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IL-24 Gene Transfer Sensitizes Melanoma Cells to Erlotinib through Modulation of the Apaf-1 and Akt Signaling Pathways

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Abstract

IL-24 is a novel tumor suppressor/cytokine gene expressed in normal human melanocytes but for which expression is nearly undetectable in metastatic melanoma. Overexpression of IL-24 protein has been shown to inhibit tumor cell proliferation and induce apoptosis in many melanoma cell lines, and is now considered a tumor suppressor. Erlotinib, a small-molecule epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, has been widely studied for the treatment of human lung cancer and other solid tumors, but the erlotinib-targeted therapy has not been tested in melanoma. The objective of this study is to investigate the potency of erlotinib in suppressing the growth of human melanoma cells and whether IL-24 could enhance the antitumor activity of erlotinib. In cell viability and apoptosis assays, treatment of erlotinib dose-dependently inhibited growth of different melanoma cell lines and when combined with adenoviral vector-mediated IL-24 gene therapy, a significant increase in cell growth inhibition and apoptosis induction resulted ($P < 0.05$). Immunoblot assay showed that combination treatment of erlotinib and IL-24 considerably increased the cleavage of caspase-3 and -9 and the expression of Apaf-1 protein in melanoma cells, inducing activation of the Apaf-1-dependent apoptotic pathways. Moreover, this combination treatment markedly inhibited phosphorylation of the EGFR, PI3K and Akt proteins, inactivating the Akt-dependent cell survival signaling pathway. These results demonstrate that a combination of IL-24-mediated molecular therapy and EGFR inhibitors such as erlotinib may be a promising treatment strategy for human melanoma and will serve as a basis for guiding the combination treatment designs in future preclinical and clinical trials.

Keywords

IL-24; erlotinib; EGFR; Apaf-1; Akt; melanoma

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INTRODUCTION

Melanoma is one of the most prevalent cancers with incidence in the US increasing at a rate faster than any other cancer [1]. While melanoma can often be cured at an early stage by surgery, for those patients in whom metastasis has occurred, mortality is high because of its aggressiveness and the lack of effective systemic therapy. Thus, there is an urgent need to develop innovative therapeutic strategies for the treatment of this lethal disease. New cancer treatments designed to restore the function of defective genes in tumor suppressing and apoptotic pathways by gene transfer and to target alterations in key signaling pathways by “smart drugs” such as protein tyrosine kinase (PTK) inhibitors are fundamentally changing the approach to treatment of human cancers. Interleukin-24 (IL-24), originally called melanoma differentiation associated gene 7 (MDA-7), is a recently defined cytokine belonging to the IL-10 family with both tumor suppressor and pro-inflammatory properties [2-4]. IL-24 is expressed in normal melanocytes and monocytes as well as early stages of melanoma, but its expression is lost during melanoma progression [5, 6]. Numerous studies have demonstrated the potent antitumor functions of IL-24 in a broad spectrum of human cancers, including melanoma [7-12]. A combination of IL-24 and the epidermal growth factor receptor (EGFR)-targeted therapy, such as the anti-Her-2 monoclonal antibody Herceptin or the small-molecule EGFR inhibitor Gefitinib, significantly increases antitumor activity against human non-small-cell lung cancers (NSCLC) and breast cancers [13-15]. Previous studies also showed that IL-24 exerts its antitumor function mainly through inhibition of the EGFR and PI3K signaling and induction of the expression of double-stranded RNA-activated protein kinase (PKR) in human NSCLC and breast cancer cells [16-19]. However, the precise molecular mechanisms and signaling pathways of IL-24 for melanoma suppression remain largely unclear, and no information has been available about the combined effect of IL-24 with the EGFR-targeted therapy on melanoma suppression. As part of our efforts to examine effect(s) of currently available rationale drugs for melanoma, we initiated the study of targeting the EGFR alone, and in combination with IL-24.

