p16 Protein and Gigaxonin Are Associated with the Ubiquitination of NFκB in Cisplatin-induced Senescence of Cancer Cells*

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Mysore S. Veena1, Reason Wilken1, Jun-Ying Zheng1, Ankur Gholkar1, Natarajan Venkatesan1, Darshni Vira1, Sameer Ahmed1, Saroj K. Basak1, Clifton L. Dalgard1, Sandhiya Ravichandran1, Raj K. Batra2,3,4,5,6, Noriyuki Kasahara1,2, David Elashoff2,3, Michael C. Fishbein4, Julian P. Whitelegge5, Jorge Z. Torres5,9, Marilene B. Wang6,7,8, and Eri S. Srivatsan5,9,10

From the 1Department of Surgery, Veterans Affairs Greater Los Angeles Healthcare System/David Geffen School of Medicine at UCLA, Los Angeles, California 90073, 2Department of Chemistry and Biochemistry, UCLA, Los Angeles, California 90095, 3Department of Head and Neck Surgery, David Geffen School of Medicine at University of California Los Angeles, Los Angeles, California 90095, 4Departments of Anatomy, Physiology, and Genetics, Uniformed Services University, Bethesda, Maryland 20814, 5Division of Pulmonary and Critical Care Medicine, Department of Medicine, Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, California 90073, 6Department of Medicine and 7Pathology and Laboratory Medicine, David Geffen School of Medicine at University of California Los Angeles, Los Angeles, California, 8Department of Psychiatry and Biobehavioral Sciences, UCLA, Los Angeles, California 90095, and 9Jonsson Comprehensive Cancer Center, UCLA, Los Angeles, California 90095

Background: Molecular mechanism of p16-mediated cellular senescence in cisplatin-treated cells is not known.
Results: Cisplatin treatment leads to p16 nuclear transport and association with gigaxonin for the ubiquitination of NFκB.
Conclusion: A protein associated with neural disease is involved in cisplatin-mediated cellular senescence.
Significance: Nuclear expression of p16 and gigaxonin is a useful marker of cancer cell chemosensitivity.

The molecular mechanism of p16-mediated senescence in cisplatin-treated cancer cells is not fully understood. Here we show that cisplatin treatment of head and neck cancer cells results in nuclear transport of p16 leading to a molecular modification of NFκB. Chromatin immunoprecipitation assays show that this modification is associated with the inhibition of NFκB interacting with its DNA binding sequences, leading to decreased expression of NFκB-transcribed proteins. LCMS proteomic analysis of LAP-TAP-purified proteins from HeLa cells containing a tetracycline-inducible GFP-S peptide-NFκB expression system identified gigaxonin, an ubiquitin E3 ligase adaptor, as an NFκB-interacting protein. Immunoblotting and siRNA studies confirmed the NFκB-gigaxonin interaction and the dependence of this binding on p16-NFκB binding. Using gel shift assays, we have confirmed p16-NFκB and gigaxonin-NFκB interactions. Furthermore, we have observed increased NFκB ubiquitination with cisplatin treatment that is abolished in the absence of p16 and gigaxonin expression. Analysis of 103 primary tumors has shown that increased nuclear p16 expression correlates with enhanced survival of head and neck cancer patients (p < 0.0000542), indicating the importance of nuclear p16 expression in prognosis. Finally, p16 expression is associated with reduced cytokine expression and the presence of human papilloma virus in chemoradiation-sensitive basaloid tumors. However, the absence of p16 expression is associated with enhanced cytokine expression and the absence of human papilloma virus in aggressive tumors. These results clearly demonstrate that nuclear p16 and gigaxonin play an important role in chemosensitivity of head and neck cancers through ubiquitination of NFκB.

Head and neck squamous cell carcinoma (HNSCC)2 is the sixth most common form of cancer worldwide and represents ~5% of all cancers diagnosed annually in the United States (1, 2). Every year >42,000 cases of oral, laryngeal, and pharyngeal cancer are diagnosed and >12,000 individuals die of the disease (3). The diagnosis and treatment of head and neck cancer presents several unique challenges. By virtue of their inexpressive location, many cases of HNSCC are not discovered until the cancer is at a later stage, not uncommonly until after spread to lymph nodes in the neck. Early stage tumors may be treated primarily with surgery or radiotherapy, but more advanced cancers often require multimodality therapy with surgery, radiation, and chemotherapy, which can result in very high morbidity (4).

Platinum-based agents form the backbone of the standard chemotherapeutic regimens for head and neck cancer. Cisplatin (cis-diaminedichloroplatinum) is a widely used drug in the class of platinum-based chemotherapies, and the adverse

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1 To whom correspondence should be addressed: Dept. of Surgery, VAGLAHS/David Geffen School of Medicine at UCLA, Bldg. 304, Rm. E2-218, 11301 Wilshire Blvd., Los Angeles, CA 90073. Tel.: 310-268-3217; Fax: 310-268-3190; E-mail: esrivats@ucla.edu.

2 The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HPV, human papilloma virus 16; TEV, tobacco etch virus; CSC, cancer stem cell; GALV, gibbon ape leukemia virus; CDDP, cisplatin; EB retinoblas-toma; EGFR, EGF receptor; LAP-TAP, localization and affinity purification (LAP = EGF-TEV-S-peptide)-tandem affinity purification; ALDH1, aldehyde dehydrogenase 1; BMI-1, B cell specific Moloney murine leukemia virus integration site-1.
effects of cisplatin are significant and include renal toxicity, nerve damage, hearing loss, and bone marrow suppression (5). The efficacy of cisplatin in HNSCC is greatly increased when combined with other chemotherapeutic agents, such as taxanes (paclitaxel and docetaxel) and 5-fluorouracil (5, 6). The precise molecular mechanism of cisplatin is unknown, but there is evidence that cisplatin may work through a p16- and p53-dependent mechanism (7). p16 and p53 are both tumor suppressor genes that function in cell cycle regulation, and mutations of these genes are linked to cancer development. Data suggest that cisplatin inhibits HNSCC growth through p16-mediated cell cycle arrest, and decreased expression of p16 has been linked to cisplatin resistance (7).

NFκB is an inducible transcription factor that regulates the expression of genes involved in inflammation as well as the control of cell proliferation and survival (8–10). NFκB is a heterodimeric protein and is retained in the cytoplasm in an inactive form by IkB (inhibitor of NF-κB), which is composed of α and β subunits. Upon receipt of the appropriate chemical signals such as TNF-α that initiate NF-κB activation, several steps are required to free NF-κB from this inhibitory binding. IkB must be phosphorylated at its α subunit by IkK (inhibitor κ B kinase, composed of α, β, and γ subunits), which results in ubiquitination and degradation of the phosphorylated IkBα and the release of NF-κB from its stationary location in the cytoplasm. The unbound NF-κB is then transported to the nucleus where it could bind to DNA and activate transcription.

