Deficient TP53 Expression, Function, and Cisplatin Sensitivity Are Restored by Quinacrine in Head and Neck Cancer

Jay Friedman, Liesl Nottingham, Praveen Duggal, Francisco G. Pernas, Bin Yan, Xin Ping Yang, Zhong Chen, and Carter Van Waes

Abstract

Purpose: To determine the nature and potential pharmacologic reversibility of deficient TP53 expression and function in head and neck squamous cell carcinomas (HNSCC) with wild-type *TP53*, previously associated with decreased sensitivity to cisplatin therapy.

Experimental Design: *TP53* genotype, mRNA and protein expression, TP53-induced p21 expression, and TP53 DNA–binding and reporter gene function were determined in a panel of nine previously characterized HNSCC cell lines from the University of Michigan squamous cell carcinoma (UM-SCC) series. The genotoxic drug doxorubicin and the anti-inflammatory and antimalarial drug quinacrine, previously identified as inducers of TP53, were used to examine the nature and potential reversibility of deficient TP53 expression and function. The specific role of inducible TP53 on function and cellular proliferation was confirmed using selective TP53 inhibitor pifithrin-α or short hairpin RNA knockdown. The capability of quinacrine to sensitize HNSCC to the cytotoxic effects of cisplatin was assessed.

Results: UM-SCC cell lines with wild-type *TP53* genotype underexpressed TP53 mRNA and protein when compared with normal human keratinocytes or UM-SCC with mutant TP53. Although doxorubicin failed to induce TP53 expression or functional activity, quinacrine induced TP53 mRNA and protein expression, increased TP53 reporter activity and p21 protein expression, and induced growth inhibition in these wild-type *TP53* cell lines. Quinacrine-induced TP53 reporter activity and growth suppression were attenuated by pifithrin-α and TP53 short hairpin RNA knockdown. Furthermore, quinacrine sensitized UM-SCC to cisplatin *in vitro*.

Conclusions: Deficient TP53 mRNA and protein expression underlies decreased function in a subset of HNSCC with wild-type *TP53* and can be restored together with cisplatin sensitization by quinacrine.

TP53, a tumor suppressor gene important in regulating cell cycle arrest, apoptosis, and therapeutic sensitivity, represents one of the most common targets for alterations underlying the development of cancer, including head and neck squamous cell carcinomas (HNSCC; refs. 1–5). Mutation of *TP53* occurs in ~40% to 50% of HNSCC, resulting in altered TP53 expression and function (3–5). In HNSCC that retain wild-type *TP53* genotype, approximately one half expressed detectable TP53 protein, whereas approximately one half exhibited deficient expression by immunohistochemistry (6). In the latter subset of HNSCC that retain wild-type *TP53* genotype, the alterations resulting in a defect in TP53 expression or function have been shown to include protein inactivation after infection with human papilloma virus (HPV), defects of *p16INK4a* and other components of the DNA damage response pathway, or unknown mechanisms. As a consequence, the differing nature and effects of these multiple alterations on TP53 expression and function likely contributed to the frequent discordance of results between studies using immunohistochemistry, genotyping, or other methods to define the role of TP53 in tumorigenesis and therapeutic resistance in HNSCC and other cancers (6–9).

Some previous studies in HNSCC provide evidence that lack of TP53 expression may be associated with worse prognosis, whereas overexpression of TP53 protein detected by immunohistochemistry predicts better response and organ preservation with cisplatin or doxorubicin chemotherapy and radiation (7–9). Further study of HNSCC lines in which wild-type and

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Received 6/28/07; revised 8/29/07; accepted 9/4/07.

Grant support: National Institute on Deafness and Other Communication Disorders Intramural project DC-00016.

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doi:10.1158/1078-0432.CCR-07-1591

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mutant *TP53* genotype were determined and correlated with TP53 expression and cisplatin sensitivity provides evidence that HNSCC with wild-type *TP53* genotype more often show lack of TP53 protein expression and lack of sensitivity to cisplatin therapy. It has been shown that the average IC_{50} for inhibition of cell growth in a series of University of Michigan squamous cell carcinoma (UM-SCC) cell lines with wild-type *TP53* is ~2-fold greater compared with cell lines with mutant *TP53* (10). These observations indicate that a significant subset of HNSCC exhibits wild-type *TP53* genotype and deficient protein expression and is likely to show greater therapeutic resistance to chemotherapy and radiation.

The nature of the deficiency in TP53 expression and function in many HNSCC in this subset is poorly defined, as is the potential for reversibility. HPV infection and expression of E6 protein that inactivates TP53 protein are observed in only a small subset of ~10% to 20% of HNSCC, which arise predominantly in the oropharynx, in association with sexual transmission (11, 12). Transcriptional inactivation by mutation or hypermethylation of the TP53 promoter itself is reportedly rare (13). Hypermethylation or mutation of coding sequence within the *p16INK4a/p14ARF* or *ATM* loci involved in TP53-dependent and TP53-independent DNA damage responses occurs more commonly (13–16). Pharmacologic reversal of DNA hypermethylation by demethylating agents, such as 5-azacytidine, has been reported to increase expression of p16INK4a and TP53 in some HNSCC cell lines (17, 18), but the broader relevance of demethylating agents for restoration of TP53 expression and function in HNSCC has not been defined. Defining the nature of alterations within this subset and development of approaches to reverse alterations resulting in defects in TP53 expression and function could potentially restore tumor suppressor function and sensitization of these HNSCC to cytotoxic therapies dependent on apoptosis.

In this study, we examined the expression and function of TP53 in a panel of nine HNSCC lines from the UM-SCC series, which included subsets exhibiting deficiency or variable expression of TP53 similar to that observed in a series of 24 HNSCC tissue specimens. We identified four UM-SCC lines with wild-type *TP53*, which exhibited a deficiency in TP53 mRNA and protein expression and function. These UM-SCC

