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A signal network involving coactivated NF- κ B and STAT3 and altered p53 modulates BAX/BCL-XL expression and promotes cell survival of head and neck squamous cell carcinomas

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Abrogation of apoptosis to sustain cell survival is an essential step in development of cancer. Aberrant activation of signal transcription factors NF- κ B or STAT3, alterations in p53 status, or BCL/BAX family expression have each been reported to affect cell survival in cancer, including head and neck squamous cell carcinomas (HNSCC). However, molecular targeting of these alterations individually has yielded disappointing results. In our study, we examined the hypothesis that alterations in a signal network involving NF- κ B, STAT3 and p53 modulates expression of proapoptotic BAX and antiapoptotic BCL-XL proteins, and promotes cell survival of HNSCC. We found that NF- κ B and STAT3 are coactivated together, and with cytokine stimulation or siRNA knock-down, both modulate BAX/BCL-XL. Greater modulation among HNSCC lines expressing low wt p53 than those overexpressing mt p53 protein suggested that decreased p53 expression might enhance activation of NF- κ B, STAT3 and BCL-XL. Reexpression of wt p53 suppressed NF- κ B and STAT3 nuclear binding activity, and BCL-XL expression, while inducing p21 and BAX. Over-expression of p53 together with inhibition of NF- κ B or STAT3 induced greater increase in the BAX/BCL-XL ratio and apoptosis than modulation of these transcription factors individually. Conversely, NF- κ B or STAT3 inducing cytokines decreased the BAX/BCL-XL ratio. Thus, a network involving signal coactivation of NF- κ B and STAT3, differentially modified by p53 inactivation or mutation, promotes altered BAX/BCL-XL expression and cell survival in HNSCC. Inhibition of signal activation of both NF- κ B and STAT3 together with reexpression of p53 could be the most effective strategy to restore BAX/BCL-XL regulation and for cytotoxic therapy of HNSCC.

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Key words: NF- κ B; STAT3; p53; BCL-XL; BAX; HNSCC

Abrogation of apoptosis and sustained cell survival is an essential step in development of cancer.¹ Inactivation of tumor suppressor genes that are proapoptotic and activation of oncogenes that activate prosurvival signal pathways have both been implicated at each stage of pathogenesis of malignancy, and resistance to cytotoxic therapies. Activation of prosurvival signal transcription factors NF- κ B and STAT3, and dysfunction of the tumor suppressor p53 are among the most common molecular alterations affecting cell survival and therapeutic sensitivity identified in cancers.^{2–4} Activation of NF- κ B and STAT3, and disruption of p53 function have been independently shown to occur in the majority of head and neck squamous cell carcinomas (HNSCC),^{5–8} and each of these alterations contributes significantly to pathogenesis and/or prognosis of patients with HNSCC. However, the potential interplay among the alterations of these 3 pathways in HNSCC and other cancers are not well understood.

Constitutive activation of NF- κ B1/Rel A (p50/p65) is detected in the majority of HNSCC cell lines, and found to promote cell survival, tumor formation and resistance to apoptosis.^{9–12} Increased nuclear staining for NF- κ B in premalignant squamous dysplasias and HNSCCs has been associated with malignant progression and decreased survival.¹³ Likewise, activation of STAT3 has been demonstrated to promote cell survival of HNSCC lines, and its activation in patient tumor specimens is associated with poor prognosis.^{7,8} Such aberrant activation of NF- κ B and STAT3 was shown to be promoted by autocrine and/or paracrine expression of several growth factors, cytokines and receptors in HNSCC.

Ligand/receptor kinase pathways involving tumor necrosis factor (TNF)- α /TNFRII, interleukin (IL)-1 α /IL-1R and transforming growth factor (TGF)- α /epidermal growth factor receptor (EGFR) each can contribute to NF- κ B activation^{11,14,15}, and TGF- α /EGFR and IL-6/IL-6R can induce STAT3 activation.^{7,16–18} The capability for TGF/EGFR to activate both NF- κ B and STAT3, and for NF- κ B-dependent IL-6 expression to promote STAT3 activation,^{11,16,17,19} creates the potential for a network involving these signal-activated transcription factors to promote expression of prosurvival molecules in HNSCC.

Conversely, mutation (mt) or inactivation of wild-type (wt) p53 occurs in ~50% of HNSCC, and is often accompanied by overexpression of mt or under-expression of wt p53 protein.⁵ Mutation or inactivation of p53 is observed in premalignant and malignant epithelia, suggesting p53 dysfunction is an important early event in pathogenesis of HNSCC.^{4,5} Mutation of p53 may result in overexpression of molecules that exhibit loss, retention or gain of function. Interestingly, HNSCC with the wt p53 genotype but absent or with low wt p53 protein expression more often demonstrate greater resistance to apoptosis induced by chemotherapy drug cisplatin than those with mt p53, and are associated with decreased prognosis in patients receiving cisplatin and radiation therapy.^{20–23} The potential interaction of p53 inactivation or mutation, upon activation of NF- κ B, STAT3 and target molecules, and their effects on cell survival in HNSCC has not been directly examined.

Functions of the apoptotic pathways are mediated through regulation of expression of a family of proapoptotic and antiapoptotic proteins, and the ratio of expressed pro and antiapoptotic proteins has been reported to determine whether cells survive or undergo apoptosis.^{24,25} p53 regulates transcription of antiproliferative and proapoptotic proteins such as p21 and BAX,^{26–28} and NF- κ B and STAT3 can both upmodulate transcription of antiapoptotic proteins such as BCL-XL that control cell growth and survival.^{29–34} A low relative ratio of BAX to BCL-XL has been detected in HNSCC,³⁵ and overexpression of BCL-XL has been shown to suppress the initiation of apoptosis and promote resistance to anticancer drugs and radiation therapy.^{24,36} Previous studies demonstrate that BCL-XL is overexpressed in ~50–75% of HNSCC.^{37–39} Together with low p53 expression, increased BCL-XL expression is observed in HNSCC that are most resistant to cisplatin chemotherapy and radiation therapy,^{22,39} and inhibitors mimicking the BH3 domain of BCL-XL and BCL-2 have been shown to sensitize HNSCC to apoptosis induced by cisplatin.³⁹ Although p53, NF- κ B, STAT3, BCL-XL and BAX are individually implicated in death and survival in HNSCC and other cancers, the regulatory relationship among those important survival factors are not well understood. Evidence suggests that the death mechanisms and

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therapeutic response of cancer cells are not dependent on a single factor alone. Here we present a more complex model of cancer cell survival whereby a signal network including activation of NF- κ B and STAT3 together with dysfunction of p53, modulated BAX/BCL-XL expression, and cell survival in HNSCC. Reexpression of p53 together with inhibition of signal activation of NF- κ B and STAT3 most effectively restored BAX/BCL-XL regulation and optimally induced apoptosis of HNSCC.

Material and methods

Cells lines

The UM-SCC-1, -9, -11B and -38 HNSCC cell lines from the University of Michigan UM-SCC series were kindly provided by Dr. T. E. Carey (University of Michigan, Ann Arbor, MI) and described previously.⁴⁰ The p53 genotype status of UM-SCC cells (wild-type, UM-SCC-1 and 9; or mutant p53, UM-SCC-11B and 38) was determined by bi-directional DNA sequencing of exons 5–9 which contain the hot spots for mutation, and expression of p53 protein was established by immunostaining,⁴¹ and by Western blot (our study). The p53 status and mis-sense mutations found in these cells are consistent with the available information that was independently determined previously.^{22,39} The cell lines were maintained in Eagle's minimal essential media supplemented with 10% fetal bovine serum and penicillin/streptomycin. Human keratinocytes (HKCs) were grown in keratinocyte serum-free medium 154CF containing 0.08 mM of calcium chloride and supplemented with human keratinocyte growth supplements (HKGS, Cascade Biologics, Portland, OR). The final concentration of HKGS in the complete medium are: 0.2% (v/v) of bovine pituitary extract, 5 μ g/ml of bovine insulin, 0.18 μ g/ml of hydrocortisone and 5 μ g/ml of transferrin, and 0.2 ng/ml of human recombinant EGF. All HKC were used within 5 passages. The cultures were incubated in a humidified cell culture incubator at 37°C and 5% of CO₂.