NFκB is involved in cellular responses to stressful stimuli such as cytokines, UV irradiation, free radicals (including cigarette smoke), hypoxia, and infectious agents (11, 12). Activation of NFκB is increased in many cancers and is associated with various steps in the development of malignancy such as expression of anti-apoptotic genes, angiogenesis, tumor promotion, and metastasis (9). A variety of cancers, including HNSCC, have demonstrated constitutive expression of NFκB (13–15). As a result, modulation of NFκB has emerged as a potential therapeutic target in anti-cancer research. Here we show that cisplatin treatment leads to nuclear transport of p16, resulting in the recruitment of gigaxonin for the ubiquitination of NFκB.

**Experimental Procedures**

**Primary Tumor samples**—Primary HNSCC tumors were obtained from the UCLA Medical Center and the cooperative human tissue network of the National Institutes of Health. Human tissues were obtained after the approval from the Institutional Review Board committees of the West Los Angeles VA Medical Center and UCLA. A total of 116 tumors were analyzed. Immunohistochemistry was performed on 103 tumors. Thirteen tumors and two normal tissues were used for Western blot analysis.

**HNSCC Cell Lines**—The HNSCC cell lines CCL23, CAL27, UM-SCC1, and UM-SCC14A representing laryngeal, tongue, and oral cavity carcinomas were used. Although CCL23 and CAL27 cell lines were obtained from the American Type Culture Collection, cell lines UM-SCC1 and UM-SCC14A were obtained from Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI). MTT growth viability assays were carried out using the established protocol.

**β-Galactosidase Senescence Assay**—CCL23 cells grown to 50–60% confluence in 6-well plates were treated with cisplatin (6 μg/ml) for 4 h and then in fresh medium for 72 h. Untreated and treated cells were stained for β-galactosidase using the standard protocol.

**Vector Construct**—Well characterized 390 amino acid-NFκB cdNA (Clontech) was amplified and cloned into pDONR221 using the Gateway BP Clonase II (Invitrogen) and subsequently into pGLAP1-N-term EGF-TEV-S tag vector using the Gateway LR Clonase II (Invitrogen) as previously described (16). Stable cell lines were generated by transfecting HeLa Flp-In T-REx cells with pGLAP1-NFκB vector, using FuGENE 6 transfection reagent (Promega Scientific). Positive clones were selected by treating cells with 400 μg/ml hygromycin B and checked for protein expression with 0.2 μg/ml doxycycline (16).

**Cisplatin Treatment**—Stable cell lines expressing NFκB were plated in serum-free media for 24 h and treated with complete media containing 3 or 6 μg of cisplatin or no cisplatin along with 0.2 μg/ml doxycycline and treated for 4 h. Post-treatment the cells were washed 3 times with PBS and further grown for 24 h in complete medium containing 0.2 μg/ml doxycycline.

**Immunoprecipitation**—Cytoplasmic and nuclear protein extracts were prepared with the cisplatin-treated and non-treated NFκB cell lines using the Subcellular Protein Fractionation kit (Thermo Scientific). Protein concentrations in the lysates (nuclear/cytoplasmic extracts) were determined by the standard protocol.

**PCR of the p16 Gene**—DNA (500 ng) isolated from SiHa (human papilloma virus 16 (HPV 16)-containing cervical cancer cell line), CCL23, CAL27, and UM-SCC1 cell lines were used for the PCR using a prior denaturation at 95 °C for 5 min. PCR conditions used included denaturation at 94 °C for 30 s, a step down annealing for 30 s, and extension at 72 °C for 45 s with a final extension at 72 °C for 7 min. A step-down annealing temperature of 60 °C to 57 °C for 5 cycles, each followed by 15 cycles at 56 °C for exon 1, and a step-down annealing temperature of 57 °C to 53 °C for 3 cycles, each followed by 19 cycles at 52 °C for exon 2, were used. Primers exon 1 forward 5′ GAA AGA GGA GGG GCT GG 3′, exon 1 reverse 5′ TGG CTC TTT GGA 3′, exon 2 forward 5′ GCG CTA CCT GAT TCC CAA TTC 3′, exon 2 forward 5′ TGG CTC TGA CCA TTC TGT TC 3′, and exon 2 reverse 5′ TTT GGA AGC TCT CAG GGT AC 3′ were used. PCR products of 340 and 382 bp for exons 1 and 2, respectively, were verified on 10% polyacrylamide gels. Sequencing was performed using Sanger sequencing protocol in the Nextgen sequencer (Invitrogen), and the sequences were compared with the NCBI blast database.

**Quantitative RT-PCR for CD44**—Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA), and concentration was determined by spectrophotometry. Reverse transcription of 1.0 μg of total RNA was completed using the iScript cDNA Synthesis kit (Bio-Rad). Quantitative PCR was performed on a CFX384 Real-Time PCR Detection System (Bio-
Rad) using SsoFast EvaGreen Supermix (Bio-Rad) with 300 nM primer concentration and an ~5-ng cDNA input. Raw Ct values were calculated as an average of four technical replicates. Relative gene expression values were calculated after normalizing to the values of the housekeeping gene LDHA (lactate dehydrogenase A) and relative to control samples. Primers used were CD44 forward 5’-CCC AGA TGG AGA AAG CTC TG-3’, CD44 reverse 5’-GTT GTG TGC TGC ACA GAT GG-3’, lactate dehydrogenase A forward 5’-CTG ACA CCT CTG AGC CAC CA-3’, and lactate dehydrogenase A reverse-5’ AAA CAT CCA CCT GGC TCA AGG GG-3’.

Western Blot Analysis— Twenty μg of protein and prestained protein markers were subjected to SDS-PAGE in 10% gels under reducing conditions, and proteins were electrophoresed to polyvinylidene difluoride membranes (Millipore, Inc.). In the case of LAP-TAP-purified samples, the immunoprecipitated and unbound samples were resolved in 4–20% Tris-glycine SDS gels followed by Western transfer and hybridization of proteins in blots to anti-NFκB, anti-p16, anti-cyclin D1, anti-CDK4 (Santa Cruz Biotechnology), anti-gigaxonin (Sigma), anti-histone H3 (Abcam Biotechnologies), anti-GAPDH (GeneTex), and anti-comm D1 (gift of Dr. Ezra Burstsein) antibodies. After hybridization with the secondary antibody, membranes were developed as described (17).

