p73 Expression Is Associated with the Cellular Radiosensitivity in Cervical Cancer after Radiotherapy

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ABSTRACT

Apoptosis is one of the causes of cell death in cervical cancer following radiotherapy (S. S. Liu et al., Eur. J. Cancer, 37: 1104–1110, 2001). By studying the gene expression profile with cDNA apoptotic array, the p73 gene was found overexpressed in radiosensitive cervical cancers when compared with radioresistant ones. To investigate the role of the p73 gene in relation to clinical assessment of radiosensitivity in cervical cancer based on the findings of residual tumor cells in biopsy samples after completion of radiotherapy, we studied the protein expression of p73 in 59 cervical cancers after radiotherapy and 68 normal cervixes using immunohistochemistry. The expression of p73 was found to be significantly increased in cancer samples and, more importantly, in those samples sensitive to radiotherapy (P < 0.001). The overexpression of p73 actually predicted a better prognosis in cervical cancer patients (P < 0.001). To investigate the possible involvement of p73 downstream genes, the protein expressions of p21 and Bax were studied. The expression of p21, but not Bax, was found to be positively correlated with the expression of p73 (P = 0.001). Furthermore, the epigenetic regulation of p73 expression via DNA methylation was also investigated in 103 cervical cancers and 124 normals. Hypermethylation of p73 gene was observed in 38.8% of cervical cancers, and it was significantly associated with reduced or absent p73 expression (P < 0.001). Reactivation of p73 expression in two cervical cancer cell lines by demethylation treatment supported the role of methylation in the regulation of p73 expression. Our findings suggested that p73 expression was related to the radiosensitivity of cervical cancer cells and may play an important role in the regulation of cellular radiosensitivity.

INTRODUCTION

Carcinoma of the uterine cervix is the second commonest female cancer worldwide. Radiotherapy is the most effective therapy for advanced stages cervical cancer. It is equally effective as radical surgery in treating early tumors, and it reduces local recurrences after surgery for patients with high-risk features (1). Resistance of cancer to radiotherapy is one of the reasons for treatment failure. It is well known that the response of tumor to ionizing radiation varies widely, which may be explained by differences in tumor cell death-inducing effects. One cellular mechanism common to various therapeutic regimens, including radiation, is tumor cell death via apoptosis. Our previous study has demonstrated a close relationship between spontaneous apoptosis and patient survival (2). Apoptosis may thus play a role in the response of cervical cancer to radiation treatment.

Apoptosis is known to be activated or inactivated by a variety of genes. Deregulation of genes involved in the activation or execution of the apoptotic process may lead to radioresistance in cells. In our pilot study, using cDNA expression array, the expressions of human apoptotic genes in radiosensitive and radioresistant cervical cancers were profiled. Different patterns of gene expression were observed between these two groups of cancers. Among the differentially expressed genes identified, p73 mRNA expression was found to be decreased or even absent in radioresistant cervical cancers when compared with radiosensitive cancers. This difference in p73 gene expression has led to a further study based on: (a) cervical cancer is often associated with human papillomavirus (HPV), its oncoproteins E6 and E7 disrupt the functions of tumor suppressor genes such as p53 and Rb, resulting in genomic instability and development of additional genetic alteration (3–5); (b) p73 is a member of p53 tumor suppressor gene family with substantial structural and functional homology (6). It activates the transcription of p53-responsive genes and induces apoptosis in a p53-like manner (7). Because p53 function is impaired in cervical cancer, it will be interesting to know whether p73, if its function is intact in cervical cancer, can compensate the impaired p53 function to trigger p53-independent apoptosis or cell cycle effectors of cancer cells in response to radiation. To confirm the pilot findings and to further investigate the relationship between p73 expression and radiosensitivity, the present study was conducted in a large group of cervical cancer patients who had undergone radiotherapy.

p73 shares a remarkable homology in DNA sequence and protein structure with p53 (6). It maps to chromosome 1p36, a region that is frequently deleted in many types of human tumors. Therefore, it has been postulated to be a new candidate tumor
suppressor gene. The p73 gene encodes two different proteins that are expressed under the control of two independent promoters and that have opposite activities: the transcriptionally active full-length TAp73 and the NH2-terminally truncated dominant-negative ΔNp73 (8). TAp73 has been reported to be involved in the cellular response to DNA damage induced by radiation and chemotherapeutic agents (9–11). It could, when overexpressed in cells, activate the transcription of p53-responsive genes such as p21, Bax, Mdm2, and GADD45 and inhibit cell growth in a p53-like manner by inducing apoptosis (7, 12, 13). In contrast to p53, however, mutation of p73 was rarely found in most human cancers (14–17).

