

Mutant p53 (G199V) Gains Antiapoptotic Function through Signal Transducer and Activator of Transcription 3 in Anaplastic Thyroid Cancer Cells

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Abstract

In the present study, we identified a missense mutation (G199V) in KAT-18 cell line established from primary cultures of anaplastic thyroid cancer (ATC). Notably, knockdown of this mutant (mt) p53 reduced cell viability and exerted antitumor activity equivalent to high doses of several chemotherapeutic agents. We showed that p53 knockdown had an antitumor effect via the induction of apoptosis. We further examined the underlying mechanism by which mt p53 (G199V) gains antiapoptotic function in KAT-18 cells. Microarray analysis revealed that p53 knockdown modified the expression of numerous apoptosis-related genes. Importantly, p53 knockdown led to downregulation of signal transducer and activator of transcription-3 (STAT3) gene expression. We further observed that p53 knockdown induced the downregulation of STAT3 protein. We also observed that a STAT3 inhibitor augmented the reduction of cell viability induced by p53 knockdown, whereas interleukin-6 treatment alleviated this effect. In addition, overexpression of STAT3 protected ATC cells against cell death induced by p53 knockdown. Taken together, these data show that mt p53 (G199V) gains antiapoptotic function mediated by STAT3 in ATC cells. Inhibition of the function of mt p53 (G199V) could be a novel and useful therapeutic strategy for decreasing the extent and severity of toxicity due to chemotherapeutic agents. (Mol Cancer Res 2009; 7(10):1645–54)

Introduction

Although it is generally believed that p53 loses its tumor suppressor function because of a mutation in *p53*, certain types of p53 mutations are gain-of-function (GOF) mutations. More specifically, some GOF mutants have been shown to have oncogenic functions that wild-type (wt) p53 does not possess. Whether various mutant (mt) p53 proteins have specific functions that support cellular growth in tumors has recently been a subject of intense research (1–4). Those studies have led to the concept that mt p53 may acquire novel oncogenic “GOF” activities in tumors.

Anaplastic thyroid carcinoma (ATC) accounts for less than 5% of all thyroid cancers (5, 6), but it is the most malignant thyroid neoplasm and is almost invariably lethal (7, 8). Surgical excision of ATC is rarely feasible, and although patients can be cured by radioiodine ablation, ATC is unfortunately the least radiosensitive of all thyroid tumors. Chemotherapy is used to treat ATC if the tumor is not responsive to I-131, inoperable, and unaffected by external radiotherapy. The importance of chemotherapy in the management of ATC cannot be understated because >50% of ATC patients have metastatic disease at presentation. Unfortunately, most series that have studied the effects of chemotherapeutic agents on anaplastic carcinoma have been unsuccessful at lessening the mortality of ATC patients.

It is currently thought that ATC represents a terminal dedifferentiation of a preexisting differentiated carcinoma. Mutation of the *p53* gene seems to be associated with this dedifferentiation process. Molecular genetic studies have shown that *p53* is rarely mutated in well-differentiated thyroid carcinomas, occasionally mutated in poorly differentiated thyroid carcinoma, and frequently mutated in ATC. Thus, p53 mutations are thought to be a late step in thyroid tumor progression and seem to play an important role in the dedifferentiation of thyroid carcinomas to ATC (9–13). In addition, p53 mutations are thought to be associated with reduced chemosensitivity and radiosensitivity of cancer cells. Therefore, understanding how mt p53 affects the response of ATC cells to treatment is important for utilization of mt p53 as a target for novel cancer therapies. However, much less is currently known about the types of p53 mutations that are present in ATC tumor cells. Furthermore, the roles of these mutations in ATC tumor cells remain to be elucidated.

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The present study identified a missense mutation (G199V) in KAT-18 cell line that was established from primary cultures of ATC. We further examined whether the mt p53 exhibits anti-apoptotic function in ATC cells. This study shows that mt p53 (G199V) gains an antiapoptotic function through the signal transducer and activator of transcription-3 (STAT3) pathway.

Results

Knockdown of p53 Reduces the Viability of KAT-18 Cells

Knockdown of p53 by p53-specific antisense (AS) oligonucleotide or small interfering RNA (siRNA) against p53 reduced the viability of KAT-18 cells in a time-dependent manner. The viability of KAT-18 cells 24 hours after p53 knockdown was ~50% of the control KAT-18 cells. Western blot analysis showed that knockdown of p53 markedly downregulated p53 protein levels in cells that exhibited reduced viability (Fig. 1).

Knockdown of p53 Exerts Antitumor Activity Similar to That with High Doses of Several Chemotherapeutic Agents

Because p53 knockdown by p53-specific AS oligonucleotide efficiently reduced the viability of these highly chemoresistant cells, we next examined at which dose chemotherapeutic agents were able to similarly reduce cell viability. Our data show that p53 knockdown had a growth inhibitory effect equivalent to that of treatment with high doses of several chemotherapeutic agents in KAT-18 cells (Fig. 2).

p53 Mutation Analysis Identifies One Missense Mutation in KAT-18 Cells

To analyze the genetic status of the p53 gene in KAT-18 cells, we used direct sequencing to analyze the DNA sequence at this locus. A transcript derived from 11 exons in the TP53 gene was 2,513 bp in length (GenBank accession no. FJ207420). Database analysis revealed that the p53 cDNA sequence had high similarity with reported p53 cDNA sequences. However, we found one missense mutation in exon 5, resulting in substitution of glycine by valine at amino acid position 199 (G199V) in the p53 protein. Sequence analysis of the cloned open reading frame (ORF) cDNA obtained from the KAT-18 cell line confirmed that this mutation resulted in the substitution of glycine by valine at amino acid position 199 (G199V). A search of the Genbank database for the TP53 ORF mutation revealed that the G199V mutation of TP53 has been reported in sporadic cancers (GenBank accession no. P04637); however, detailed information about this variant was not found in the literature.