Reagents, antibodies and plasmids

Human recombinant IL-6 and TNF- α were purchased from R&D Systems (Minneapolis, MN). Antibodies for BCL-XL (sc-7195), BAX (sc-493), p53 (sc-98, mouse monoclonal antibody against N-terminus 32–79), β -tubulin (sc-9935), mouse monoclonal anti-STAT3 antibody (sc-8019), anti-phospho-tyrosine (PY99), and Protein G PLUS Agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for NF- κ B (p65)-Biotin and p65 RNAi plasmid were obtained from Imgenex (San Diego, CA). Antibody for phospho-p65 (Ser536, cat No. 3031) and phospho-STAT3 (Tyr705, cat No. 9138) was purchased from Cell Signaling (Beverly, MA). Mouse monoclonal anti-human TP53 (DO-1, IgG2a) was purchased from Calbiochem, EMD Biosciences (San Diego, CA). The plasmid containing human intronless p53 (pORF5-p53) and control plasmid (pORF5) were obtained from InvivoGen (San Diego, CA). The STAT3 β dominant negative (DN) (pIRES-STAT3 β) and backbone plasmid (pIRES-EGFP) encoding enhanced green fluorescence protein (EGFP) were kindly provided from Dr. James Turkson and Dr. Richard Jove of the University of South Florida College of Medicine in Tampa, Florida. The construction and characterization of pIRES-STAT3 has been described previously.⁴² The fragment of human BCL-XL promoter sequence (–1,498 to +427) was PCR amplified, sequencing confirmed and constructed with luciferase reporter gene plasmid using pBV-Luc by Dr. Lin Zhang (University of Pittsburgh School of Medicine, Pittsburgh, PA), and pBV-Luc plasmid was created by Molecular Genetics Laboratory of the Johns Hopkins Oncology Center. The Bicinchoninic Acid Protein Assay and Super Signal West Pico Chemiluminescent Detection kits were obtained from Pierce (Rockford, IL).

Western blot and immunoprecipitation

Whole cell lysates were harvested by Nuclear Extraction kit (Active motif, Carlsbad CA). Twenty micrograms of whole-cell lysates were mixed with Laemmli loading buffer (containing β -

mercaptoethanol) and heated at 100°C for 5 min. The samples were loaded onto 10% Tris-Glycine precast gels and electrophoresis was performed at 140 V for 90 min. The proteins were transferred to 0.45 μ m nitrocellulose membranes (Invitrogen, Carlsbad, CA) for 2 hr at 20 V at room temperature using the Invitrogen Gel Blot Module. Immunoblotting was conducted according to manufacturer's specifications. Primary and secondary antibodies were diluted in 5% nonfatmilk prepared from TBS-T at 1:500 and 1:2,000, respectively. For immunoprecipitation of phospho-p65, 500 μ g of whole-cell lysate were used. Each blot was incubated with Pierce Super Signal West Pico substrate and exposed to Kodak X-OMAT film. The intensity of the protein bands were measured and quantified by densitometry using Kodak 1D image analysis software (Kodak, Rochester, NY).

Transient transfection, electrophoretic mobility shift assay and quantitative binding assays

To examine the effects of p53, p65RNAi and STAT3 β DN, transient transfection was performed. The transfection efficiency of ~80–90% was reached by pretesting with transfection of the plasmid containing LacZ gene followed by β -gal staining. UM-SCC-1, -9, -11B and -38 were seeded at 100-mm dishes. On the following day the cells were transfected with plasmids using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. For stimulation experiments, 3 hr after transfection, the cells were stimulated with 10 ng/ml of IL-6 or 10 ng/ml of TNF- α for 24 hr. The cell lysates were then harvested to perform Western blot or binding assay.

To detect p53 and p65 binding activities, nuclear extracts were obtained using Nuclear Extraction kit (Active Motif). Twenty microgram of nuclear extracts were used in each reaction. The binding activity was evaluated using TransAM kits for STAT family, p53 and NF- κ B (Active Motif) according to manufacturer's protocol. The optical density was measured at wavelength of 450 nm by a microplate reader (Biotek, Winooski, VT).

Electrophoretic mobility shift assay (EMSA) was performed after previously published protocol.^{9,10} Nuclear extract (5 μ g) was used for EMSA with ³²P-NF- κ B responsive element oligonucleotide (sense, 5'AGTTGAGGGGACTTTCCAGGC-3'; antisense, 3'TCAACTCCCCTGAAAGGGTCCG-5'; Promega, Madison, WI). ³²P-OCT-1 was used as a positive control for loading.

DNA flow cytometry

UM-SCC cells transfected with plasmids containing wild-type p53, p65 RNAi or STAT3 β DN were incubated for 72 hr prior to harvesting. Both live monolayer cells and dead nonadhesive cells were collected and counted by haemocytometer with trypan blue solution (Invitrogen). Cells were stained with propidium iodide (PI) of Cycletest Plus DNA Reagent Kit (Becton Dickinson, San Jose, CA) after the manufacturer's suggestions. DNA staining for cell cycle and apoptosis was quantified by FACScan flow cytometer using CellQuest software (Becton Dickinson). Cell cycle and DNA degradation of cells were analyzed by ModFit LT software (Verity Software House, Topsham, MA).

Tissue array, H&E staining and immunohistochemistry

Exemption from IRB review for study of commercially available tissue arrays without patient identifiers was obtained from the Office of Human Subjects Research, NIH. Formalin fixed and paraffin embedded HNSCC tissue arrays were obtained from Cybrdi (Gaithersburg, MD). Each array contained HNSCC tumor tissues from 20 individuals spotted in triplicates, plus normal mucosa tissues from 6 normal subjects, spotted in duplicates. Each array spot was 1.5 mm in diameter and verified by a pathologist with histological H&E and immunohistochemical pan cytokeratin staining. The tissues were sectioned 5 μ m in thickness. H&E staining and immunohistochemistry of BCL-XL (1:250) were performed by VECTORSTAIN ABC systems (Vector Laborato-

ries, Burlingame, CA). Antigen retrieval was applied in the staining procedure.

MTT assay

UM-SCC-1, -9, -11B or -38 cells were transfected with siRNA for BCL-XL and control oligos at 100 nM (Dharmacon, Chicago, IL). Cells were grown in antibiotic-free medium during transfection. The next day, 5×10^3 transfected cells were plated in each well of a 96-well microtiter plate and incubated overnight. Cell density was determined using an MTT cell proliferation assay at Days 1, 3 and 5 after transfection (Boehringer Mannheim, Indianapolis, IN). Colorimetric optical density was measured at 570 nm by a microplate reader (Biotek, Winooski, VT). Cell toxicity was also monitored each day.

Results

BCL-XL is overexpressed in HNSCC and plays an important role in cell survival

BCL-XL protein has been previously shown as a critical cell survival factor in cancers including HNSCC,^{31–34,36} as well as an important down stream gene regulated by NF- κ B or STAT3.^{12,29–34} As shown in Figure 1a, over-expression of BCL-XL protein was observed in a panel of 4 UM-SCC lines with differing p53 alterations (UM-SCC-1 and 9 cells, with inactivated wt p53; UM-SCC-11B and 38 cells, with mt p53)⁴¹ when compared to HKCs. The expression levels of BCL-XL in these and subsequent experiments below were not consistently different between cell lines with either p53 status. Overexpression of BCL-XL protein in UM-SCC cells was consistent with that observed in HNSCC specimens relative to normal mucosa, as determined by immunohistochemistry using a tissue array containing HNSCC tumor tissues from 20 individuals and normal mucosa from 6 subjects (Fig. 1b). Overall, 15 of 20 (75%) of the HNSCC specimens showed increased staining relative to normal mucosa, regardless of clinical grades and p53 staining status (Chen Z, unpublished observation). The prevalence of BCL-XL staining is consistent with the presence of increased staining BCL-XL in HNSCC reported in other patient series.^{37–39} Next, the functionality of BCL-XL was investigated by knocking down BCL-XL using siRNAs, and cell proliferation of UM-SCC cells was tested by MTT assay. Figure 1c shows that BCL-XL siRNA strongly inhibited the increase in cell density in all cell lines relative to controls. These data confirm that BCL-XL is one of the functionally important BCL family members in cell lines derived from HNSCC.