Purification and LCMS—The NFκB cell line was cultured and induced with 0.2 μg/ml doxycycline for 24 h, post with or without cisplatin treatment. The cells were lysed, and the lysate was subjected to LAP-TAP purification (16). Briefly, NFκB was immunoprecipitated by incubating the protein extracts with GFP antibody-coupled protein-A beads. After incubation, unbound supernatant was removed, and the beads with GFP-bound proteins were thoroughly washed. Proteins were then eluted by boiling the beads with reducing sample buffer. The solubilized proteins were treated with TEV enzyme and then precipitated with S-tag peptide. The beads containing S-tag pulldown proteins were again washed, and the bound proteins were solubilized by boiling with reducing sample buffer. This double immunoprecipitation method improves specificity of NFκB-bound proteins. The eluted samples were resolved on 4–20% Tris-glycine SDS gels and cut into slices that were then digested with trypsin for LCMS analysis. The proteomic MASCOT program was used to identify proteins with and without modification settings. For the control, p16 or gigaxonin siRNAs transfections, cells at 70% confluence were transfected without modification settings. For the control, p16 or gigaxonin siRNAs transfections, cells at 70% confluence were transfected without modification settings. For the control, p16 or gigaxonin siRNAs transfections, cells at 70% confluence were transfected without modification settings. For the control, p16 or gigaxonin siRNAs transfections, cells at 70% confluence were transfected without modification settings. For the control, p16 or gigaxonin siRNAs transfections, cells at 70% confluence were transfected without modification settings. For the control, p16 or gigaxonin siRNAs transfections, cells at 70% confluence were transfected without modification settings. For the control, p16 or gigaxonin siRNAs transfections, cells at 70% confluence were transfected without modification settings. For the control, p16 or gigaxonin siRNAs transfections, cells at 70% confluence were transfected without modification settings. For the control, p16 or gigaxonin siRNAs transfections, cells at 70% confluence were transfected without modification settings. For the control, p16 or gigaxonin siRNAs transfections, cells at 70% confluence were transfected without modification settings. For the control, p16 or gigaxonin siRNAs transfections, cells at 70% confluence were transfected without modification settings. For the control, p16 or gigaxonin siRNAs transfections, cells at 70% confluence were transfected without modification settings. For the control, p16 or gigaxonin siRNAs transfections, cells at 70% confluence were transfected without modification settings.

Gel Shift Assay— Gel shift assays were carried out as described (18). Oligonucleotides representing the consensus NFκB binding site were used in the assay. An NF-κB oligonucleotide sequence containing a mutation at the binding site and oligonucleotide sequences representing the activator protein 1 binding site were used as controls. For supershift assays, the lysates were incubated with anti-EGFR (U. S. Biologicals), anti-p16, anti-p21 (Santa Cruz Biotechnology), or anti-gigaxonin (Sigma) antibody for 15 min at room temperature before the addition of the labeled oligonucleotide.

Ubiquitination Assay— HeLa or CCL23 cells were grown in minimum Eagle’s medium to 75% confluence and treated with cisplatin (6 μg/ml) for 4 h. Cisplatin-free media was added, and the cells were incubated for 20 h. Cells were then treated with protease inhibitor (MG-132, 10 μM) and deubiquitinase inhibitor (N-ethylmaleimide, 10 μM) for 4 h, and the lysates were prepared in the ice bath by incubation with the lysis buffer (50 mM HEPES, 200 mM KCl, 1 mM EGTA, 1 mM MgCl2, 0.5 mM DTT, 0.5% Nonidet P-40 containing protease and phosphatase inhibitors) for 15 min. The lysate was spun at 15,000 rpm for 10 min, and the supernatants were used for protein concentration measurements and immunoblot analysis. Proteins were immunoprecipitated with anti-NFκB antibody (Santa Cruz Biotechnology) and hybridized to the control IgG or multiplex hybridization to NFκB and anti-ubiquitin antibodies (BML-PW8810-0100, Enzo Life Sciences). After secondary antibody (IR680 and IR800; Li COR, Corp.) hybridization, membranes were developed as described (17). For the siRNA studies, cisplatin treatment was initiated 24 h after siRNA transfections.

Chromatin Immunoprecipitation (ChIP) Assay— Chromatin immunoprecipitation assays were performed using the assay kit (17). Immunoprecipitations were carried out using NFκB (Calbiochem) and IgG (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. DNA isolated from the input as well as the immunoprecipitated samples were amplified using IL-8 and β-actin primers. PCR products were separated on 10% polyacrylamide gels and stained with ethidium bromide, and images were captured using the Kodak Gel documentation system.

Immunofluorescence— Cells grown to 50–60% confluence were subjected to cisplatin treatment, and immunofluorescence was performed as described (7).

Immunohistochemistry— Paraffin sections (5 μm) of tumor samples were dewaxed and hybridized to p16 antibody after standard immunohistochemical protocol (19). Two pathologists were involved in independent scoring of hybridization intensities. The percentage expression was calculated with respect to the total number of cells present in the slide. Intensity was determined 1+ to 4+ as minimum to maximum intensity respectively. At least two slides were evaluated for most of the samples.

Statistical Analysis— The p values for the MTT growth assays were calculated using Student’s t test at 95% confidence interval. Results are presented as the means ± S.D. For the quantitative RT-PCR, statistical analysis for differential expression was performed by one-way analysis of variance with multiple pairwise comparisons with Sidak correction. The log-rank test and Cox proportional hazards regression analysis was used to assess the relationship of nuclear p16 expression to overall survival.