The transcription of p73 can be regulated by the promoter and the exon 1, which is rich in CpG dinucleotides (18). Methylation of the cytosine residues at the CpG dinucleotides within this region plays an important role in inactivating the gene expression because this epigenetic modification has been commonly observed in many tumor suppressor genes in various human cancers. Transcriptional silencing of the p73 gene through methylation has been demonstrated in leukemias, lymphomas, brain tumors, and ovarian cell lines (19–25) but not in breast, renal, and colon cancers (19).

The aim of this study was to investigate the relationship between the p73 gene expression and the radiosensitivity of cervical cancer cells. We assessed the p73 protein expression in cervical carcinomas with different degrees of radiosensitivity to determine whether the expression of the p73 gene was related to the cellular radiosensitivity. In addition, with the notion that p73 can activate some p53-responsive genes, expressions of p21 and Bax were also studied and correlated with p73 expression. Furthermore, the mechanism underlying the regulation of p73 gene expression was investigated.

MATERIALS AND METHODS

Cervical Tissue Specimens. A total of 115 patients with cervical carcinomas was recruited at the Department of Obstetrics and Gynaecology, Queen Mary Hospital, the University of Hong Kong. All patients were treated with radiotherapy, and some had additional treatment of chemotherapy and/or surgery. The average age of patients was 53.8 years (ranged from 29 to 84 years). The stage of carcinoma was diagnosed according to the criteria of the International Federation of Gynaecology and Obstetrics classification (26): 52 (45.2%) early stage (I and IIa) and 63 (54.8%) advanced stage (IIb and above). Histological cell types of the tumors were squamous cell carcinomas (95 of 115), and adenocarcinoma and adenosquamous carcinomas (20 of 115). All specimens were obtained before the treatment. Histological assessment of frozen tissue section confirmed that all cancer specimens consisted of at least 70% of tumor cells. Among the 115 cancer samples, 59 of them had matched normal controls, including endometrium, myometrium, or blood lymphocytes. Normal cervical tissues from 68 patients undergoing hysterectomy for benign gynecological diseases were also included as normal controls. Their age ranged from 37 to 89 years, with a mean age of 62.5 years.

Cervical cancer samples were divided into radiosensitive and radioresistant groups based on the histological findings of residual tumor cells in the cervical biopsy specimens taken after the completion of radiotherapy. Fifty-nine samples were radioresistant, and 56 were radiosensitive.

Cell Lines and Demethylation Treatment. Four cervical cancer cell lines, C4–1, SiHa, HeLa, and Caski, were included in this study. To induce p73 expression, SiHa and Caski cell lines were treated with a demethylation reagent, 5-aza-2’-deoxycytidine (Sigma Co., St. Louis, MO) at 1.0, 3.0, and 5.0 μM for 3 days to achieve demethylation.

RNA Preparation and cDNA Array Hybridization. Total RNA was extracted from cervical tissues and cell lines using TRIzol reagent (Life Technologies, Inc.) as recommended by the manufacturer and described previously (27). Gene expression was analyzed using the Atlas human cDNA apoptosis array (Clontech Laboratories, Palo Alto, CA). The membrane was immobilized with cDNAs of 205 apoptotic genes and 9 housekeeping genes. Twelve cervical cancers and 5 normal cervical samples were recruited for gene profiling. The procedures and gene expression analysis were performed as described previously (27).

Reverse Transcription-PCR. The semiquantitative reverse transcription-PCR was used to confirm the differential gene expression identified in cDNA array study and to assess expression levels of p73 in cell lines. Three μg of total RNA were reverse transcribed with oligo-dT and Moloney murine leukemia virus reverse transcriptase according to manufacturer’s protocol (SuperScript first-stand synthesis system for reverse transcription-PCR; Life Technologies, Inc.). The cDNA was then amplified by p73-specific primers flanking from exon 1 to 3 and normalized with internal control glyceraldehyde-3-phosphate dehydrogenase (Table 1).

