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Different Expression of p16^{INK4a} and p14^{ARF} in cervical and lung cancers

J.-G. LI, L. LI[1], S.-W. ZHANG

Center of Reproductive Medicine, General Hospital of Beijing PLA Military Region, Dongcheng District, Beijing, China
[1]Daping Hospital and Research Institute of Surgery of the Third Military Medical University, Yuzhong District, Chongqing, China

Abstract. – AIM: This study aimed to assess clinical significance of expression of p16^{INK4a} and p14^{ARF} proteins in cervical and lung cancers.

MATERIALS AND METHODS: Expression of these proteins was examined in 50 cervical cancer specimens (42 hr-HPV-associated cervical cancer and 8 non-hr-HPV-associated cervical cancer) and 127 lung cancer specimens (34 squamous cell carcinomas, 33 adenocarcinomas, 36 bronchioloalveolar carcinomas, and 24 small cell lung cancers) by immunohistochemistry.

RESULTS: Overexpression of both p16INK4a and p14ARF was found in 100% cervical cancer specimens and in, respectively, 61.42% and 30.79% of lung cancer specimens. Thus, expression ratio of p16INK4a and p14ARF was significantly higher in cervical cancer than in lung cancer ($p < 0.01$). Both proteins were unexpressed in 38 lung cancer specimens (29.92%), and there was no correlation between the expressions of these proteins.

CONCLUSIONS: Different patterns of p16INK4a and p14ARF expression in cervical and lung cancer patients suggest different involvement of these proteins in the development of either cancer type. We propose p16^{INK4a} and p14^{ARF} as biomarkers in clinical assessment for cervical cancer.

Key Words:
Cervical cancer, Lung cancer, Immunohistochemistry, p16^{INK4a}, p14^{ARF}.

Abbreviations

ARF = Alternative reading frame; CDK4 = Cyclin-dependent kinase 4; CIN = Cervical intraepithelial neoplasia; E_2F = E2A binding factor; G1 = Presynthetic phase; G2 = Postsynthetic phase; HPV = Human papilloma virus; hr-HPV = High-risk HPV types; M = Mitotic period; MDM2 = Murine double minute 2; S = Synthesis period; SCC = Squamous carcinoma of the cervix.

Introduction

Deregulation of cell cycle is one of the important features of cancer cells[1]. Cell cycle regulatory proteins may play important role in cancer development. Two of these proteins, p16^{INK4a} and p14^{ARF}, are encoded by the tumor suppressor gene INK4a/ARF and exert their functions through, respectively, cyclinD-CDK4-pRb-E2F and MDM2-p53 pathways[2].

In the present study, we carried out immunohistochemistry assays to evaluate the expression of p16^{INK4a} and p14^{ARF} in specimens of cervical cancer with and without evidence of high-risk Human papilloma virus (hr-HPV) infection and in various types of lung cancer. The choice of these types of cancer was defined by their high prevalence. Thus, cervical cancer is ranked the second most common cancer in women[3] and has the highest mortality rate among all gynecological malignancies[1,4]. Lung cancer is also a very common cancer.

Our study adds to the understanding of the role of cell cycle regulatory proteins p16^{INK4a} and p14^{ARF} in the development and progression of cervical and lung cancers.

Materials and Methods

Patients

Fifty samples of cervical secretions were collected during biopsy or surgical removal in the Daping Hospital between November 2004 and December 2006. The samples were stored in paraffin wrap and subsequently examined for the evidence of hr-HPV infection detected by the Hybrid Capture II assay. Forty-two patients were hr-HPV positive and 8 hr-HPV negative. The patient age ranged from 22 to 61 years (mean age of 42.18 years). Forty-seven patients had cervical

Corresponding Author: Li Li, MD; e-mail: cqlili2011@163.com

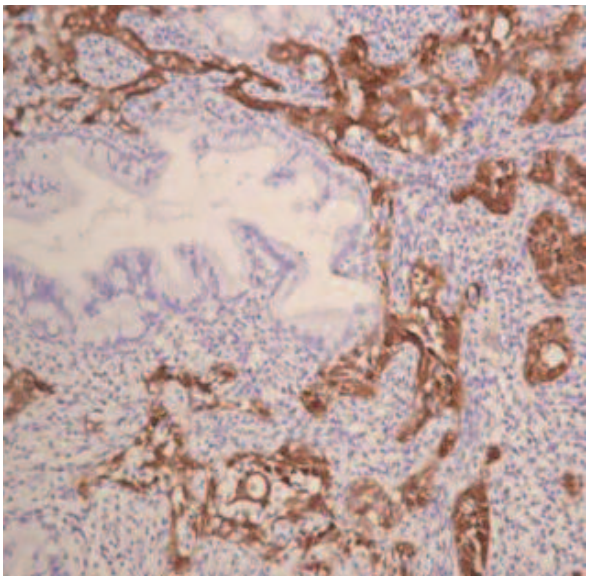

Figure 1. Positive expression of $p16^{INK4a}$ in cervical adenocarcinoma (× 200).