EGFR signaling is involved in many cellular processes including cell proliferation, motility, adhesion, and angiogenesis through the activation of three pathways: phosphatidylinositol-3 kinase (PI3K)/Akt pathway, the Jak/STAT pathway and the ras/raf pathway [20-22]. EGFR is expressed or highly expressed in a variety of human solid tumors including melanoma, and is therefore a promising target for cancer therapy [23-29]. Erlotinib (Tarceva) is a small-molecule orally active selective EGFR inhibitor that binds competitively to the ATP-binding site at the kinase domain of EGFR and inhibits the activities of EGFR tyrosine kinases. Erlotinib has been approved by the FDA as the second/third tier treatment for patients with advanced NSCLC and is being studied for the treatment of various cancers either as single-agent therapy or in combination with chemotherapy and/or radiation [30-38]. However, drug resistance of this EGFR inhibitor develops rapidly in patients [39-41]. A recent study has demonstrated that combined treatment of NSCLC cells with erlotinib and the GST-fusion protein of MDA-7/IL-24 synergistically inhibits tumor cell growth and induces apoptosis and suggested that this combination therapy is a promising treatment strategy for NSCLC patients with acquired resistance to EGFR inhibitors [42]. To date, most of the current efforts for melanoma treatment have focused on mitogen-activated protein kinase (MAPK)

inhibitors or immunotherapy approaches [43-45], and the erlotinib-mediated EGFR-targeted therapy has not been tested in melanoma. Based on the findings that EGFR-associated PTK signaling pathway is involved in melanoma tumorigenesis [46-51] and that IL-24 can induce apoptosis and inhibit EGFR-associated PTK cell survival signaling pathway [13-19, 43], we hypothesize that an integrated treatment by combining the IL-24-mediated molecular therapy and the erlotinib-mediated EGFR-targeted therapy can increase the inhibition of melanoma growth and enhance the sensitivities of melanoma cells to erlotinib treatment. In this study, we evaluated the combined effect of IL-24 gene transfer and erlotinib on cell growth inhibition and apoptosis induction in various melanoma cells and examined their mutual mechanisms of action for melanoma suppression. We found that combination of IL-24 and erlotinib significantly increase antitumor activity by simultaneously activating the Apaf-1 apoptotic pathway and inactivating the Akt cell survival signaling pathway. Our findings therefore suggest that the novel combination of IL-24 and erlotinib is a promising treatment approach for melanoma treatment.

METHODS

Cell lines and cell culture

Eight human melanoma cell lines at different stage [WM35 (early-stage), WM793 (advanced-stage), A375 (metastatic), MeWo (metastatic), Skmel-2 (metastatic), SB2 (advanced-stage), Mel-2 (metastatic) and Mel-3 (metastatic)] and human epithelial carcinoma cell line (A431) were used in our experiments. A375 and MeWo cell lines were obtained from the American Type Culture Collection (Rockville, MD). WM35 and WM793 cell lines were kindly provided by Dr. Robert Kerbel at Sunnybrook Health Sciences Centre (Toronto, Canada). SB2 cell line was provided by Dr. Jeffrey Gershenwald at M. D. Anderson Cancer Center. Skmel-2 cell line was obtained from the NCI, Division of Cancer Treatment and Diagnosis. Mel-2 and Mel-3 cell lines were established in our laboratory from melanoma metastasis to the lymph node and dermis, respectively. Passage 6 (Mel-2) and passage 3 (Mel-3) were used in our experiments. All cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 µg/ml glutamine, penicillin (100 units/mL), and streptomycin (100 µg/mL) (Invitrogen, Carlsbad, CA). All cells were grown at 37°C in an atmosphere of 5% CO₂.