Knockdown of p53 Reduces the Viability of KAT-18 Cells by Inducing Apoptosis

The observed reduction in cell viability of KAT-18 cells following p53 knockdown suggests that mt p53 (G199V) may have a certain activity in these tumor cells. Thus, we hypothesized that this p53 mutation may confer a GOF activity. Most of the studies conducted thus far on GOF activity of mt p53 proteins have been done by overexpression of exogenous mutant proteins. However, the concept that mt p53 proteins are oncogenic has been questioned in part because of the artificial systems used (14). A few studies were done in a more phys-

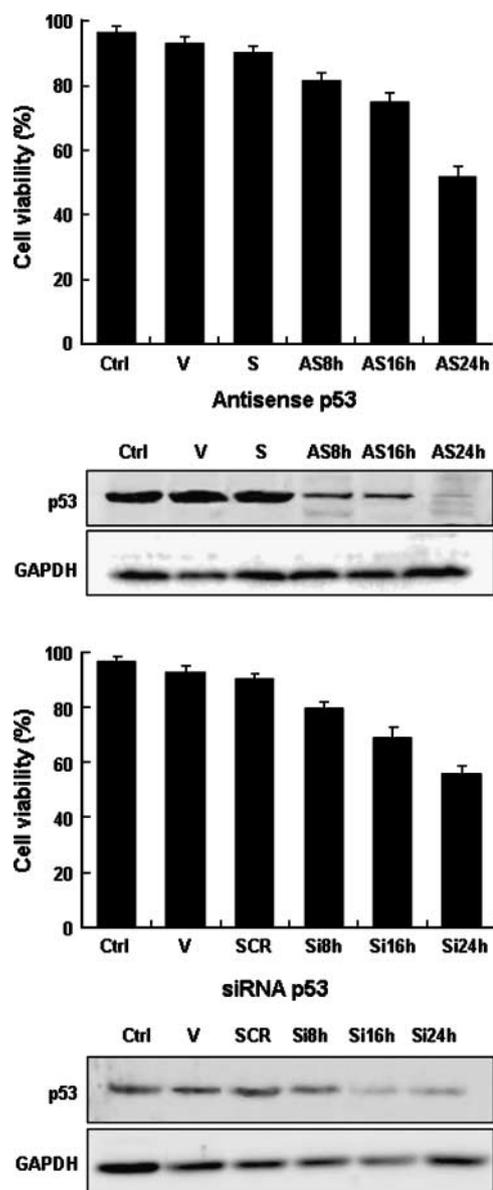


FIGURE 1. Knockdown of p53 reduces the viability of KAT-18 cells. Ctrl, untreated control; V, vehicle; S, sense p53; AS, antisense p53; Si, siRNA against p53; SCR, scrambled control. Knockdown of p53 by either p53-specific AS oligonucleotide or siRNA significantly reduces the viability of KAT-18 cells compared with control treatments (Ctrl, V, and S or SCR). Columns, mean ($n = 4$); bars, SD. The Western blot data below each viability assay graph were obtained from cells at 24 h after treatment and show marked downregulation of p53. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a loading control.

ologic context (e.g., by knockdown of endogenously expressed mt p53 proteins in cancer cell lines; refs. 15-17). In this study, we knocked down mt p53 in KAT-18 cells using p53-specific AS oligonucleotide to evaluate the underlying molecular mechanism of action of mt p53.

Increased cell death subsequent to p53 knockdown was detected by analysis of a number of apoptotic markers, as described below. Flow cytometry indicated that p53 knockdown induced the accumulation of apoptotic cells with subdiploid

DNA content (Fig. 3A). Hoechst staining showed that p53 knockdown induced nuclear condensation (Fig. 3B). Although ladderlike DNA fragments were not observed on agarose gels following p53 knockdown, pulsed-field gel electrophoresis (PFGE) revealed disintegration of nuclear DNA into giant fragments of 1 to 2 Mbp and high molecular weight fragments of 200 to 800 kbp (Fig. 3C), indicating the induction of stage I apoptosis (14). p53 knockdown induced degradation of procaspase-3, -8, and -12 and a caspase substrate, poly(ADP-ribose) polymerase. p53 knockdown also induced the degradation of caspase-activated DNase and the accumulation of its cleavage product. In addition, p53 knockdown led to downregulation of two antiapoptotic factors, 14-3-3 and X-chromosome linked inhibitor of apoptosis (Fig. 3D). To examine the effects of p53 knockdown on mitochondrial events, we measured the cellular metabolic activity by assessment of the mitochondrial membrane potential. Our data indicate that p53 knockdown reduced the mitochondrial membrane potential (Fig. 3E). Confocal microscopy showed that p53 knockdown induced the translocation of cytochrome *c* from the mitochondria to the cytosol (Fig. 3F). Taken together, these results suggest that mt p53 knockdown induces apoptosis of KAT-18 cells, and that mt p53 (G199V) may have an antiapoptotic function in KAT-18 cells. We next examined whether the antiapoptotic GOF activity of mt p53 (G199V) is cell selective. We transiently overexpressed *p53G199V* in a p53-null cancer cell line, HCT-116. Mt p53 (G199V), in contrast to wt p53, protected HCT-116 cells against apoptosis induced by three chemotherapeutic agents, which indicates that the gain of antiapoptotic function of this mutation is not specific for the cell line (Supplementary Fig. S1).