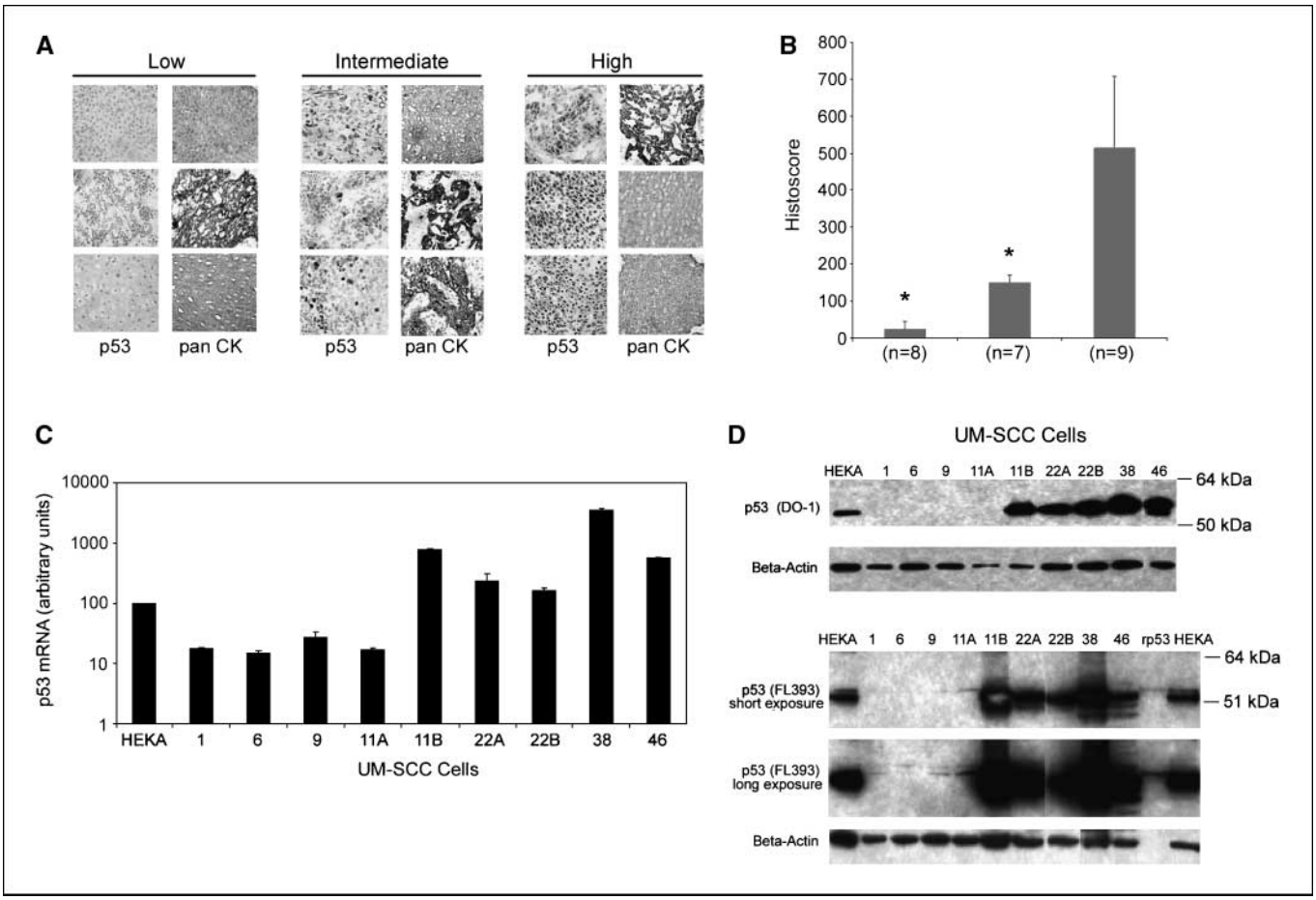

Fig. 1. TP53 expression in HNSCC tissue specimens and UM-SCC cell lines. *A,* immunostaining of TP53 (DO-1) and pan-cytokeratin (*pan CK*) representative of range observed in 24 individual HNSCC tissue specimens includes minimal or none (*left*), intermediate (*middle*), or high (*right*) TP53 protein expression levels. *B,* TP53 immunostaining for each specimen was scored, and the samples were grouped by low, intermediate, and high intensity of TP53 staining. *, $P < 0.05$, statistically significant difference when comparing groups with low or intermediate TP53 staining with the group with the highest TP53 staining (Student's *t* test). *C,* TP53 mRNA expression in UM-SCC. Total RNA was isolated from HEKA, wild-type TP53 cell lines (UM-SCC-1, UM-SCC-6, UM-SCC-9, and UM-SCC-11A), and mutant TP53 cell lines (UM-SCC-11B, UM-SCC-22A, UM-SCC-22B, UM-SCC-38, and UM-SCC-46), and TP53 mRNA levels were determined by real-time reverse transcription-PCR. *D,* TP53 protein expression in UM-SCC. Fifteen micrograms of whole-cell extracts were run on Tris-Glycine gel, and TP53 protein levels were determined by Western blot analysis using a mouse monoclonal antibody (*DO-1*; *top*). Ten micrograms of cell extracts were run on Bis-Tris gel, and TP53 protein was detected using a goat polyclonal antibody (*FL393*; *bottom*). One nanogram of recombinant TP53 (*rp53*) was loaded as the positive control.

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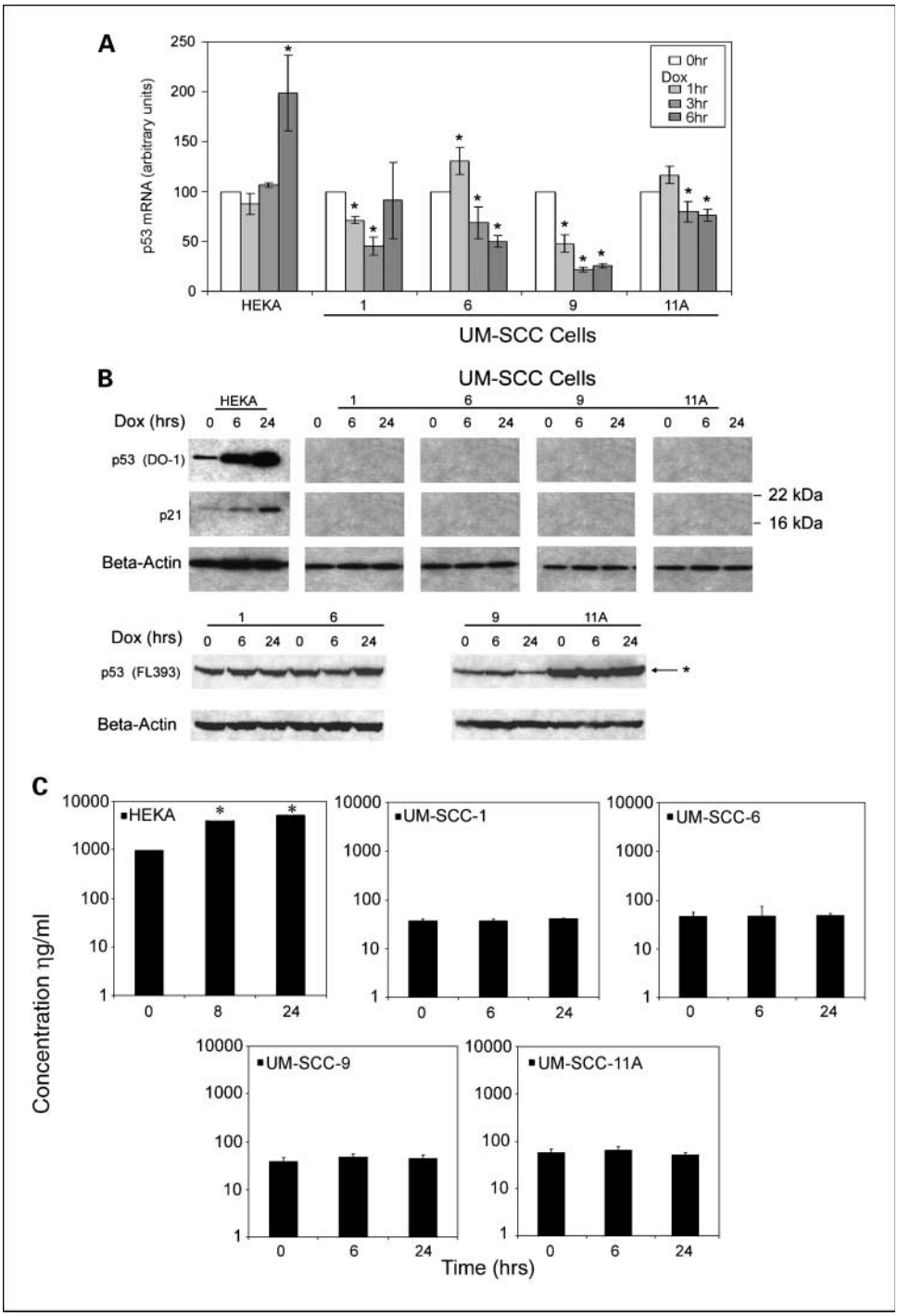

Fig. 2. Effect of doxorubicin on TP53 mRNA and protein and on p21 protein expression. *A,* TP53 mRNA levels in HEKA cells and wild-type *TP53* UM-SCC cell lines 1, 3, and 6 h after treatment with 0.5 μg/mL doxorubicin (*Dox*). *, $P < 0.05$, the levels at the time points that are significantly different from 0 h for HEKA or each individual cell line (Student's *t* test). *B, top,* effects of 0.5 μg/mL doxorubicin on TP53 and p21 protein levels in HEKA cells and wild-type *TP53* UM-SCC cell lines 6 and 24 h after treatment. Fifteen micrograms of protein were loaded into each lane. *Bottom,* TP53 protein levels in wild-type *TP53* UM-SCC cell lines 6 and 24 h after treatment with 0.5 μg/mL doxorubicin. Forty micrograms of protein were loaded into each lane. *, upon longer exposure, recombinant TP53 protein comigrates with band detected with FL393 antibody. *C,* quantitative measure of total TP53 protein in HEKA and wild-type *TP53* UM-SCC cell lines after treatment with 0.5 μg/mL doxorubicin using TP53 ELISA. In this particular experiment, 8- and 24-h time points were assayed for HEKA and 6- and 24-h time points were assayed for UM-SCC cell lines.

previously tested negative for HPV (19),[1] and we found no evidence for mutations or hypermethylation of the TP53 promoter by sequence analysis.[2] We examined the effects of doxorubicin, a DNA-damaging agent and a classic inducer of TP53 (20, 21), and observed no significant induction of TP53 expression. Quinacrine, an anti-inflammatory and antimalarial aminoacridine derivative capable of inducing wild-type *TP53* in renal cell and colorectal carcinoma cells through inhibition of nuclear factor-κB (NF-κB) activation or ubiquitination of TP53 (22, 23), overcame the deficient expression and function of TP53 in the wild-type *TP53* cell lines. Quinacrine inhibited growth of UM-SCC, and the combination of quinacrine with cisplatin showed greater inhibitory effects than either drug alone in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Inhibition of TP53 function by pifithrin-α or short hairpin RNA (shRNA) attenuated these effects. This study indicates that a subset of HNSCC with wild-type *TP53* genotype has undergone a reversible inactivation of

[1] C. Bradford, personal communication.
[2] J. Friedman, unpublished results.