squamous cell carcinoma (SCC) and 3 patients had adenocarcinoma of cervix. The SCC patients were categorized based on pathology results into high, medium, low grade and in situ SCC with, respectively, 10, 19, 8 and 10 cases in each category.

One hundred and twenty-seven paraffin-embedded specimens of lung tissue were collected from patients with lung cancer at the Department of Pathology of Xinqiao Hospital between 1997 and 2003. Eighty-nine specimens were collected from male patients and 38 specimens from female patients. The patient age ranged from 27 to 74 years (mean of 53.85 years). Thirty-four patients had squamous cell carcinoma, 33 patients – adenocarcinoma, 36 patients – bronchioloalveolar carcinoma, and 24 patients had small cell lung cancer.

All specimens were fixed in 10% formalin, dehydrated, and embedded in paraffin. Wax blocks were sliced into 5 sections of 2 μm thickness. One section was stained with hematoxylin-eosin to confirm the diagnosis. Two sections were subjected to immunohistochemistry assays. The remaining two sections were stored for potential future studies.

Reagents

Biotinylated primary mouse anti-$p16^{INK4a}$ monoclonal antibody (MAB-0223) and primary rabbit anti-$p14^{ARF}$ polyclonal antibody (RAB-0532) as well as secondary streptavidin-peroxidase conjugate were purchased from Fuzhou Maixin Biotechnology Development (FuZhou, China). The broad-spectrum assay kit and positive controls were purchased from the same manufacturer. In negative control specimens, primary antibodies were omitted. Immunohistochemistry assays were carried out using the manufacturer's protocol.

Interpretation of the Results

Positive staining was mainly located in the nucleus and was ranked semi-quantitatively based on staining intensity into strongly positive (+++; diffuse yellow-brown staining), positive (++; diffuse or large patchy brown staining), weakly positive (+; small pieces or focal reaction), and negative (-; no staining).

Statistical Analysis

The SPSS10.0 statistical software (SPSS Inc., Chicago, IL, USA) was used to perform the chi-square and Pearson correlation analyses. Differences with the p value < 0.05 were considered statistically significant.

Results

Expression of $p16^{INK4a}$ in Cervical and Lung Cancers

All 50 specimens of cervical cancer were found $p16^{INK4a}$ positive, leading to a 100% expression ratio (Figure 1). In lung cancer specimens, the expression of $p16^{INK4a}$ was found in 78

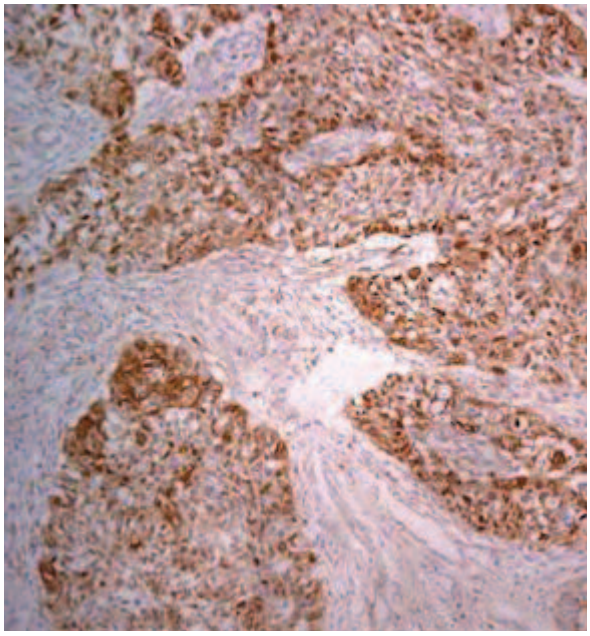

Figure 2. Positive expression of $p16^{INK4a}$ in lung squamous cell carcinoma (× 200).