Western Blot Analysis. Protein was extracted from tissues and cell lines using radioimmunoprecipitation assay buffer as described previously (28). Western blot was performed with 50 μg of protein and the polyclonal antibody p73 (raised against the p73 α and β isoforms, Ab6, Neomarkers; Lab Vision Corporation, Fremont, CA) in a dilution of 1:250, and then visualized with the ECL chemiluminescence detection kit (enhanced chemiluminescence; Amersham, Arlington Heights, IL) and autoradiography. The membranes were also reprobed with β-actin (Sigma) after stripping.

Immunohistochemistry. Three-step streptavidin-biotin complex immunohistochemical assays were performed for detection of p73, p21, and Bax expressions as described previously (2). Antigen retrieval was performed by microwave pretreatment in 0.01 m citrate buffer for 9 min (p73 and p21) or 12 min (Bax). Antibodies of p73 (Ab6, Neomarkers), p21 (Ab1; Calbiochem, Boston, MA) and Bax (Ab1, Neomarkers) were diluted in 1:50, 1:30, and 1:50, respectively. Positive and negative controls were included in each experiment.

Sections were examined at high power (×400), and 10 fields were chosen randomly. The nuclear positivity of p73 staining was assessed quantitatively, and the cytoplasmic staining was excluded as p73 is a nuclear protein. In each case, the immunoreactivity of p73 and p21 was estimated by scoring the percentage of positive nuclei. The cytoplasmic staining of Bax was scored with respect to the sum of the values of both
Table 1  Primer sequences and PCR conditions used in reverse transcription-PCR, methylation-specific PCR and human papillomavirus (HPV) typing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5'-3')</th>
<th>Annealing temperature (°C)</th>
<th>PCR cycles</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p73</td>
<td>ACGGCTGCCCCAACCAGCAGG</td>
<td>53</td>
<td>33</td>
<td>231</td>
</tr>
<tr>
<td></td>
<td>GCGGTTACATGCCCCCTACA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>CACCATCCTCAAGGAGCAGG</td>
<td>60</td>
<td>20</td>
<td>372</td>
</tr>
<tr>
<td>p73 methylation</td>
<td>GACGGAGCTAAAATGAAAATGGGCCG</td>
<td>60</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>p73 unmethylation</td>
<td>AGGGGATGCTAAAATGAAAATGGGCCG</td>
<td>62</td>
<td>35</td>
<td>69</td>
</tr>
<tr>
<td>HPV-16</td>
<td>ATGCTAAAGAAGACGTAGAG</td>
<td>58</td>
<td>35</td>
<td>100</td>
</tr>
<tr>
<td>HPV-18</td>
<td>GATTTCTACACATAGACTGGG</td>
<td>58</td>
<td>35</td>
<td>127</td>
</tr>
<tr>
<td>HPV-31</td>
<td>TGTCAAGAGACCTGTGTGCC</td>
<td>55</td>
<td>35</td>
<td>232</td>
</tr>
<tr>
<td>HPV-52</td>
<td>GAGCTGTCGGGTTAATGCTC</td>
<td>55</td>
<td>35</td>
<td>231</td>
</tr>
<tr>
<td>HPV-58</td>
<td>TGTCACACACATAGACTGCC</td>
<td>55</td>
<td>35</td>
<td>244</td>
</tr>
<tr>
<td>HPV-E6</td>
<td>TGTCGACACATAGACTGCC</td>
<td>59</td>
<td>35</td>
<td>250</td>
</tr>
<tr>
<td>β-Globin</td>
<td>GAAGAGCCAAGCAGGTAC</td>
<td>56</td>
<td>30</td>
<td>268</td>
</tr>
</tbody>
</table>

percentage and intensity of the positive cells as described previously (2). Each case with positive staining in >50% of cancer cells was considered as strongly positive for statistical analysis in this study.

Sodium Bisulfite Modification of DNA and Methylation-Specific PCR. Genomic DNA was prepared from the tissue specimens and the cultured cells using conventional proteinase K digestion and phenol/chloroform extraction method. The method for sodium bisulfite modification of DNA was described previously (29). Briefly, genomic DNA (1 μg) was denatured in NaOH and treated with sodium bisulfite and hydroquinone. Sodium bisulfite converts unmethylated cytosine to uracil while leaving the methylated cytosine intact. This allows their differentiation by methylation-specific PCR.