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TP53 expression and provides a rationale for preclinical and clinical studies specifically targeting HNSCC that display wild-type *TP53*, to assess safety and efficacy of quinacrine in combination therapy.

Materials and Methods

Immunohistochemical analysis of HNSCC tissue specimens. Detailed procedure for immunohistochemistry is described in Supplementary Methods.

Cell lines. A panel of nine HNSCC cell lines from the UM-SCC series was obtained from Dr. T.E. Carey (University of Michigan, Ann Arbor, MI). These UM-SCC cell lines were previously screened and found to be negative for HPV (19)[1] and, upon extensive characterization in previous studies in our laboratory, were found to reflect molecular and phenotypic alterations important in the pathogenicity of HNSCC (24–29). The tumor, treatment, and outcome characteristics of patients providing UM-SCC cell lines are shown in Supplementary Fig. S1. The *TP53* mutation status of these cell lines was analyzed by bidirectional DNA sequencing of exons 1 to 9 for mutation (Supplementary Fig. S2; ref. 27). In addition, standard and bisulfite sequencing for mutation or methylation from -1,000 bp to the transcriptional start site was done. UM-SCC cell lines and normal keratinocytes were cultured as previously described (24, 27).

Reagents. The reagents were obtained from the following manufacturers: Sigma (doxorubicin), Calbiochem (quinacrine and pifithrin-α), Active Motif (recombinant TP53 protein), and Bedford Laboratories (cisplatin).

Western blot. Whole-cell lysates were obtained using a Nuclear Extraction kit from Active Motif, mixed with a SDS protein gel-loading solution (Quality Biological, Inc.), heated at 85°C for 2 min, and subjected to electrophoresis using Tris-Glycine precast gels (Invitrogen) at 135 V for 100 min. Alternatively, when running Bis-Tris precast gels, the whole-cell lysate was mixed with NuPAGE LDS sample buffer and NuPAGE sample reducing agent (Invitrogen) and heated at 70°C for 10 min; electrophoresis was conducted at 200 V for 50 min. The Invitrogen Gel Blot Module was used to transfer proteins onto a 0.45-μm nitrocellulose membrane for 100 min at 20 V or 70 min at 30 V at 4°C for Tris-Glycine or Bis-Tris gels, respectively. Primary antibodies were diluted in a 5% nonfat powdered milk solution prepared from Tween 20-TBS: mouse monoclonal anti-TP53 antibody, 1:1,000 (clone DO-1; Calbiochem-EMD Biosciences); goat polyclonal anti-TP53 antibody, 1:300 (FL393; Santa Cruz Biotechnology); goat polyclonal anti-p21 antibody, 1:250 (Santa Cruz); and rabbit polyclonal anti–β-actin antibody, 1:2,000 (Cell Signaling Technology). Each blot was incubated with Pierce Super Signal West Pico substrate (Pierce Biotechnology Inc.) and exposed to Kodak X-OMAT film.

Quantitation of total TP53 by ELISA. Total TP53 protein was determined by a TP53 ELISA (Invitrogen) using 10 μg of whole-cell lysates. The absorbance for each sample was measured by a microplate reader at a wavelength of 450 nm. Data were calculated from triplicates and presented as mean ± SD.

TP53-binding assay. Nuclear extracts were isolated using Nuclear Extraction kit (Active Motif), and the binding activity of TP53 was determined by a TransAM TP53 Transcription Factor Assay (Active Motif) with 10 μg of nuclear extracts. Five micrograms of MCF-7 nuclear extract served as the positive control. The absorbance was measured by a microplate reader at a wavelength of 450 nm. Data were calculated from triplicates and presented as mean ± SD.

Real-time reverse transcription-PCR. RNA isolation and cDNA synthesis were done as previously described (29). Real-time reverse transcription-PCR for TP53 and 18S rRNA was achieved with Assays-on-Demand Gene Expression Assay from Applied Biosystems. Amplification conditions were as follows: activation of enzymes for 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 15 s at 95°C and 1 min at 60°C. Thermal cycling and fluorescence detection were done using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). TP53 values were normalized to 18S rRNA. Each sample was assayed in triplicate, and data were presented as mean ± SD.

Reporter gene assay. The TP53 reporter vector containing the firefly luciferase reporter gene was obtained from Panomics, Inc. The expression vector containing the human TP53 open reading frame (pORF-hTP53) and the empty control vector (pORF-mcs) were obtained from InvivoGen. Control shRNA and TP53 shRNA vectors were a kind gift from the laboratory of Dr. Curtis Harris (NIH, Bethesda, MD). UM-SCC-9, UM-SCC-11A, UM-SCC-11B, and UM-SCC-38 were

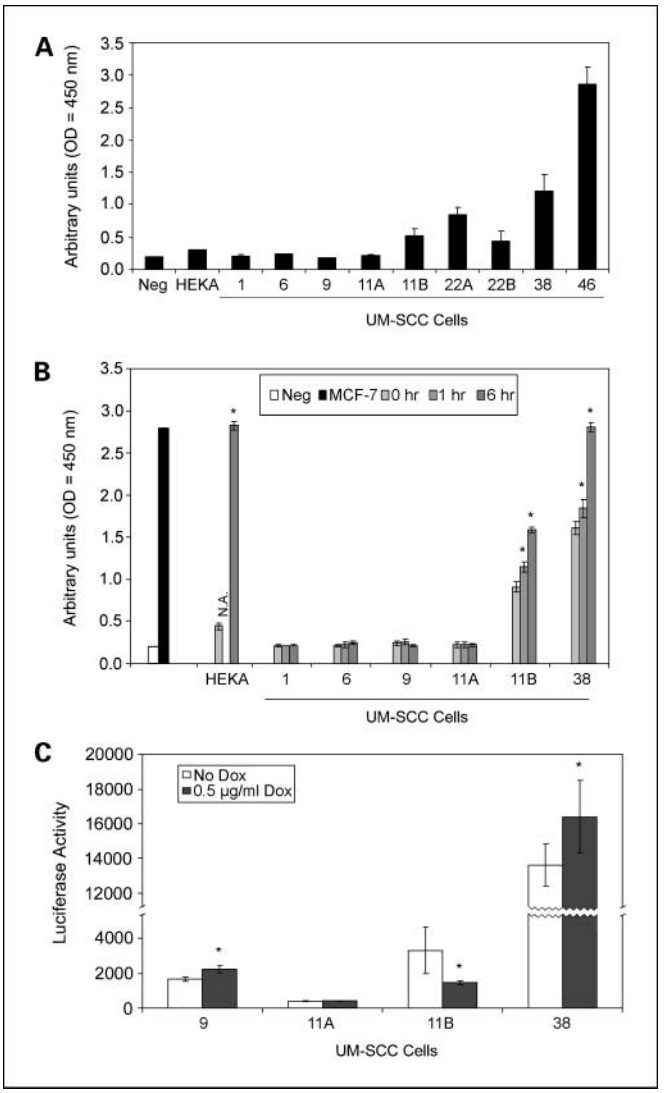

Fig. 3. Basal and doxorubicin-induced TP53 DNA binding and reporter gene transactivation in UM-SCC cell lines. *A,* nuclear extracts were collected from HEKA, wild-type *TP53* UM-SCC cell lines (UM-SCC-1, UM-SCC-6, UM-SCC-9, and UM-SCC-11A), and mutant *TP53* UMSCC cell lines (UM-SCC-11B, UM-SCC-22A, UM-SCC-22B, UM-SCC-38, and UM-SCC-46), and basal TP53-binding activity was determined. *B,* TP53-binding activity in HEKA and wild-type TP53 UM-SCC cell lines 1 and 6 h after treatment with 0.5 μg/mL doxorubicin. One-hour time point for HEKA was not assayed for binding activity (*N.A.*). *, $P < 0.05$, time points that are significantly different from 0 h either for HEKA or each respective cell line (Student's *t* test). The extract from MCF-7 cells was used as the positive control. *C,* effect of 0.5 μg/mL doxorubicin on cell reporter gene activity. UM-SCC-9, UM-SCC-11A, UM-SCC-11B, and UM-SCC-38 cells were transfected with the TP53 reporter vector, and the reporter activity was assayed 24 h after doxorubicin treatment. *, $P < 0.05$, doxorubicin-induced significant alteration of TP53 reporter activity (Student's *t* test).

