Original Article
Increased expression of oncogene-induced senescence markers during cervical squamous cell cancer development

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Received October 14, 2014; Accepted December 1, 2014; Epub December 1, 2014; Published December 15, 2014

Abstract: Purpose: To investigate the expression of p15INK4b, p16INK4a and p21Waf1/Cip1 in specimens from cases of normal cervical epithelium (NCE), cervical intraepithelial neoplasia (CIN) and squamous cell carcinoma (SCC), and to evaluate whether there is evidence implicating oncogene-induced senescence (OIS) in cervical squamous cell cancer development. Methods: The immunohistochemical expression of p15INK4b, p16INK4a and p21Waf1/Cip1 were investigated in formalin-fixed paraffin-embedded specimens from 19 NCE, 51 CIN and 21 SCC cases, respectively. Comparisons among different groups for each marker were performed with Chi-square test. Results: The expression of p15INK4b, p16INK4a and p21Waf1/Cip1 were significantly higher in both CIN and SCC compared to NCE. Furthermore, the expression of p15INK4b and p21Waf1/Cip1 was significantly higher in CIN Ш compared to CIN І, and these expressions were statistically higher in CIN Ш compared to CIN І. Conclusions: The results suggested that the senescence programs mediated by p15INK4b, p16INK4a and p21Waf1/Cip1 were activated during the stage of CIN and SCC, and demonstrated that senescence may play important role in preventing from NCE to SCC.

Keywords: Cervical cancer, senescence, carcinogenesis

Introduction

Cervical cancer is the second only to breast cancer in women as the most common of gynecologic malignancies, and it remains one of the most important causes of mortality in women worldwide [1]. More than 90% of cervical cancer are SCC in pathologic classification. The direct precursor of cervical SCC is represented by CIN, that is usually detected and managed through the Papanicolaou (Pap) test cytological screening and/or high-risk human papillomavirus (HPV) DNA testing [2]. Most of CIN І has complete regression during the 2-year follow-up period. In contrast, high-grade CIN (CIN ІІ and CIN ІІІ) carries a significant risk of progression to invasive carcinoma. So one of the focuses on cervical cancer research has always been the mechanism of the initiation and development of CIN and SCC.

It was recently demonstrated that cellular apoptosis and senescence are assumed to be two main mechanisms that prevent from cancer development for cells with accumulated somatic mutations. Senescence is defined by a process that keeps the stable form of cell cycle arrest at G1 phase [3], which can be subdivided into two distinct categories: replicative and premature senescence [4, 5]. OIS, as one type of stress-induced senescence, has emerged as a barrier to carcinogenesis [6]. Senescent cells are characterized by a flat and large morphology with vacuoles, and with an increase in SAβ-gal [7]. Previous studies have revealed that the ARF/p53/p21 and p16/Rb/E2F pathways play important role in inducing cellular senescence [8]. The regulatory proteins involved in these pathways are cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs). Among the CDKIs, p15INK4b, p16INK4a and
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p21\textsuperscript{Waf1/Cip1} have been identified to be important in maintaining senescence [7, 9]. The p16\textsuperscript{INK4a} negatively regulates the cell cycle through competitive binding of CDK4 and 6, thereby inhibiting their binding to cyclin D1. The p15\textsuperscript{INK4b} is located centromeric to the p16/p14 gene locus p14\textsuperscript{ARF}, which is a tumor suppressor and causes cell cycle arrest through transforming growth factor β [10]. The p21\textsuperscript{Waf1/Cip1} is involved in controlling CDKs activity, and results in cell cycle arrest at the G1- to S-phase transition. Its effector functions are predominantly induced by p53 and it is considered to be a mediator of the tumor-suppressor activity of p53. However, p21\textsuperscript{Waf1/Cip1} can also be induced in a p53-independent manner [11].

More recent evidence has revealed that senescence markers p15\textsuperscript{INK4b}, p16\textsuperscript{INK4a} and p21\textsuperscript{Waf1/Cip1} had different expression level in many types of premalignant lesions and cancers, indicating senescence may play important role in cancer development. However, these studies have reported conflicting results of senescence markers expression in different cancers [10, 12, 13]. In the present study, we investigate the expression of p15\textsuperscript{INK4b}, p16\textsuperscript{INK4a} and p21\textsuperscript{Waf1/Cip1} in specimens from cases of NCE, CIN (including CIN I, CIN Π and CIN І) and SCC, and evaluate whether there is evidence implicating OIS in cervical squamous cell cancer development.