The methylation status of the p73 gene was determined by specific primer sequences which were designed to amplify the 5′-CpG island (18, 19, 21, 22, 30). Unmethylated and methylated fragments were amplified under different PCR conditions (Table 1). Direct sequencing on bisulfite-treated tumor DNA was performed to confirm the result of the CpG methylation sites of p73 gene.

HPV Detection and Typing. DNA from 92 cervical cancer samples were examined for HPV infection. Specific primers for HPV-16, HPV-18, HPV-31, HPV-52, and HPV-58, as well as consensus primers E6 were used in PCR amplification (Table 1). The E6 consensus primers were used for screening and positive samples were additionally typed by the HPV type-specific primers.

Statistical Analysis. Statistical analysis was performed using Statistical Package for the Social Sciences 10.0 (SPSS, Inc., Chicago, IL). χ² and Kaplan-Meier tests were used for the comparison and survival analysis, respectively. P of <0.05 was considered to be statistically significant.

RESULTS

Differential Expression of p73 in Cervical Cancers of Different Radiosensitivity. Tissue samples from 12 cervical cancers (6 radiosensitive and 6 radioresistant) and 5 normal cervices were selected for apoptosis array experiment. Gene expression patterns were compared between cancers and normals, and genes that differentially expressed were identified in radiosensitive and radioresistant cancers. p73 gene showed an increased expression in radiosensitive cancers but reduced or even absent expression in radioresistant samples (Fig. 1A). This observation was then confirmed by the semiquantitative reverse transcription-PCR (Fig. 1B). Because the specific primers were flanking from exon 1 to 3 of the p73 gene, the PCR amplified product was the full-length TAp73. Subsequently, Western blot analysis also demonstrated that p73 protein expression was concomitant with the RNA expression level for the samples studied and was higher in radiosensitive cancers (Fig. 1, B and C). Such findings suggested that p73 expression might be related to radiosensitivity of cervical cancer cells. To further elucidate the potential correlation between the p73 expression and the radiosensitivity of cervical cancer cells, the study was carried out on more cervical cancer specimens from patients with known radiotherapeutic outcome.

The p73 protein expression was evaluated in 59 cervical cancers and 68 normal cervices using immunohistochemistry (Fig. 2, A and B). Majority of the cancer cases (58 of 59, 98.3%) exhibited p73 expression, whereas nearly half of the normal cervices (33 of 68, 48.5%) displayed no p73 expression (Table 2). Only 3 of 68 (4.4%) normal tissues showed strong p73 expression (>50% of normal epithelia cells showed positive p73 staining), and a significant difference (χ² test, P < 0.001) was found when compared with cancer group.

Regarding patient survival, increased p73 expression was
Expression Patterns of p21 and Bax in Cervical Cancers and Normal Cervices. To investigate the possible involvement of p73 downstream genes, the protein expressions of p21 and Bax genes were also examined in the same batch of cervical cancer and normal cervical samples by immunohistochemistry (Fig. 2C–F). All cancer samples showed positive p21 expression, but the immunoreactivity was mainly <50% (Table 2). A similar staining pattern was also found in normal cervices, whereas 26.9% of samples (18 of 67) were p21 negative. No significant difference was found between cancers and normal cervices ($\chi^2$ test, $P > 0.05$). Similar to p21, positive Bax staining was found in most of cervical cancers, and 78% of cancer samples (46 of 59) had the Bax immunoreactivity <50% (Table 2). In normal cervices, 65.7% of the cases (44 of 67) had positive Bax expression, but most of the expression was <50%, which was similar to cancer cases. Hence, there was no significant difference of Bax expression found between cancers and normal cervical tissues ($\chi^2$ test, $P > 0.05$).

p73 protein expression was compared with that of p21 and Bax in cervical cancers. Cancer samples with decreased p73 expression (<50%) were associated with decreased p21 expression ($\chi^2$ test, $P = 0.001$) (Table 4). However, no correlation was found between p73 and Bax expressions in cancers (results not shown).