Constitutive coactivation and inducible phosphorylation and DNA binding activity of NF- κ B p65 and STAT3 in UM-SCC cell lines with wt or mt p53

We and others previously showed that NF- κ B p65 and STAT3 are each constitutively activated when compared to nonmalignant keratinocytes, and individually contribute to regulating survival and outcome of head and neck cancers.^{7–17,43} In our study, we compared the DNA binding activity of these 2 factors in nuclear extracts from HKCs and 4 UM-SCC lines in the same experiment by EMSA. The NF- κ B and STAT3 binding activities detected by EMSA were increased in 3 UM-SCC cells except in UM-SCC-9, when compared to the minimal activation observed in HKC (Fig. 2a). Further, similar relatively high, intermediate or low activation of both factors was observed in a wider panel of cell lines (Yeh, unpublished data), consistent with previous studies that both NF- κ B and STAT3 may be coactivated as a result of alterations in common upstream signaling molecules such as EGFR,^{14,36} or by cross-talk such as NF- κ B induced activation of STAT3 mediated by IL-6.¹⁹

We next examined the basal activation and signal inducibility of NF- κ B and STAT3 in these UM-SCC by Western blot analysis. All 4 cell lines exhibited detectable basal constitutive activation of

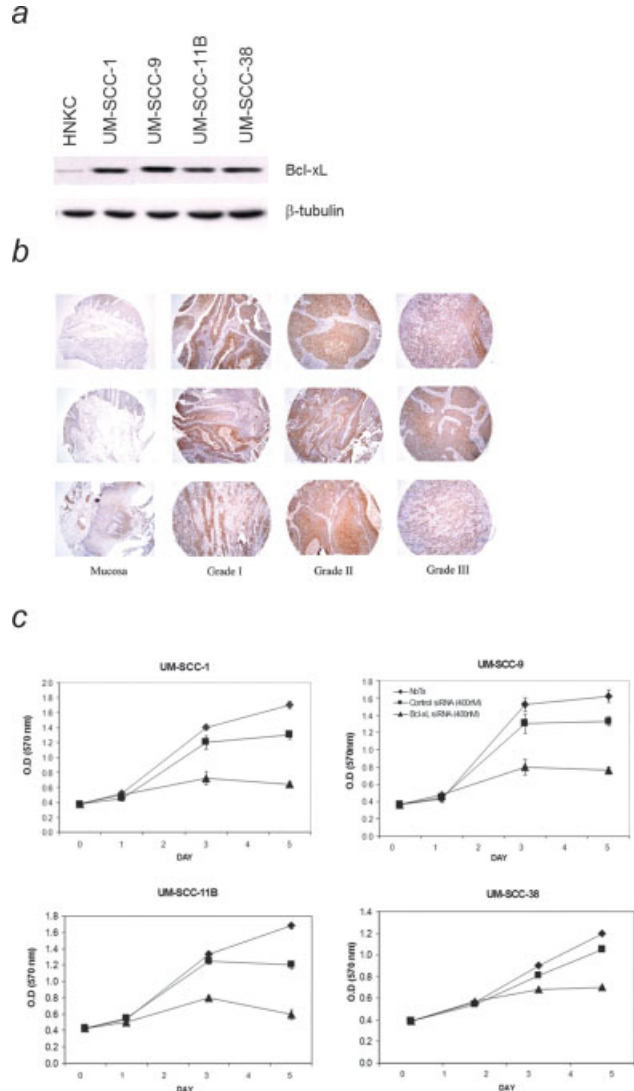


FIGURE 1 – BCL-XL is overexpressed and affects cell survival of HNSCC. (a) Whole cell lysates were harvested from cultured HKC and UM-SCC cell lines and Western blot analyses of BCL-XL were performed. β -tubulin was used as the loading control. (b) Immunohistochemical staining of BCL-XL on tissue array sections containing human normal mucosa (left column) and HNSCC tumors with different clinical grades ($\times 400$). (c) Cultured UM-SCC cells were transfected with BCL-XL siRNA and control oligonucleotides, and the cell density was determined on Days 0, 1, 3 and 5 by MTT assay. The data are presented as the mean plus standard deviation from quadruplicates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

both NF- κ B and STAT3, as indicated by NF- κ B p65 and STAT3 protein phosphorylation and DNA binding activity (Figs. 2b and 2c). Using a classical stimulus for each pathway, TNF- α or IL-6, significant induction of NF- κ B or STAT3 activation was detected, as indicated by increased phosphorylation and DNA binding activity of NF- κ B RELA (p65) and STAT3 (Figs. 2b and 2c). The levels of constitutive and cytokine-inducible phospho-proteins detected by Western blot were consistent with the levels of p65 and STAT3 DNA binding activity (Fig. 2). Together, these data provide evidence for constitutive coactivation and intact cytokine-inducible signal phosphorylation and DNA binding activity of NF- κ B p65 and STAT3 pathways in UM-SCC cell lines with either dysfunctional wt or mt p53 expression.

Effects of modulation of NF- κ B p65 and STAT3 on BCL-XL and BAX protein expression in UM-SCC cell lines with different p53 status

Next, we examined the effects of modulating NF- κ B or STAT3 activation on expression of BCL-XL and BAX in these cell lines with different p53 status. We tested if BCL-XL and BAX activity could be modulated by the cytokine stimuli above, or by knocking

down p65 through siRNA, or over-expression of STAT3 β , a truncated dominant negative (DN) form of STAT3.⁴² Knocking down p65 by siRNA significantly decreased p65 protein level in both UM-SCC-1 and -11B cell lines, and transfection of STAT3 β resulted in expression of the expected protein of faster mobility (Fig. 3a). We confirmed that knocking down NF- κ B p65 could also significantly affect BCL-XL gene expression, as cotransfection of UM-SCC cells with p65 siRNA and a BCL-XL promoter reporter construct resulted in partial inhibition of BCL-XL reporter activity in UM-SCC-1 and -11B cells (TL Lee, preliminary data, not shown). Grandis et al., have established that STAT3 also modulates BCL-XL in HNSCC.³⁶

At baseline, Figures 3b–3e shows that the level of BCL-XL protein relative to constitutive protein β -tubulin was similar between the cell lines with deficient wt and mt p53, consistent with Figure 1a. By comparison, a relatively lower level of the p53 regulated protein BAX was detected in UM-SCC-1 and -9 cells (Figs. 3b and 3d) when compared to UM-SCC-11B and -38 cells (Figs. 3c and 3e). This is consistent with relative lack of detectable wt p53 and p21 protein and increase in expression of mt p53 and p21 protein in these cell lines⁴¹ (Fig. 4a below). TNF- α or IL-6 was used to stimulate NF- κ B or STAT3 activation, and siRNA for p65 or a STAT3 β DN plasmid was used to specifically inhibit the function of these transcription factors. TNF- α stimulated BCL-XL expression, while p65 siRNA inhibited both constitutive and TNF- α stimulated BCL-XL expression, as expected (Figs. 3b–3c). However, greater modulation was observed in cell lines UM-SCC-1 and -9 exhibiting deficient expression of wt p53 than in UM-SCC-11B and -38 which express mt p53 (Figs. 3b–3c). Conversely, BAX expression was inhibited by TNF- α in UM-SCC-1 and 9 cells, but not in UM-SCC-11B and -38 cells that over-express mt p53 and relatively greater basal BAX protein expression (Figs. 3b–3c). In contrast, p65 siRNA significantly inhibited BCL-XL and increased BAX expression in both UM-SCC-1 and -9 cells, resulting in an increased BAX/BCL-XL ratio (Fig. 3b), but these effects, while present, were significantly dampened in UM-SCC-11B and -38 cells with mt p53 protein (Fig. 3c). There was no change in either BAX or BCL-XL with nonspecific siRNA controls. With IL-6 induction or expression of DN STAT3 β , similar and significant reciprocal effects on BCL-XL and BAX expression were observed in UM-SCC-1 and -9 cells with deficient wt p53 status (Fig. 3d), while dampened effects were observed in those with mt p53 (Fig. 3e). These observations suggest that cytokine