Materials and methods

The pathology database in the department of pathology, the Second Affiliated Hospital of Soochow University, was retrospectively reviewed. All investigations were approved by the local ethics committee, and waived the need for written informed consent. We recruited specimens from 19 cases of NCE, 51 cases of CIN and 21 cases of SCC. Furthermore, there were 18 CIN I, 16 CIN Π, and 17 CIN І in total of 51 specimens of CIN.

Assessment of p15\textsuperscript{INK4b}, p16\textsuperscript{INK4a} and p21\textsuperscript{Waf1/Cip1} expression and statistical analysis

All slides were evaluated independently by two experienced pathologist (Zhang Y and Li F), and five high-power fields were selected randomly for each slide. The percentage of positive-stain-
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Figure 1. Expression of OIS markers in NCE, CIN and SCC (magnification × 200).

Table 3. Expression of OIS markers in cases of CIN I, CIN II and CIN III

<table>
<thead>
<tr>
<th></th>
<th>p15 (INK4b)</th>
<th>p16 (INK4a)</th>
<th>p21 (Waf1/Cip1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low (%)</td>
<td>High (%)</td>
<td>Low (%)</td>
</tr>
<tr>
<td>CIN I</td>
<td>18 (100.0)</td>
<td>0 (0)</td>
<td>8 (44.4)</td>
</tr>
<tr>
<td>CIN II</td>
<td>16 6 (37.5)</td>
<td>10 (62.5)</td>
<td>6 (37.5)</td>
</tr>
<tr>
<td>CIN III</td>
<td>17 0 (0)</td>
<td>17 (100.0)</td>
<td>2 (11.8)</td>
</tr>
</tbody>
</table>

Table 4. Statistical results of expression differences of OIS markers among CIN I, CIN II and CIN III

<table>
<thead>
<tr>
<th></th>
<th>p15 (INK4b)</th>
<th>p16 (INK4a)</th>
<th>p21 (Waf1/Cip1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>χ²</td>
<td>P</td>
<td>χ²</td>
</tr>
<tr>
<td>CIN I vs. CIN II</td>
<td>15.938</td>
<td>0.000</td>
<td>0.423</td>
</tr>
<tr>
<td>CIN I vs. CIN III</td>
<td>35.000</td>
<td>0.000</td>
<td>4.575</td>
</tr>
<tr>
<td>CIN II vs. CIN III</td>
<td>7.792</td>
<td>0.005</td>
<td>2.169</td>
</tr>
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The intensity of staining was graded on a scale of 0-2, with negative to weak intensity as grade 0, weak-moderate intensity as grade 1, and moderate to strong intensity as grade 2. For each marker, the score of percentage and intensity was multiplied. The final score between 0-2 was determined as low expression, and score higher than 2 was determined as high expression.

Figure 1. Expression of OIS markers in NCE, CIN and SCC (magnification × 200).
Comparisons among different groups for each marker were performed with Chi-square test. For all tests, a two-sided $P < 0.05$ was considered significant.

**Results**

Expression differences of p15\textsuperscript{INK4b}, p16\textsuperscript{INK4a}, and p21\textsuperscript{Waf1/Cip1} among NCE, CIN, and SCC

The expression of p21\textsuperscript{Waf1/Cip1} was predominantly within the nucleus, while the expression of p15\textsuperscript{INK4b} and p16\textsuperscript{INK4a} was predominantly within the cytoplasm. The p15\textsuperscript{INK4b} expression level was low in all of NCE, and its expression was high in CIN (52.9%) and SCC (100.0%), respectively. The expression p16\textsuperscript{INK4a} and p21\textsuperscript{Waf1/Cip1} were significantly higher in CIN and SCC compared to NCE. However, this expression was no statistically differences between CIN and SCC (Tables 1 and 2; Figure 1).