RESULTS

Nuclear p16 Expression Correlates with Cisplatin Sensitivity in HNSCC Cell Lines— Cell growth assays showed CCL23 and CAL27 to be sensitive to cisplatin treatment, UM-SCC14A to be intermediate sensitive, and UM-SCC1 to be resistant (Fig. 1A). Immunofluorescence studies demonstrated base-line cytoplasmic and nuclear p16 expression in untreated CCL23
cells (Fig. 1B). After cisplatin treatment, p16 localization shifted to the nucleus. In contrast, immunofluorescence studies in UM-SCC1 cells showed p16 localization to the cytoplasm (Fig. 1B). No shift to nuclear p16 localization was observed after cisplatin treatment (data not shown). The presence of increased nuclear p16 expression in CCL23 cells after cisplatin treatment was confirmed by Western blot analysis of both nuclear and cytoplasmic cell extracts (Fig. 1, C and D). Increased p16 expression was accompanied by a molecular modification, the appearance of a higher molecular weight band, in NFkB of the nuclear extract (Fig. 1C). This shift was not seen in the cytoplasmic extract (Fig. 1D). The retinoblastoma protein (pRb) is the product of the Rb tumor suppressor gene and is also an important regulatory molecule controlling entry into the cell cycle. Phosphorylation of Rb is associated with release of the transcription factor E2F and increased transcription of genes involved in cell division (20, 21). In addition to increased nuclear expression of p53, decreased nuclear phospho-Rb expression was also seen in the cisplatin-treated CCL23 cell extracts (Fig. 1C). Immunofluorescence studies of CCL23 cells treated with cisplatin also demonstrated reduced phosphorylation of Rb (Fig. 1E).

**Increased p16 Expression Correlates with Decreased Cyclin D1 Expression**—Western blot analysis of the different HNSCC cell lines has demonstrated that CCL23 expresses p16, whereas p16 expression was reduced or absent in the other HNSCC cell lines (Fig. 2A). Although CAL27 cells were sensitive to cisplatin treatment, p16 expression was not seen. Therefore, we investigated the genomic status of the p16 gene. PCR analysis followed by sequencing of the two exons showed nonsense mutation at codon 69, resulting in the conversion of glutamic acid to a stop codon (E69*, GAG205UAG). We could not detect a shorter p16 protein in the Western blots possibly due to degradation. In CAL27 cells we have attributed cisplatin sensitivity to the nuclear expression of p53 (7). Analysis of cyclin D1 expression showed an inverse relationship to p16 expression, with CCL23 expressing low levels of Cyclin D1 (Fig. 2E). Increased expression of cyclin D1 upon cisplatin treatment (Fig. 2F). Cyclin D1 levels were not altered in the resistant cell lines CCL23 and CAL27 (Fig. 2C). Cyclin D1 levels were not altered in the resistant cell lines UM-SCC1 and UM-SCC12 (Fig. 2D).

**Increased Cancer Stem Cell (CSC) Marker, CD44, Expression Correlates to Cisplatin Resistance**—Cisplatin resistance has also been shown to be related to the formation of CSCs. Therefore, we investigated the expression of the CSC marker CD44 in CCL23 and UM-SCC1 cells. Expression of CD44 was 5-fold higher in UM-SCC1 cells in comparison with that of CCL23 cells (Fig. 2E). CCL23 cells selected in 8 μg/ml cisplatin showed a 2.5-fold increased expression, confirming an increase in CSCs after cisplatin treatment. Increase in CD44 expression was minimal in UM-SCC1 cells selected in 3 μg/ml cisplatin. Additionally, our earlier studies have also shown increased secretion of cytokines IL-6 and IL-8 in the resistant cell lines, indicating increased presence of CSCs in the resistant cell lines (Table 1). These studies, therefore, indicated an increased NFkB transcription activity in cisplatin-resistant cells.

**Mobility Shift of NFkB Complex after Cisplatin Treatment**—It has been established that cisplatin treatment leads to senescence through the activation of p16 and p21 proteins. However,
the molecular mechanism is not known. As expected, cisplatin treatment led to the expression of senescence marker β-galactosidase around the nucleus in CCL23 cells confirming cisplatin sensitivity (Fig. 3A). Because there is an indication in the literature for the association of exogenously expressed p16 and NF-κB (22), we wanted to determine whether an in vivo association also exists between p16 and NF-κB. A gel mobility shift assay was performed using the lysates collected from control (untreated) and cisplatin-treated CCL23 cells and the consensus NF-κB binding site oligonucleotide probe. The presence of a band with CCL23 lysate indicated the binding of the oligo probe to the NF-κB complex (Fig. 3B). Reduced intensity of this band with the inclusion of the cold oligonucleotide and not with cold mutant oligonucleotide or a nonspecific activator protein 1 binding site oligonucleotide confirmed the specificity of binding to NF-κB. Treatment of CCL23 cells with cisplatin for 8 h showed a shift of NF-κB-bound oligonucleotide to a higher molecular weight band. A near complete shifting to the higher

**TABLE 1**

Cisplatin sensitivity and the expression of cell cycle and cytokine genes in HNSCC cell lines

| Cell line | Cisplatin sensitivitya | Status (PCR data) | Western | IF | Cyclin D1 expression Westerna | Status (PCR data) | Western | IF | Rb Status | IL-6 expressiond | IL-8 expressiond |
|-----------|------------------------|-------------------|---------|---|-----------------------------|-------------------|---------|---|-----------|----------------|----------------|
| CCL23e    | Most sensitive         | Wild              | +        | + | +                          | Wild              | 3       | + | Wild      | 85             | 95             |
| CAL27f    | Sensitive              | Mutantg           | 0        | - | -                          | Mutanth           | 5       | + | Wild      | 300            | 300            |
| UM-SCC1   | Least sensitive        | Wild              | 0        | + | -                          | Wild/mutanti      | 2       | + | Wild      | 475            | 2000           |
| UM-SCC14A | Less sensitive         | ND                 | 0        | - | -                          | Mutanti           | 1       | + | Wild      | 600            | 450            |

* In relationship to the sensitivity of CCL23 cells.
* Western blot intensity measured as 1 being the lowest and 5 being the highest expression.
* Inferred from the presence of hypo and hyper phosphorylated forms of the Rb protein.
* Expression level in the cell supernatant (pg/ml) measured by the ELISA assay (59).
* HPV 18-containing cell line. All other cell lines are HPV negative.
* p53 expression in CAL 27 cells (7).
* Nonsense exon 2 mutation in codon 69 resulting in the conversion of glutamic acid to a stop codon (E69*, GAG205UAG); present investigation.
* Missense exon 6 mutation in codon 193 resulting in the conversion of histidine to a leucine (H193L, CAC578CUC) (7).
* Exon 3 and exon 8 skipping in UM-SCC1 and UM-SCC14A cell lines respectively (24).
molecular weight band was seen with an 18-h cisplatin treatment. This mobility shift was abolished with the inclusion of cold NFκB oligo confirming the specificity of this protein-DNA binding. The mobility shift of NFκB-bound oligonucleotide was not seen with the lysate from the untreated or cisplatin-treated UM-SCC1 cells (Fig. 3C). Thus, our results suggested an association between nuclear p16 expression and binding of proteins to NFκB as seen in mobility shift assays in cisplatin-treated cells.