Methylation-Dependent p73 Expression in Cervical Cancers and Normal Cervices. Epigenetic silencing of p73 gene via methylation of promoter and exon one CpG islands has been demonstrated in some tumors. To understand whether hypermethylation leads to reduction or absence of p73 expression in cervical cancers, the methylation status of the CpG island of p73 gene was evaluated in 103 cervical cancers and 59 matched normal controls, as well as 65 normal cervices (Fig. 4A). All cancer samples showed unmethylated band, which may be because of the contamination of normal cells. Direct sequencing of methylation-positive and -negative PCR products confirmed the methylated and unmethylated alleles in CpG island of p73 gene (Fig. 4B). It was found that p73 was frequently methylated in 38.8% of cervical cancers (40 of 103) versus 3.2% in normal controls (4 of 124), including both matched controls and normal cervixes ($\chi^2$ test, $P < 0.001$; Table 5). Consistently, p73 expression was down-regulated in most of cancer samples with p73 hypermethylation. A significant association was observed between decreased p73 expression and p73 hypermethylation ($\chi^2$ test, $P < 0.001$; Table 5). These data indicated that DNA methylation might be an important mechanism in regulating the expression of the p73 gene in cervical cancers.

In relation to radiosensitivity of cancer tissues, 58% of radioresistant tissues (29 of 50) had p73 methylation, which was significantly higher than radiosensitive tissues (11 of 53, 20.8%; $\chi^2$ test, $P < 0.001$; Table 5). p73 protein expression was down-regulated in most of cancer samples with p73 hypermethylation. p73 expression was significantly associated with a better survival of cervical cancer patients (log-rank test, $P < 0.001$; Fig. 3). More importantly, there was significantly increased p73 expression found in radiosensitive cancer samples (20 of 26, 76.9%) when compared with radioresistant samples (8 of 33, 24.2%; $\chi^2$ test, $P < 0.001$; Table 3). Taken together, p73 expression was likely to be related to radiosensitivity of cervical cancer cells.
Moreover, p73 hypermethylation was also found to correlate with the adverse outcome of the patients (log-rank test, \( P < 0.001 \); Fig. 5). Therefore, p73 hypermethylation may also carry prognostic significance in cervical cancer patients after radiotherapy.

To further strengthen our hypothesis, an in vitro study was performed on three cervical cancer cell lines. Methylated p73 gene was detected in two cervical cancer cell lines, SiHa (complete methylation) and Caski (partial methylation), and both cell lines showed absence of p73 expression. Methylated p73 was not found in C4-1 cell line, which had detectable p73 expression (Fig. 6A). This observation was concomitant with our findings in the tissue samples that methylation was correlated with reduced expression of p73. The demethylation agent, 5-aza-2’-deoxycytidine, being able to alter the CpG methylation, was used to treat SiHa and Caski cells to assess the methylation effect on p73 gene expression. After 3 days of treatment with 5-aza-2’-deoxycytidine, SiHa and Caski expressed p73 mRNA and protein levels comparable with untreated C4-1 cell (Fig. 6B). This again demonstrated that DNA hypermethylation was important for regulating the expression of p73 gene.

\( \chi^2 \) test, \( P < 0.001 \); Table 5). Moreover, p73 hypermethylation was also found to correlate with the adverse outcome of the patients (log-rank test, \( P < 0.001 \); Fig. 5). Therefore, p73 hypermethylation may also carry prognostic significance in cervical cancer patients after radiotherapy.

**DISCUSSION**

p73 is a member of the p53 tumor suppressor protein family, which is implicated in development, cellular differentiation, apoptosis, and tumor suppression (8). However, the type of stimuli inducing p73 and the mechanism of activity regulation are yet to be identified. In this study, we demonstrated the overexpression of p73 gene in cervical cancers and, more importantly, in radiosensitive cancers that had significantly higher p73 expression than radioresistant cancers. Furthermore, p73 expression also had prognostic significance in predicting the outcome of patients after radiotherapy. To our knowledge, this is the first evidence showing an association between the p73 expression and the radiosensitivity of cancer cells. These findings suggested that p73 might be important in regulating radiation response of cervical cancer cells. However, its function and the underlying mechanism need to be further elucidated.