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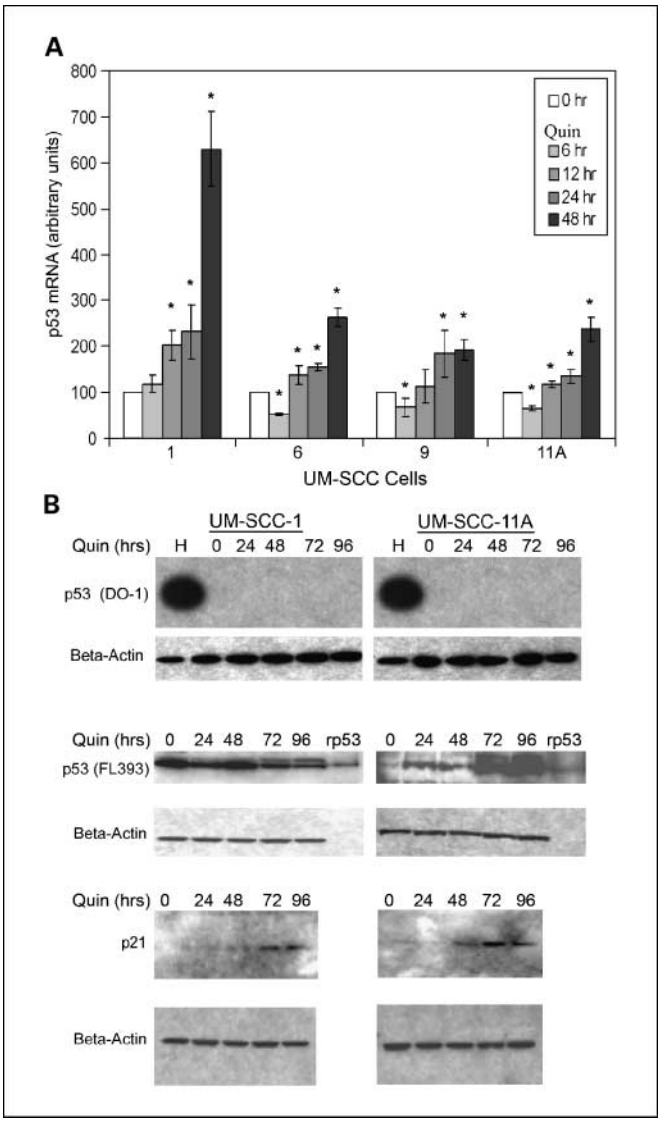

Fig. 4. Increase in TP53 mRNA levels and TP53 and p21 protein levels after quinacrine treatment. *A*, TP53 mRNA levels in wild-type *TP53* UM-SCC cell lines 6, 12, 24, and 48 h after treatment with 2.5 μmol/L quinacrine (*Quin*). *, $P < 0.05$, time points that are significantly different from 0 h for each respective cell line (Student's *t* test). *B*, TP53 and p21 protein levels in wild-type *TP53* UMSCC cell lines 24, 48, 72, and 96 h after treatment with 5 μmol/L quinacrine. The Western blots that were probed with the DO-1 antibody had baseline HEKA (*H*) whole-cell extract loaded into lane 1 to serve as a positive control for this antibody. The Western blots that were probed with the FL393 antibody had recombinant TP53 loaded into lane 6 to serve as a positive control for this antibody.

cultured and transfected, and cellular extracts were collected as previously described (28). Reporter gene activity was assayed by the chemiluminescent detection protocol from the Dual-Light System kit, and measurements were obtained using the Wallac VICTOR^2 1420 Multilabel Counter. Each sample was assayed in triplicate, and data were presented as mean ± SD.

MTT cell proliferation assay. UM-SCC-11A cells were initially plated in quadruplicate onto a 96-well plate at 5×10^3 cells per well in 100 μL of complete MEM. The next day, cells were exposed to quinacrine, pifithrin-α, cisplatin, or in combination, and cell proliferation was measured using a MTT Cell Proliferation kit (Roche Diagnostics). The absorbance was measured by a microplate reader at a wavelength of 570 nm.

To test TP53 shRNA effects on cell proliferation, UM-SCC-11A cells were transfected with either TP53 or control shRNA vector in 24-well plates for 24 h. Then, the cells were trypsinized and transferred to a 96-well plate at 5×10^3 per well with or without quinacrine. Cell growth rates were analyzed by MTT in quadruplicate, and data were presented as mean ± SD.

Results

Low expression of TP53 mRNA and protein is observed in a subset of HNSCC tissues and UM-SCC cell lines. Survey of TP53 protein expression in a panel of 24 HNSCC tissues revealed variable TP53 immunohistochemical staining intensity with the anti-TP53 antibody DO-1 in the tumor epithelia highlighted by pan-cytokeratin staining (Fig. 1A). Tumor specimen immunostaining intensity was quantified by histoscore as described previously (30) and was classified according to low, intermediate, and high expression of TP53 protein (Fig. 1A). The specimens exhibited a distribution of low staining (8 of 24; 33%), intermediate staining (7 of 24; 29%), and high staining (9 of 24; 38%) for TP53, consistent with the range observed in previous studies (6). The histoscores for the low and intermediate TP53 staining groups were significantly different from the high staining group (Fig. 1B; Student's *t* test, $P < 0.05$). We further analyzed a panel of nine HNSCC cell lines and identified subsets that expressed lower or higher levels of TP53 mRNA and protein than HEKA (Fig. 1C and D). The UM-SCC lines with low baseline TP53 mRNA levels were found to be those with wild-type *TP53* genotype by sequence analysis (UM-SCC-1, UM-SCC-6, UM-SCC-9, and UM-SCC-11A), whereas the cell lines which expressed TP53 mRNA at higher levels were those with mutant *TP53* genotype (UM-SCC-11B, UM-SCC-22A, UM-SCC-22B, UM-SCC-38, and UM-SCC-46; Fig. 1C; Supplementary Table S1). To show TP53 protein in these cell lines and determine if there is a consistent pattern between TP53 mRNA and protein levels in the wild-type and mutant *TP53* cell lines, we used Western blot analysis with two commercially available antibodies against TP53. We used a mouse monoclonal anti-TP53 antibody (DO-1) that recognizes a single epitope (amino acids 21-25) and a goat polyclonal antibody (FL393) that recognizes multiple epitopes within the full-length TP53 protein. By Western blot analysis for TP53 protein using different antibodies, TP53 protein was undetectable (Fig. 1D; *top*) or weakly detected (Fig. 1D; *bottom*), consistent with the low levels of mRNA detected in the wild-type *TP53* cell lines. When the monoclonal antibody DO-1 was used, TP53 protein was undetectable in those cell lines with wild-type *TP53* status (Fig. 1D; *top*). However, very low levels of TP53 protein that comigrated with recombinant TP53, which served as a positive control, could be detected in wild-type *TP53* cell lines when using a goat polyclonal antibody against full-length TP53 protein (FL393) and a longer exposure time (Fig. 1D; *bottom*). The undetectability of TP53 protein by monoclonal DO-1, which recognizes an NH_2-terminal epitope of TP53, compared with a polyclonal antibody, did not seem to be due to the splicing or mutation of the NH_2 terminus of the *TP53* gene because the band detected by the polyclonal antibody comigrated with recombinant TP53 protein, and DNA sequencing of *TP53* exons 1 to 3 or 4 to 9 did not detect any mutations (27).[3] The cell lines with mutant *TP53* genotype expressed comparable or higher

[3] J. Friedman, unpublished data.