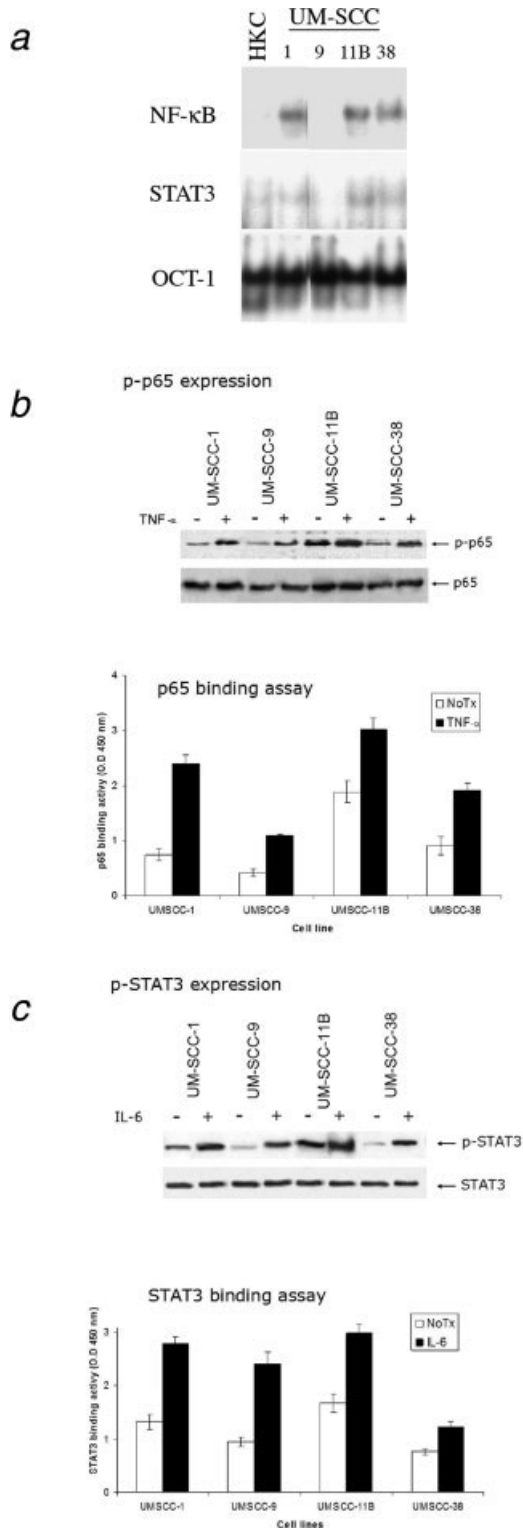


FIGURE 2 – NF- κ B p65 and STAT3 binding activity in UM-SCC cell lines associated with constitutive and inducible phosphorylation by TNF α and IL-6 stimuli. (a) Basal NF- κ B p65 and STAT3 binding activity tested by EMSA. Nuclear extracts (5 μ g) were isolated from each cultured HKC or UM-SCC cell lines, and used for binding to radiolabeled oligonucleotide containing NF- κ B or STAT3 response element. OCT binding activity was measured as a control for nuclear extract integrity and loading. (b) Cultured UM-SCC cells were treated with 10 ng/ml of recombinant human TNF- α for 30 min, and whole cell lysates were harvested. Immunoprecipitation of the cell lysates were carried out by a biotinylated antibody against total p65 and analyzed by Western blot (upper panel). Cultured UM-SCC cells were treated with TNF- α under the same condition as described earlier. Nuclear extracts were harvested, and p65 binding activity was measured using TransAM NF- κ B p65 binding kit (Active Motif, lower panel). The data are presented as mean plus standard deviation from triplicates. (c) Cultured UM-SCC cells were treated with 10 ng/ml of recombinant human IL-6 for 30 min, and the whole cell lysates were harvested. STAT3 activation was detected by an antibody against phospho STAT3, and an antibody against total STAT3 was used as a control (upper panel). Cultured UM-SCC cells were treated with IL-6 under the same condition as described earlier, nuclear extracts were harvested, and STAT3 binding activity was measured using TransAM STAT3 binding kit (Active Motif, lower panel). The data are presented as the mean plus standard deviation from triplicates.

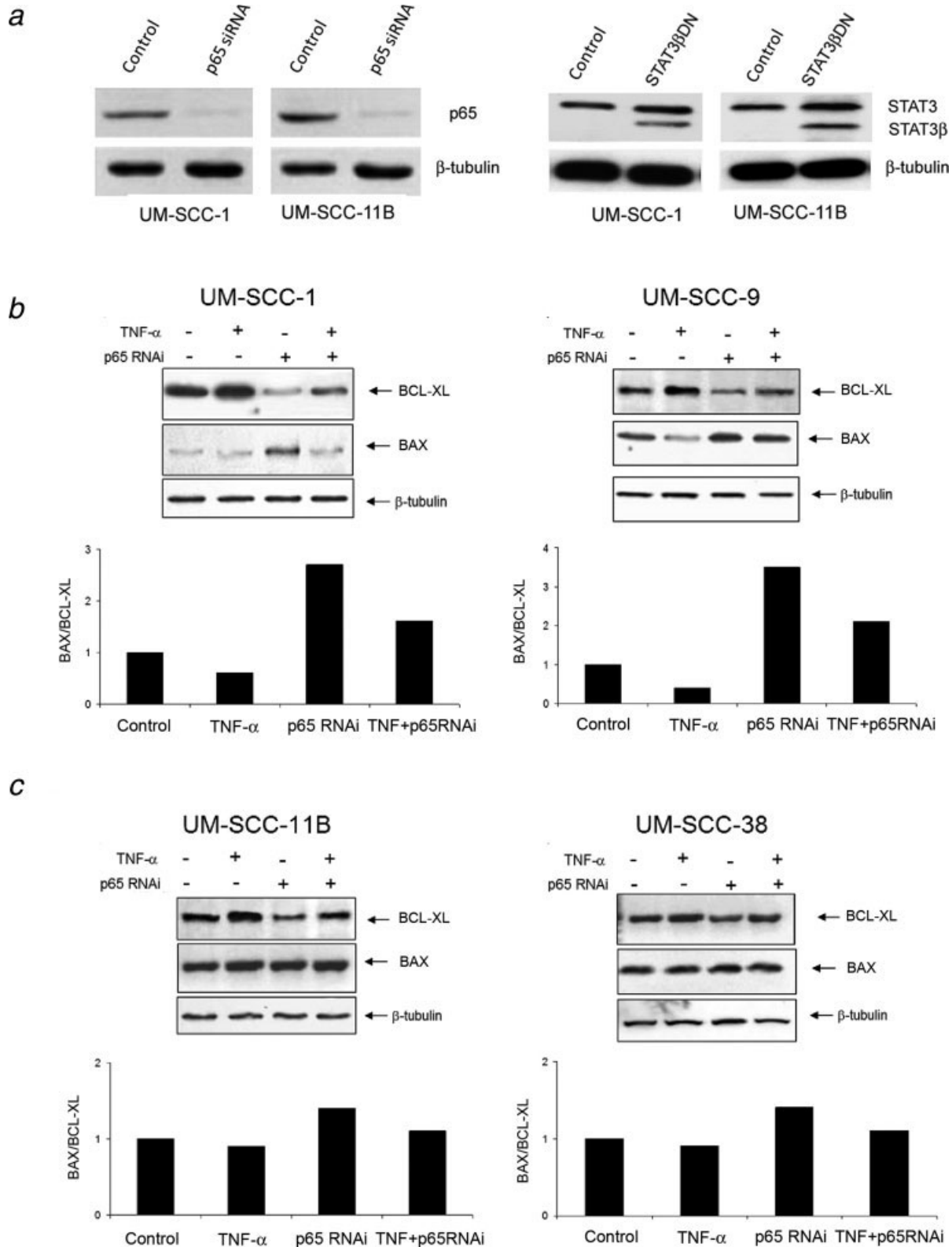


FIGURE 3 – Baseline and modulation of BCL-XL and BAX protein expression in UM-SCC cells by TNF- α /p65 siRNA and IL6/STAT3 β . (a) Cultured UM-SCC cells with wt p53 (UM-SCC-1) or mt p53 (UM-SCC-11B) were transiently transfected with p65 RNAi or control plasmid, or Stat3 β dominant negative or control plasmid for 48 hr. Whole cell lysates were collected and analyzed for the protein expression of NF- κ B p65 or STAT3 by Western blot. Cultured UM-SCC cells with wt p53 (UM-SCC-1 and UM-SCC-9), or (c) and (e) mt p53 (UM-SCC-11B and UM-SCC-38) were transiently transfected with p65 RNAi or a control plasmid (b and c), or Stat3 β dominant negative or a control plasmid (d and e) for 48 hr. The cells were treated either by TNF- α (10 ng/ml, b and c), or IL-6 (10 ng/ml, d and e) for 24 hr. Whole cell lysates were collected and analyzed for the protein expression of BCL-XL and BAX by Western blot. The band intensity was obtained by densitometry, and the data were calculated and presented as ratios in a bar graph from 1 representative experiment.