**Table 2** Immunohistochemical analysis of p73, p21, and Bax in cervical cancers and normal cervic

<table>
<thead>
<tr>
<th>Immunohistochemistry</th>
<th>p73</th>
<th>p21</th>
<th>Bax</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor</td>
<td>Normal</td>
<td>Tumor</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 50%(^{a})</td>
<td>28 (47.5%)</td>
<td>3 (4.4%)</td>
<td>9 (15.3%)</td>
</tr>
<tr>
<td>≥ 50%(^{b})</td>
<td>30 (50.8%)</td>
<td>32 (47.1%)</td>
<td>50 (84.7%)</td>
</tr>
<tr>
<td>Negative(^{c})</td>
<td>1 (1.7%)</td>
<td>33 (48.5%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Total</td>
<td>59</td>
<td>68</td>
<td>59</td>
</tr>
</tbody>
</table>

\( \chi^2 \) test (\(^{a}\) versus \(^{b}\)), \( P < 0.001 \).

\( \chi^2 \) test (\(^{a}\)), \( P = 0.001 \).

\( \chi^2 \) test (\(^{a}\)), \( P = 0.001 \).

\( \chi^2 \) test (\(^{a}\) versus \(^{b}\)), \( P > 0.05 \) and \( P > 0.05 \), respectively.

**Table 3** p73 expression in association with p21 expression in cervical cancers\(^{a}\)

<table>
<thead>
<tr>
<th></th>
<th>≤ 50%</th>
<th>&gt; 50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>p73 &gt; 50%</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>p73 ≤ 50%</td>
<td>31</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^{a}\) \( \chi^2 \) test, \( P = 0.001 \).

\(^{a}\) IHC, immunohistochemistry.

**Table 4** p73 expression in association with p21 expression in cervical cancers\(^{a}\)

**Table 5** p73 expression in RR and RS cervical cancers\(^{a}\)

<table>
<thead>
<tr>
<th>IHC</th>
<th>RR</th>
<th>RS</th>
</tr>
</thead>
<tbody>
<tr>
<td>p73 &gt;50%</td>
<td>8 (24.2%)</td>
<td>20 (76.9%)</td>
</tr>
<tr>
<td>≤50%</td>
<td>25 (75.8%)</td>
<td>6 (23.1%)</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td>26</td>
</tr>
</tbody>
</table>

\(^{a}\) \( \chi^2 \) test, \( P = 0.001 \).

\(^{a}\) RR, radioresistant, RS, radiosensitive, IHC, immunohistochemistry.

**HPV Infection in Cervical Cancers.** High-risk HPV infections were detected in 79 of 92 (85.9%) cancer samples, and 72 of them (78.3%) were HPV type 16 or/and 18 positive, and 7 of them (7.6%) were other high-risk type, including HPV-31, HPV-52, and HPV-58. Thirteen cancer samples showed negative HPV result. No correlation between p73 expression and HPV infection was observed (\( \chi^2 \) test, \( P = 0.61 \)). Moreover, HPV status correlated neither with tumor radiosensitivity nor with patient survival (\( \chi^2 \) test, \( P = 0.56 \) and \( P = 0.98 \), respectively).
apoptosis in response to DNA damage such as radiation (6, 7, 31). However, the molecular mechanisms by which p73 triggers cell death have not been studied in great detail. There is an overall assumption that p73 adapts pathways that are almost identical to those described for p53 (8). p73 is able to drive, with different activities, the transcription of several apoptotic effectors that are also induced by p53, including p21, GADD45, Bax, NOXA, p53AIP1, and CD95 (13, 32–34). In the present study, p21 expression was detected in all cancer samples, but the expression level of them was mainly /H1102150%. An association was found between the expressions of p73 and p21. It had previously been reported that the level of p21 induced by p73 was several times less than that by p53 (13). On the other hand, Bax transactivation via p73 was still controversial (13, 35). Our results showed no significant correlation between the expressions of Bax and p73. Substantially, Bax might not be the effective downstream gene of p73 in cervical cancer. Additional studies on p73-responsive genes may shed some light on the p73 function in regulating cellular response to radiation.