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baseline levels of TP53 protein when compared with HEKA (Fig. 1D). Together, these results show that a subset of HNSCC specimens and UM-SCC lines exhibit low TP53 protein expression and that the low TP53 protein expression is associated with correspondingly low *TP53* mRNA expression in UM-SCC with wild-type *TP53* genotype.

Doxorubicin fails to induce TP53 mRNA and protein and p21 protein in the wild-type* TP53 *UM-SCC cell lines. Doxorubicin is a DNA-damaging agent that is classically used to show TP53 inducibility (20, 21). Doxorubicin- and cisplatin-based regimens have been used in the treatment of nasopharygeal carcinoma, and lack of TP53 staining has been associated with decreased response (8). As expected, TP53 mRNA levels in normal HEKA controls were significantly induced 6 h after doxorubicin treatment (Fig. 2A). In contrast, most UM-SCC cell lines with wild-type *TP53* genotype showed no change or significant decline in TP53 mRNA levels 6 h after treatment (Fig. 2A). Only UM-SCC-6 cells showed a small increase in TP53 mRNA levels 1 h after doxorubicin treatment, but this was followed by a rapid decline in TP53 mRNA levels (Fig. 2A). Upon analysis of protein expression by Western blot, doxorubicin induced a parallel increase in expression of TP53, detectable by the DO-1 antibody, and its target p21, in HEKA cells, but not in the UM-SCC lines with wild-type *TP53* genotype (Fig. 2B; *top*). Very low levels of TP53 protein could be detected when a higher concentration of protein was loaded and probed with a polyclonal antibody against full-length TP53 protein (FL393; Fig. 2B; *bottom*). However, the TP53 protein detected did not increase after doxorubicin treatment (Fig. 2B; *bottom*). The DO-1 antibody did not detect any basal or inducible TP53 protein when more cell extract was loaded into the gel (data not shown).

To further confirm the Western blot data and obtain a quantitative measurement of TP53 protein, we used a TP53 ELISA using whole-cell extract from HEKA and wild-type *TP53* UM-SCC cell lines after doxorubicin treatment (Fig. 2C). Consistent with the data presented in Fig. 2B, doxorubicin induced increased TP53 protein in HEKA but failed to induce TP53 in the wild-type *TP53* UM-SCC cell lines. The median basal TP53 protein level in wild-type TP53 UM-SCC cells was about 20-fold lower than that detected in HEKA cells, consistent with Western blots shown above.

Doxorubicin fails to induce functional TP53 binding and transactivation in wild-type* TP53 *UM-SCC cell lines. TP53 acts as a transcription factor that binds to DNA and regulates many genes important in cell cycle and apoptosis (31, 32). The basal-binding activity of TP53 in the panel of 10 UM-SCC cell lines was examined, and cells with wild-type *TP53* genotype showed minimal baseline TP53-binding activity close to levels observed for the negative control. In contrast, the mutant *TP53* cell lines had higher basal-binding activity, indicating the retention of the DNA-binding function by the missense mutant TP53 proteins expressed by these lines (Fig. 3A). After doxorubicin treatment, TP53-binding activity was significantly induced in HEKA and two UM-SCC cells with mutant *TP53* genotype (UM-SCC-11B and UM-SCC-38; Fig. 3B). However, doxorubicin failed to induce TP53-binding activity in any of the wild-type *TP53* UM-SCC cell lines (Fig. 3B). We further assessed doxorubicin-induced TP53 transactivation by TP53 reporter gene assay in two of the lines with wild-type *TP53* genotype, UM-SCC-9 and UM-SCC-11A, and two lines with mutant *TP53*, UM-SCC-11B and UM-SCC-38. Minimal or no change in TP53 reporter activity was observed in the cell lines with wild-type *TP53* genotype and UM-SCC-11B with mutant *TP53*, in contrast to a significant increase in TP53 reporter activity in UM-SCC-38 cells. Together, these data indicate that the cell lines with wild-type *TP53* genotype exhibit abnormal basal and doxorubicin-induced binding and transactivating activities.

Quinacrine induces TP53 mRNA and protein in the wild-type* TP53 *UM-SCC cell lines. Based on recent studies showing that quinacrine and other aminoacridine derivatives may activate TP53 in other cancer types (22, 23), we examined if quinacrine could potentially reactivate TP53 in HNSCC. In pilot experiments, quinacrine was titrated for growth-inhibitory activity and cytotoxicity in UM-SCC cell lines,[4] and subcytotoxic concentrations (2.5 and 5 μmol/L) were used to assess its effect on TP53 mRNA and protein expression. Quinacrine (2.5 μmol/L) induced a sustained and significant increase in TP53 mRNA levels between 12 and 48 h after treatment in most cell lines, especially in UM-SCC-1 cells (Fig. 4A). Although induction of TP53 protein by quinacrine at 5 μmol/L was not detectable by the antibody DO-1, polyclonal antibody FL393 detected a band that comigrates with recombinant TP53 protein at baseline, is increased between 24 and 48 h, and forms a doublet between 72 and 96 h (Fig. 4B). Quinacrine had more of a profound effect on TP53 protein expression in UM-SCC-11A when compared with UM-SCC-1, despite the greater increase in mRNA expression. Expression of p21 levels were increased by 48 h and peaked along with the appearance of the TP53 doublet by 72 to 96 h after quinacrine treatment in both cell lines (Fig. 4B).

Quinacrine induces functional TP53 and suppresses cellular proliferation in wild-type* TP53 *UM-SCC cell lines. We further determined if quinacrine can induce functional TP53 in wild-type *TP53* UM-SCC lines by using a TP53 reporter vector. As a control, we first cotransfected the TP53 reporter vector and a wild-type TP53 expression vector. After transfection of the TP53 expression vector in UM-SCC-11A, TP53 reporter activity was significantly increased, and this was abolished by a selective TP53 inhibitor, pifithrin-α (33), in a dose-dependent manner (Fig. 5A; *left*). Quinacrine (5 μmol/L) induced TP53 reporter activity at lower levels, which was attenuated significantly by pifithrin-α in a dose-dependent manner (Fig. 5A; *middle*). To further confirm that the quinacrine-induced TP53 reporter activity was due to TP53 and not other family members or nonspecific effects, cells were transfected with a control and TP53-specific shRNA (34). The TP53-specific shRNA reduced basal- and quinacrine-induced TP53 reporter activity relative to a scrambled control shRNA, consistent with the effects seen with pifithrin-α (Fig. 5A, *right*). We further examined the effect of quinacrine on cellular proliferation in UM-SCC-11A cells (Fig. 5B). Quinacrine at 5 μmol/L showed minimal effect on cellular proliferation, whereas increased dosage (10 μmol/L) significantly decreased proliferation (Fig. 5B). Quinacrine at 10 μmol/L also caused cell rounding, reduced cell size, blebbing, and detachment, which are changes in cell morphology consistent with cell death

[4] L. Nottingham, unpublished data.