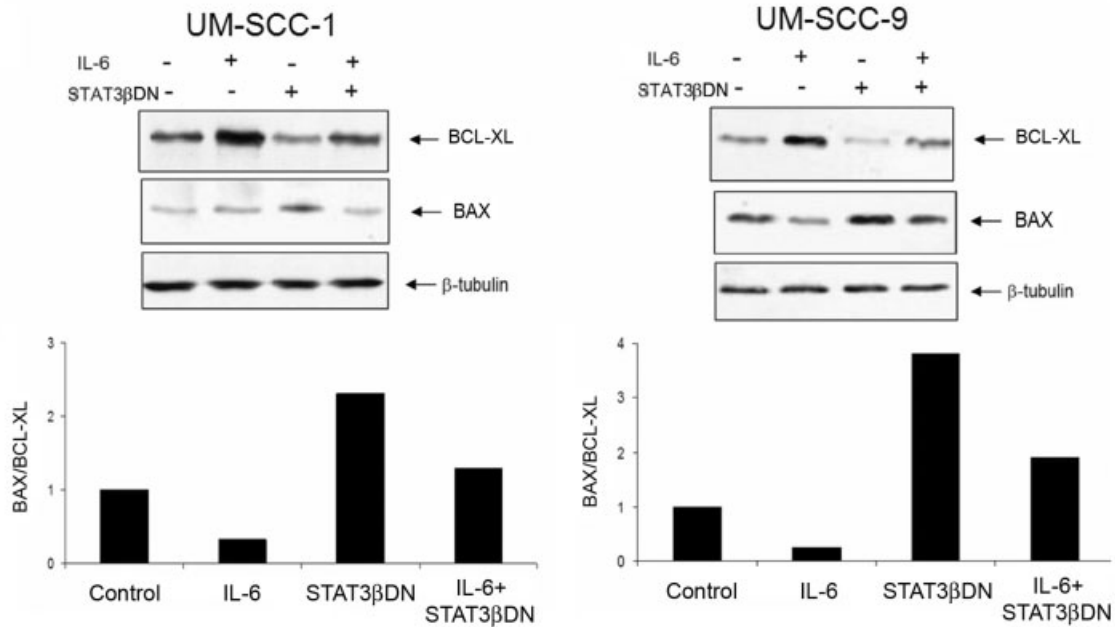
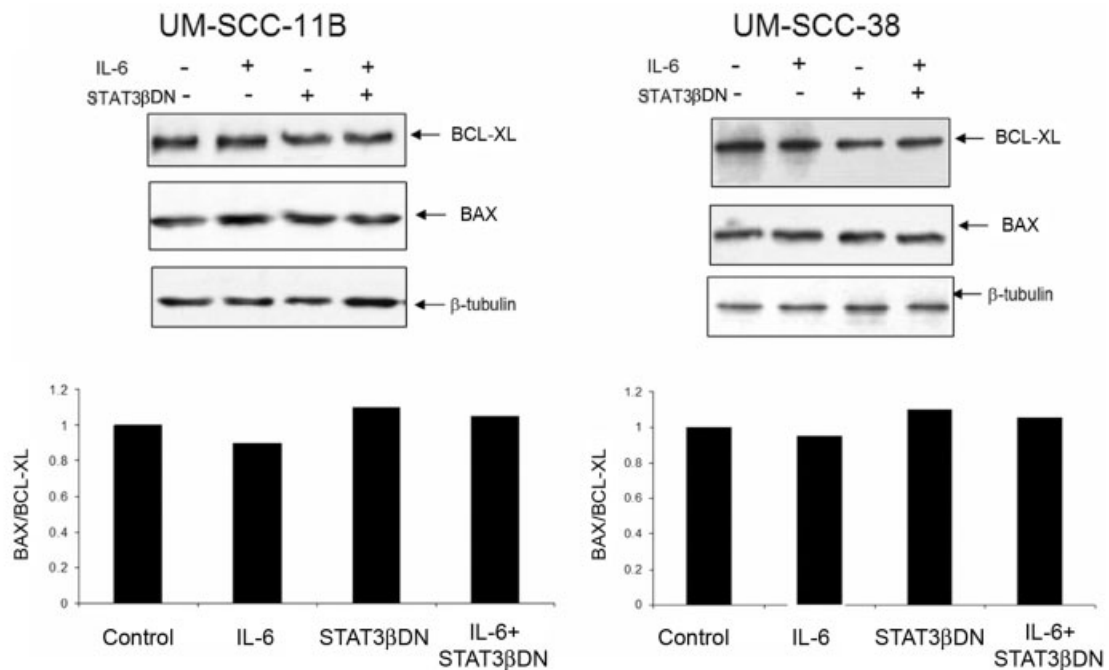
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FIGURE 3 – CONTINUED.

stimulation and genetic inhibition of NF- κ B and STAT3 are differentially affected in UM-SCC as a result of lack of expression or mutation of p53.

Reexpression of wt p53 in UM-SCC cells suppresses DNA binding activity of NF- κ B and STAT3, and expression of BCL-XL

On the basis of the earlier findings, we next compared the expression of p53 and another p53 regulated protein p21, and asked if NF- κ B and STAT3 activation and BCL-XL expression observed in UM-SCC cells which differ in p53 status, may be

inhibited by transient transfection and reexpression of wt p53. The baseline expression of p53, p21 and BCL-XL in the cell lines was compared by Western blot. As shown in Figure 4a, p53 protein expression in UM-SCC-1 and -9 cells was below the level detectable with antibody DO-1, and consistent with this, also lacked expression of p53 regulated protein p21. In UM-SCC-11B and -38 cells, both p53 and p21 proteins were detectable. Thus, UM-SCC-1 and -9 retain wt p53 sequence but are deficient for p53, p21 and BAX protein expression (Figs. 4a, 3b and 3d), while UM-SCC-11B and -38 containing missense mt p53 express relatively higher levels of p53, p21 and BAX (Figs. 4a, 3c and 3e), consistent with

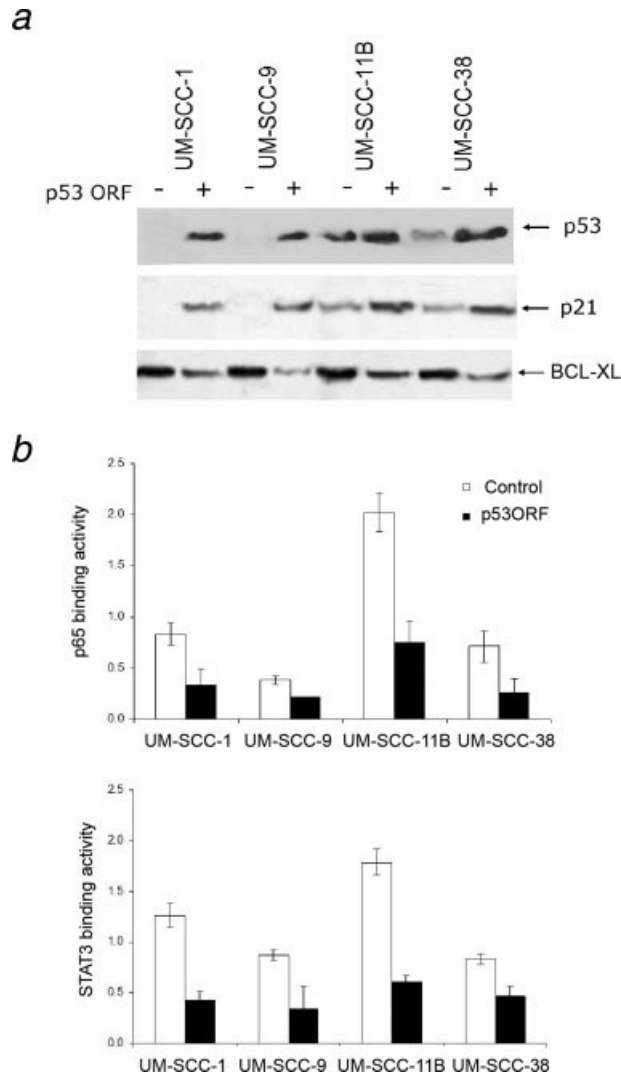


FIGURE 4 – Effects of overexpression of exogenous wt p53 on p21 and BCL-XL protein expression and NF- κ B and STAT3 DNA binding activity in UM-SCC cells. Cultured UM-SCC cells were transiently transfected with an expression vector containing wt p53 gene (p53ORF) or the control vector for 48 hr. (a) Whole cell lysates were harvested and analyzed by Western blot of p53 protein expression and its downstream target protein p21 and BCL-XL. (b) Nuclear extracts were harvested, and NF- κ B p65 and STAT3 activities were measured using ELISA based binding assay. The data are presented as the mean plus standard deviation from triplicates.

residual function of these p53 missense mutants for expression of p21 and BAX. In contrast, baseline expression of BCL-XL protein was similar in all 4 cell lines (Figs. 1a, 3 and 4a) with different p53 status (Fig. 4a).