Nuclear p16 Binding to NFκB—Western blot analysis of CCL23 cells showed increased nuclear expression of CDK4 and NFκB proteins when p16 activity was inhibited through the addition of p16 small-interfering RNA (p16 siRNA) (Fig. 4A). However, when CDK4 expression was abolished with the addition of CDK4 siRNA, the expression of NFκB was also reduced. Furthermore, immunoprecipitation studies with antibody against NFκB (p65 subunit) confirmed the binding of NFκB to both p16 and CDK4 proteins (Fig. 4B). Although NFκB binding to CDK4 was not affected in the presence of p16 siRNA, there was reduced binding to p16 in the presence of CDK4 siRNA. These results, therefore, suggested that p16 might interact with NFκB through a CDK4-dependent mechanism and that p16 plays a role in the negative regulation of NFκB.

Cisplatin Treatment Decreases NFκB Nuclear Expression—Immunofluorescence studies of NFκB expression in untreated CCL23 cells demonstrated cytoplasmic expression of the transcription factor at base line (top left panel in Fig. 4C), which underwent a shift to increased nuclear expression after treatment with TNF-α for 1 h. Treatment of CCL23 cells with cisplatin for 6 h resulted in decreased NFκB expression. Additionally, treatment with TNF-α for the final hour of the 6-h cisplatin treatment period still resulted in decreased nuclear translocation of NFκB. The decreased NFκB nuclear translocation after cisplatin treatment can, therefore, be correlated with the increased nuclear expression of p16 at the same time points (see Fig. 1B).

Nuclear p16 Expression Is Associated with Molecular Modification of NFκB—We have seen increased p16 expression in cisplatin-treated cells correlating with the appearance of a higher molecular weight NFκB band (Fig. 1D). To determine whether this association is present in untreated cycling HNSCC cells, nuclear expression of p16 and NFκB was evaluated at various time points of the cell cycle. We could observe an association between increased p16 expression and higher molecular weight NFκB band and reduced p16 expression and lower molecular weight NFκB (Fig. 5A). To confirm this association, we measured p16 and NFκB nuclear expression at different times after cisplatin treatment. We noticed increased p16 expression at 4 and 8 h that was associated with the presence of the higher molecular weight NFκB (Fig. 5B). At later times, reduced p16 expression was associated with the lower molecular weight NFκB bands. There was no appreciable difference in p16 expression in the cytoplasmic fraction of cisplatin-treated samples (Fig. 5C). Also, a single cytoplasmic 65-kDa NFκB protein was observed. These results, therefore, point to a strong association between nuclear p16 expression and molecular modification of NFκB in the nucleus of CCL23 cells. Reduced nuclear p16 expression at 12 h and later periods could be attributed to increased cell death seen 24 h after cisplatin treatment (data not shown).

To determine whether NFκB molecular modification has an effect on its transcription activity, we performed ChIP assays using the promoter sequences of IL-8, a cytokine transcribed by NFκB. Although the control input DNAs showed the 182-bp PCR product for all the cisplatin-treated samples, reduced PCR product was seen for the 4-h time period (high nuclear p16 expression; see Fig. 5B) in the NFκB-immunoprecipitated samples (Fig. 5D). These results, therefore, demonstrated removal of NFκB from its binding sites by p16 and reloading of NFκB to the DNA sites with reduction in p16 expression. The presence of 272-bp β-actin PCR product in the input and not in NFκB-immunoprecipitated samples confirmed the specificity of the ChIP assay. Additional proof for specificity was provided by the absence of IL-8 PCR product in the control IgG-immunoprecipitated samples.
p16 Associates with Gigaxonin for the Ubiquitination of NFκB

To determine the proteins that are associated with the NFκB modification, we generated a doxycycline-inducible localization and affinity purification (LAP-EGFP-TEV-S-peptide)-tagged-NFκB HeLa stable cell line that expresses NFκB from a single specific loci within the genome (16). The LAP-NFκB cell line was induced with doxycycline and treated with and without cisplatin. Nuclear protein extracts were prepared, and LAP-NFκB was tandem affinity-purified (16). Western blotting and immunoprecipitation studies confirmed the...
induction of the 50-kDa protein representing the inserted 390-amino acid NFκB and its interaction with CDK4 and p16 proteins (data not shown). We then performed the LAP-TAP assay to purify the NFκB-bound proteins. Here, the LAP-TAP-purified total cell lysate immunoprecipitates of the doxycycline-induced control and cisplatin-treated cells were separated on 4–20% PAGE gels and silver-stained to identify NFκB-bound proteins. We observed the presence of 16-, 34-, and 50-kDa proteins, indicating the presence of p16, CDK4, and NFκB in the complex (Fig. 6A). Bands of 65 and 75 kDa were seen in control and cisplatin-treated samples, and an additional 80-kDa band was seen in cisplatin-treated samples. A smear above 50 kDa was noticed in both the control and cisplatin samples indicating the presence of ubiquitinated products.

To identify the interacting proteins, LAP-TAP-purified eluates were run to a length of 2 cm in a 4–20% SDS-PAGE gel and four 5-mm gel slices were excised, trypsinized, and analyzed by LC-MS/MS. The LAP-TAP investigation identified matches to p65 form of NFκB, ankyrin repeats reflecting the sequences present in CDK4 and p16, and chaperone Bip protein reflecting cisplatin effect (Table 2). Among the interacting proteins, we also identified gigaxonin, an ubiquitin E3 ligase adaptor involved in the ubiquitination of neuronal intermediate filaments (23). Western blot analysis confirmed the expression of gigaxonin along with NFκB, CDK4, and p16 proteins in the cytoplasmic and nuclear fractions of protein lysates of doxycycline-induced control and cisplatin-treated HeLa Flp-In T-REX EGFP-S-NFκB cells (Fig. 6B). Although the cytoplasmic gigaxonin showed interactions with NFκB in the S-peptide pulldown assays of the control and cisplatin-treated samples, nuclear interaction was seen only in cisplatin-treated samples. Although CDK4-NFκB interaction was seen in the untreated nuclear extracts, binding of p16 to the complex again occurred only in cisplatin-treated samples. Specificity of these interactions was confirmed by the absence of NFκB binding to cytoplasmic GAPDH or nuclear histone H3 (Fig. 6B). Additional confirmation was provided by the absence of protein bands in the immunoblots of wild type HeLa Flp-In T-Rex cells (Fig. 6C).