Table I. Expression of p16^{INK4a} in cervical and lung cancers.

Cancer type	Number of specimens	-	+	++	+++	Positive expression ratio (%)	*p*
Cervical cancer	50	0	1	19	30	100.00	0.000
Lung cancer	127	49	8	23	47	61.42	

Footnote: The staining was ranked semi-quantitatively into strongly positive (+++; diffuse yellow-brown staining), positive (++; diffuse or large patchy brown staining), weakly positive (+; small pieces or focal reaction), and negative (-; no staining).

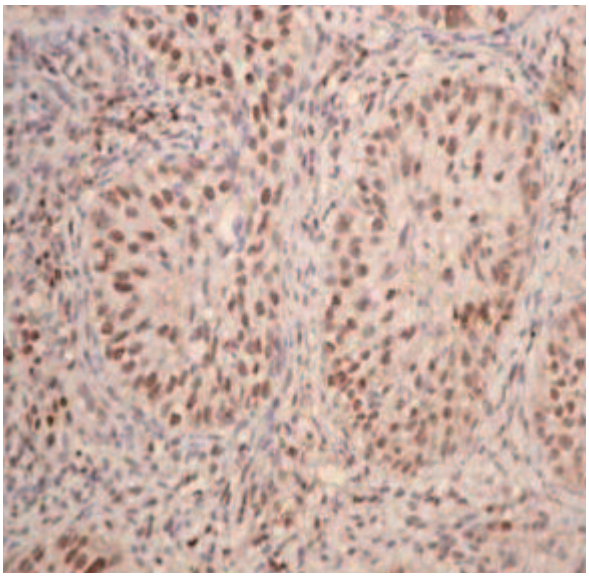

Figure 3. Positive expression of p14^{ARF} in cervical squamous cell carcinoma (× 200).

out of 127 specimens (Figure 2), giving the expression ratio of 61.42%. The expression ratio of p16^{INK4a} in cervical cancer was significantly higher than in lung cancer ($p < 0.01$; Table I).

Expression of p14^{ARF} in Cervical and Lung Cancers

All 50 specimens of cervical cancer were positive for p14^{ARF}, providing a 100% expression ratio (Figure 3). Thirty-nine of 127 patients with lung cancer expressed p14^{ARF} (Figure 4). Thus, the expression rate in lung cancer was 30.79%. Again, the expression ratio of p14^{ARF} was significantly higher in cervical cancer compared with lung cancer ($p < 0.01$; Table II).

Co-expression of p16^{INK4a} and p14^{ARF} in Lung Cancer Specimens

Both p16^{INK4a} and p14^{ARF} were found unexpressed in 38 out of 127 lung cancer specimens, leading to the ratio of 29.92% (38/127). Eleven specimens expressed only p14^{ARF}, accounting for an 8.66% expression ratio (11/127). Fifty specimens expressed exclusively p16^{INK4a}, accounting for a 39.37% expression ratio (50/127). The total ratio of absent expression of either p16^{INK4a} or p14^{ARF}, or both proteins, was 77.95% (99/127). Pearson correlation analysis did not reveal any significant correlation between expressions of p16^{INK4a} and p14^{ARF}.

Expression of p16^{INK4a} and p14^{ARF} Proteins in hr-HPV Positive and Negative Specimens

We further stratified the cervical cancer specimens into those with or without hr-HPV infection. It was found that specimens expressed both p16^{INK4a} and p14^{ARF} regardless of the presence of hr-HPV.

Figure 4. Positive expression of p14^{ARF} in alveolar cell carcinoma (× 200).

Table II. Expression of $p14^{ARF}$ in cervical and lung cancers.

Cancer type	Number of specimens	-	+	++	+++	Positive expression ratio (%)	p
Cervical cancer	50	0	5	30	15	100.00	0.000
Lung cancer	127	88	28	8	3	30.79	

Footnote: The staining was ranked semi-quantitatively into strongly positive (+++; diffuse yellow-brown staining), positive (++; diffuse or large patchy brown staining), weakly positive (+; small pieces or focal reaction), and negative (-; no staining).

