Loss of Imprinting and Elevated Expression of Wild-Type p73 in Human Gastric Adenocarcinoma

Min-Ju Kang, Bum-Joon Park, Do-Sun Byun, Jae-II Park, Hyo-Jong Kim, Jae-Hoon Park, and Sung-Gil Chi

INTRODUCTION

Although gastric cancer is one of the most common malignancies worldwide, the pathogenesis of this disease and the molecular genetic events that contribute to its development are poorly understood (1). Recently, the first homologue of p53, termed p73, has been cloned at 1p36 (2). p73 shares remarkable sequence identity to the DNA-binding, transactivation, and oligomerization domains of p53. Transient overexpression of p73 induces Go cell cycle arrest and apoptosis in a p53-like manner and activates the transcription of p53-responsive genes, such as p21\textsuperscript{Waf1} (3, 4). In addition, p73 is expressed monoallelically in neuroblastoma cell lines and tumor specimens, as well as in normal peripheral blood, supporting the notion that p73 is paternally imprinted with regard to the discrete deletions in neuroblastomas (2).

Despite a striking similarity to p53, p73 is expressed at low levels in all normal tissues and is not induced by UV irradiation or actinomycin D, which is known to induce p53, and differently regulates cellular p53 target genes (3, 4). In addition, p73 inactivation is not required for virus-induced tumor development, and none of the p53-inactivating viral oncoproteins, such as adenovirus E1B 55K, SV40 T antigen, and human papillomavirus E6, destabilize p73 (5, 6). Recent studies demonstrated that the tyrosine kinase c-Abl phosphorylates p73 and stimulates p73-mediated transactivation and mismatch repair-dependent apoptosis (7–9). The ability of c-Abl to phosphorylate p73 is markedly increased by γ-irradiation or cisplatin, suggesting that p73 participates in the apoptotic response to DNA damage through a c-Abl-dependent mechanism. Together, these studies indicate that p53 and p73 are not functionally equivalent and involved in distinct cellular pathways.

The observation that p73 is monoallelically expressed by imprinting raised the interesting possibility that functional inactivation of p73 would require only a single event leading to preferential loss or mutation of the expressed allele (2). However, mutations of p73 have been found to be extremely rare in primary human cancers, including tumors showing loss of heterozygosity (4) at 1p36 (10–12). Furthermore, biallelic overexpression of p73 has been frequently observed in diverse human tumors, including lung, prostate, and kidney cancer, with the transcriptional activation of the silent allele (13–16). These observations suggest that p73 is not a tumor suppressor gene to fit a two-hit model of tumorigenesis or is not the relevant target of 1p36 deletions.

To investigate the penetrance of p73 in gastric carcinogenesis, we analyzed expression level, allele-specific expression, and mutational alteration of p73 in 75 tissues and five cell lines. Here, we demonstrate that p73 is not a target of genetic alteration in gastric carcinogenesis and also show that wild-type p73 is frequently overexpressed in carcinoma tissues by the transcriptional induction of an active allele and/or the activation of a silent allele.
MATERIALS AND METHODS

Gastric Tissues, Cell Lines, and 5 Aza-dC4 Treatment.
A total of 75 gastric tissues (including 39 adenocarcinomas, 3 adenomas, 6 hamartomas, 7 hyperplastic polyps, and 20 noncancerous tissues) were obtained from 39 gastric cancer patients and 16 noncancer patients by surgical resection in the Kyung Hee University Medical Center (Seoul, Korea). Tissue specimens were snap-frozen in liquid N2 and stored at −70°C until used. Five gastric carcinoma cell lines (SNU-1, SNU-5, SNU-16, AGS, and KATO-III) were obtained from Korea Cell Line Bank (Seoul National University, Seoul, Korea) or American Type Culture Collection (Manassas, VA). Five human cell lines, derived from neuroblastoma (IMR32 and SK-N-SH), breast carcinoma (MCF7), and leukemia (HL60 and U937) were included to validate the quantitative RT-PCR approach for \( p73 \) expression. To achieve demethylation, cell lines were treated with 5 Aza-dC (Sigma Chemical Co., St. Louis, MO) at a concentration of 2 \( \mu \)M for 2–5 days.