Recombinant vectors and agents

The recombinant adenoviral vectors Ad-IL-24 and Ad-Luc, containing IL-24 and luciferase genes, respectively, were prepared in the Gene Therapy Core Lab at the University of Texas M.D. Anderson Cancer Center. The recombinant expressing plasmid vector containing dominant negative-Akt (dn-Akt) (T308A, S473A) and the DOTAP:cholesterol (DC) nanoparticles were provided by Dr. Lin Ji (M. D. Anderson Cancer Center). Erlotinib was bought from LC Laboratories (Woburn, MA). The human IL-24 protein was overexpressed in HEK cells and purified in our laboratory.

Transient transfection

DOTAP:cholesterol (DC)-based nanoparticles were used to transiently transfect recombinant expressing plasmid vectors or the siRNA duplexes in our experiments. In brief,

DC nanoparticles (2 μ L) and plasmid vector (4 μ g) or siRNA nucleoligos (0.2 nmol) were mixed, and the mixture was added to each well of a 6-well plate with 2 ml medium and incubated at 37 °C for 72 hours. The transfection efficiency was assessed by a parallel transfection with an equal amount of enhanced green fluorescent protein (GFP)–expressing plasmid vector in each cell line.

Cell viability assay

Cell growth inhibition was determined by a MTT assay (Roche Diagnosis, Indianapolis, IN). Briefly, melanoma cell lines plated in 96-well plates (5000 cells/well) were co-treated with the Ad-IL-24 (2000 vp/cell) or the purified IL-24 protein (100 ng/ml) plus erlotinib at various doses. At 72 hours after treatment, cell viability was determined. Each experiment was carried out in triplicate wells for each drug concentration and carried out independently 3 times. The IC₂₀ and IC₅₀ values, defined as the concentration needed for a 20% or 50% reduction in absorbance, were calculated from the survival curves.

Apoptosis assay

Apoptosis was measured by flow cytometry using a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) or a propidium iodide (PI) staining-based fluorescence-activated cell sorter (FACS) as described previously [51, 52]. In brief, melanoma cell lines plated in 6-well plates (200,000 cells/well) were co-treated with Ad-IL-24 vector (2000 vp/cell) and erlotinib at the IC₂₀ dose. At 72 hours after treatment, cells were fixed in 1 % paraformaldehyde, permeabilized with 70% ethanol, and washed with PBS. Apoptosis was measured with an APO-BRDU kit (Phoenix Flow System, San Diego, CA). DNA fragmentation was analyzed by flow cytometry. The apoptosis was calculated in terms of the FITC-positive or Sub-G1 values in cells. Experiments were repeated three times for each treatment.

siRNAs

The siRNAs targeting Apaf-1 and Akt genes were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CO). Cells plated in 96-well plates (5000 cells/well) or 6-well plates (200,000 cells/well) were transfected with siRNA duplexes (100 nM) by using DOTAP:cholesterol (DC)-based nanoparticles. At 48 or 72 hours after treatment, cell viability or apoptosis were tested by MTT and FACS analysis, respectively.

Western blot analysis

Melanoma cell lines plated in 6-well plates (200,000 cells/well) were co-treated with Ad-IL-24 vector (2000 vp/cell) and erlotinib at the IC₂₀ dose. At 72 hours after treatment, cell lysates were harvested and subjected to Western blotting. Western blot was probed with antibodies against pTyr1068-EGFR, EGFR, pTyr458-PI3K, PI3K, pSer473-AKT, AKT, caspase-3, caspase-9, Apaf-1, β -actin (Cell Signaling Technology, Beverly, MA). The protein bands were visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Statistical analysis

The *t*-test was used to compare the values of the test and control samples. $P < 0.05$ was considered to a statistically significant difference. The experiments were done three times, and mean values and standard deviation were calculated. Statistica 6.1 software was used for the statistical analyses. IC₂₀ and IC₅₀ values of erlotinib were measured using CurveExpert software.