To determine whether gigaxonin was recruited by p16 for NFκB ubiquitination, we performed siRNA studies. (LAP = EGFP-TEV-S-peptide)-tagged NFκB HeLa stable cell line was treated for 12 h with control, p16, or gigaxonin siRNA. Cells
were then treated with a medium containing cisplatin (6 μg/ml) and doxycycline (0.2 μg/ml) for 4 h and then with doxycycline (0.2 μg/ml) alone medium for 32 h for a total of 48 h post-siRNA treatment. Western blot analysis of the protein lysates showed reduced expression of p16 and gigaxonin in the respective siRNA-treated samples confirming down-regulation of these two proteins with the siRNAs (Fig. 6D). There was also a reduced expression of comm D1 (component of the comm D1-cullin E3 ubiquitin system; Ref. 11) and p16 in gigaxonin siRNA-treated samples. The S-tag pulldown assays showed a reduction in the binding of gigaxonin and comm D1 in p16 siRNA-treated samples. Interaction of NFκB with all the examined proteins was reduced in gigaxonin siRNA samples possibly due to dysregulated cytoskeletal structure in the absence of gigaxonin. Again, there was no binding of any of the proteins to S-peptide pulldown assays in the control cells in the absence of gigaxonin. We, therefore, hypothesize that the absence of a clear reduction in p16 and NFκB interaction with the siRNA could be attributable to residual p16 in treated cells. This is also reflected in partial and not complete loss of gigaxonin and comm D1 binding to NFκB.

To further confirm the interaction of p16 and gigaxonin to NFκB, we performed gel shift assays. As shown earlier in Fig. 3B, whole cell lysates of CCL23 cells were incubated with the 32P-labeled NFκB oligonucleotides, and incubation of NFκB oligonucleotides without the lysates were used as controls. Precipitation of the protein lysate with anti-p16 or anti-gigaxonin antibody resulted in a supershift of the NFκB bound oligonucleotides (Fig. 7A). The supershift was, however, not observed with the anti-EGFR antibody used as an antibody isotype control. Supershift was also not observed with anti-p21 antibody, indicating the specificity of interaction between NFκB and p16 and NFκB and gigaxonin.

Because gigaxonin is an E3 ubiquitin ligase, we investigated the ubiquitination of NFκB in HeLa Flp-In T-Rex (cell line used in the LAP-TAP proteomic analysis) and CCL23 cells. Protein lysates were immunoprecipitated with NFκB and hybridized to anti-NFκB and anti-ubiquitin antibodies through a multiplex hybridization. NFκB ubiquitination was observed in green and that of ubiquitin in red. The analysis showed increased hybridization of the higher molecular weight bands of NFκB to the ubiquitin antibody in cisplatin treated cells in comparison to that of untreated control cells (Fig. 7B). Increased green and red signals pointing to enhanced NFκB ubiquitination was clearly observed in the multiplex hybridization (Fig. 7C). Hybridization to the ubiquitin antibody was lost in the absence of p16 and gigaxonin expression confirming the role of p16 and gigaxonin in the ubiquitination of NFκB. We, therefore, hypothesize that cisplatin treatment leads to nuclear translocation of p16, resulting in the recruitment of gigaxonin-comm D1 ubiquitin complex for the ubiquitination of NFκB (Fig. 7D). This would then explain reduced NFκB-DNA interaction leading to decreased transcription of cytokines and growth factors. Our results could also imply that there is a direct interaction between gigaxonin and NFκB in the nucleus that gets amplified after cisplatin treatment.

**Introduction of Ectopic p16 Expression Is Not Sufficient to Increase Sensitivity of Cisplatin-resistant Cell Lines—**To determine whether nuclear p16 expression was sufficient to induce cisplatin sensitivity, we performed transfection of p16 into two HNSCC cell lines: UM-SCC-14A (aggressive and cisplatin-resistant with the absence of p16 expression) and CCL23 (cisplatin-sensitive containing nuclear and cytoplasmic p16 expression) using the GALV (gibbon ape leukemia virus) retroviral vector system. Western blot analysis confirmed the expression of nuclear p16 in UM-SCC-14A-GALVp16 and enhanced

| Gene ID     | Score | Mass   | Tryptic peptide matches (non-duplicates/duplicates) | Sequences searched | Gene     | Modification observed |
|-------------|-------|--------|-----------------------------------------------------|--------------------|----------|-----------------------|
| gi/189504   | 405   | 60,705 | 39 Peptides (11/28)                                  | 11                 | NFκB     | None                  |
| gi/15559457 | 402   | 28,216 | 38 Peptides (17/21)                                  | 15                 | ReA      | None                  |
| gi/16753192 | 92    | 11,391 | 4 Peptides (2/2)                                     | 2                  | Dermcidin | None                  |
| gi/19438424 | 49    | 59,042 | 5 Peptides (5/0)                                     | 5                  | Unnamed   | None                  |
| gi/6470150  | 50    | 71,002 | 5 Peptides (5/0)                                     | 5                  | BiP protein, partial | None                  |
| gi/553734   | 47    | 2,269  | 118 Peptides (1/117)                                 | 1                  | Putative protein | None                  |
| gi/54781221 | 46    | 13,399 | 2 Peptides (1/1)                                     | 1                  | IGG k-variable region VK1 | None                  |
| gi/11343200 | 40    | 118,740| 4 Peptides (2/2)                                    | 2                  | POTE ankyrin domain family member J | None                  |
| gi/46057811 | 37    | 11,752 | 29 Peptides (1/26)                                   | 1                  | Alternative protein C3P2RB | None                  |
| gi/3046606  | 36    | 1,955  | 1 Peptide (1/0)                                     | 1                  | Type II interleukin 1 receptor antagonist | Acetyl (K)             |
| gi/13413226 | 35    | 122,882| 3 Peptides (2/1)                                    | 2                  | POTE ankyrin domain family member E | Met (O)                |
| gi/2853301  | 31    | 100,236| 1 Peptide (1/0)                                     | 1                  | Mucin, partial | None                  |
| gi/38051823 | 28    | 93,263 | 1 Peptide (1/0)                                     | 1                  | Plasminogen | None                  |
| gi/72534660 | 27    | 27,578 | 22 Peptides (2/21)                                  | 1                  | Serine/arginine-rich splicing factor 7 isofrom 1 | None                  |
| gi/107719   | 26    | 18,742 | 7 Peptides (1/4)                                    | 1                  | Sphingomyelin phosphodiesterase | None                  |
| gi/54114933 | 25    | 70,002 | 4 Peptides (1/3)                                    | 1                  | Dis3 mitotic control homologue (s. cerevisiae)-like 2 | None                  |
| gi/11546731 | 25    | 68,678 | 28 Peptides (4/24)                                  | 3                  | Gigaxonin (597 aa) | Met (O), Ac-Lys, Tyr(P) |
| gi/13600656 | 25    | 470,227| 4 Peptides (4/0)                                   | 4                  | DNA-dependent protein kinase catalytic subunit | None                  |
| gi/11958734 | 25    | 101,103| 2 Peptides (1/1)                                   | 2                  | Piwi-like 4 (Drosophila), isofrom CRA_b | Ac-Lys                |
| gi/5734135  | 22    | 94,777 | 1 Peptide (1/0)                                    | 1                  | ATP cassette binding transporter 1 | None                  |
| gi/5262601  | 20    | 113,916| 1 Peptide (1/0)                                   | 1                  | Hypothetical protein | Ac-Lys                |
| gi/4505725  | 20    | 143,804| 4 Peptides (3/1)                                   | 3                  | Peroxisome biogenesis factor 1 | Met (O)               |
| gi/7706681  | 19    | 139,359| 2 Peptides (2/0)                                   | 2                  | DNA repair protein REV1 isofrom 1 | None                  |
| gi/41017504 | 19    | 260,688| 3 Peptides (2/1)                                   | 2                  | Horserin | None                  |
| gi/11956910 | 17    | 11,437 | 23 Peptides (2/21)                                  | 1                  | hCG1820599 | Tyr(P)               |