Expression differences of p15\textsuperscript{INK4b}, p16\textsuperscript{INK4a}, and p21\textsuperscript{Waf1/Cip1} among CIN I, CIN П, and CIN Ш

The expression of p15\textsuperscript{INK4b} and p21\textsuperscript{Waf1/Cip1} was significantly higher in CIN П (62.5% and 62.5%) compared to CIN І (0% and 22.2%), and these expression were statistically higher in CIN Ш (100.0% and 94.1%) compared to CIN П, respectively. The p16\textsuperscript{INK4a} expression was no significantly different between CIN І (55.6%) and CIN П (62.5%) group, and between CIN П and CIN Ш (88.2%) group. However, its expression was significantly higher in CIN Ш compared to CIN І (Tables 3 and 4; Figure 2).
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Discussion

Recent studies have revealed that OIS plays important role in limiting the progression of premalignant lesions to invasive cancer during tumor initiation [6]. Elucidation of a number of potential biomarkers for detecting senescent cells has facilitated to evaluate the role of OIS in cancer development. Now SAβ-gal seems to be a reliable marker of senescent cells in culture [5, 14], but it fails to demonstrate senescent cells in vivo models [15, 16]. Other markers of senescence involving signaling pathway were studied. Previous studies have revealed that the ARF/p53/p21 and p16/Rb/E2F pathways play important role in inducing cellular senescence [8]. The senescent-associated genes, including p15\(^{INK4b}\), p16\(^{INK4a}\) and p21\(^{WAF1/CIP1}\), involve into these processes. Several studies showed that p15\(^{INK4b}\), p16\(^{INK4a}\) and p21\(^{WAF1/CIP1}\) are upregulated in premalignant lesions and early stage of cancer, but widely downregulated in the corresponding cancers, including thyroid, hepatocellular, breast, pancreatic carcinoma and glioma [7, 17, 18]. However, Bai et al [10] found that the expression of p15\(^{INK4b}\) and p16\(^{INK4a}\) were almost completely negative in the normal esophageal epithelium. The p15\(^{INK4b}\) and p16\(^{INK4a}\) was found to be expressed in 73% and 73% of the esophageal intraepithelial dysplasia (EID), and 92% and 88% of the esophageal squamous cell carcinoma (ESCC). Similarly, Feng et al [13] found that p15\(^{INK4b}\) and p16\(^{INK4a}\) were also overexpressed in both CIN and cervical SCC. Van de Putte et al [12] found that p21\(^{WAF1/CIP1}\) had no expression in normal cervical squamous epithelium, while its high expression were detected in 20% cervical SCC. In the present study, p15\(^{INK4b}\), p16\(^{INK4a}\) and p21\(^{WAF1/CIP1}\) expression were significantly higher in both CIN and SCC compared to NCE. Furthermore, the expression of p15\(^{INK4b}\) and p21\(^{WAF1/CIP1}\) was significantly higher in CIN II compared to CIN I, and these expression were statistically higher in CIN \(\Pi\) compared to CIN \(\Pi\), respectively. The p16\(^{INK4a}\) expression was significantly higher in CIN \(\Pi\) compared to CIN I. These results suggested that the senescence programs mediated by p15\(^{INK4b}\), p16\(^{INK4a}\) and p21\(^{WAF1/CIP1}\) were also activated as reflected in the overexpression of these markers in cervical dysplasia and SCC, and the ARF/p53/p21 and p16/Rb/E2F pathways were activated during the dysplasia stage of cervical carcinogenesis and remained intact in most cervical SCC. In addition, these results suggested that the expression of these senescence markers may exist tissue-specific, and different cancer tissues have different expression level.

In conclusion, the results showed that the senescence programs mediated by p15\(^{INK4b}\), p16\(^{INK4a}\) and p21\(^{WAF1/CIP1}\) were activated during the stage of CIN and SCC, and demonstrated that senescence may play important role in preventing from NCE to SCC. However, the exact mechanism is still unclear, and the further study is needed.

Acknowledgements

This study was supported by grants from Jiangsu Natural Science Funding (BK20141185) and Jiangsu Province’s Key Medical Person (RC2011144).

Disclosure of conflict of interest

None.

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References

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