Microarray Analysis Shows a p53-Specific AS Oligonucleotide-Induced Modification of Apoptosis-Related Genes Expression

When compared with the untreated control KAT-18 cells (Gene Expression Omnibus accession no. GSE12912), 2,926 genes were found to have altered expression levels of 2-fold or more in cells treated with AS p53 oligonucleotide. Among those genes, 33 apoptosis pathway activators and 20 apoptosis pathway repressors were identified (Table 1).

p53 Knockdown Downregulates STAT3 and Phospho-STAT3 (Ser727 and Tyr705)

Notably, STAT3 is downregulated by p53 knockdown. Although STAT3 is known to have an antiapoptotic function, this activity has not been documented in the context of a GOF mt p53. Thus, we examined whether STAT3 plays a role in the antiapoptotic GOF activity of mt p53 (G199V). First, the gene expression of *STAT3* was examined by real-time PCR in KAT-18 cells. Real-time PCR showed that transfection of the p53-specific AS oligonucleotide led to reduced expression of *STAT3* in KAT-18 cells (Fig. 4A). In addition, the luciferase assay showed that p53-specific AS oligonucleotide reduced induction of the *STAT3* promoter (Fig. 4B). These data indicate that *STAT3* is regulated at the transcriptional level. We next examined whether AS p53 oligonucleotide altered *STAT3* protein expression and phosphorylation. Western blot assays showed that *STAT3* protein levels were lower in the presence of p53-specific AS oligonucleotide. Corresponding downregulation of Janus kinase 2, *STAT5*, and *STAT6* was also shown (Fig. 4C). Western blot analysis using phosphorylation site-specific antibodies showed that p53-specific AS oligonucleotide exposure

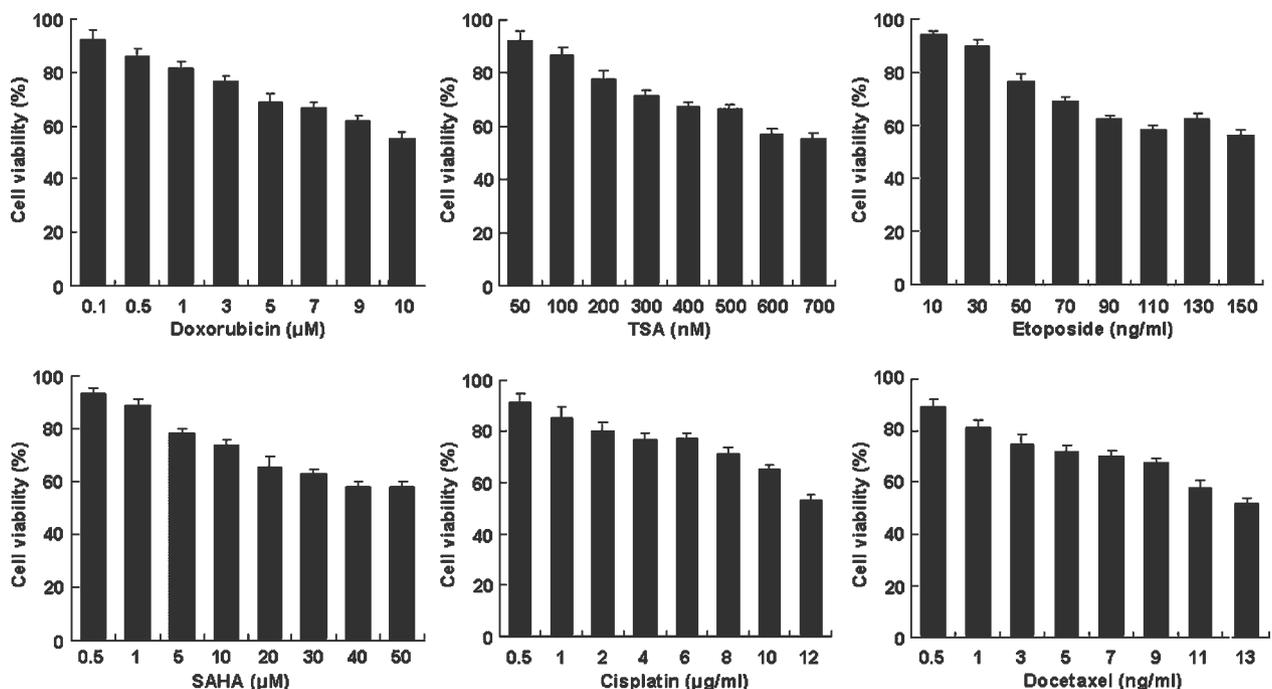


FIGURE 2. KAT-18 cells are moderately responsive to many chemotherapeutic agents. Cells were treated for 24 h with six chemotherapeutic agents at various doses and viability was determined with the Vi-Cell cell counter. Columns, mean ($n = 4$); bars, SD.