**TABLE 2**

Proteomic MASCOT search matches for NFκB-interacting proteins

aa, amino acids. Met (O), oxidation of the methionine residue.
expression in CCL23-GALVp16 cells (Fig. 8A). Immunofluorescence studies demonstrated nuclear localization of the p16 protein in the transfected cell lines (Fig. 8B). Growth assays demonstrated a decreased rate of proliferation in CCL23-GALVp16 cells correlating with increased p16 expression. Cell growth, however, was not affected in UM-SCC14A-GALVp16 cells in comparison to the parental cells (Fig. 8C). These results suggested that although nuclear p16 expression is an important factor, the effect may also require other p16-interacting proteins such as gigaxonin for the induction of cisplatin sensitivity.

Nuclear p53 Expression Is Also Associated with Cisplatin Sensitivity in HNSCC Cell Lines—We have previously shown that although the CAL27 cells lacked p16 expression and contained a mutant p53, the cells were sensitive to cisplatin (7). We showed that this was due to the nuclear localization of the p53 protein in this cell line. It is likely then that nuclear p53 is required for the cisplatin-mediated cell growth inhibition. Western blot studies indicated wild type and mutated forms of p53 expression in UM-SCC14A cells and close to background level expression in UM-SCC1 cells and close to background level expression in UM-SCC14A cells (Fig. 9B). Thus, we hypothesize that nuclear expression of p16 and/or p53 are responsible for the cisplatin-induced cell growth inhibition of head and neck cancer cells. Because we previously showed that cisplatin-induced growth arrest could involve apoptosis (7), p53-mediated growth inhibition of CAL27 might be related to apoptotic cell death.

Nuclear p16 Expression Correlates with a Favorable Clinical Prognosis in HNSCC Patients—We and others have shown inactivation of p16 by homozygous deletion and DNA methylation in 70% of HNSCC tumors (25). Little is known about the tumors with p16 expression, specifically in relationship to cytoplasmic and nuclear expression. Analysis of 103 head and neck tumors by immunohistochemistry showed p16 protein expression in 28 samples. Ten samples had cytoplasmic expression with 1% nuclear expression. The remaining 18 samples contained nuclear expression, with 11 having 20% and 7 having expression between 4 and 20%. Expression intensity mostly correlated with percent expression, i.e., higher nuclear expression had higher intensity (3+ to 4+), and lower expression had <2+ intensity. The relationship to survival was, therefore, calculated with respect to the percentage of expression. The analyses

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showed significantly longer overall survival for patients with higher levels of nuclear p16 expression ($p < 0.0000542$), an effect likely related to increased cisplatin sensitivity (Fig. 10A). Western blot analysis of an independent set of primary tumors showed an inverse relationship between the expression of p16 and that of CDK4 and NFκB (Fig. 10B). Although tumors with p16 expression showed reduced expression of CDK4 and NFκB (as seen in tumor samples 18 and 40), loss of p16 was accompanied by overexpression of CDK4 and NFκB (demonstrated in tumor samples 26, 54, 91, 222, 277, and 295).

**Association of HPV with Nuclear p16 Expression in HNSCC Tumors**—In recent years, HPVs have also been implicated in head and neck cancers (26, 27). Tumors with HPV positivity, known as basaloid (laryngeal and pharyngeal) tumors, are shown to have a better prognosis, i.e. increased sensitivity to chemoradiation therapies (27). To determine the relationship between p16 expression and the presence of HPVs, five each of basaloid, primary, and recurrent head and neck tumors were analyzed for the expression of p16, IL-8, macrophage marker CD68, and cancer stem cell markers (related to tumor aggres-
siveness) BMI-1, CD44, and ALDH-1. Four of the basaloid tumors contained enhanced p16 nuclear expression accompanied by lower expression of CD68, IL-8, and cancer stem cell markers (Fig. 11A and Table 3). Primary and recurrent tumors were devoid of p16 expression but showed higher expression of all the examined markers (Fig. 11B). Recurrent tumors also showed expression of CD68 and IL-8 in the tumor clusters indicating a tumor immune response (Fig. 12). These results suggested a direct correlation between HPV and p16 expression and an inverse relationship between HPV and the expression of IL-8 and CD68, indicating an inverse relationship between HPV and tumor-associated immune response.

**DISCUSSION**

Platinum-based agents are the standard of care in chemotherapeutic regimens for HNSCC. However, cisplatin and its related drugs alone are not effective for the treatment of head and neck cancers. The efficacy of cisplatin in HNSCC is significantly increased in combination with other chemotherapeutic agents and/or radiation therapy (28–30). A key event mediating the cellular toxicity of cisplatin is the formation of DNA cross-links. After adduct formation via replacement of the chloride ligands of the drug with DNA bases, further replication and transcription is halted, and cells are stimulated to undergo apoptosis (31). In addition to triggering apoptosis, several studies have shown that cisplatin induces cellular senescence, as evidenced by expression of the senescence-associated marker β-galactosidase (32, 33). However, the mechanism of cisplatin-induced cellular senescence is not known. Investigations into the mode of action of cisplatin have, therefore, focused on key regulators of the cell cycle and apoptosis such as p16 and p53 proteins (34–36).
Studies correlating the level of p16 expression and response to platinum-based chemotherapy in various cancers have pointed to an association between p16 deletion and cisplatin resistance (37). A retrospective investigation of ovarian cancer patients revealed a significantly higher frequency of p16 deletion in tumors that did not respond to cisplatin chemotherapy (37). It was demonstrated in non-small cell lung cancer that p16 is involved in mediating G1 cell cycle arrest and apoptosis in response to treatment with irradiation, DNA topoisomerase inhibitors, and cisplatin (38, 39). We have previously shown that cisplatin treatment in HNSCC cells was associated with increased expression of p16 and p53, suggesting that cisplatin-induced cell cycle arrest operates through the p16/p53-dependent pathways (7).