RESULTS

Increased inhibition of tumor cell growth by IL-24 and erlotinib combination

To study whether erlotinib inhibits growth of melanoma, we examined the effect of erlotinib on cell viability in melanoma cell lines at different stages by MTT assay (Table 1). As shown in Fig. 1A, treatment with erlotinib alone dose-dependently decreased the cell viability of melanoma cell lines. The IC₅₀ and IC₂₀ values of erlotinib for melanoma growth suppression were measured (Table 1).

We next evaluated the combined effects of the Ad-IL-24 and erlotinib on tumor cell growth (Fig. 1B). Treatment with erlotinib and the control vector Ad-Luc (2000 vp/cell) produced a dose-dependent inhibition of cell growth in all four melanoma cell lines. Cotreatment of melanoma cells with Ad-IL-24 (2000 vp/cell) and erlotinib at various doses significantly increased antitumor activity by comparison with the treatment with erlotinib plus the control vector Ad-Luc ($P < 0.05$), indicating that IL-24-mediated molecular therapy enhanced antitumor activity induced by erlotinib. To further evaluate the degree of IL-24-mediated enhancement of tumor suppression, we determined the changes in IC₅₀ values of erlotinib in melanoma cell lines cotreated with Ad-IL-24 and erlotinib. Treatment of Ad-IL-24 resulted in a 2- to 5- fold reduction of the IC₅₀ value of erlotinib when compared to the treatment with the control vector Ad-Luc (Table 1). To confirm these findings, we also cotreated melanoma cells with the purified human IL-24 protein (100 ng/ml) and erlotinib (at the dose of IC₂₀) and found that the combination treatment also significantly increased inhibition of melanoma cell growth ($P < 0.005$) (Fig. 1C). These results indicate that IL-24 modulates the sensitivity of melanoma to the EGFR inhibitor erlotinib and suggests that a combination treatment with IL-24-mediated molecular therapy and erlotinib may be a novel treatment strategy for human melanoma.

Enhanced induction of apoptosis by IL-24 and erlotinib combination

To determine whether the enhancement of antitumor activity induced by IL-24 and erlotinib combination is associated with the increase of apoptosis, we also evaluated induction of apoptosis by a FACS analysis. In all cells examined, treatment with Ad-IL-24 or erlotinib alone at the IC₂₀ dose also induced some apoptosis at 72 hours after treatment. However, the combination treatment with both agents markedly increased apoptosis (Fig. 2A).

Activation of caspases is an important event in the apoptosis signaling pathway. To determine whether the enhanced apoptosis induced by combination of IL-24 and erlotinib is related to the activation of caspases, we next analyzed the combined effects of IL-24 and erlotinib on the activities of two key caspases, caspase-3 and caspase-9 in A375 cells by

Western blot analysis (Fig. 2B). Cleavage of both caspase-3 and caspase-9 were increased in the cells cotreated with Ad-IL-24 and erlotinib by comparison with the cells treated with the control vector Ad-Luc, as indicated by the increased intensity of the cleaved bands (p37 and p17 for caspase-9; p19 for caspase-3) on the Western blot (Fig. 2C). These results demonstrate that the increased apoptosis induced by combination of IL-24 and erlotinib is mediated by the caspase activation.

Activation of Apaf-1 apoptotic pathways by IL-24 and erlotinib combination

The activation of apoptotic protease activating factor-1 (Apaf-1)-dependent pathway plays a key role in mitochondrial-associated apoptosis by various antitumor agents. For example, decreased expression of Apaf-1 has been shown to be associated with progression of melanoma [53]. To determine whether the increased apoptosis by the IL-24 and erlotinib combination is implicated in induction of the Apaf-1 signaling in melanoma cells, we analyzed the effect of the combination therapy on Apaf-1 protein expression by Western blot (Fig. 3A). Cotreatment with Ad-IL-24 and erlotinib considerably increased the expression of Apaf-1 proteins (Fig. 3A), indicating that the enhancement of apoptosis induced by combination of IL-24 and erlotinib is also through activation of Apaf-1 apoptotic pathway.

