Reduced expression of ΔNp63α in cervical squamous cell carcinoma

Abstract

Purpose: As a member of the p53 family, p63 is considered to be an important differentiation regulation transcriptional factor, but the roles of p63 in many epithelial tumorigenesis and metastasis processes are still not clear. This study was designed to investigate the expression of p63 and its isoform in normal tissues and squamous cell cancer tissues of uterine cervix, and its significance in cancer cell differentiation.

Methods: The expression of p63 was assessed in cervical tissue and cell lines by immunohistochemistry, RT-PCR and Western Blotting. The relationships between p63 protein, various clinico-pathological features, and the differentiation marker involucrin were analyzed.

Results: ΔNp63α is the predominant isoform expressed in cervical epithelial tissues, and it is decreased in moderately or poorly differentiated cervical squamous carcinoma, as well as in the HeLa, SiHa and C33A cervical cancer cell lines. The expression level of ΔNp63α was positively correlated with that of involucrin in cervical squamous cancer tissue, and the expression of ΔNp63α is decreased with the degree of tumour invasion.

Conclusion: The decrease of ΔNp63α in cervical squamous cell cancer appears to be associated with the tumour progression, and ΔNp63α may be a sensitive marker for cervical squamous cancer differentiation.
p63, one of the p53 family members, is the crucial regulator of cell differentiation [1]. While p53 is the key guardian or “molecular police-man” of the genome, the complex and controversial subject of p63 involvement in cancer remains poorly defined. Unregulated p63 (over- or under-expression) may be detrimental to proper epithelial cell differentiation and tumour progression. Although it has been reported that p63 is over-expressed in many squamous cell carcinomas, the differentiation of these cancers in the context of p63 expression levels is not completely understood [2,3]. In addition, it is still unknown whether p63 over-expression regulates cell proliferation and apoptosis, which results in carcinoma pathogenesis.

Based on different N-terminus and C-terminus regions, p63 produces different protein isoforms, including TaP63α, TaP63β, TA p63γ, ΔNp63α, ΔNp63β, ΔNp63γ, ΔNp63δ, and ΔNp63ε [4,5]. These isoforms are involved in epithelial stratification and terminal differentiation [6-8]. In this paper, we demonstrate that ΔNp63α is the most abundantly expressed isoform in cervical tissues but it is expressed only at reduced or undetectable levels in moderately or poorly differentiated cervical squamous cancer tissues and some cervical cancer cell lines. Involucrin is an epithelial cell differentiation marker [9-11] and the expression levels of involucrin and ΔNp63α were found to be positively correlated in cervical cancer tissues, suggesting that ΔNp63α might be also involved in regulation of epithelial carcinoma cell differentiation.

Materials and Methods

Patient Samples

A total of 183 formalin-fixed cervical tissue samples were used in this study, which was conducted at the Anhui Provincial Hospital, Hefei, China. Informed consent from the patients was obtained as part of an institutionally approved protocol. The cervical cancer stages were determined by at least two certified gynaecological pathologists based on a modified International Federation of Gynaecology Obstetrics (FIGO) staging system for cervical cancer that was published in 2000. A total of 104 cases of non-metastatic squamous epithelial carcinomas consisted of type Ia (21 patients), Ib (28 patients) and Ila (55 patients). Of the 104 cases that were analysed, 29 cases were diagnosed as well differentiated squamous cell cancer, 29 cases were diagnosed as moderately differentiated squamous cell cancer, and 46 cases were diagnosed as poorly differentiated squamous cell cancer. Additionally, 79 cases of normal cervical tissue from patients who underwent hysterectomy for reasons other than neoplasms of the cervix or endometrium were collected and used as normal controls in this study.

Part of each freshly excised tissue sample was flash frozen at -80°C for protein analyses, and the remaining part of each tissue sample was paraffin-embedded for pathological diagnosis and Immunohistochemical analysis. Paraffin embedded tissue blocks were cut into 1-2 μm thick serial sections. One section was stained with hematoxylin and eosin (H&E) for standard histopathological diagnosis, and the remaining serial sections were processed for IHC staining for the detection of p63 and involucrin. Each H&E-stained and IHC-processed slide was reviewed to verify the histopathological diagnosis.