Table 5  Methylation analysis of p73 gene in normal controls and cervical cancers and radioresistant and radiosensitive cancers, as well as the correlation with p73 expression in cancers

<table>
<thead>
<tr>
<th></th>
<th>Methylation (+)</th>
<th>Methylation (-)</th>
<th>Total</th>
<th>χ²</th>
</tr>
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<tbody>
<tr>
<td>Normal</td>
<td>4 (3.2%)</td>
<td>120 (96.8%)</td>
<td>124</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cancer</td>
<td>40 (38.8%)</td>
<td>63 (61.2%)</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>Radioresistant</td>
<td>29 (58.0%)</td>
<td>21 (42.0%)</td>
<td>50</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Radiosensitive</td>
<td>11 (20.8%)</td>
<td>42 (79.2%)</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>p73 &gt; 50%</td>
<td>5</td>
<td>15</td>
<td>20</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>≤ 50%</td>
<td>23</td>
<td>4</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 5 Cumulative patient survival as a function of p73 methylation stratified as methylated or unmethylated DNA.

identical to those described for p53 (8). p73 is able to drive, with different activities, the transcription of several apoptotic effectors that are also induced by p53, including p21, GADD45, Bax, NOXA, p53AIP1, and CD95 (13, 32–34). In the present study, p21 expression was detected in all cancer samples, but the expression level of them was mainly <50%. An association was found between the expressions of p73 and p21. It had previously been reported that the level of p21 induced by p73 was several times less than that by p53 (13). On the other hand, Bax transactivation via p73 was still controversial (13, 35). Our results showed no significant correlation between the expressions of Bax and p73. Substantially, Bax might not be the effective downstream gene of p73 in cervical cancer. Additional studies on p73-responsive genes may shed some light on the p73 function in regulating cellular response to radiation.

Fig. 6 Demethylation study in cancer cell lines. A, methylation-specific PCR analysis of C4-1 and pre- and post-5-aza-2’-deoxycytidine-treated SiHa and Caski cells. 5-Aza-2’-deoxycytidine concentration was applied at 1.0, 3.0, and 5.0 μM. DMSO represented the negative control for 5-aza-2’-deoxycytidine. B, detection of p73 mRNA and protein expressions in C4-1 and pre- and post-5-aza-2’-deoxycytidine-treated SiHa and Caski cells by reverse transcription-PCR and Western blot, respectively.
The similarity of p73 to p53 suggested that p73 might be a new candidate tumor suppressor gene (6). However, it has thus far not been classified as a classical two-hit tumor suppressor because its mutations or homozygous deletion is absent or very rarely found (14, 15, 36–38). It has been suggested that p73 was an imprinted gene, with loss of protein expression in cancer through selective loss of the normally expressed allele rather than an inactivating mutation. However, the fact that the p73 gene has monoallelic or biallelic expression in different tissue types (6, 39) makes it more difficult to understand the role of this gene in cancer.

In the present study, the epigenetic modification of p73 via CpG island hypermethylation represents a critical alternative mechanism for inactivation of this gene in cervical cancer. The methylation analysis showed a high incidence of p73 hypermethylation (38.8%) in cervical cancer. This hypermethylation seemed to be tumor-specific because it was rarely observed in the normal controls studied (3.2%), and it was significantly correlated with decreased or loss of p73 expression in cancers. In addition, radioresistant cancer samples had significantly higher methylation rate than radiosensitive ones. In vitro demethylation assay was successful in restoring the expression of p73 on the cervical cancer cell lines that were previously found to have methylated p73 and lack of p73 mRNA and protein expressions. Such results additionally confirmed the role of hypermethylation in silencing of p73 gene expression in cervical cancer. Epigenetic silencing of p73 gene via promoter and exon 1 hypermethylation was a common event in some leukemias, lymphomas, and brain tumors, as well as ovarian cell lines (19–25) but not in cancers of breast, renal, and colon (19). Overall findings on our methylation study suggested that hypermethylation might also be one of the important mechanisms in suppressing the p73 gene expression in cervical cancer. However, because a small proportion of the cancer samples with decreased p73 gene expression were not found to have aberrant methylation pattern, additional mechanisms and/or factors may be involved leading to loss of p73 expression.

