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• BRIEF REPORTS •

Expressions of PCNA, p53, p21^{WAF-1} and cell proliferation in fetal esophageal epithelia: Comparative study with adult esophageal lesions from subjects at high-incidence area for esophageal cancer in Henan, North China

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Abstract

AIM: To characterize the expression of p53, p21^{WAF-1} and proliferation-cell-nuclear-antigen (PCNA) in fetal esophageal epithelia and to determine the role of these genes in proliferation of fetal and adult esophageal epithelial cells.

METHODS: Immunohistochemical avdin-biotin peroxidase complex (ABC) method was applied to 31 cases of fetal esophageal specimens and 194 cases of adult esophageal specimens to detect the expression of p53, p21^{WAF-1} and PCNA in fetal and adult esophageal epithelia.

RESULTS: Both the PCNA positive immunostaining cell number and PCNA positive immunostaining rate in fetal esophageal epithelia (506±239) were significantly higher than those in adults, including normal epithelia (200±113) and epithelia with basal cell hyperplasia (BCH) (286±150) (P<0.05, t test). However, the number of PCNA positive immunostaining cells in adult esophageal dysplasia (719±389) and squamous cell carcinoma (SCC) (1261±545) was apparently higher than that in fetal esophageal epithelia (506±239) (P<0.05, t test). The positive immunostaining rate of P53 was 10 % (3/31) in fetal esophageal epithelia, which was significantly lower than that in adult normal esophageal epithelia (50 %), adult epithelia with basal cell hyperplasia (62 %), dysplasia (73 %) and squamous cell carcinoma (86 %) (P<0.05, Fisher's exact test). No p21^{WAF-1} positive immunostaining cells were observed in fetal esophageal epithelia. However, p21^{WAF-1} positive immunostaining cells were observed in adult esophagus with 39 % (11/28) in normal, 38 % (14/37) in BCH, 27 % (3/11) in DYS and 14 % (1/7) in SCC.

CONCLUSION: PCNA could act as an indicator accurately reflecting the high proliferation status of fetal esophageal epithelium. p53 may play an important role in growth and differentiation of fetal esophageal epithelium. p21^{WAF-1} may have no physiological function in development of fetal esophageal epithelium.

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INTRODUCTION

Fetal esophageal epithelium is characterized by cellular hyperproliferation. Tumor suppressor genes have been known to suppress malignant cell proliferation through encoding corresponding proteins that inhibit cell cycle. But it is still not clear whether this inhibition effect of tumor suppressor genes is also involved in the proliferative activity of fetal epithelium.

Tumor suppressor proteins p53 and p21^{WAF-1} play important roles in regulating G1 phase progression, which is the key modulation point in the cell cycle[1-8]. Proliferating cell nuclear antigen (PCNA) acts as a good marker for cell proliferation and can reflect the status of epithelium growth[9,10]. To detect the expression of these proteins would help to explore fetal esophageal epithelium proliferation status and characteristics, and to further understand the role of tumor suppressor genes in fetal esophageal epithelium growth control.

Most previous studies about fetal esophagus development focused on the influences of certain chemical factors, such as nitrosamine[11,12], and biological factors, such as alternariol[13]. There are, however, few reports about the proliferation characteristics and control of cell cycle in growth of fetal esophageal epithelium.

In this study, immunohistochemical avdin-biotin peroxidase complex (ABC) method was applied to investigate the expression of PCNA, p53 and p21^{WAF-1} in fetal esophageal epithelium and adult esophageal epithelium with different histopathological subtypes. Comparison between the expression of the above proteins in fetal and adult esophageal epithelium would provide important evidences for characteristics of fetal esophageal epithelium proliferation and the mechanisms of its cell cycle control.

MATERIALS AND METHODS

Tissue collection and processing

Thirty-one cases of fetal esophageal specimens were collected from Runan County, Taikang County, Lankao County and Zhengzhou City. The ages of the fetuses ranged from 4 months to 10 months (Table 1). No history of drug using and family history of tumor were found among all the parents of these fetuses. 194 cases of adult esophageal specimens were collected in the same areas for control of PCNA expression, among which 83 adult esophageal specimens were used for the control of p53 and p21^{WAF-1} expression. All specimens were fixed in 85 % ethanol, embedded with paraffin and serially sectioned at

5 μm. The sections were mounted onto the histostick-coated slides. Four or five adjacent ribbons were collected for histopathological diagnosis (hematoxylin and eosin stain) and immunohistochemical staining.

Table 1 Distribution of fetus sex and age

Sex	Age (months)						Mean age (months)
	4	5	6	7	8	>9	
Male	0	2	4	3	0	0	6.11±0.78
Female	2	5	4	7	3	1	6.36±1.47[a]
Total	2	7	8	10	3	1	6.29±1.30

[a]P>0.05, t-test, no significant difference was detected in the distribution of age between male and female fetuses.