We then asked if forced over-expression of wt p53 protein affects NF- κ B p65 and STAT3 activation and BCL-XL expression. We transfected the cells with an expression vector encoding the wt p53 open reading frame (p53 ORF), and examined p53, p21 and BCL-XL protein expression by Western blot. Transfection of cells with p53 ORF plasmid resulted in a detectable increase in expression of p53 protein in all 4 cell lines (Fig. 4a). The increase in intensity of the p53 band in UM-SCC-11B and 38 is consistent with coexpression of wt p53 protein with the missense mutants of p53 protein that retain similar mobility in these cells. Forced expression of wt exogenous p53 protein induced p21 expression in

all cell lines, indicating that transfected wt p53 protein was functional. Notably, expression of wt p53 protein also partially repressed expression of BCL-XL in all of the cell lines (Fig. 4a). Expression of wt p53 also suppressed DNA binding activity of both NF- κ B and STAT3 in all 4 cell lines (Fig. 4b), providing evidence that wt p53 may inhibit activation of NF- κ B and STAT3 in cells of either p53 status. Thus, these findings indicate that NF- κ B p65 and STAT3 DNA binding activity and expression of their target antiapoptotic protein, BCL-XL, can be partially suppressed after reexpression of wt p53 protein in UM-SCC cells with different alterations in p53. Together, these observations provide evidence for reciprocal cross-talk between p53, NF- κ B and STAT3 that affects modulation of BCL-XL.

Expression of exogenous wt p53 and modulation of NF- κ B and STAT3 coregulate BCL-XL and BAX expression in cells with over-expression of mutant p53 protein

Since p53 similarly inhibited NF- κ B, STAT3 and BCL-XL in cells with wt or mt p53, we compared the individual and combined effects of expressing wt p53 and/or regulating NF- κ B or STAT3 activity on BCL-XL and BAX expression in the 2 UM-SCC lines with mt p53 status. Figure 5 shows that overexpression of wt p53 protein partially inhibited BCL-XL while enhancing BAX expression, consistent with the effects observed in Figure 4 above for BCL-XL and p21. Expression of wt p53 together with inhibition of NF- κ B or STAT3 most strongly inhibited BCL-XL and increased BAX expression, and increased the BAX/BCL-XL ratio. Conversely, potent stimulus of NF- κ B by TNF- α or STAT3 by IL-6 attenuated the effects of wt p53 alone or the combination with p65RNAi or STAT3 β DN. No change in BAX and BCL-XL was observed with a nonspecific siRNA control (data not shown). These findings provide evidence that over-expression of wt p53 protein with inhibition of NF- κ B and STAT3 can together increase the BAX/BCL-XL ratio in cells with mt p53 status.

Induction of cell cycle alterations and apoptosis by overexpression of wt p53 and inhibition of NF- κ B or STAT3

We examined the effects of individual or combined modulation of p53, NF- κ B or STAT3 pathways on the cell cycle and cell death by DNA with flow cytometry. Cells were mock transfected or with p53, STAT3 β DN or p65 siRNA vectors alone, or in combination, and DNA fluorescence analysis was performed after 72 hr. Figure 6 shows that under control conditions, the UM-SCC lines exhibited a relatively low percentage of apoptotic cells, as measured by sub G0/G1 DNA staining. Interestingly, a slightly higher percentage of cells undergo spontaneous apoptosis in the 2 cell lines expressing mt p53, p21 and BAX proteins (9–13%, UM-SCC-11B and 38). Forced expression of wt p53 protein alone slightly increased the proportion undergoing apoptosis in UM-SCC cells by ~10–14% (except in UM-SCC-1, only ~3%). p65 RNAi blocked all 4 cell lines in the G2/M phase, and increased cell death to ~20–48%, most significantly in UM-SCC-11B cells. Suppression of STAT3 activity by the DN construct also blocked all 4 lines in the G2/M phase of the cell cycle, and most strongly increased cell apoptosis in UM-SCC 11B and 38 cells with mt p53. Expression of wt p53 protein along with inhibition of either p65 or STAT3 more strongly enhanced cell apoptosis by DNA staining criteria (34–65%). The induced apoptosis reached ~60% in 3 UM-SCC cell lines except in UM-SCC-9 cells. Together, these data indicate that reexpression of p53 protein with inhibition of either NF- κ B or STAT3 induces massive cell death, with the greatest effect observed in UM-SCCs expressing mt p53, and higher levels of p21 and BAX proteins.

Discussion

In our study, we provide evidence that a network involving signal coactivation of NF- κ B and STAT3, differentially modified by p53 inactivation or mutation, modulates altered BAX/BCL-XL expression and cell survival in HNSCC. NF- κ B and STAT3 are

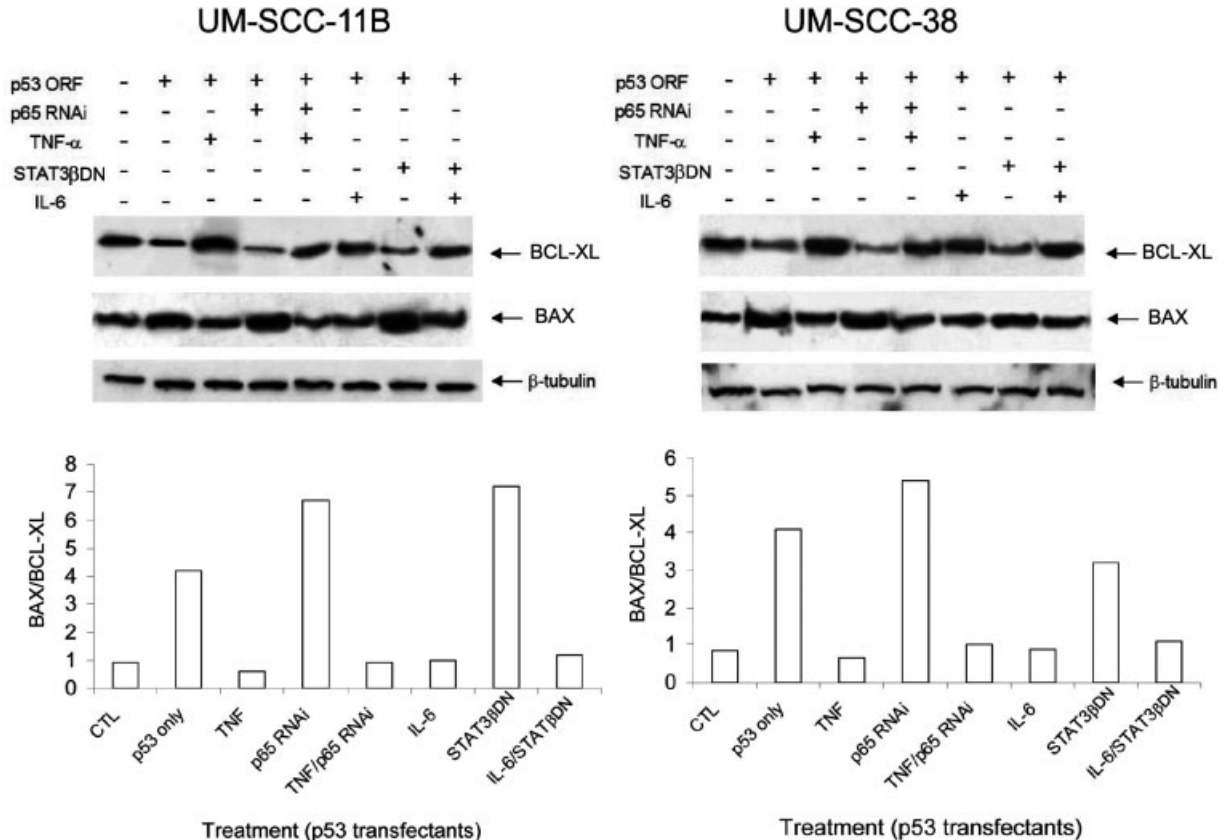


FIGURE 5 – Effect of overexpression of exogenous wt p53 and modulation of NF- κ B and STAT3 activity on coregulation of BCL-XL and BAX expression in UM-SCC cells with mt p53. UM-SCC-11B and 38 cells (mt p53) were transiently cotransfected with a wt p53 expression vector, in combination with either a p65 RNAi plasmid, or a STAT3 β dominant negative plasmid for 48 hr. The transfected cells were treated with either 10 ng/ml TNF- α or IL-6 for 24 hr. Whole cell lysates were collected and analyzed for the protein expression of BCL-XL and BAX by Western blot. The band intensity was obtained by densitometry, and the data were calculated and presented as a ratio in a bar graph from 1 representative experiment.

constitutively coactivated and further inducible by cytokines in UM-SCC cell lines, and modulation of NF- κ B and STAT3 up-regulated BCL-XL relative to BAX, with stronger effects observed in UM-SCC cells with deficient expression of wt p53. Conversely, wt p53 downmodulated p65 and STAT3 binding activity, and expression of BCL-XL relative to BAX. These and the DNA flow cytometric data suggest that modulation of expression of these important prosurvival and proapoptotic mediators in UM-SCC cells is controlled by a network of signal pathways, and the blockage of any 1 component alone is insufficient to achieve the level of cell apoptosis attainable when they are targeted together.