To further verify the role of Apaf-1 pathway in IL-24 and erlotinib-mediated tumor suppression and apoptosis, we blocked the expression of Apaf-1 protein by transfection of the Apaf-1-specific siRNA (si-Apaf-1) mediated by DC-nanoparticles and evaluated the effect of the si-Apaf-1 on cell growth and apoptosis induced by IL-24 and erlotinib combination. By comparison with the scrambled non-specific control siRNA (si-NSC), transfection of si-Apaf-1 in the melanoma cells cotreated with Ad-IL-24 and erlotinib significantly attenuated the antitumor activity induced by IL-24 and erlotinib combination in A375 and WM793 cells, resulting in a >30% increase of cell viability (Fig. 3B) and >5% decrease of apoptosis (Fig. 3C). These results therefore demonstrate that the enhancement of antitumor activity induced by IL-24 and erlotinib combination is mediated by upregulating the Apaf-1-dependent apoptotic pathways.

Inactivation of Akt signaling pathway by IL-24 and erlotinib combination

Activation of the EGFR triggers its downstream Akt signaling pathways that regulate many cellular processes involved in tumor survival and growth as well as chemotherapy resistance. To assess whether the development and progression of melanoma and the enhanced antitumor activity by IL-24 and erlotinib combination are also associated with the Akt-dependent signaling pathway, we analyzed the changes in levels of the total and phosphorylated proteins involved in the EGFR and Akt-dependent pathway in melanoma cell lines by Western blot analysis. Endogenous expression of the total and phosphorylated EGFR, PI3K and Akt proteins were detected in all eight melanoma cell lines at different stages and passages (Fig. 4A). By comparison with either agent alone or the combination of control vector Ad-Luc and erlotinib, a combination of Ad-IL-24 with erlotinib markedly decreased the levels of phosphorylated proteins involved in the Akt signaling pathway (Fig. 4C), whereas the total protein levels of these signaling molecules were not changed (Fig. 4B). These results therefore indicate that the enhancement of antitumor activity induced by

IL-24 and erlotinib combination may be also mediated by inactivating the Akt-dependent cell survival signaling pathway in melanoma cells.

To validate association of the Akt signaling pathway with the enhanced antitumor activity mediated by IL-24 and erlotinib combination, we inhibited the expression of the endogenous Akt protein by transfecting melanoma cells with an Akt-specific siRNA (si-Akt) using DC-nanoparticles and examined its effect on growth inhibition and apoptosis induction. By comparison with the scrambled non-specific control siRNA (si-NSC), transfection of the Ad-IL-24 and erlotinib-cotreated melanoma cells with the Akt siRNA (si-Akt) significantly increased the antitumor activity mediated by IL-24 and erlotinib combination, resulting in a decrease of cell viability (Fig. 5A) and an increase of apoptosis (Fig. 5B). Treatment with the Akt siRNA (si-Akt) alone in A375 and WM793 cells also caused a slight inhibition of cell viability (Fig. 5A) and induction of apoptosis (Fig. 5B). To further confirm the involvement of the Akt pathway in IL-24 and erlotinib combination-mediated tumor suppression, we also blocked the function of Akt by transfecting a dominant-negative Akt-expressing plasmid vector (dn-Akt, T308A, S473A) using DC-nanoparticles in melanoma cells cotreated with Ad-IL-24 and erlotinib. Transfection of the dn-Akt vector significantly increased the tumor suppressing activity mediated by IL-24 and erlotinib combination in melanoma A375 and WM793 cells, causing an increase of cell growth inhibition (Fig. 5C) and apoptosis induction (Fig. 5D) by comparison with the transfection with the empty control vector (EV). Taken together, these results showed that the enhancement of antitumor activity induced by IL-24 and erlotinib combination is also mediated by inactivating the Akt-dependent cell survival signaling pathway.