Discussion

The INK4a/ARF gene is located in band 9p21 on the short arm of human chromosome 9. Two promoters are located in this gene locus and are responsible for expression of two transcripts which encode for two different proteins, $p16^{INK4a}$ and $p14^{ARF}$. These two transcripts share some exons within their reading frames and are, therefore, similar but not identical. The INK4a transcript contains three exons (exon 1α, exon 2, and exon 3) and two introns. The three exons encode cyclin-dependent kinase 4 (CDK4) inhibitor protein, or $p16^{INK4a}$, with molecular weight of 16 kD; the protein comprises 156 amino acids. This protein, competing with cyclin D, binds to CDK4 and inhibits its catalytic activity. It, thereby suppresses phosphorylation of retinoblastoma protein, reduces release of the extension factor E2F (E2A binding protein) and eventually halts the cell division in the G1 (presynthetic) phase. The ARF transcript is produced by an alternative reading frame of the INK4a gene. ARF transcript shares Exon 2 and exon 3 with the INK4a, but has a unique third exon, exon1β, located approximately 20 kb upstream of exon1α. These three exons encode $p14^{ARF}$ protein which consists of 133 amino acids and has molecular weight of 14 kD. $p14^{ARF}$ binds to oncoprotein MDM2 (murine double minute 2) and accelerates its degradation, restores and stabilizes the expression level of tumor protein p53, and enhances the effect of p53 in the restriction point of G1 → S (synthesis) and G2 (postsynthetic phase) → M (mitotic period), resulting in cell cycle arrest in G1 and G2 phases[2].

INK4a/ARF is one of the few gene loci in mammalian cells that have overlapping coding regions. The functional similarity and the synergic actions of $p16^{INK4a}$ and $p14^{ARF}$ define the important biological significance of this gene locus. In recent years, it has been repeatedly reported that INK4a/ARF gene expression is depleted in glioma, melanoma, and nasopharyngeal, lung, breast, head and neck, esophageal, stomach and pancreatic cancers, leukemia, and other malignancies. The mechanisms that lead to the functional inactivation of this gene include gene deletion, mutation and methylation[2,5].

As shown below, there are also reports that both $p16^{INK4a}$ and $p14^{ARF}$ are overexpressed in some cancers. Thus, it was suggested that expression of $p16^{INK4a}$ expression can be a diagnostic marker of cervical cancers. Our findings indicate that expression of $p16^{INK4a}$ or $p14^{ARF}$ coincides with cervical cancer. By contrast, there was no significant correlation between expressions $p16^{INK4a}$ and $p14^{ARF}$ in lung cancer, suggesting that expression of these two proteins in lung cancer are unrelated.

Human Papilloma Virus (hr-HPV) E6/E7 oncogenes are thought to be the major cause of cervical cancer. Ninety percent of patients with cervical cancer have hr-HPV infection[6]. Overexpression of $p16^{INK4a}$ and $p14^{ARF}$ in cervical cancer was linked to hr-HPV infection by a number of studies[6-8]. Ishikawa et al[6] showed that the ratio of infections increases as cervical neoplasia progresses to cancer, and that overexpression of $p16^{INK4a}$ follows the same pattern. Others researchers found no $p16^{INK4a}$ expression in normal cervical tissue, while the protein was expressed

Table III. Co-expression of $p16^{INK4a}$ and $p14^{ARF}$ in lung cancer.

$p14^{ARF}$	$p16^{INK4a}$	
	-	+ – +++
-	38	50
+ – +++	11	28

Footnote: No significant correlation between expressions of two proteins was found using Pearson analysis ($p = 0.11$).

in abnormally developed squamous and glandular epithelial cells. Kanao et al[8] found overexpressed p14^{ARF} mRNA in 100% of HPV infected samples and overexpressed p16^{INK4a} mRNA in 81% of these samples. In other studies[7], all specimens from hr-HRV infected patients expressed p16^{INK4a}, while p16^{INK4a} expression in non-infected patients was very low. It was suggested that p16^{INK4a} can be used to detect individual dyskaryotic cells as the result of hr-HPV infection in ThinPrep smears[7]. However, we found overexpressed p16^{INK4a} and p14^{ARF} not only in hr-HPV positive but also in negative specimens. Other studies also reported that hr-HPV infection is discrepant from expressions p16^{INK4a} and p14^{ARF} in cervical cancer[9,10].

Conclusions

INK4a/ARF is a tumor suppressor gene which is often down-regulated in lung cancers and constitutively overexpressed in cervical cancer. It can be speculated that the role of this gene is different in cervical cancer development than in lung cancer. Further researches are needed to clarify the role of INK4a/ARF gene in cervical cancer and the mechanisms of its action. We propose p16^{INK4a} and p14^{ARF} as biomarkers in clinical assessment for cervical cancer.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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