Quantitative PCR Analysis. Our PCR-based strategies and sequences of oligonucleotide primers used for quantitation of expression and genomic status of \( p73 \) were described previously (15). Briefly, expression of \( p73 \) was analyzed using primers \( p73-1 \) and \( p73-14 \), and \( GAPDH \) was used as an endogenous expression standard. For detection of alternatively spliced \( \alpha \) and \( \beta \) variants, primers \( p73-11 \) and \( p73-9 \) were used. For quantitative DNA/PCR analysis, intron-specific primers \( p73-E2S \) and \( p73-12 \) were used for amplification of the exon 2 region of the gene, and primers \( G3 \) (sense, 5'-AACCAGTTGAGATGAGCAACAGC-3') and \( G5 \) (antisense, 5'-GAGTCCTTCCAGGATACCAAAG-3') were used for amplification of the intron 5 region of \( GAPDH \). Quantitation was achieved by densitometric scanning of the ethidium bromide-stained gels, and absolute area integrations of the curves representing each specimen were compared after adjustment for \( GAPDH \). Integration and analysis were performed using Molecular Analyst software program (Bio-Rad, Hercules, CA).

Allelic Expression of \( p73 \). Allelotyping assay using a \( SstI \) polymorphism in exon 2 was performed as described previously (15). For allelotyping using a \( BsmI \) polymorphism, the exon 5 region was amplified by primers \( p73-15 \) (sense, 5'-ACTCCCGCTTGTAAGAAAC-3') and \( p73-2 \) (antisense, 5'-TGCTGAGCAGACTTCTTGCT-3') for transcripts, and \( p73-15 \) (see above) and \( p73-E5AS \) (antisense, 5'-TGCTGTCGGGATGCTGGGCAA-3') for genomic DNA. Twenty microliters of the PCR products were digested with \( BsmI \) (Boehringer Mannheim, Mannheim, Germany) overnight and resolved on a 3% agarose gel.

Nonisotopic RT-PCR-SSCP Analysis. Nonisotopic RT-PCR-SSCP analysis of the entire coding region of the \( p73 \) transcript was carried out as reported previously (15).

RESULTS

\( p73 \) Expression in Gastric Carcinoma Cell Lines. To explore the candidacy of \( p73 \) as a suppressor in gastric carcinogenesis, we initially evaluated expression levels of \( p73 \) mRNA in five gastric carcinoma cell lines (SNU-1, SNU-5, SNU-16, AGS, and KATO-III). As we reported previously, \( p73 \) expression levels in five \( p73 \)-characterized human cell lines were examined for validation of our quantitative RT-PCR approach (15). IMR32, SK-N-SH, and MCF7, which carry one, two, and three alleles of \( p73 \), respectively, were included as expression controls, and U937 and HL60 were chosen to exemplify nonexpressor (2). As shown in Fig. 1A, predicted levels of \( p73 \) expression were observed in three expressor cell lines, whereas no expression was detected in U937 and HL60, indicating that the expression level determined by our RT-PCR assay is well consistent with previously characterized genomic and expression status of the gene. Interestingly, \( p73 \) expression was not detectable in gastric cell lines, except SNU-16. To address that the absence of mRNA expression results from gene deletion, genomic level of \( p73 \) was determined by quantitative DNA/PCR. However, no significant difference was recognized in \( p73 \) gene levels among the five cell lines. Allelotyping...
p73

Analysis of the gene also demonstrated that AGS is heterozygous for Syl and BanI polymorphisms and SNU-1 and KATO-III are heterozygous for a BanI polymorphism (Figs. 1 and 2). Thus, our result shows that p73 mRNA is undetectable in four of the five gastric cell lines despite no evidence for an allelic deletion of the gene.

Monoallelic Expression of p73 in Normal Tissues. Previous study demonstrated that p73 expression is extremely low in normal tissues, possibly by the transcriptional silencing of one allele due to genomic imprinting (2). To define whether p73 is imprinted in the stomach, we analyzed the expression level and allelic origin of p73 transcripts in 33 noncancerous tissues including 20 normal tissues, 3 hamartomas, and 7 hyperplastic polyps. As shown in Fig. 1A, all of the noncancerous tissues showed undetectably low expression of p73. For allelotyping analysis of p73 transcripts in these tissues, exons 2 and 5 regions of the transcripts were amplified using nest-PCR approach and subjected to digestion with Syl and BanI, respectively, to define the allelic origin of the induced transcripts.

Overexpression of Wild-Type p73 in Gastric Carcinomas. Compared with noncancerous tissues, significantly increased expression of p73 was found in 37 of 39 (94.9%) carcinomas and 2 of 3 (66.7%) adenomas (Fig. 1A). Furthermore, tumor-specific overexpression was identified in 14 of 16 (87.5%) matched sets. Whereas p73a variant mRNA was clearly observed, p73b mRNA was nearly undetectable (data not shown). Among the 39 overexpressors, 21 (53.8%) were heterozygous for Syl or BanI and 5 (23.8%) of these were found to express p73 biallelically (Fig. 1B). p73 expression showed no correlation with the histopathological characteristics of the tumors. Quantitative DNA/PCR and RT-PCR-SSCP analyses showed no allelic deletion and mutations, except the previously described polymorphisms (17). Collectively, these data suggest that wild-type p73 is frequently overexpressed in gastric carcinoma tissues, possibly due to the induction of an active allele and/or the transcriptional activation of a silent allele.