Tumor suppression by erlotinib via targeting EGFR signaling

To determine whether the antitumor effects of erlotinib at such high concentrations are EGFR specific in melanoma cells, we first blocked the EGFR signaling with an EGFR-specific monoclonal antibody cetuximab (Cetx), and then evaluate the effects of erlotinib (Erl) on the cetuximab-mediated antitumor activity. Melanoma cell lines (A375 and WM793) were treated with cetuximab (80 ng/ml) for 48 hours, and then followed by addition of erlotinib (40 μ M). After 48 hours, cell viability and apoptosis were determined. The human epithelial carcinoma cell line A431 that highly expresses EGFR protein was used as a positive control cell line. As shown in Figure 6, treatment with erlotinib or cetuximab alone inhibited cell viability (Fig. 6A) and induced apoptosis (Fig. 6B, 6C). However, treatment first with cetuximab to block EGFR signaling, then followed by addition of erlotinib, did not markedly change the levels of cell viability (Fig. 6A) and apoptosis (Fig. 6B, 6C) induced by cetuximab in all cell lines tested. These results show that the antitumor effect of erlotinib in melanoma cells is EGFR specific.

DISCUSSION

In the present study, we studied the effect of the EGFR inhibitor erlotinib on the growth of human melanoma and found that erlotinib dose-dependently inhibited tumor cell growth in different melanoma cell lines. We also demonstrated that the novel combination of IL-24 and erlotinib increased the effectiveness of erlotinib. Treatment with Ad-IL-24 or purified

IL-24 protein in combination with erlotinib significantly inhibited tumor cell growth and increased apoptosis induction in various melanoma cell lines. These results show that this novel combination treatment is a promising therapeutic approach for melanoma treatment. To our knowledge, this is the first report regarding EGFR tyrosine kinase inhibitor erlotinib and its combination with IL-24 tumor suppressor for the treatment of melanoma. These results will serve as a basis for guiding the combination treatment designs in future preclinical and clinical trials.

Mutations in apoptotic pathways and decreased susceptibility to apoptosis are associated with cellular resistance to chemotherapeutic drugs in various human cancers. The IL-24/MDA7 protein has been found to physically interact with dsRNA-dependent protein kinase (PKR), a potent growth inhibitory protein, in human lung cancer cells. This direct interaction between PKR and IL-24 is important for PKR-mediated apoptosis [18]. Previous studies also showed that overexpression of IL-24 in melanoma cells activated the p38 mitogen-activated protein kinase (MAPK) pathway, resulting in up-regulated expression of the genes associated with cell growth-arrest and apoptotic processes [54]. However, the precise molecular mechanisms and signaling pathways of IL-24 for tumor suppression and apoptosis induction remain largely unclear in melanoma cells. Our current study has demonstrated that the enhancement of apoptosis by the IL-24 and erlotinib combination is mediated by activation of the caspase- and Apaf-1-dependent apoptotic pathway in melanoma cells. Apaf-1 is an important signaling protein involved in the activation of caspase-9 during apoptosis. Apaf-1 forms a complex with caspase-9 in the presence of cytochrome c and dATP, ultimately leading to activation of caspase-9 and caspase-3 and subsequently inducing apoptosis [55, 56]. Our study provides new insights into understanding the molecular mechanisms of IL-24 for tumor suppression.

Previous studies have shown that EGFR signaling pathway is involved in the development and progression of melanoma [46-50]. EGFR tyrosine kinase inhibitor erlotinib has been used to treat various solid tumors, but have not been tested in melanoma. We hypothesized that inhibition of EGFR-dependent signaling pathway by erlotinib would also suppress the growth of melanoma cells, and that a combination treatment consisting of IL-24 and erlotinib would enhance antitumor activity by simultaneously activating the tumor suppression and apoptotic pathways and inhibiting the anti-apoptotic and cell survival signaling pathways in melanoma cells. The results from our study support our hypothesis and have important implications in exploring novel therapeutic strategies for melanoma. Although sensitivity to erlotinib varied in our panel of melanoma cell lines, our study demonstrates that IL-24-mediated molecular therapy increases the response to erlotinib, and suggests a potential of this combination as a promising strategy for the treatment of melanoma.