Individually, the functions of BCL-XL and BAX have been studied extensively in HNSCC and other cancers, providing evidence of their opposing function in cell survival and apoptosis. BCL-XL has been shown as an important component in cell survival and therapeutic resistance in HNSCC.^{34,35–39} In our study, we confirmed the over-expression of BCL-XL protein in all UM-SCC cell lines tested (Fig. 1a) and in 75% HNSCC tissues (Fig. 1b), which is consistent with other independent investigations.^{37–39} Knocking down BCL-XL expression by siRNA significantly suppressed cell proliferation (Fig. 1c). Consistent with this, Carey and coworkers recently published a study showing that a small molecule BH3 mimetic, (–)-gossypol, which binds to the BH3 domain of BCL-2 and BCL-XL, significantly induced cell apoptosis in 70% of cisplatin-resistant cells in UM-SCC lines with wt p53 and BCL-XL over-expression.³⁹ Knocking down BCL-XL expression by siRNA in our study also blocked the cell proliferation

to a similar degree, especially in the cell lines with wt p53 status (UM-SCC-1 and -9, Fig. 1c).

It has been established that the ratio of the antiapoptotic or proapoptotic BCL family members as well as absolute level of expression are critical to determine the fate of cell survival.^{44,45} BCL-XL and BAX retain structural similarity, but exhibit opposing and competitive effects. Specifically, BAX accelerates the opening of mitochondria channels, dysregulates the mitochondrial membrane potential, and promotes the release of cytochrome c during apoptosis, whereas BCL-XL closes the channels by binding to it directly.⁴⁶ BAX has been shown to be a target gene for p53 transcriptional regulation, since multiple functional p53 binding sites have been identified in the promoter region.²⁸ Consistent with this, the UM-SCC cells with mt p53 that retain function showed higher basal BAX protein expression (Fig. 3), and exhibited higher spontaneous cell apoptosis (Figs. 6a and 6b). Thus, our data are consistent with a model that, although similar over-expression of BCL-XL protein is observed in the UM-SCC cell lines, differences in the expression level and function of p53 and BAX can affect the basal level of BCL-XL/BAX ratio and apoptosis.

Over-expression of BCL-XL protein in HNSCC or other cancers have been shown to be modulated individually through NF- κ B or STAT3.^{29–34,42} However, regulation of BCL-XL expression by the coactivation of these 2 factors has not been investigated in HNSCC cells in the same study. Here we showed that cytokine induced activation and inhibition of NF- κ B and STAT3, that both

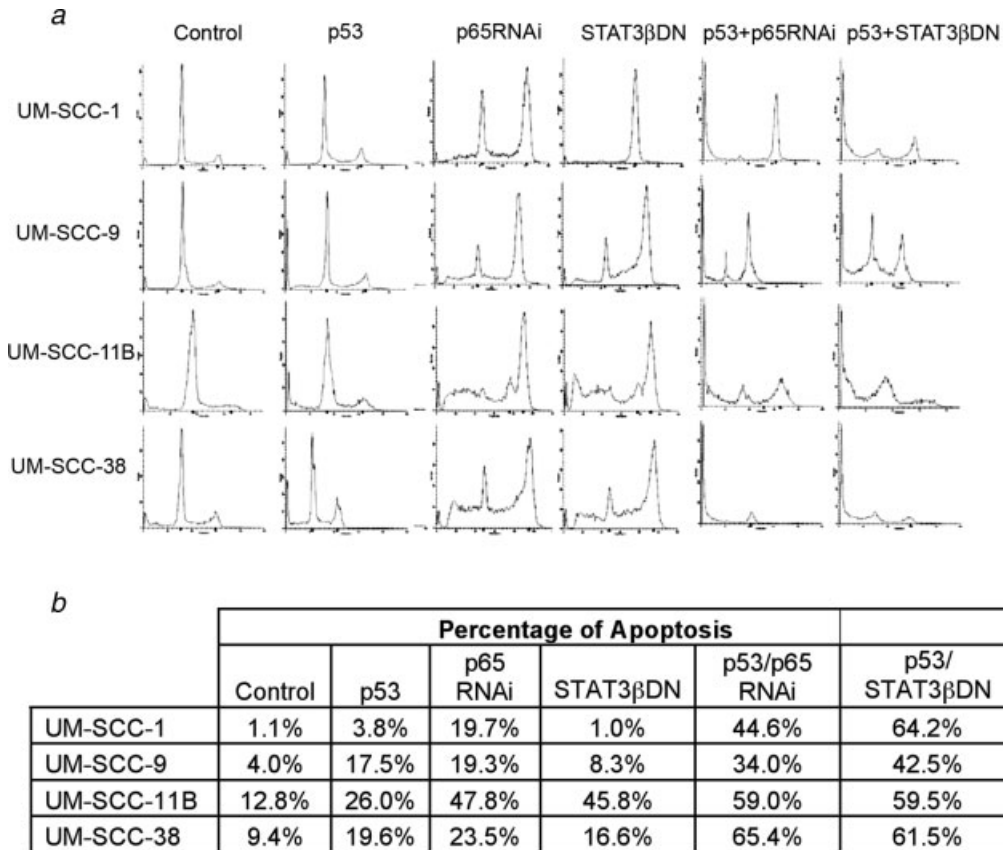


FIGURE 6 – Induction of cell cycle alterations and apoptosis by overexpression of wt p53 and inhibition of NF- κ B or STAT3. UM-SCC cells were transiently transfected with a wt p53 expression vector, a p65 RNAi or a STAT3 β dominant negative plasmid alone or in combination for 72 hr. Cells were harvested and stained with PI, and cell cycle and sub-G0 apoptotic profiles were analyzed by flow cytometry (a). Percentage of sub-G0 apoptotic cell population under different transfection conditions was calculated (b). The data was from 1 representative of repeated experiments.

modulated BCL-XL (Fig. 2, 3, 5). Previously, activation of either NF- κ B or STAT3 has been shown to affect the expression of BCL-XL, which has been identified as a critical component for death pathway and resistance to chemotherapy.^{29–34,36–39} NF- κ B and STAT binding sites have been identified to coexist in the proximal BCL-XL promoter region, with 9 putative NF- κ B and 2 STAT3 binding motifs predicted with high probability by computational analysis (–3,000 to +1,000 bp from the transcription starting site, Bin Yan, unpublished data). Further, NF- κ B or STAT3 have been previously shown to bind and transactivate BCL-XL gene.^{29,32,33} We confirmed that siRNA knockdown of NF- κ B downmodulated BCL-XL protein (Fig. 3b) as well as a reporter gene driven by BCL-XL promoter (TL Lee, data not shown). We also showed previously that knocking down of NF- κ B p65 by siRNA significantly suppressed BCL-XL mRNA expression and induced apoptosis in UM-SCC cell lines,³⁰ and inhibition of proteasome dependent activation of NF- κ B by bortezomib significantly suppressed BCL-XL gene expression in HNSCC patient tissue specimens.¹² Independently, Grandis and coworkers have shown that a STAT3 decoy oligonucleotide significantly suppresses BCL-XL mRNA expression in HNSCC cell lines, and blocks their cellular proliferation.⁴⁷ These data indicating dual regulation of BCL-XL by these signal transcription factors support our observation that blocking NF- κ B or STAT3 alone only partially suppresses BCL-XL expression (Figs. 3 and 5) and incompletely induced cell death in HNSCC (Fig. 6). The contribution of both pathways may explain the incomplete cytotoxic effects observed with inhibition of either pathway alone, and suggest tar-

geting both may be important for optimal therapy, as in the models shown by Fig. 7a and b.

In addition to NF- κ B and STAT3 mediated cell survival and their effects on BCL-XL expression, p53 status and forced expression also affected NF- κ B and STAT3 modulated BAX/BCL-XL expression (Fig. 5) with cell survival (Fig. 6). A reciprocal induction of increased BAX protein expression was observed with NF- κ B or STAT3 knock down, which was greatest in UM-SCC cell lines with deficient expression of wt p53 protein. This observation suggests a reciprocal interaction of NF- κ B or STAT3 with mutant as well as wt p53 family members. Consistent with this hypothesis, reexpression of wt p53 protein in cells with either p53 alteration partially suppressed NF- κ B and STAT3 activation, inhibited expression of their target antiapoptotic gene, BCL-XL, and induced p21 and BAX expression (Figs. 4 and 5). The expression of wt p53 also helped further restore the BAX/BCL-XL expression regulated by NF- κ B and STAT3 in UM-SCC cells with mt p53 status (Fig. 5), as p53 induced significantly greater cell apoptosis in combination with specific genetic inhibition of NF- κ B or STAT3 than when using individual reagents alone (Fig. 6). Thus, reexpression of p53 together with inhibition of signal activation of NF- κ B and STAT3 may be a more effective way to restore BAX/BCL-XL regulation and optimally induce apoptosis of HNSCC, as illustrated in our working model in Figure 7b.