Induction of p73 by Serum Deprivation. To explore the implication of hypermethylation in the transcriptional silencing of the p73 gene, we treated the four nonexpressor cell lines with the demethylating agent 5 Aza-dC (2 μM) treatment. Expression of p73 mRNA was analyzed by RT-PCR analysis. Exons 2 and 5 regions of the p73 transcripts were amplified using nest-PCR approach and digested with Syl and BanI, respectively, to define the allelic origin of the transcripts induced by 5 Aza-dC treatment.
sequence alteration of p53 was detected in 8 of 23 (34.8%) carcinomas, and 6 (75.0%) of these mutant p53-carrying specimens showed low or nearly undetectable levels of p21^{WAF1} expression. However, no association of p73 expression and the mutational status of p53 or expression levels of p21^{WAF1} were recognized in these carcinomas, as well as the five cell lines we examined (data not shown).

**DISCUSSION**

In the present study, we demonstrate that wild-type p73 is frequently overexpressed in gastric carcinoma tissues through the monoallelic induction of an active allele and/or the activation of a silent allele, suggesting that, unlike p53, p73 may not be a target of genetic alteration in gastric carcinogenesis and that overexpression of p73 rather than as tumor suppressor may contribute to the tumorigenesis. This result is consistent with recent reports of more intense expression of p73 in lung, prostate, and bladder tumors than in normal tissues and loss of genomic imprinting in renal cell carcinomas (13–16).

Genomic imprinting of p73 raises the interesting possibility that loss of the transcriptionally active allele by a single event might be sufficient to contribute to human carcinogenesis (2). In this study, allelotyping analysis revealed the monoallelic expression of p73 in normal gastric tissues and 5 Aza-dC treatment led to the transcriptional activation of a silent allele, suggesting the genomic imprinting of p73 in the stomach. However, no allelic deletion or mutation of p73 was observed and p73 expression is markedly increased in carcinomas, and biallelic expression was found in a subset of tumors. Interestingly, the transcriptional induction of an active allele was followed by the activation of a silent allele by 5 Aza-dC treatment, resulting in a significant elevation of p73. This finding suggests that the release of p73 imprinting could lead to a biallelic overexpression of p73 in tumor cells, possibly by the autoregulation of gene transcription. Collectively, these results indicate that p73 is unlikely to be a tumor suppressor gene that conforms to a two-hit model of tumorigenesis.

There are currently no genetic evidences that inactivation of p73 is required for transformation or malignant progression of human tumors, except recent reports of the epigenetic silencing of p73 in specific types of hematological malignancies, such as acute lymphoblastic leukemias or lymphomas and Burkitt’s lymphomas (18, 19). Recently, Yoshikawa et al. (17) examined the mutational alterations of p73 in 54 human cancer cell lines and found 3 lung cell lines carrying p73 mutations. However, no evidences for p73 mutations in primary lung cancers raise the possibility that the mutations occur in cell culture. Our RT-PCR-SSCP analysis also suggests that mutations in p73 might be rare in gastric cancer. In addition, no correlation of p73 expression with the mutational status of p53 or expression levels of p21^{WAF1} was recognized. Thus, our result is inconsistent with the hypothesis that disruption of normal p53 function results in compensatory or deleterious up-regulation of p73 or that overexpressed wild-type p73 may mimic mutant p53, thus acting as a dominant-negative factor in wild-type p53-carrying tumor cells (2, 14). Two of the three lung cell lines with p73 mutations have been also reported to carry p53 mutations (17).

The question then arises as to why wild-type p73 is elevated in a variety of solid tumors. Our observation of the dramatic induction of p73 by serum starvation or clump formation of cells raises the possibility that p73 overexpression is associated with unfavorable growth conditions within outgrowing tumors. Recent study also showed that p73 expression is physiological condition dependent and that monoallelic expression of p73 is not strictly maintained in tumors (20). In this context, we speculate that the physiological stresses accompanied by cancerous outgrowth of solid tumors, such as hypoxia, nutrient deprivation, or imbalances between growth-regulating signals, can trigger the transcription of p73, leading to apoptosis or growth inhibition of tumor cells. However, the absence of mutational inactivation of p73 in human tumors suggests that p73 induction may not be sufficient to suppress the malignant progression of tumors.

Taken together, we show here that wild-type p73 is frequently overexpressed in gastric carcinoma tissues, which argues that inactivation of p73 is not a target of genetic alteration in gastric carcinogenesis. Further work will be required to ascertain the biological significance of elevated p73 in the growth and apoptosis of gastric tumor cells.

**REFERENCES**

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