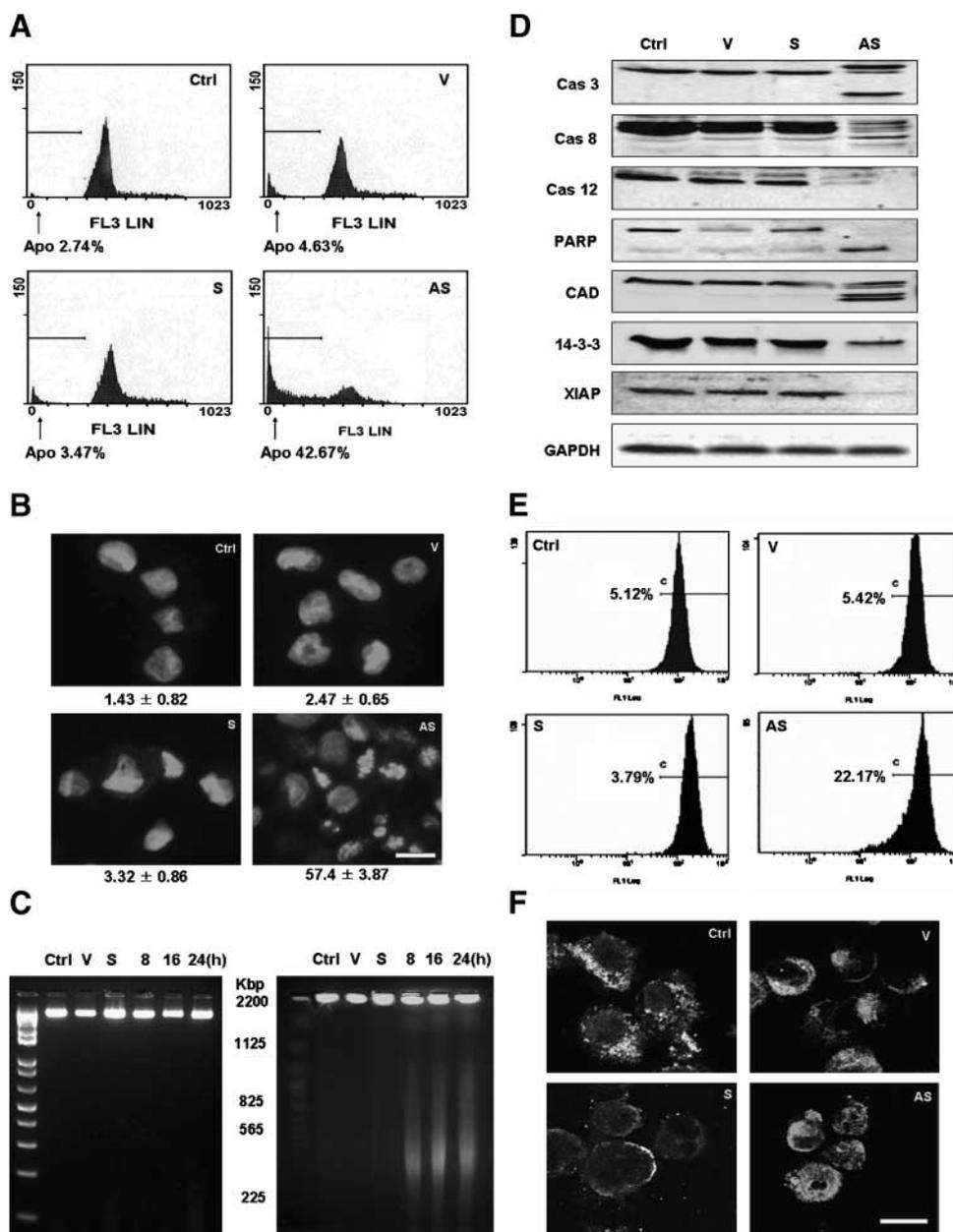


FIGURE 3. Knockdown of p53 induces apoptosis in KAT-18 cells. Bar, 10 μ m. **A.** Representative histograms showing cell cycle progression and induction of apoptosis. The apoptotic population was significantly increased in KAT-18 cells treated with p53-specific AS oligonucleotide compared with controls (*Ctrl*, *V*, and *S*). **B.** Hoechst staining of cells harvested 24 h after p53-specific AS oligonucleotide treatment. Numbers represent the percentage of apoptotic cells presenting condensed or fragmented nuclei. Cells with condensed or fragmented nuclei were significantly increased in KAT-18 cells treated with p53-specific AS oligonucleotide compared with controls (*Ctrl*, *V*, and *S*). **C.** DNA electrophoresis (*left*) and PFGE (*right*). Although p53-specific AS oligonucleotide-treated cells did not show ladderlike DNA fragments on agarose gel, PFGE revealed disintegration of nuclear DNA into giant fragments of 1 to 2 Mbp and high molecular weight fragments of 200 to 800 kbp. **D.** Western blot assay for representative apoptosis-related proteins. p53-specific AS oligonucleotide treatment induced the degradation of procaspase-3, -8, and -12, poly(ADP-ribose) polymerase (*PARP*), and caspase-activated DNase (*CAD*) and the production of their cleavage products. p53 knockdown led to downregulation of 14-3-3 and X-chromosome linked inhibitor of apoptosis (*XIAP*) expression. GAPDH is shown as a loading control. **E.** Flow cytometry showing reduction of the mitochondrial membrane potential (*MMP*) in cells undergoing apoptosis. Mitochondrial retention of DiOC₆(3) decreased in KAT-18 cells treated with p53-specific AS oligonucleotide. The percentage of cells with decreased staining is indicated in the center of each graph. **F.** p53 knockdown induced the translocation of cytochrome *c* from mitochondria to cytosol. Cas, caspase. See Fig. 1 for other definitions.

resulted in reduced phosphorylation of STAT3 at Ser727 and Tyr705. The cellular activation of signaling ELISA assay also showed that p53-specific AS oligonucleotide treatment down-regulated phosphorylated STAT3 in KAT-18 cells (Fig. 4D). Interestingly, phosphorylated STAT3 seems to be affected by p53

depletion even stronger than STAT3, which indicates that STAT3 is further regulated at the posttranscriptional level. Confocal microscopy showed that STAT3 and phospho-STAT3 (Ser727 and Tyr705) proteins, which were distributed in the cytosol and the nucleus in control KAT-18 cells, were markedly

depleted in cells treated with p53-specific AS oligonucleotide (Fig. 4E). To this end, we examined whether the p53-specific AS oligonucleotide decreases STAT3 protein in other cells not carrying mt p53 (G199V). Our data show that p53-specific AS oligonucleotide does not decrease STAT3 protein in HT-29, HL-60, and ARPE-19 cells (Supplementary Fig. S2).

Mt p53 (G199V) Gains Antiapoptotic Function through STAT3

We next asked whether STAT3 is required for p53-specific AS oligonucleotide-induced apoptosis in KAT-18 cells. To answer this question, we first examined the effect of a STAT3 inhibitor on the induction of apoptosis by p53-specific AS oligonucleotide in KAT-18 cells. Because STAT3 inhibitor (1.5 mmol/L) slightly reduced the viability of KAT-18 cells, we assayed the effect of cotreatment with both STAT3 inhibitor (1.5 mmol/L) and p53-specific AS oligonucleotide on the induction of apoptosis in KAT-18 cells. We found that STAT3 inhibitor modestly augmented cell death in combination with p53 knockdown (Fig. 5A; Supplementary Fig. S3A). We next asked whether interleukin-6 (IL-6), which is known to activate STAT3, protected KAT-18 cells against apoptosis. Our data show that IL-6 treatment significantly alleviated the induction of apoptosis caused by p53 knockdown. Conversely, IFN- γ , which is known to regulate STAT3 activity in certain contexts, did not significantly alter the induction of apoptosis in p53-specific AS oligonucleotide-treated cells (Fig. 5B and C; Supplementary Fig. S3B and C). Importantly, overexpression

of STAT3 alleviated the induction of apoptosis in KAT-18 cells caused by p53 knockdown (Fig. 5D). To corroborate that mt p53 (G199V) is involved in the upregulation of STAT3, we examined the effect of the transient transfection of mt p53 (G199V) in ARPE-19 cells. Noticeably, the transient transfection of mt p53 (G199V) upregulated STAT3 in ARPE-19 cells (Fig. 5E).

