enhanced. These results indicated that c-FLIP_{p43} may promote the activation of NF- κ B_{p65} in a dose-dependent manner. These observations were consistent with those of Kataoka *et al* (3), which found that c-FLIP_L was cleaved into c-FLIP_{p43} by caspase-8 to promote the activation of NF- κ B. Caspase 8 activity is thus required for FLIP_L-induced NF- κ B activation. FLIP_L may be specifically processed by caspase 8 and be cleaved into FLIP_{p43} and FLIP_{p12}. In caspase-8 deficient cells, c-FLIP_{p43} was not able to provoke NF- κ B activation without complemented procaspase-8 or the intermediate cleavage products caspase-8_{p43}. FLIP_L appears to have a molecular switch role to regulate cell death and cell growth by inhibiting the activation of caspase 8, as well as proliferative signaling pathways, including the NF- κ B pathway (7). Transfection of c-FLIP_{p43} promoted NF- κ B activation and increased the expression of NF- κ B in A375 cells. This may explain that overexpression of FLIP_{p43} maintains the NF- κ B activation and downregulation of c-FLIP induces apoptosis in the A875 malignant melanoma cell line (6).

Melanoma progression is a multistep process. It involves multiple genetic mutations and multimolecular activation, and NF- κ B has also been considered to be involved (36). It has been reported that NF- κ B activation may inhibit apoptosis in tumor cells and further promote tumor formation and chemoresistance (37). Huang *et al* (38) found that the constitutive expression RelA (p65) a member of the NF- κ B family was relevant to the metastasis of melanoma. These results indicated that c-FLIP_{p43} may be one inducer that leads to the activation of NF- κ B and thus promotes melanoma progression.

In conclusion, transfection of the c-FLIP_{p43} expression vector may induce the protein expression of NF- κ B_{p65} and promote the activation of the NF- κ B signaling pathway in the A375 melanoma cell line. Targeting c-FLIP_{p43} may be a promising potential strategy for melanoma treatment, particularly in TRAIL-resistant cell lines. Furthermore, the combination therapy of c-FLIP_{p43} regulation with TRAIL-related treatment (39), inhibitors of the NF- κ B signaling pathway or conventional chemotherapy may be an effective treatment option and requires further investigation to elucidate its clinical utility.

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