Immunohistochemical staining

The tissue sections were deparaffinised in xylene for 10 min, fixed using 100% ethanol for 5 min, and then dehydrated with graded ethanol. Endogenous peroxide activity was quenched by incubation with 3% hydrogen peroxide in methanol for 10 min. The sections were washed twice with PBS and incubated with the primary antibody overnight at 4°C. They were rinsed twice with PBS and incubated with the corresponding secondary antibody for 30 min at 37°C. The sections then were washed with PBS and incubated for 1 min with high-sensitivity substrate (DAB). Finally, the slides were counterstained with 10% hematoxylin, and photographs were captured using a microscope with a digital camera. The A549 lung epithelial cell line, which constitutively expresses moderate levels of ΔNp63α, was used as the positive control. Formalin-fixed, paraffin-embedded blocks of each positive control cell line were prepared, and sections were cut from each block and included in each staining reaction. Brownish granules in the nucleus confirmed the presence of p63 antibodies and in the cytoplasm confirmed the presence of involucrin antibody. IHC results were scored as positive (>50%), low (10% to 50%), or negative (<10%) for degree of epithelial cell layer staining by at least two certified gynaecological pathologists.

Antibodies and Cell Culture

Several commercially available primary antibodies were tested for reactivity against paraffin embedded positive control cell lines. All antibodies were diluted at 1:100 for Immunohistochemical staining, and diluted at 1:1000 for Western blotting. Antibodies that yielded the strongest reactivity were then chosen for use in the current study; these included anti-p63 monoclonal antibody (4A4, Santa Cruz, CA), which recognises all six p63 isoforms, anti-ΔN p63 monoclonal antibody (poly6190, Biolegend, San Diego, CA), which specifically recognizes ΔN isoforms of p63, anti-TA p63 monoclonal antibody (poly6189, Biolegend, San Diego, CA), which specifically

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Clin Invest Med • Vol 34, no 3, June 2011
recognizes TA-isofoms of p63, anti-p63α monoclonal antibody (H-129, Santa Cruz, CA), which specifically recognizing α-isofoms of p63, anti-p63γ monoclonal antibody (C-18, Santa Cruz, CA), which specifically recognizes γ-isofoms of p63, and anti-involucrin (SY5, Santa Cruz, CA, USA). The secondary antibody for immunohistochemical staining was purchased from a PicTure™-PV6000 Kit (Zymed Laboratories Inc., South San Francisco, CA). The following cell lines were grown in DMEM with 10% foetal bovine serum (Invitrogen Corporation, CA) 100 U/mL penicillin, and 100 U/mL streptomycin; HeLa, SiHa, CaSk, C-33A, ME-180 and MS751 human cervical cancer cell lines and A549 human lung epithelial cells (American Type Culture Collection).

**Immunoblotting**

Proteins were transferred to polyvinylidene fluoride (PVDF) membranes and probed with the indicated primary antibodies. Membranes were then incubated with a 1:5000 dilution of a peroxidase-conjugated corresponding secondary antibody (Sigma, St. Louis, MO). Blots were developed using an Enhanced Chemiluminescence kit (Pierce, Thermo Scientific, UK).

**Reverse transcription and quantitative PCR**

For PCR, 1 μg of total RNA, extracted from the tissues or cells, was converted to cDNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). PCR amplification was conducted using the following primer sets: TA (497 bp), sense: GT CCCAGAGCACAGACAA, antisense: GCAATTTGG CAGTAGATTT; ΔN (308 bp), sense: GACTCAATT TA GTGAGCCACAGT, antisense: GCAA TTTGGCAGT AGA CAGT AGAGTTT; CCCAGAGCACACAGACAA, antisense: GCAA TTTGGCACAGT AGAGTTC; GTGAGCCACAGT, antisense: GCAA TTTGGCAGT AGA CAGT AGAGTTT; CCCAGAGCACACAGACAA, antisense: GCAA TTTGGCACAGT AGAGTTC; GTGAGCCACAGT, antisense: GCAA TTTGGCAGT AGA CAGT AGAGTTT; CCCAGAGCACACAGACAA, antisense: GCAA TTTGGCACAGT AGAGTTC.

**TABLE 1. Expression of p63 in cervical tissues.**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>p63α</th>
<th>Low / Neg</th>
<th>Positive</th>
<th>P&lt;0.001</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>79</td>
<td>0</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>104</td>
<td>69</td>
<td>35</td>
<td></td>
<td></td>
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</tbody>
</table>

a Sections cut from formalin fixed, paraffin embedded biopsy specimens.
b Normal tissue included inflammation.
c Diagnosis made from adjacent serial section stained with hematoxylin and eosin.
d Staining with anti-p63 (4A4).

c CAATTTTAATCAT, antisense: CGTCAGATTGTTTTGG AGTT.