Discussion

Numerous studies have shown that the introduction of p53 mutants into cells lacking endogenous p53 results in increased oncogenicity. A variety of mutant human p53 alleles (p53-Ala143, p53-His175, p53-Trp248, p53-His273, and p53-Gly281) have been found to enhance tumorigenic potential, as determined by increased tumorigenicity when injected into nude mice (3, 4). In addition, several mutants (p53-His175, p53-Gln213, and p53-Gln248) have been shown to enhance metastatic capacity when injected into severe combined immunodeficiency mice, whereas other mutants (p53-Cys273 and p53-His234) had no effect on metastatic capacity in this same model (18). Furthermore, transgenic mice designed to express murine p53-His172 exhibit increased susceptibility to chemical carcinogenesis (19).

The molecular mechanisms that underlie the GOF activities of mt p53 are highly complex. Reports thus far indicate that the GOF activity is linked to the ability of mt p53 to modulate the expression of several genes, such as *MDR-1* (4, 20), *c-myc* (21),

Table 1. AS p53 Oligonucleotide-Induced Modification of Expression of Apoptosis-Related Genes

Activated Gene	Fold Change (AS*/control)	Repressed Gene	Fold Change (AS/control)
<i>SERPINB2</i>	9.7805	<i>TNFSF10</i>	-9.4317
<i>SPATA3</i>	4.4796	<i>TP53</i>	-9.063
<i>SPHK1</i>	4.1911	<i>DAPK1</i>	-6.1304
<i>PPP1R15A</i>	3.7702	<i>TNFRSF1A</i>	-5.5697
<i>THAP1</i>	3.4045	<i>BNIP3</i>	-5.1213
<i>IL6</i>	3.2589	<i>TNFRSF9</i>	-5.0876
<i>BAG1</i>	3.1076	<i>SCARB1</i>	-4.8935
<i>FLJ39616</i>	3.0146	<i>GFRA1</i>	-4.1561
<i>MOAP1</i>	2.9053	<i>STK25</i>	-3.3605
<i>TNFRSF10D</i>	2.8171	<i>FXR2</i>	-3.1267
<i>KIRREL2</i>	2.4332	<i>BNIP3L</i>	-3.0589
<i>MST4</i>	2.2512	<i>STAT6</i>	-3.0511
<i>CARD6</i>	2.2496	<i>MAGED1</i>	-2.9045
<i>SGKL</i>	2.2042	<i>AVEN</i>	-2.6139
<i>BCL2L11</i>	2.1961	<i>DAP</i>	-2.5864
<i>WWOX</i>	2.1883	<i>STAT5B</i>	-2.5504
<i>GADD45A</i>	2.1771	<i>ARHGEF1</i>	-2.5022
<i>GFRA3</i>	2.1092	<i>NALP1</i>	-2.4995
<i>BCL2A1</i>	2.1031	<i>PDCD6</i>	-2.357
<i>HSPC159</i>	2.0089	<i>STAT3</i>	-2.342
		<i>TRADD</i>	-2.3269
		<i>CAMKK2</i>	-2.3087
		<i>BCL2L1</i>	-2.2924
		<i>MLLT7</i>	-2.2343
		<i>CLU</i>	-2.2199
		<i>RAI</i>	-2.1889
		<i>MAGED2</i>	-2.1317
		<i>TRAF5</i>	-2.117
		<i>C20orf158;DATF1</i>	-2.1089
		<i>BOK</i>	-2.0966
		<i>MGC4171;MAPK3</i>	-2.0909
		<i>SCARB2</i>	-2.0811
		<i>APG5L</i>	-2.0409

*AS, p53-specific AS oligonucleotide treatment.