A strong positive association between erlotinib toxicity and patient survival has been reported for several epithelial malignancies such as including lung cancer, head and neck cancer, and ovarian cancer. The toxicities experienced by patients taking erlotinib are multifactorial and determined by distinct parameters in different tissues. The most common adverse effects of erlotinib are skin rash and diarrhea. [57-59]. Adenovirus-mediated IL-24/MDA-7 gene therapy alone has been shown to inhibit the growth and kills a broad spectrum

of cancer cells, with no toxicities or other deleterious effects in normal human epithelial or fibroblast cells [60-62]. A recent study in our laboratory has also shown that IL-24 plays an important role in the resolution of skin wound healing (Poindexter, N., submitted for publication). Therefore, IL-24 may prove useful to regulate the severity of the skin rash and provides additional rationale to explore the dose and scheduling in patients experiencing toxicities of erlotinib.

The persistent activity of the Akt signaling pathway contributes to resistance of tumor cells to EGFR inhibitors. In this study, we also identified the combined effect of IL-24 and erlotinib on EGFR and Akt signaling and found that the combination of IL-24 and erlotinib led to an enhanced inactivation of the Akt signaling pathway. Phosphorylated Akt is a mediator of EGFR-induced cell survival and clinical response to EGFR inhibitors, and a reduction of phosphorylated Akt expression is thought to be an important event in sensitizing tumor cells to EGFR inhibitor treatment. Our current study showed that combination of IL-24 and erlotinib significantly increased the inhibition of phosphorylation of the EGFR, PI3K, and Akt proteins. Therefore, our findings revealed a novel pathway for IL-24 in melanoma cells.

In summary, we demonstrated that treatment with Ad-IL-24 in combination with erlotinib significantly inhibited tumor cell growth and induced apoptosis in melanoma cells. The enhancement of antitumor activity by IL-24 and erlotinib combination is associated with the activation of the Apaf-1-dependent apoptotic pathway and the inactivation of the Akt-dependent cell survival signaling pathway. Our findings provide new insights into the molecular mechanisms of IL-24 and suggest that a combination of IL-24 and erlotinib may be an effective treatment strategy for human melanoma.

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Figure 1.

Combined effects of IL-24 and erlotinib (Erl) on melanoma cell growth. The melanoma cell lines were treated with erlotinib alone at various doses (**A**), or cotreated with Ad-IL-24 (2000 vp/cell) plus erlotinib (**B**), or with the purified IL-24 protein (100 ng/ml) plus erlotinib at the dose of IC_{20} (**C**). The Ad-Luc vector was used for negative control. At 72 hours after treatment, the cell viability was determined by MTT assay. Cells treated with PBS were used as the referent group with cell viability set at 100%. The percent cell viability in each treatment group was calculated relative to cells treated with PBS. *, $P < 0.05$; **, $P < 0.005$, significant differences between treatment groups.

Figure 2.

Combined effects of IL-24 and erlotinib (Erl) on apoptosis induction. The human melanoma cell lines were cotreated with Ad-IL-24 vector (2000 vp/cell) and erlotinib at the dose of IC_{20} . The Ad-Luc-infected cells were used as negative controls. At 72 hours after treatment, the apoptosis was determined by FACS analysis (A). The activation of caspases in A375 cells was analyzed by detecting the cleavage products of caspase-9 and caspase-3 using Western blot analysis (B). Densitometry of the cleaved caspase protein bands (p35, p17 and p19) from a representative of blots was analyzed (C). The apoptosis are represented by relative percentages of apoptotic cells.

Figure 3.