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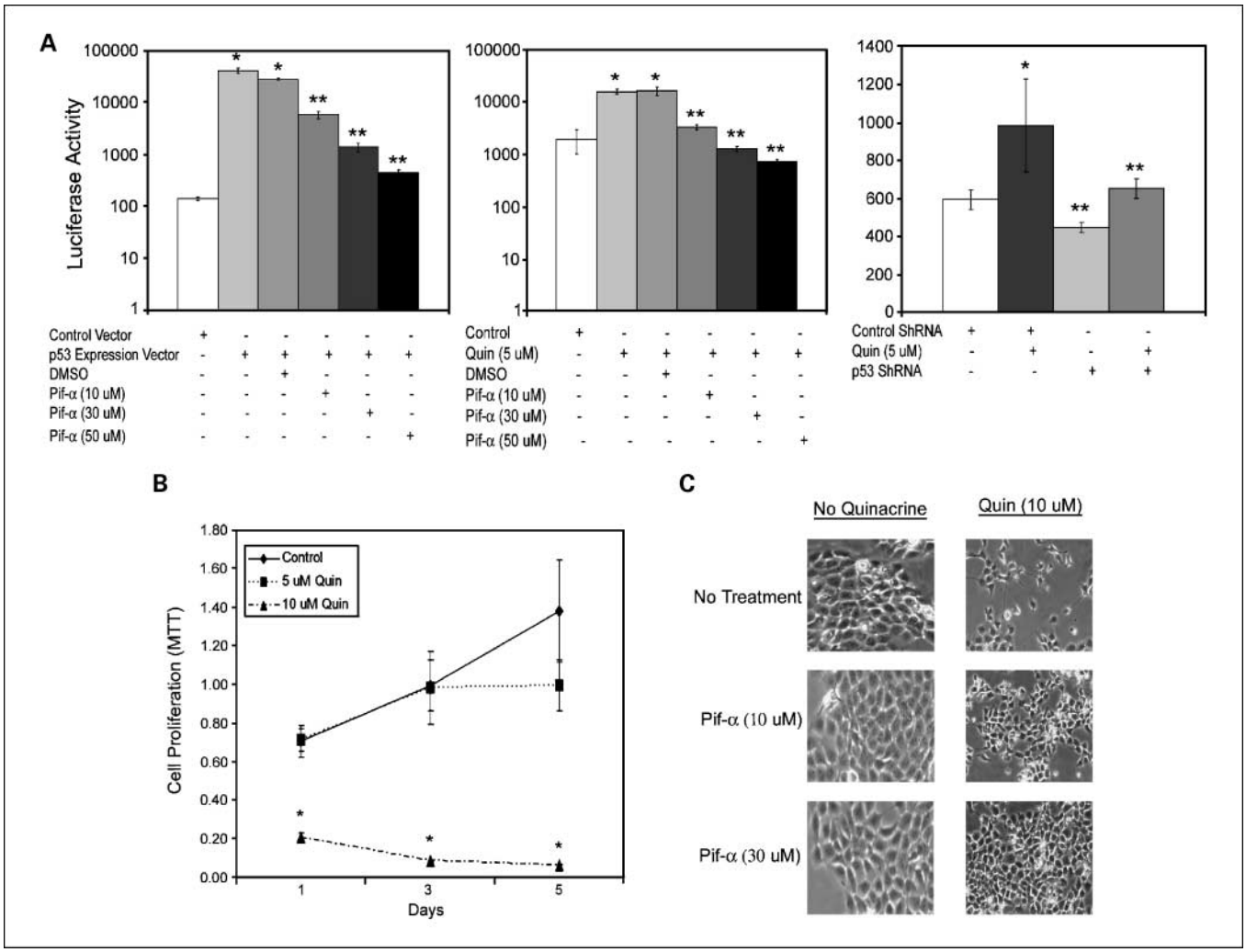

Fig. 5. Restoration of TP53 activity by quinacrine mediates decrease in cell proliferation of UM-SCC-11A. *A, left,* control overexpression of TP53 and inhibition of TP53 reporter activity. Transfection of TP53 expression vector augments TP53 reporter activity at 24 h, which is inhibited by the selective TP53 inhibitor, pifithrin-α (*Pif-α*). UM-SCC-11A cells were cotransfected with a TP53 reporter vector plus a TP53 expression or control vector. After transfection for 4 h, the cells were treated by different concentrations of pifithrin-α or just DMSO (the vehicle for pifithrin-α) for 24 h. *, $P < 0.05$, exogenous wild-type TP53 expression significantly increased reporter activity when compared with controls (Student's *t* test). **, $P < 0.05$, pifithrin-α significantly suppressed TP53 reporter activity in a dose-dependent manner (Student's *t* test). *Middle,* quinacrine increases TP53 reporter activity at 24 h, which is inhibited by pifithrin-α. UM-SCC-11A cells were transfected with a TP53 reporter vector and treated with different drugs as described above. *, $P < 0.05$, quinacrine significantly enhanced TP53 reporter activity (Student's *t* test). **, $P < 0.05$, pifithrin-α significantly suppressed quinacrine-induced TP53 reporter activity in a dose-dependent manner (Student's *t* test). *Right,* utilization of TP53 shRNA inhibits basal and inducible TP53 reporter activity at 24 h. UM-SCC-11A cells were cotransfected for 24 h with a TP53 reporter vector in combination with either a TP53 shRNA or a control vector. After transfection, the cells were treated with 5 μmol/L quinacrine for 24 h. *, $P < 0.05$, quinacrine significantly induced TP53 reporter activity (Student's *t* test). **, $P < 0.05$, TP53 shRNA attenuated both basal- and quinacrine-induced TP53 reporter activity (Student's *t* test). *B,* MTT assay for cell proliferation was done in UM-SCC-11A after either 5 or 10 μmol/L quinacrine treatment for 1, 3, and 5 d. Quinacrine at 10 μmol/L significantly decreased cellular proliferation at all time points. *C,* phase contrast photomicrographs showed the effects of quinacrine, pifithrin-α, or the combination on cell proliferation in UM-SCC-11A. UM-SCC-11A cells were pretreated with DMSO, 10 μmol/L pifithrin-α, or 30 μmol/L pifithrin-α for 5 h. After 5 h of pretreatment, the medium was replaced with fresh medium with drugs alone, or in combination for 24 h, and cells were photographed at ×4 magnification.

(Fig. 5C, *top*). To dissect if these quinacrine-mediated effects are dependent on TP53 activation, we used pifithrin-α or TP53 shRNA either alone or in combination with quinacrine and quantified cell proliferation and density by MTT assay. Exposure of quinacrine alone for 24 h significantly decreased cellular proliferation, and the effect was abolished with the use of 10 to 30 μmol/L pifithrin-α (Fig. 5C and D). Morphologically, the effect of quinacrine on cell density but not cell size was attenuated with an increased concentration of pifithrin-α (Fig. 5C and D). In addition, TP53 shRNA alone enhanced baseline cell proliferation and partially reversed quinacrine-induced cell growth inhibition (Fig. 5E). Taken together, our data suggest that quinacrine can reverse the defect in expression and increase TP53 expression and function in the UM-SCC lines with wild-type *TP53* genotype. In addition, these results indicate that the quinacrine-mediated effects on cellular proliferation are due, at least in part, to TP53 activation and function.

Quinacrine and cisplatin show combined inhibitory activity in UM-SCC-11A cells. Based on the aforementioned results, we examined if quinacrine can further sensitize UM-SCC cells to cisplatin, a DNA-damaging and chemotherapy agent frequently used in the treatment of HNSCC patients in the clinical setting, and to which wild-type *TP53* HNSCC are reported to be

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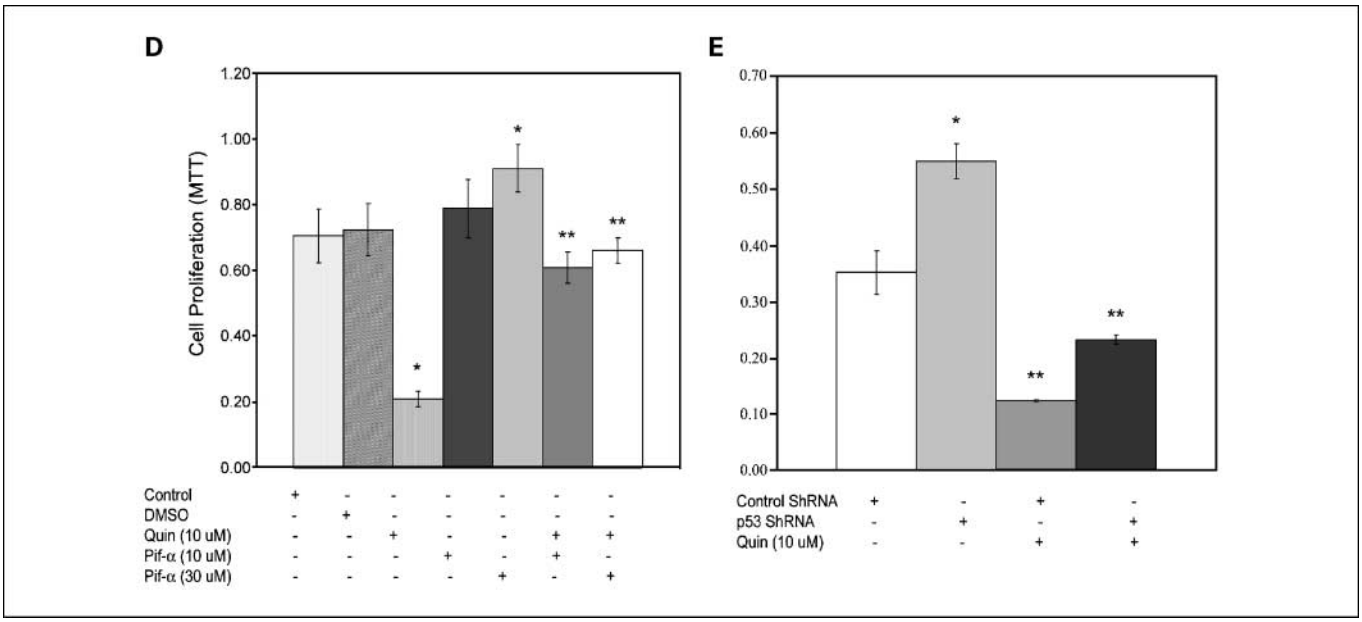