**Statistical analysis**

SPSS version 13.0 software was used for all statistical analyses. The significance of the differences between two groups was assessed by independent t-test. A P value of <0.05 was considered to be significant.

**Results**

**Low expression of p63 in cervical squamous carcinoma, and ΔNp63α is the predominant isofom expressed in cervical tissues**

Of the 183 biopsy samples, 62% (n=114) showed positive expression of p63. The staining reactions were confined to the basal and parabasal layers in the normal cervical epithelium. The p63 expression was down-regulated in 66.3% (n=69) cervical squamous cancer samples, and weak p63 cytoplasmic staining was observed in some samples (Fig. 1A). The tissues with positive expression of p63 varied significantly between normal cervical tissues and cervical squamous cancer tissues (Table 1). Also, histological grades and clinical stages showed a significant correlation with p63 expression level (p<0.05) (Table 2). As previously reported, anti-p63 monoclonal antibody (4A4) recognises all six p63 isotypes [12], we further investigated which of the p63 isotypes were expressed in the cervical tissue. Since only p63α and ΔNp63 protein were detected

**TABLE 2. Clinicopathologic characteristics versus p63 in human cervical squamous carcinomas.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>No.</th>
<th>p63 expression</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low / Neg</td>
<td>Positive</td>
</tr>
<tr>
<td>Ages</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50 yrs</td>
<td>61</td>
<td>42</td>
<td>19</td>
</tr>
<tr>
<td>&gt;50 yrs</td>
<td>43</td>
<td>27</td>
<td>16</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High differentiation</td>
<td>29</td>
<td>0</td>
<td>29</td>
</tr>
<tr>
<td>Moderate differentiation</td>
<td>29</td>
<td>24</td>
<td>5</td>
</tr>
<tr>
<td>Poor differentiation</td>
<td>46</td>
<td>45</td>
<td>1</td>
</tr>
<tr>
<td>clinical stage</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Ia</td>
<td>21</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Ib</td>
<td>28</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>Iia</td>
<td>55</td>
<td>43</td>
<td>12</td>
</tr>
</tbody>
</table>

a Sections cut from formalin fixed, paraffin embedded biopsy specimens.
b Diagnosis made from adjacent serial section stained with hematoxylin and eosin.
c Staining with anti-p63(4A4).
in the tissues (Fig. 1B), we concluded that ΔNp63α was the predominant isoform expressed in cervical tissues.

ΔNp63α is largely expressed in some cervical cancer cells
As ΔNp63α is the predominant isoform expressed in cervical tissue, the expression of ΔNp63α was then examined in cervical cancer cell lines. α, γ, TA, and ΔN transcripts were found to be expressed in HeLa, MS751, ME-180, SiHa, C-33A and CaSki cells; and p63α and ΔNp63 were the main transcripts in ME-180, MS751 and CaSki cell lines but were only weakly expressed in the HeLa, SiHa, and C-33A cell lines (Fig. 2A-B). Using the various p63 antibodies, ΔNp63α was found to be the major isoform expressed in cervical cancer cells (Fig. 2C), whereas Western blotting indicated that TAp63 and p63γ isoforms were not expressed in these cells by. Since ΔNp63α was detected in ME-180, MS751 and CaSki cells, the coding region of ΔNp63α was then sequenced in these cells: no mutation sites were observed.

Loss of expression of ΔNp63α is related to the differentiation status of cervical squamous cancers
ΔNp63α has been reported to play a role in normal epithelial cell differentiation, and involucrin is an early differentiation marker for the keratinocytes of the epidermis [13]. Thus, the expression levels of ΔNp63α were further explored in both normal cervical tissues and cervical cancer tissues using involucrin as a marker. The results showed that ΔNp63α was expressed in the basal and superbasal layer cells, whereas involucrin was expressed in superbasal layer cells and stratified squamous epithelial cells. Interestingly, both ΔNp63α and involucrin were expressed in well-differentiated cervical squamous cancers, whereas their expression was lost in poorly differentiated cervical squamous cancers (Fig. 3). To further illustrate these phenomena, 10 cases that involved cervical squamous cancer patients were selected, and the slides with both normal tissue and cancer tissue were analysed; the expression of ΔNp63α was indeed positive related with that of involucrin during the invasion of cervical squamous cancer, as shown in Fig. 4.
FIGURE 2. *Expression of ΔNp63α in some cervical cancer cell lines.* (A) RT-PCR analysis of the expression of ΔN and TA p63 isoforms in cervical cancer cell lines. (B) RT-PCR analysis of α, β, and γ isoforms of p63 transcripts in cervical cancer cell lines. (C) Western blotting for p63 in cervical cancer cell lines using different antibodies against peptides representing ΔN and α isoforms of p63.