Multiple studies have shown that functional p53 is necessary for chemotherapy-induced apoptosis; several investigations in HNSCC have found that cell lines with p53 mutations demonstrate increased sensitivity to cisplatin (40–42). These findings may not actually be in conflict, as p53 mutations that do not affect nuclear localization of the protein were noted to increase sensitivity to cisplatin, whereas mutations associated with loss of nuclear p53 predicted a poor response to chemotherapy (40–42). Overall, the data in multiple cancers including HNSCC support the role of functional p53 expression in predicting a positive response to platinum-based chemotherapy.

The regulation of cyclin D1 expression is controlled through NFκB, an inducible transcription factor that also regulates a host of gene products involved in inflammation and cellular proliferation including cyclooxygenase-2, IκBα, TNF-α, cyclin E, ICAM-1, c-myc, Bcl-2, MMP-9, inducible nitric-oxide synthase (iNOS), and interleukins including IL-6 and IL-8 (8–10). NFκB is a nuclear factor that has been widely studied for its role in cancer development and growth. Investigations have shown an increased inflammatory response in association with the growth and metastasis of both hematologic and solid malignancies (43–45). It has also been shown that the up-regulated expression of tumor-promoting cytokines IL-6, IL-8, and TNF-α in various cancers is the result of increased activation of NFκB (46). In addition, it has also been shown that NFκB expression is associated with the development of cancer stem cells related to chemoradiation resistance in several malignancies including head and neck, colon, and prostate cancers (47). Therefore, understanding the control of NFκB activation pathways represents an important target in the area of cancer prevention and therapy.

Studies in animal models of human cancer have supported the role of the inflammatory cascade in cancer progression. Investigations in melanoma, lung, and prostate cancers have shown that modulation of NFκB activity via inhibition of IκBα/IκBβ has the potential to suppress tumor growth and metastasis (48, 49). In head and neck cancer, we and others have demonstrated that inhibition of IκBβ leads to suppression of HNSCC growth (50). However, the precise molecular mechanism of this NFκB-mediated growth-suppressive effect of cisplatin in human cancers is not yet understood.

In the present investigation we provide a mechanism for cellular apoptosis and senescence mediated by cisplatin treatment of head and neck cancers. This involves targeting of NFκB for ubiquitination by p16 through the recruitment of gigaxonin (Fig. 7D). Gigaxonin was identified because of mutations of the GAN gene (coding for gigaxonin) in giant axonal neuropathies, an early onset neuronal disorder (23, 51). Gigaxonin belongs to the BTB-KELCH family of adaptor proteins where the KELCH domain interacts with the protein targeted for degradation and BTB domain interacts with the E3 ubiquitin ligase complex. In the absence of a functional gigaxonin, aggregates of vimentin intermediate filaments in fibroblasts and aggregates of peripherin and neurofilament intermediate filaments in neurons have been documented (23). These studies have implicated gigaxonin in the ubiquitination of intermediate filaments for the for-
| Sample | Tumor | ALDH | BMI-1 | CD 44 | CD 68 | IL-8 | p16 |
|--------|-------|------|-------|-------|-------|------|------|
|  1     | Primary | 70%, Cytoplasmic | Membrane, diffuse | Mostly at periphery of tumor nests (100%) | Scattered IL-8 inflammatory cells mostly at periphery of tumor nests (100%) | Not done | Negative |
|  2     | Primary | 100%, Diffuse | Membrane, diffuse | Mostly at periphery of tumor nests (100%) | Not done | Not done | Positive |
|  3     | Primary | Scattered, focal positivity | Membrane, diffuse | Mostly at periphery of tumor nests (100%) | Not done | Not done | Negative |
|  4     | Primary | 5%, Scattered, individual cells | Membrane, diffuse | Mostly at periphery of tumor nests (100%) | Not done | Not done | Positive |
|  5     | Primary | 5%, Scattered | Membrane, diffuse | Mostly at periphery of tumor nests (100%) | Not done | Not done | Negative |
|  6     | Recurrent | Faint, weak, more at periphery | Membrane, diffuse | Mostly at periphery of tumor nests (100%) | Scattered IL-8 inflammatory cells within center (50%) and at periphery (70%) of tumor nests | Not done | Negative |
|  7     | Recurrent | Strong at periphery and basal layer, cytoplasmic in tumor | Membrane, diffuse | Mostly at periphery of tumor nests (100%) | Scattered IL-8 inflammatory cells mostly at periphery of tumor nests (100%) | Not done | Negative |
|  8     | Recurrent | Strong at periphery and basal layer, cytoplasmic in tumor | Membrane, diffuse | Mostly at periphery of tumor nests (100%) | Scattered IL-8 inflammatory cells mostly at periphery of tumor nests (100%) | Not done | Negative |
|  9     | Basaloid | 50–74%, Cytoplasmic, moderate-to-strong | Membrane, strong | Mostly at periphery of tumor nests (100%) | Not done | Not done | Positive |
| 10     | Basaloid | 50–75%, Cytoplasmic, weak-to-moderate | Membrane, strong | Mostly at periphery of tumor nests (100%) | Not done | Not done | Positive |
| 11     | Basaloid | 25–49%, Membranous, weak-to-moderate | Membrane, strong | Mostly at periphery of tumor nests (100%) | Not done | Not done | Negative |
relationship between NFκB activation and development of tumor promoting M2 phenotype is not known. We believe that the gigaxonin effect on intermediate filaments and microtubule assembly could activate autophagy. In the absence of gigaxonin, microtubule reorganization could result in the suppression of autophagy and the development of tumor-promoting M2 phenotype. We, therefore, hypothesize that nuclear expression of p16 and gigaxonin could serve as useful markers of chemosensitivity in head and neck cancers.

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