Fig. 5 *Continued. D,* cell proliferation was measured by MTT under the same experimental conditions as described above. *, $P < 0.05$, the treatment of the drug alone is significantly different from the control sample (Student's *t* test). **, $P < 0.05$, pifithrin-α significantly reversed quinacrine-mediated suppression in cellular proliferation (Student's *t* test). *E,* 10 μmol/L quinacrine decrease cellular proliferation in UM-SCC-11A at 24 h, which is partially reversed by TP53 shRNA. *, $P < 0.05$, treatments that are significantly different from the control shRNA sample (Student's *t* test). **, $P < 0.05$, TP53 shRNA significantly reversed quinacrine-mediated suppression in cellular proliferation (Student's *t* test).

relatively more resistant. Figure 6A to D show that by days 3 and 5, subcytotoxic concentrations of quinacrine (5 μmol/L) significantly sensitize UM-SCC-11A cells to increasing concentrations of cisplatin, causing a further reduction in cellular density when compared with treatment with quinacrine or cisplatin as single agents. These results show that quinacrine can sensitize UM-SCC cells with wild-type *TP53* status to the antiproliferative and cytotoxic effects of DNA-damaging agents, such as cisplatin.

Discussion

The majority of HNSCC that retain wild-type *TP53* genotype have previously been shown to exhibit low immunostaining for TP53 protein (6), and this subset has also been linked with greater resistance to chemotherapy with DNA-damaging agents doxorubicin or cisplatin in previous reports (7–10). In the present study, we identified a subset of UM-SCC lines exhibiting deficiency or variable expression of TP53, in a range similar to that observed in HNSCC tissue specimens (Fig. 1; refs. 6–9). We found that the UM-SCC cell lines with the wild-type *TP53* genotype exhibit a deficiency in mRNA expression at baseline and in response to doxorubicin relative to normal keratinocytes (Figs. 1 and 2). This altered mRNA expression underlies the deficiency in protein expression, DNA-binding, and reporter gene function of TP53 observed in these HNSCC (Figs. 1-3). The identification of such a subset with an underlying defect in mRNA expression may help explain discrepancies in the results from different clinical studies in which attempts have been made to correlate *TP53* genotyping and immunostaining with the outcome in HNSCC (6–9). The identification of HNSCC with wild-type *TP53* and decreased mRNA expression distinguishes them from other defects in wild-type *TP53*, such as TP53 protein inactivation by expression of E6 protein from HPV infection (35, 36) or enhanced expression of endogenous inhibitor HDM2, which can be enhanced by the aberrant activation of transcription factor NF-κB (37). Consistent with a distinct mechanism(s) involving mRNA expression, HPV infection was not detected after the derivation of this panel of UM-SCC cell lines (18),[1] nor was overexpression of HDM2 mRNA detected in array studies.[5]

The apparent defect in TP53 mRNA and protein expression was reversible because quinacrine was shown to induce TP53 mRNA and protein and evidence of functional activity, including TP53-inducible protein p21, TP53 reporter gene activity, growth inhibition, and cytotoxicity (Figs. 4 and 5). The effects of quinacrine are at least partly dependent on the restoration of TP53 expression and function because the selective TP53 inhibitor pfithrin-α and shRNA attenuated this quinacrine-induced TP53 expression, function, and growth arrest (Fig. 5). Further, the combination of quinacrine with cisplatin showed enhanced effects (Fig. 6). Together, these data provide evidence that a reversible inactivation of wild-type TP53 mRNA transcription may underlie the decrease in protein expression and at least partially contribute to the TP53 functional defects, as well as cisplatin resistance and poor clinical outcome in the subset of HNSCC with wild-type *TP53* genotype observed in previous chemotherapy and radiotherapy clinical studies.

We have not yet defined the mechanism(s) for the inactivation or reversible effects of quinacrine on TP53 mRNA and protein expression in HNSCC. We have preliminary data indicating that several mechanisms previously implicated in the

[5] Z. Chen, unpublished observation.

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inactivation of TP53 mRNA expression are not likely to be primarily responsible for the low expression of TP53 mRNA and protein observed. Transcriptional inactivation by mutation or hypermethylation of the TP53 promoter itself is reportedly rare in HNSCC (13). Sequence analysis of the promoter region of the *TP53* gene from -1,000 bp to +300 bp of the transcription starting site in the four UM-SCC lines with wild-type *TP53* provided no evidence for direct mutation or hypermethylation.[2] Deficient expression of TP53 mRNA has also been previously reported in breast cancer with wild-type *TP53* genotype due to hypermethylation of the *HOXA5* gene promoter, which functions as the transcription factor activating TP53 mRNA expression (38). We tested *HOXA5* gene expression in wild-type *TP53* UM-SCC cell lines by real-time reverse transcription-PCR, and there was no significant decrease of HOXA5 mRNA levels observed in tumor cell lines.[2]Interestingly, however, TP53 mRNA expression, but not protein expression, could be enhanced by treating cells with 5-azacytidine,[2] suggesting that hypermethylation of the promoter regions of other factors controlling *TP53* gene