FIGURE 3. *Expression of ΔNp63α and involucrin in cervical squamous cancers and cells.* (A) Hematoxylin and eosin (H&E) staining (top), p63 immunoreactivity (middle) and involucrin immunoreactivity (bottom) in normal cervical tissue and cervical squamous cancer tissue. Bar indicates 20 μm. Inv indicates involucrin. p63 was stained with anti-p63 (4A4). (B) The expression of ΔNp63α and involucrin were examined by Western blotting. Inv indicates involucrin. p63 was immunoblotting with anti-p63 (4A4).
FIGURE 4. Slide with both normal tissue and cancer tissue. Each slide contained the following surgical specimens: 1. normal squamous epithelia; 2. superficial tumour tissues; 3. tumour tissues. These samples represent the progression of human cervical carcinoma. (A) H&E staining of cervical squamous cancer. Bar indicates 2 mm. (B) H&E staining, p63 immunoreactivity and involucrin immunoreactivity of cervical squamous cancer. Bar indicates 20 μm. The enlarged figures are 5 fold of their left boxed area. Inv indicates involucrin. p63 was stained with anti-p63 (4A4)
Discussion

The critical functions of p63 are to promote epithelial tissue differentiation. Here, ΔNp63α is shown to be the predominant isotype expressed in cervical epithelial tissues, which is consistent with other studies using basal/progenitor cells of prostate, urogenital tract and skin [14-16]. Although α, γ, TA, and ΔN transcripts expression was observed in the cervical cancer cell lines, only ΔNp63α protein expression was detected in MS751, ME-180, CaSki cells by Western blotting. The findings of low expression for TA and γ transcripts, and the reports of quickly degradation for TAp63 protein [17,18], might explain the reason for negative detection of TA and γ isoforms.

Unlike p53, which is mutated or inactivated in many tumours, p63 is rarely mutated in cancers. Investigations into the role of p63 in cancer are ongoing and recent studies support the suppressive activities roles of ΔNp63α in neoplastic transformation and tumour progression: firstly, ΔNp63α can inhibit the Wnt signal pathway of epithelial-to-mesenchymal transition (EMT) [19]; secondly, Snail and Slug can initiate tumour cells EMT by inhibition the expression of ΔNp63α [20,21]; thirdly, mutant p53 can opposes p63 to empower tumor metastasis [22-24]; firstly, impaired p63 expression is associated with poor prognosis [14]. In this study, ΔNp63α was found to be down-regulated in moderately or poorly differentiated cervical squamous cancer tissue and in some cancer cells, MS751, ME-180 and CaSki cells expressed ΔNp63α as well as the early differentiation marker (involutrin), consistent with our clinical results that the expression of ΔNp63α is positively related with that of involucrin in cervical squamous cancers. Suggesting that diminished ΔNp63α is associated with differentiation of cervical squamous cancer, and might be also related with progression from superficial to invasive tumours.

The regulation of ΔNp63α expression is involved in ubiquitin-proteasome degradation mediated by HECT domain family members such as ITCH and WWP1 [25-28], the transcriptional regulation of nuclear transcription factors, and the inhibition of protein translation by miR-203 [29]. Persistent infection with high-risk human papillomavirus (HR-HPV) is closely associated with cervical cancer [30], and HR-HPV can bind to their receptors specifically expressed in cervical basal cells [31-33], the integration of HR-HPV DNA into host cells can disrupt normal cervical squamous differentiation by altering several transcriptional factors to promote the initiation of the carcinogenic process [34-38], and their coding proteins,E6 and E7 contribute to disorganised layer formation and hyperplasia in organotypic cultures [35,39], Thus, HR-HPV infection probably contribute to ΔNp63α inactivation during tumourigenesis.

Acknowledgments

This work was supported by the Anhui Provincial Natural Science Foundation Project (20090413117, 11040606M176, 11040606M178), the Provincial Natural Science Research Project of Anhui Provincial Higher University Education (KJ2010B375), and the National Natural Science Foundation of China (81001168, 81072127). WHX is supported by the National Natural Science Foundation of China (30771973, 30721002 and 81071683) and the National Basic Research Program of China (973 Program) (2007CB914503).

References