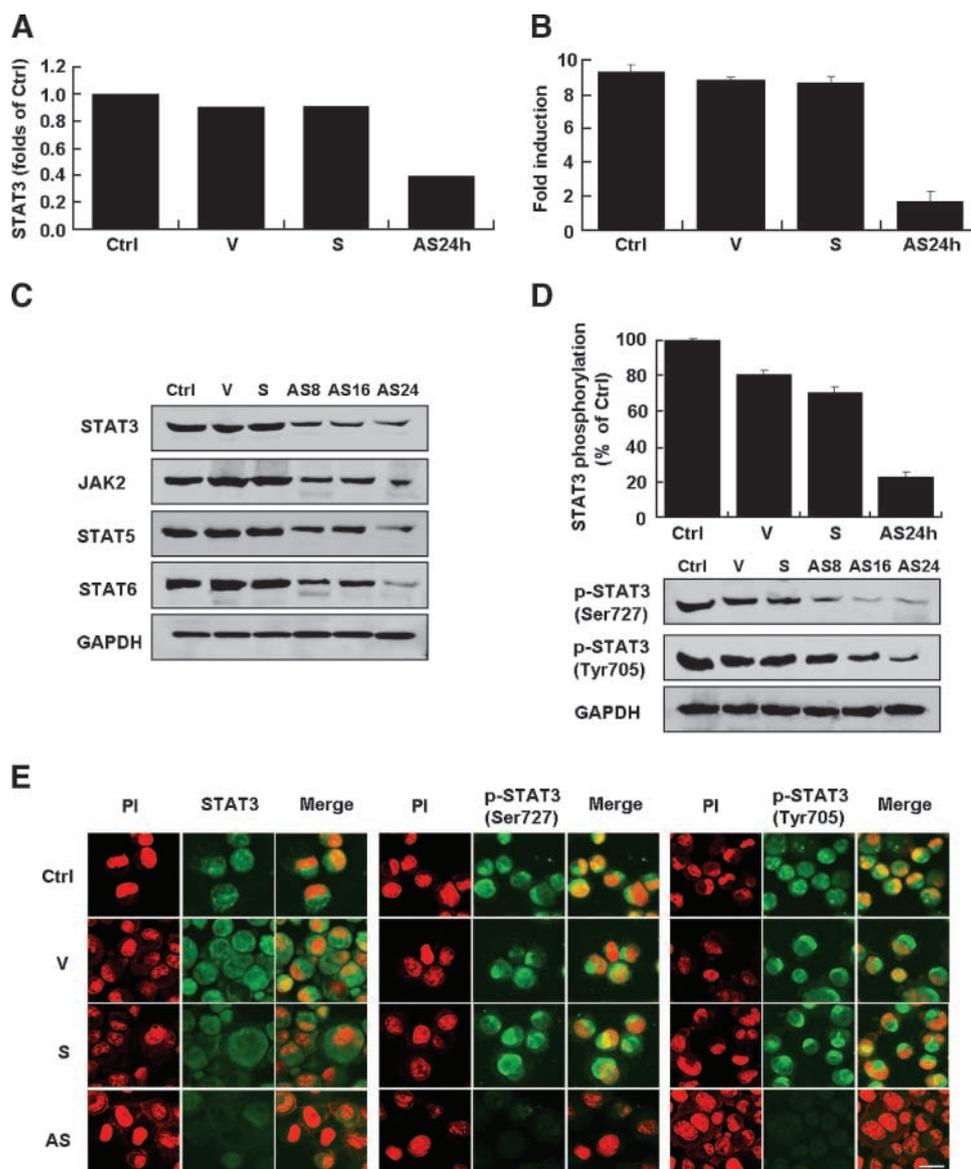


FIGURE 4. p53-specific AS oligonucleotide treatment led to reduced levels of STAT3 and phospho-STAT3 (Ser727 and Tyr705). **A.** Real-time PCR showing that expression of STAT3 was reduced in KAT-18 cells treated with p53-specific AS oligonucleotide compared with controls (*Ctrl*, *V*, and *S*) 24 h after treatment. Representative data. **B.** Luciferase assay showing that p53 knockdown significantly reduced the induction of the *STAT3* promoter compared with controls (*Ctrl*, *V*, and *S*) 24 h after treatment. **C.** Western blot assay showing STAT3 is downregulated in a time-dependent manner. Corresponding downregulation of Janus kinase 2 (*JAK2*), STAT5, and STAT6 are also shown. **D.** Western blot and cellular activation of signaling ELISA assays showing the alteration of phosphorylated STAT3. Western blot analysis shows that phosphorylation of STAT3 on Ser727 and Tyr705 was reduced in p53-specific AS oligonucleotide-treated cells. GAPDH is shown as a loading control. Cellular activation of signaling ELISA assay shows that p53-specific AS oligonucleotide treatment downregulated phosphorylated STAT3 in KAT-18 cells compared with controls (*Ctrl*, *V*, and *S*) at 24 h after treatment. **E.** Confocal microscopy showing that p53-specific AS oligonucleotide markedly depleted STAT3 and phospho-STAT3 (*Ser727* and *Tyr705*) at 24 h after treatment. Bar, 20 μ m. See Fig. 1 for other definitions.

the antiapoptotic gene *BAG-1* (22), *heat shock protein 70* (23), *IL-6* (24), and *human epidermal growth factor receptor* (25), as well as HIV-1 long terminal repeat promoters (26). Previous reports indicate that multiple mt p53 domains are required for the observed GOF activity. More specifically, the NH₂-terminally located transactivation domain and the COOH-terminally located sequences that overlap with the oligomerization domain and the nonspecific nucleic acid binding domain are required for the GOF activity observed for some

p53 mutants (3, 20, 27). The GOF activities of mt p53 can also be explained by interactions between mt p53 and certain proteins. A previous study showed that two variants of mt p53 (p53His175 and p53Gly281) associate *in vitro* and *in vivo* with p73 and markedly reduce its transcriptional activity (28). Another study showed that p53His273 and p53Trp248 can associate *in vitro* and *in vivo* with p63 through their core domains, and that the interaction with mt p53 also affects transcriptional activity. Other mt p53 proteins (p53His175, p53His273, and

p53His273/Ser309) have been shown to interact *in vivo* with NF-Y. Mt p53/NF-Y complexes have been reported to associate with NF-Y target promoters and recruit p300 in response to DNA damage, resulting in aberrant transactivation of NF-Y target genes and cell cycle deregulation (29). In addition, mt p53 proteins may inhibit normal p53-target gene expression. One study showed that the mt p53 R175H inhibits transcription at the p53-dependent CD95 (FAS/APO-1) promoter (30). However, whether such a phenomenon is general and affects the status of other wt p53 target genes is unclear.

STAT3 is a member of a family of transcription factor proteins that are involved in normal cellular responses to cytokines and growth factors (31). STAT3 signaling pathways are activated in response to cytokines and growth factors (32). STAT3 activation not only may provide a growth advantage and allow accumulation of tumor cells but may also confer resistance to conventional therapies that rely on apoptotic machinery to eliminate tumor cells (33). A growing number of tumor-derived

cell lines as well as tumor specimens from human cancers are reported to express constitutively activated STAT3 protein (34). STAT3 is most likely activated by upstream activators such as Src, Janus kinase 2, and epidermal growth factor receptor in cancer cells (35, 36). Because STAT3 is known to be a promising molecular target for cancer therapy, targeting STAT3 represents a novel therapeutic approach for cancer treatment.