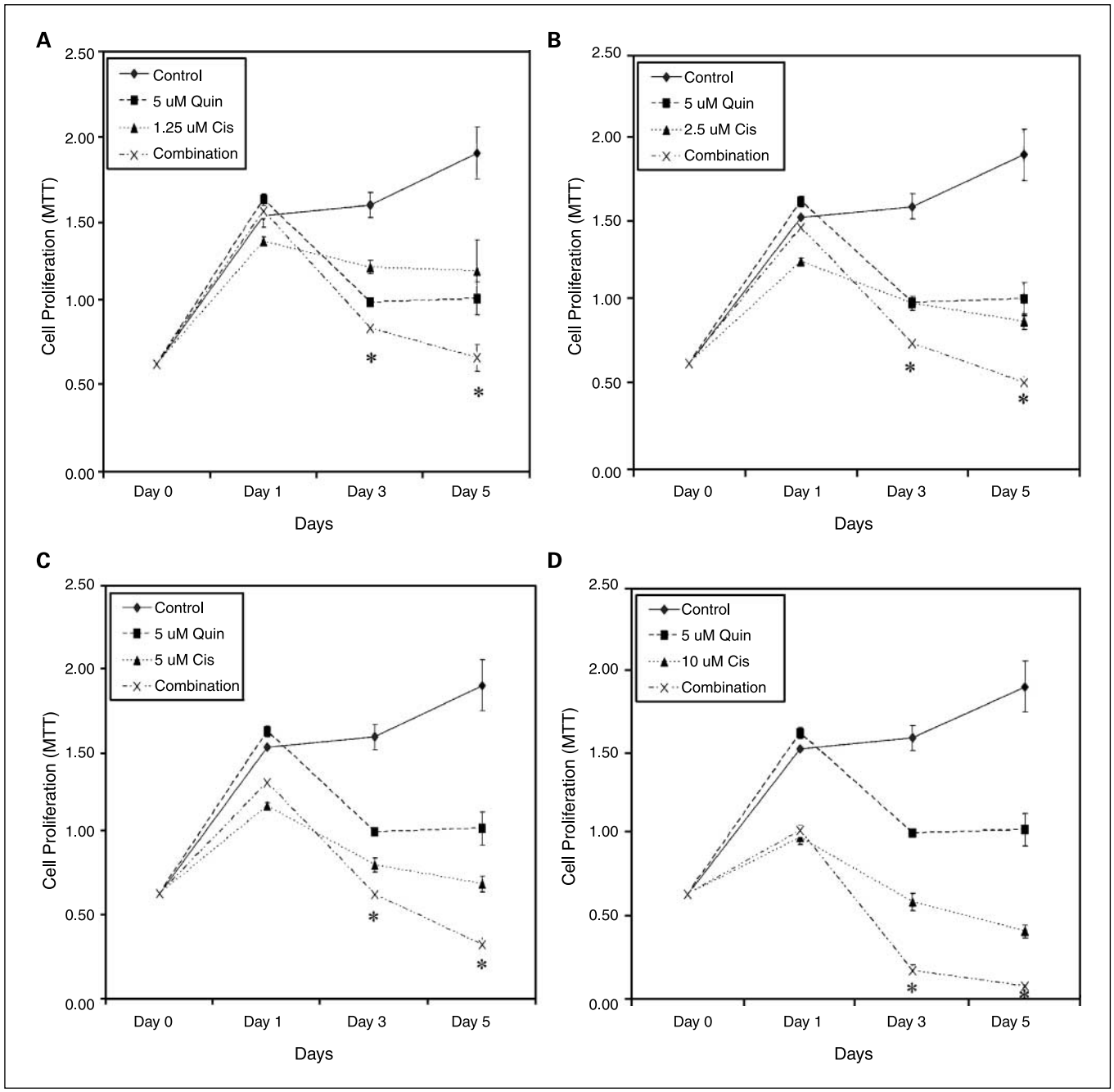

Fig. 6. Quinacrine sensitizes UM-SCC-11A to effects of cisplatin in MTT assay. MTT assay for cell proliferation was done in UM-SCC-11A after treatment by 5 μmol/L quinacrine or varying doses of cisplatin (*Cis*) alone, and combined quinacrine and cisplatin for 1, 3, and 5 d. Cisplatin at concentrations of 1.25, 2.5, 5, or 10 μmol/L was used alone or in combination with 5 μmol/L quinacrine (*A, B, C*, and *D,* respectively). *, $P < 0.05$, combined treatment was statistically significant when compared with single-drug treatment (Student's *t* test).

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expression could potentially enhance TP53 mRNA expression without affecting protein expression. By contrast, quinacrine treatment enhanced TP53 mRNA as well as protein expression and the appearance of another slower migrating species (Fig. 4B), consistent with possible posttranslational modification of TP53 protein. These observations suggest that quinacrine can enhance mRNA and protein expression, providing a protein substrate for modifications by kinases in the DNA damage response pathway, or may enhance such modifications in addition to its effects on expression. Further studies will be of interest to determine the nature and role of quinacrine in protein expression and modifications.

Besides the defects in mRNA and protein expression of TP53 in the UM-SCC cells, we have not ruled out defects in other protein-dependent mechanisms that may contribute to deficiencies in TP53 function and reversal by quinacrine (Figs. 3-5). Much effort has been previously put forth to understand other mechanisms of the inactivation of TP53 function resulting from alterations in the expression of components of the DNA damage response pathway in tumor cells that harbor wild-type *TP53* (13–16). Decreased expression or mutation of *p14ARF* or *ATM* genes has been implicated in defective TP53 function because p14ARF regulates MDM2-dependent TP53 stabilization (39), and ATM is involved in the activation of TP53 protein through S15 phosphorylation (40). Preliminary data from previous microarray studies suggest that there may be a decreased mRNA expression of the *ATM* gene in all of the UM-SCC cells included in the present study, regardless of *TP53* status,[6] whereas one cell line shows hypermethylation of the promoter of *p14ARF*.[7] Thus, effects of quinacrine on or independent of these alterations merit further study.

Inactivation of TP53 function may also occur through an aberrant increase in the activation of NF-κB, signal transducer and transcription-3, or other transcription factors, through competition for the coactivation factors necessary for TP53 function, such as CBP/p300 (41, 42). Quinacrine has been reported to reactivate TP53 expression in renal carcinoma cells through the inhibition of NF-κB activation, but excluding a mechanism involving CBP/p300 (22). These investigators showed that treatment by aminoacridines can cause a reduction of phosphorylated Ser^{536} of the p65 subunit of NF-κB, a posttranslational modification that is usually necessary for NF-κB gene transactivation and typically mediated by the inhibitor-κB kinase complex, a key upstream kinase involved in the activation of the NF-κB signaling. They propose that aminoacridines may be inhibitors of inhibitor-κB kinase activation, and the resultant lack of phosphorylation at this site converts NF-κB to a transrepressor that is active in recruiting histone deacetylases, leading to repression of unknown targets that are inhibiting TP53 expression (22). These observations imply a possible reciprocal relationship between TP53 and NF-κB, with the activation of NF-κB potentially inactivating TP53, favoring a prosurvival state. We have recently shown increased activation of NF-κB phospho-Ser^{536} p65 and TP53 inactivation in the subset of HNSCC with wild-type *TP53* (27, 43) and have preliminary data indicating that quinacrine can inhibit NF-κB reporter activity and restore TP53 expression in cells found to underexpress TP53 in the same concentration range.[8] Activation of these transcription factors may be reciprocally related and contribute to cell survival in HNSCC. Thus, mechanisms involving inhibitory cross-talk, and effects of quinacrine on them, may also merit further investigation.

Several observations indicate that our findings regarding a distinct type of defect in TP53 mRNA and protein expression and reversibility with quinacrine may be of importance in the biological development and clinical approach to therapy of HNSCC. Because mechanisms of TP53 inactivation involving HPV are limited to TP53 protein and a smaller subset of tumors, such a distinct mechanism(s) affecting mRNA expression in cells with wild-type *TP53* genotype may account for a significant portion of HNSCC arising from other sites. If common mechanisms affecting TP53 mRNA expression are born out as an important basis for deficient TP53 protein expression in a larger clinical case study, analysis of TP53 mRNA and protein expression together with testing for methylation of TP53 pathway genes and HPV could provide useful prognostic information and facilitate the selection of therapy. In this regard, several studies including a larger panel of UM-SCC lines and tumor specimens indicate that HNSCC of wild-type TP53 genotype, which underexpress TP53, are often more resistant to cytotoxic effects of chemotherapy with the DNA-damaging agent cisplatin and associated with worse prognosis after cisplatin and radiotherapy (7–10). Addition of quinacrine or other agents that restore TP53 expression in this subset of patients can enhance the antiproliferative and/or cytotoxic effects of current agents.

Quinacrine and other acridine derivatives have been used in the clinical setting as topical antibacterials and antimalarial agents throughout the 20th century (44). The first acridine derivative introduced to the clinical setting for an anticancer effect was amsacrine, specifically for use as an antileukemia drug (45). This drug showed activity in early clinical trials (46, 47). There have been other derivatives such as pyrazoloacridine that have been shown to have a wide range of *in vivo* activity in animal solid tumor models (48). In a phase I clinical study using pyrazoloacridine, responses were seen in several patients with platinum- and taxane-refractory ovarian carcinoma and in cervical and colorectal carcinomas (49). Based on the similarity of low TP53 protein expression in the wild-type *TP53* cell lines and HNSCC tissue specimens, and the effect of quinacrine on TP53 expression, function, and apoptosis, quinacrine or other acridine derivatives may serve as useful chemotherapeutic agents in the clinical setting for HNSCC patients with tumors displaying low expression of TP53 and the wild-type *TP53* genotype.

Acknowledgments

We thank Drs. Lyuba Varticovski, Anna Robles, and Curt Harris of the National Cancer Institute for the gift of TP53 shRNA; and Drs. Vartikovski of the National Cancer Institute and Carol Bradford of University of Michigan for the review and helpful comments.

[6] Z. Chen, unpublished data.
[7] T.L. Lee, unpublished data.
[8] L. Nottingham et al., unpublished observations.

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Clinical Cancer Research

Deficient TP53 Expression, Function, and Cisplatin Sensitivity Are Restored by Quinacrine in Head and Neck Cancer

Jay Friedman, Liesl Nottingham, Praveen Duggal, et al.

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