Combined effects of IL-24 and erlotinib (Erl) on Apaf-1 apoptotic pathway. **(A)**, The human melanoma cell lines A375 and WM793 were cotreated with Ad-IL-24 vector (2000 vp/cell) and erlotinib at the dose of IC_{20} . At 72 hours after treatment, the levels of Apaf-1 proteins were determined by Western blot analysis. Densitometry of the Apaf-1 protein bands was analyzed. **(B-C)**, Human melanoma cell lines were transfected with the Apaf-1 specific siRNA (si-Apaf-1) (100 nM) or the scrambled non-specific control siRNA (si-NSC), and cotreated with Ad-IL-24 vector (2000 vp/cell) and erlotinib at the dose of IC_{20} . At 72 hours after treatment, cell viability **(B)** and apoptosis **(C)** were determined by MTT and FACS analysis, respectively. The percent cell viability in each treatment group was calculated relative to cells treated with PBS, and the apoptosis was represented by relative percentages of apoptotic cells. *, $P < 0.05$; **, $P < 0.005$, significant differences between treatment groups.

Figure 4.

Combined effects of IL-24 and erlotinib (Erl) on Akt cell survival pathway. **(A)**, Endogenous expression of the total and phosphorylated Akt, PI3K and EGFR proteins in various melanoma cell lines detected by Western blot analysis. **(B, C)**, Human melanoma cell lines A375 and WM793 were cotreated with Ad-IL-24 vector (2000 vp/cell) and erlotinib at the dose of IC_{20} . The Ad-Luc-infected cells were used as negative controls. At 72 hours after treatment, expression of the total **(B)** and phosphorylated **(C)** Akt, PI3K and EGFR proteins were detected by Western blot. The densitometry of the phosphorylated protein bands was also analyzed **(C)**. This is a representative of the blots.

Figure 5.

Effects of Akt-specific siRNA and dominant-negative Akt on IL-24 and erlotinib (Erl) combination-mediated tumor suppression. The human melanoma cell lines A375 and WM793 were transfected with a Akt siRNA (si-Akt) (**A, B**) or a dominant-negative Akt (dn-Akt) expressing vector (**C, D**), and then cotreated with Ad-IL-24 vector (2000 vp/cell) and erlotinib at the dose of IC₂₀. At 72 hours after treatment, and cell viability (**A, C**) and apoptosis (**B, D**) were determined by a MTT and FACS analysis, respectively. The scrambled non-specific control siRNA (si-NSC) (**A, B**) or the empty vector (**C, D**) was used as negative controls. The percent cell viability in each treatment group was calculated relative to cells treated with PBS, and the apoptosis was represented by relative percentages of apoptotic cells. *, $P < 0.05$; **, $P < 0.005$, significant differences between treatment groups.

Figure 6.

Effects of erlotinib on cetuximab-induced tumor suppression. The A375, WM793 and A431 were first treated with cetuximab (Cetx) (80 ng/ml) for 48 hours, and then followed treatment with erlotinib (Erl) (40 μ M). After 48 hours, cell viability (**A**) and apoptosis (**B**, **C**) were determined by a MTT and FACS analysis, respectively. The percent cell viability in each treatment group was calculated relative to cells treated with PBS, and the apoptosis was represented by relative percentages of apoptotic cells.

Table 1

IC₂₀ and IC₅₀ values of erlotinib in melanoma cells treated with or without Ad-IL-24

Cell Lines	Stage	IC ₅₀ (μM)	IC ₂₀ (μM)	IC ₅₀ (μM)		Sensitizing Fold
				Luc	IL-24	
WM35	Early	176	30	171	59	2.9
WM793	Advanced	75	17	68	26	2.6
A375	Malignant	59	10	57	18	3.2
MeWo	Metastatic	45	4	52	11	4.7

* Fold changes in IC₅₀ values of erlotinib in melanoma cells infected by Ad-Luc compared with those by Ad-IL-24.