Although constitutive activation of STAT3 and inactivation of p53 are both commonly detected in human cancer cells, there is little information about the influence, direct or indirect, of p53 on the STAT3 pathway. Previous studies showed that wt p53, but not mt p53, reduces STAT3 phosphorylation and DNA binding activity in cancer cells that express constitutively active STAT3 (32, 37). Because most cancer cell lines expressing constitutively active STAT3 only harbor mt p53, these data indicate that the inability of mt p53 to phosphorylate STAT3 could facilitate the development of some cancers. On the other hand, mutation of p53 is often a late event in malignant progression

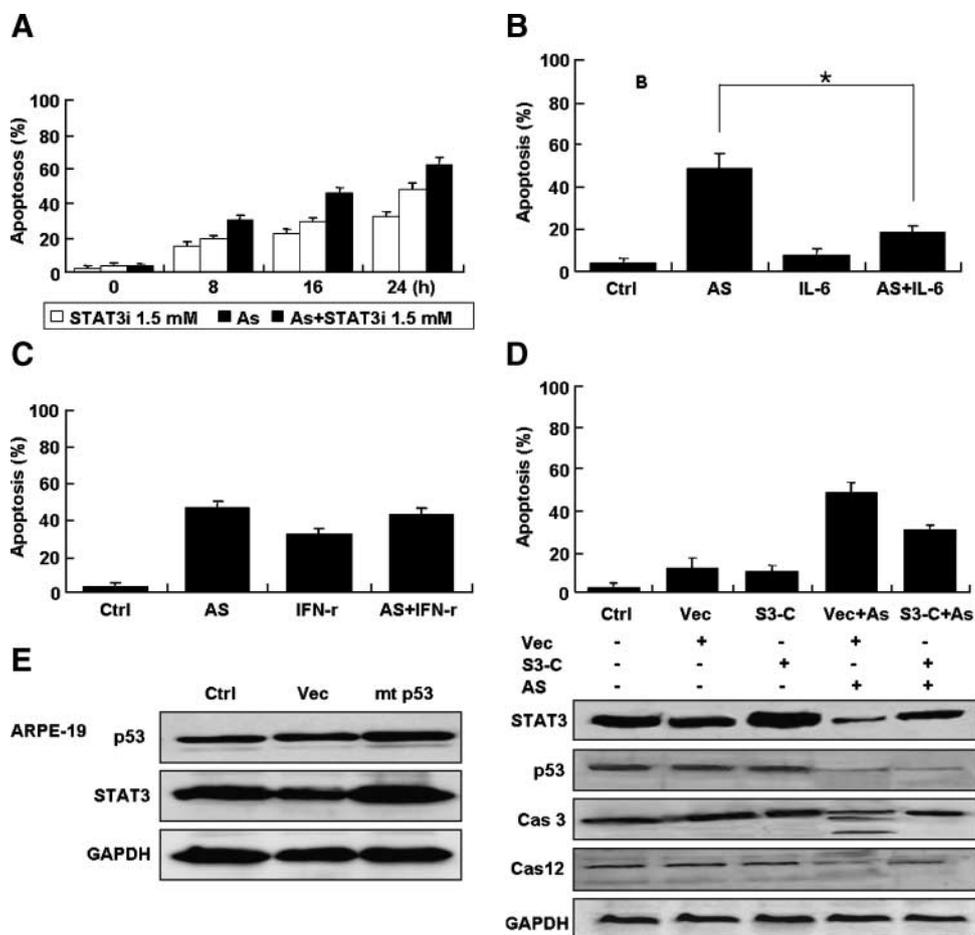


FIGURE 5. Mt p53 (G199V) gains antiapoptotic function through STAT3. Apoptosis was determined by nuclear condensation. **A.** STAT3 inhibitor (STAT3i) modestly augments the induction of apoptosis by p53 knockdown. **B.** IL-6 (20 ng/mL) significantly alleviates the induction of apoptosis by p53 knockdown at 24 h after treatment (*, $P < 0.05$). **C.** IFN- γ (30 nmol/L) does not significantly alter the induction of apoptosis by p53 knockdown at 24 h after treatment. **D.** Constitutively active STAT3 (S3-C) overexpression significantly protects KAT-18 cells against apoptosis induced by p53 knockdown at 24 h after treatment. Western blot analysis shows that the STAT3 protein level is maintained in KAT-18 cells that overexpress constitutively active STAT3, even in the case of p53-specific AS oligonucleotide treatment. **E.** Western blot assay showing that the transient transfection of pcDNA-mt p53 (G199V) plasmids induces upregulation of STAT3 in ARPE-19 cells, compared with control cells (Ctrl) or pcDNA empty vector-transfected cells (Vec). GAPDH is shown as a loading control. See Fig. 1 for other definitions.

and many clinically detectable cancers without p53 mutations exhibit reduced p53 expression. Thus, either blocking of p53 activity or silencing of p53 expression is likely to have an important role in the initiation and progression of tumorigenesis. A previous study showed that constitutively activated STAT3 inhibits the ability of endogenous p53 to regulate p53-responsive genes, indicating that repression of p53 by STAT3 is likely to have an important role in tumorigenesis as well (38). Irrespective of those studies on the cross talk between mt p53 and the STAT3 pathway, it has not been elucidated to date that mt p53 gains an antiapoptotic function through STAT3.

The present study shows that mt p53 (G199V) gains antiapoptotic function through STAT3 in ATC cells. To our knowledge, this is the first study demonstrating cross talk between mt p53 and the STAT3 pathway. However, a wealth of information remains to be uncovered with regard to the GOF activity of mt p53 (G199V). Exactly how p53 knockdown induces the down-regulation of STAT3 in ATC cells also remains to be delineated. In addition, further studies will be able to show how this p53 mutation (G199V) contributes to cancer aggressiveness. The answers to these questions will yield important insights into the pathways through which mt p53 gains antiapoptotic function in cancer cells. In addition, we here revealed that p53 knockdown exerts antitumor activity to a level similar to that achieved with high doses of several chemotherapeutic agents. These data suggest that knockdown of mt p53 (G199V) may serve as a novel strategy for reducing the extent and severity of the toxicity of chemotherapeutic agents.

In conclusion, mt p53 (G199V) gains antiapoptotic function through STAT3. Our data suggest that inhibition of mt p53 (G199V) might be a useful therapeutic strategy for ATC with this GOF p53 mutation.

Materials and Methods

Cell Line

The ATC cell line KAT-18 was kindly provided by Dr. K.B. Ain (University of Kentucky Chandler Medical Center, Lexington, KY). Using short tandem repeat analysis, it was verified that this cell line had not been cross-contaminated.