Cumulative evidence indicates that the p53 pathway may regulate cell fate, not only by directly governing expression of genes that regulate the cell cycle and apoptosis, but also by negatively modulating the activation of prosurvival signal kinases, such as

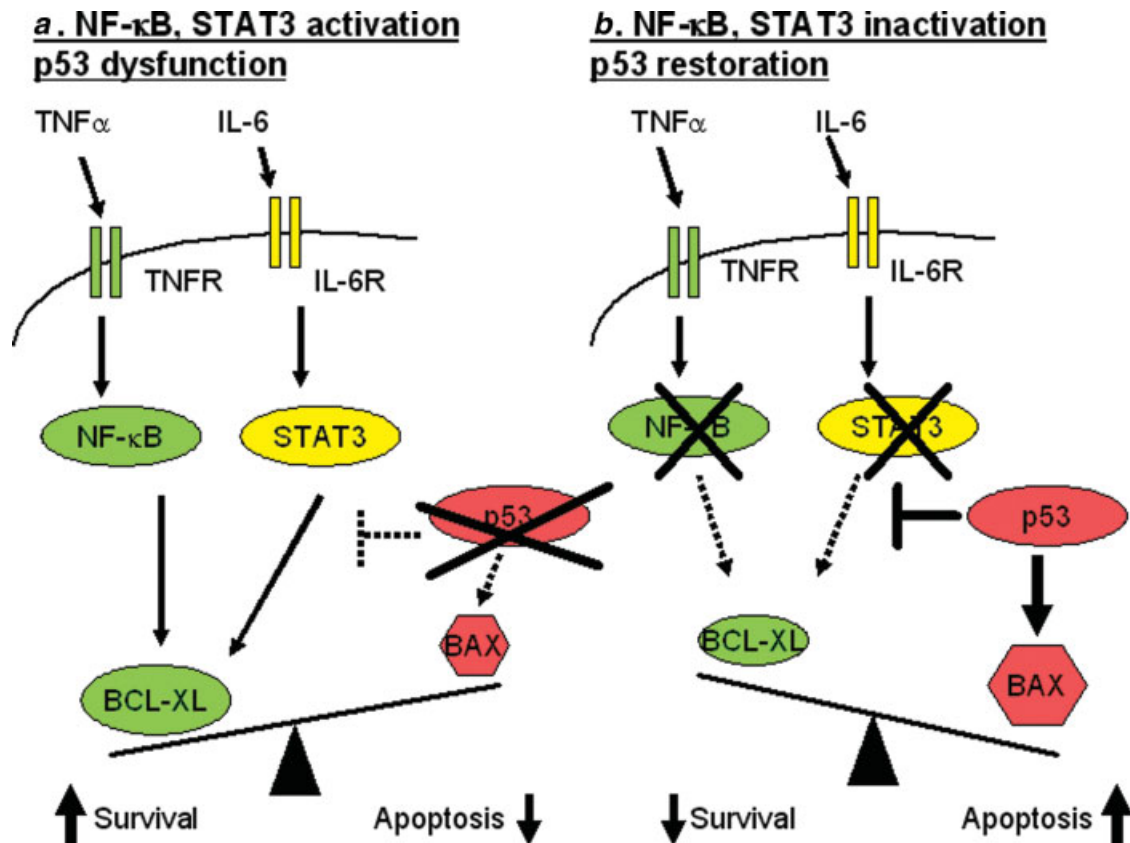


FIGURE 7 – A signal network involving NF- κ B, STAT3 and p53 modulates BAX/BCL-XL expression and cell survival of HNSCC. (a) in HNSCC, coactivation of NF- κ B and STAT3 plus dysfunctional p53 increased BCL-XL expression relative to BAX and enhanced cell survival. (b) in HNSCC, inhibition of NF- κ B and STAT3 with restoration of p53 decreased BCL-XL expression to BAX, which favors cell death. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

JNK, p38 and Akt,⁴⁸ and transcription factors including NF- κ B, STAT3^{49–54} (and our study), and AP-1.⁴⁸ Potential mechanisms for interaction between p53 and NF- κ B have been explored in other experimental cancer models. Perkins and colleagues have reported that NF- κ B is activated in p53 null osteosarcoma and fibroblast cells, and expression of wt p53 can reciprocally inhibit the activation of NF- κ B p65.^{51,52,54} They provided evidence that expression of wt p53 can compete for cofactor CBP/p300, resulting in decreased NF- κ B activation and expression of Cyclin D1 and BCL-XL.^{51,52,54} Like the effects of exogenous wt p53 on both NF- κ B and STAT3 observed in our study, both wt and mt p53 protein could more broadly affect the coactivation of NF- κ B and STAT3, since CBP/p300 is a common transactivating cofactor for these prosurvival transcription factors.^{49–54} In addition, the status of expression of other p53 family members, such as p63 and p73, may also affect such interactions and cell survival.⁵⁵ Studies are underway to examine the role of these potential mechanisms.

Interestingly, we identified distinct subsets of UM-SCC in association with their p53 status in that expression of mt p53 appeared to be associated with varying degrees of inhibitory effects on modulation of NF- κ B, STAT3 and BCL-XL (Figs. 3c and 3e), as well as retained function of p21 and BAX transcription (Figs. 3c 3e, and 4a). This appears to result in greater sensitivity of such HNSCC to cell death at baseline and with expression of wt p53, p65 RNAi or STAT3 β , such as observed in UM-SCC-11B and 38 cells (Fig. 6). Clinically, an association of p53 mutation with chemosensitivity and improved prognosis has been observed in HNSCC, which is counterintuitive but consistent with retention of function by many missense mutants of p53, as observed in our

study.^{22,39} By contrast, we examined 2 wt p53 UM-SCC cell lines with high expression of BCL-XL proteins but absent detectable wt p53 protein (Fig. 5a). These cell lines represent a subset of HNSCC patient samples collected in clinical studies with high BCL-XL and low p53 protein expression, which have been shown to exhibit poor prognosis and response to chemotherapies.^{22,39} Our findings in these cell lines may help to explain why previous clinical and pathologic studies have not found a clear relationship between p53 mutation and expression status and development of HNSCC. As part of a larger panel of UM-SCC cell lines recently studied in our laboratory, we found that this subset of cells with wt p53 by genomic sequencing express very low levels of wt p53 protein, even under conditions of genotoxic stress.⁵⁶ The distribution of this expression pattern in the HNSCC lines is consistent with ~50% prevalence found in immunohistochemical studies of HNSCC tumor tissues.^{57,58,59} Thus, the sequence defined wt p53 status does not usually lead to expressing functional wt p53 protein, and is more often associated with functional p53 null phenotypes.

The therapeutic implications of our study derive from the demonstration that all of these factors interact to regulate BCL/BAX proteins, and combining overexpression of wt p53 with inhibition of either NF- κ B or STAT3 activity was more effective in induction of apoptosis than modulating these factors individually. Knocking down either NF- κ B p65 or STAT3 alone significantly blocked progression at the G2/M phase of the cell cycle 72 hr after transfection, and had intermediate effects on apoptosis. We speculate this G2/M arrest could be due to the effects of NF- κ B on cyclins and Cdk inhibitors⁶⁰ and microtubule depolymerization,⁶¹ as well as STAT3 activity required for centrosome dupli-

cation⁶² which is an essential step for mitosis. However, the greatest induction of apoptosis was observed when over-expression of wt p53 was combined with knocking down either NF- κ B or STAT3 and BCL-XL expression. This notion is consistent with the results from recent clinical trials utilizing single reagents to target these prosurvival signal transduction pathways, such as gefitinib (EGFR/NF- κ B and STAT3),⁶³ bortezomib (proteasome/NF- κ B),⁶⁴ or to restore p53, such as ONYX.^{65,66} In these clinical trials, relatively low clinical response rates were observed among patients with solid tumors when single agents were used, likely indicating that modulating prosurvival or proapoptotic pathways individually is not as effective in inducing tumor cell death. Since function of both wt and mt p53 protein in tumors is apparently deficient as a tumor suppressor, it may be of benefit if feasible to restore wt p53 when specifically targeting

prosurvival pathways such as NF- κ B and STAT3 in patients with HNSCC.

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