Cervical cancer develops through a multistep process through cervical cancer precursor lesions that are known to originate after HPV infection (40). HPV infection was found in >85% of cervical cancers (41). High-risk HPV types 16 and 18 carry two major oncoproteins E6 and E7, which bind and inactivate p53 and pRb tumor suppressors for ubiquitin-mediated degradation (3–5, 42). Loss of p53 function leads to impairment in the control of cell cycle and induction of apoptosis in response to DNA damage. This makes cervical cancer unique among other cancers in that the predominant mechanism for inactivation of p53 is gene mutation. It is important to know whether HPV oncoproteins E6 and E7 could inactivate p73 function similar to p53. Some studies have found controversial results. Park et al. (43) had demonstrated that HPV E6 interacted with p73 both in vivo and in vitro and functionally inactivated p73. On the other hand, Marin et al. (44) have reported that viral oncoproteins, including adenovirus E1B, SV40 T, and HPV E6, did not interact with p73 protein. In fact, p73β induced growth inhibition and apoptosis in cancer cells that produce E6 (45). Furthermore, HPV E7 was found to transactivate p73 in cell lines and primary human keratinocytes (46). In the present study, ~86% of the cancer samples had detectable HPV infections, but no correlation was observed between the infections and p73 expression, suggesting that p73 response was unlikely to be affected by HPV oncoproteins in cervical cancer. We anticipated that the p73 gene might play a role, in p53-independent manner, in triggering apoptosis or cell cycle arrest effectors in p53-deficient cervical cancer cells in response to radiation. The possible role of p73 in modulating radiation response will be further investigated.

In conclusion, our study demonstrated a significant association between the overexpression of p73 and the cellular radiosensitivity in cervical cancers after radiotherapy. Therefore, p73 may play an important role in regulating the cellular response of cervical cancer to radiotherapy. The underlying mechanism on how p73 signaling the apoptotic pathway in the consequence of irradiation needs further investigation. Aberrant methylation was found to be an important epigenetic mechanism in contributing to the inappropriate silencing of p73 gene expression in cervical cancer. A better understanding of epigenetic mechanisms leading to the suppression of the gene, subsequently to radioresistance, may not only allow us to delineate the function of p73 in radiosensitivity of cancer cells but may also enable the development of new cancer therapeutic agents. On the clinical aspect, p73 expression appears to be a potential prognostic marker in evaluating the treatment regimens and predicting the outcome of the cervical cancer patients after radiotherapy.

ACKNOWLEDGMENTS

We thank Dr. Elly S. W. Ngan for helpful suggestions and critique of the manuscript.

REFERENCES

Correction: p73 Expression Is Associated with the Cellular Radiosensitivity in Cervical Cancer after Radiotherapy

In this article (Clin Cancer Res 2004;10:3309–16), which was published in the May 15, 2004 issue of Clinical Cancer Research (1), the author presented us with a replacement image for Fig. 6B, stating that several incorrect Western blot images were mistakenly included in the final figure. AACR Publications staff members reviewed all figures in the article and, through a posting on PubPeer, discovered potential reuse within the TP73 reverse transcription PCR (RT-PCR) results shown in Fig. 1B and methylation-specific PCR results shown in Fig. 4A. Because the original images were no longer available, the authors repeated the experiments, and the results were consistent with the published findings.

Radioresistant cancer  Radiosensitive cancer

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Figure 1B. The gel picture of p73 mRNA expressions in radioresistant and radiosensitive cervical cancers.

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p73 Methylation

Figure 4A. The gel picture of p73 methylation study.

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<tr>
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Figure 6B. The gel picture of p73 demethylation study.
Specific details of the repeated experiments are as follows: In Fig. 1B, the RT-PCR was performed on RNAs extracted from six radioresistant and six radiosensitive cervical cancer samples with the same p73 primers and PCR conditions described in the Materials and Methods section of the published article. In Fig. 4A, genomic DNA of four cervical cancer samples and normal control samples were bisulfate-treated and PCR-amplified with p73 methylation and unmethylation primers, respectively, following the procedure described in the Materials and Methods section of the published article. Because the authors had only mini-gel apparatus available to them, the PCR products had to be resolved in two 10% acrylamide gels (first gel: Pos, T1M, T1U, N1M, N1U, T2M, T2U, N2M, N2U; second gel: T3M, T3U, N3M, N3U, T4M, T4U, N4M, N4U, Neg). Thus, the corrected Fig. 4A gel image contains two gel pictures. In Fig. 6B, the authors repeated the experiments in the SiHa and Caski cell lines. Both cell lines were treated with 5-aza 2'-deoxycytidine of 1, 3, and 5 μM/L or DMSO alone following the procedure described in the Materials and Methods section of the published article. The mRNA and protein expressions of p73 were detected.

The corrected versions of Figs. 1B, 4A, and 6B are above. The results and conclusions put forth in this article remain unchanged. The authors regret these errors.

Reference

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p73 Expression Is Associated with the Cellular Radiosensitivity in Cervical Cancer after Radiotherapy

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