Knockdown of p53 by Transfection with a p53 AS Oligonucleotide

KAT-18 cells inoculated into six-well plates (10^5 per well) were transiently transfected with the AS oligonucleotide (5'-CCCTGCTCCCCCTGGCTCC-3') using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Briefly, the AS oligonucleotide was mixed with Lipofectamine 2000 and then incubated with Opti-MEM. The oligonucleotide-Lipofectamine 2000 complex was added to the cells and further incubated for 4 h at 37°C. As a control, the same concentration of Lipofectamine 2000 without oligonucleotide or Lipofectamine 2000 with sense oligonucleotide (5'-GGAGC-CAGGGGGGAGCAGGG-3') was added to the cells. After incubation, the medium was replaced with RPMI 1640 and cells were maintained for additional 8, 16, and 24 h. To examine the effects of a pharmacologic reagent, STAT3 inhibitor, and cytokines such as IL-6 and IFN- γ on AS p53 treatment, cells were cultured for 4 h in the presence or absence of the indicated reagents and transfected with AS p53 as described above.

Silencing of the p53 Gene

The Silencer Validated siRNA, which contains siRNA specific to the p53 mRNA (5'-GGAAAUUUGCGUGUGGA-GUtt), was purchased from Ambion. As a negative control, the same nucleotides were scrambled to form a nongenic combination. Transfection of siRNA was done by using si-PORT Amine and Opti-MEM according to the manufacturer's recommendations. Cells grown to 40% to 50% confluence in six-well plates were transfected with siRNA (1 nmol/L final concentration) per well. Transfection mixture was added to each well and incubated for 4 h. After siRNA transfection, the medium was replaced with RPMI 1640 and lysates were collected 24 h later.

Genetic Sequence Analysis of p53 in KAT-18 Cell Line

A sequence analysis of more than 7,200 bp of p53, including introns as well as all exons, was done using genomic DNA isolated from KAT-18 cells. The DNA sequence analyses were done at the Core Genotyping Facility of Macrogen (Seoul, South Korea). Sequence primers specific for each intron/exon are listed (Supplementary Table S1). Genomic DNA was amplified by PCR, and the resulting PCR products were submitted to DNA sequencing. The cycle sequencing reactions were carried out using an ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). DNA sequence analysis was done using an ABI PRISM 3730XL Analyzer (Applied Biosystems).

ORF cDNA Sequence of p53 in KAT-18 Cell Line

To identify p53 ORF cDNA mutants in KAT-18 cells, cDNA preparations were prepared from 2.0 μ g of total RNA by reverse transcription using the Superscript First Strand Synthesis Kit (Invitrogen). The resultant cDNA was then used as template for reverse transcription-PCR (RT-PCR). The used PCR primers specific for the p53 ORF are as follows: p53 ORF forward, 5'-ATGGAGGAGCCGAGTCAGAT-3', and p53 ORF reverse, 5'-GTCTGAGTCAGGCCCTTCTGTCTT-3'. For PCR, 25- μ L reaction mixtures were used, consisting of 2 μ L cDNA, 0.2 mmol/L deoxynucleotide triphosphates, 1.5 mmol/L MgCl₂, 0.25 μ mol/L gene-specific forward and reverse primers, and 2.5 units Platinum Taq DNA polymerase (Invitrogen). Reaction mixtures were heated to 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final cycle of 72°C for 5 min in a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems). Amplified products were separated on a 1.5% agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen). Gel-purified PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen) and individual clones were sequenced using standard methods.

Microarray Procedure and Data Analysis

Five micrograms of total RNA collected from wt KAT-18 cells or KAT-18 cells 24 h after p53-specific AS treatment served as the mRNA sources for microarray analysis. RNA extraction was undertaken with a Qiagen Rneasy Micro Kit (Qiagen). The collected RNA was quantified by measuring absorbance at 260 nm, and RNA purity was determined by the ratios $A_{260 \text{ nm}}/A_{280 \text{ nm}}$ and $A_{230 \text{ nm}}/A_{280 \text{ nm}}$. The RNA integrity was assessed by electrophoresis using the Agilent 2100

bioanalyzer (Agilent Technologies). The control and AS-treated samples were hybridized to the Applied Biosystems 1700 human chip that screened for more than 30,000 transcripts to show alterations in KAT-18 mRNA expression. The data for array were scanned on an Applied Biosystems 1700 Chemiluminescent Microarray Analyzer (version 1.1.0). Differences in microarray intensities were normalized and grouped using the Avadis Prophetic 3.3 version (Strand Genomics Pvt. Ltd.). Alterations in biological processes were analyzed with PANTHER. Representative experimental data among experiments repeated three times were shown.

Quantitative RT-PCR

For RT-PCR of STAT3 mRNA, total RNA was extracted from KAT-18 cells using the RNeasy Plus Mini Kit (Qiagen) following the manufacturer's instructions. Reverse transcription reactions were done with the iScript cDNA Synthesis Kit for RT-PCR (Bio-Rad) using 1 µg of fractionated cellular RNA, which was purified as described above, as template. Real-time PCR was carried out using TaqMan gene expression presynthesized reagents and iTaq Supermix with ROX (Bio-Rad). Reactions were prepared following the manufacturer's protocol. All reactions were carried out in triplicate (Bio-Rad). Specific primers were used to detect the presence of the STAT3 mRNA. Standard thermal cycling conditions included a hot start of 2 min at 50°C and 10 min at 95°C. The DNA was amplified through 50 cycles of 15 s at 95°C and 1 min at 60°C for STAT3 and β-actin, respectively. Data analysis was carried out using MJ Opticon Monotor 3 software (version 3.1) and Microsoft Excel. Expression values are presented relative to the measurements for β-actin values in the corresponding samples.

A further description of the cell viability assay, flow cytometric analysis, nuclear morphology analysis of apoptosis, DNA electrophoresis, PFGE, Western blot analysis, assay of mitochondrial membrane potential, immunofluorescence staining and confocal microscopy, luciferase reporter assay, cell-based STAT3 phosphorylation analysis, STAT3 overexpression, cDNA cloning of mt p53 and transient transfection, and statistical analysis is presented in Supplementary Appendix S1 (14, 15).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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