Histopathologic diagnosis

Histopathological diagnosis and categorization for esophageal epithelium were based on the changes in cellular morphology and tissue architecture in reference to previous reports[14-17], and the adult esophageal epithelium was correspondingly classified as normal, basal cell hyperplasia (BCH), dysplasia (DYS) and squamous cell carcinoma (SCC).

Immunohistochemical staining (IHC)

Anti-p53 antibody is a monoclonal mouse anti-serum against p53 of human origin, and recognizes both wild and mutant type p53 (Ab-6, Oncogene Science, Manhasset, NY). Anti-PCNA antibody is a monoclonal mouse anti-serum against PCNA of human origin (Mab, DAKO, Carpinteria, CA). Anti-$p21^{WAF-1}$ antibody is a monoclonal mouse anti-serum against $p21^{WAF-1}$ of human origin, and recognizes both wild and mutant type p21 (Ab-6, Oncogene Science, Manhasset, NY). The avidin-biotin-peroxidase complex method was used for the immunostaining of p53, PCNA and $p21^{WAF-1}$ as previously reported. In brief, after dewaxing, inactivating endogenous peroxidase activity and blocking cross-reactivity with normal serum (Vectastain Elite Kit; Vector, Burlingame, CA), the sections were incubated overnight at 4 ℃ with a diluted solution of the primary antibodies (1:500 for p53, 1:200 for PCNA and 1:20 for $p21^{WAF-1}$). Location of the primary antibodies was achieved by subsequent application of a biotinylated anti-primary antibody, an avidin-biotin complex conjugated to horseradish peroxidase, and diaminobenzidine (Vectastain Elite Kit, Vector, Burlingame, CA). The slides were counter-stained by hematoxylin. Negative controls were established by replacing the primary antibody with PBS and normal mouse serum. Known immunostaining-positive slides were used as positive controls.

The criteria of positive staining for p53 and $p21^{WAF-1}$ were as previously reported[6-8, 14-16]. Quatitative analysis of PCNA immunostaining results was recorded as the number of positive staining cells per mm^2 of the tissue section[17,18]. All the immunostaining slides were observed by two pathologists independently and the final concordant results were adopted.

Statistic analysis

Fisher' s exact χ^2 test and t-test were applied for the statistical analysis and two-sided P value of less than 0.05 was considered statistically significant.

RESULTS

Histopathological results

Among the cases of fetal esophageal epithelium, the number of basal cell layers ranged from 2 to 6. Five cases contained more than 10 basal cell layers and showed a high proliferation activity. As for the cases of adult esophageal epithelium, the results of histopathological diagnosis were 31 normal cases, 106 cases with BCH, 31 cases with DYS and 26 cases with SCC.

Comparison of PCNA protein expression between fetal and subtypes of adult esophageal epithelium

PCNA positive immunostaining cells were located mainly in the basal cell layer. And the positive immunoreaction occurred mainly in the nucleolus in dark brown. The mean number of PCNA positive immunostaining cells was 506±239 per mm^2 in fetal esophageal epithelia, which was significantly higher than that in adult esophageal epithelia of normal and BCH (P<0.05, t-test), but significantly lower than that in adult esophageal epithelia with DYS and SCC (P<0.05, t-test) (Table 2).

Table 2 Quantitative comparison of PCNA expression between fetal and subtypes of adult esophageal epithelia

Histopathological subtypes	Case (n)	Number of immunostaining positive cells / mm^2
Adult normal	31	200±113
Adult BCH	106	286±150
Fetal	31	506±239[a]
Adult DYS	31	719±389
Adult SCC	26	1261±545

[a]P<0.05 *vs* t-test.

Comparison of P53 protein expression between fetal and subtypes of adult esophageal epithelia

p53 positive immunostaining cells were located mainly in the basal cell layer and the positive immunoreaction occurred mainly in the nucleoli in brown.

The positive immunostaining rate of p53 was 10 % (3/31) in fetal esophageal epithelia, which was significantly lower than that of adult normal esophageal epithelia (50 %, 14/28), adult epithelia with BCH (62 %, 23/37), DYS (73 %, 8/11) and SCC (86 %, 6/7) (P<0.05, Fisher' s exact test) (Table 3).

Table 3 Comparison of p53 expression between fetal and subtypes of adult esophageal epithelia

Histopathological subtypes	Case (n)	Positive immunostaining	
		Number	Percentage (%)
Fetal	31	3	10[a]
Adult normal	28	14	50
Adult BCH	37	23	62
Adult DYS	11	8	73
Adult SCC	7	6	86

[a]P<0.05 *vs* Fisher' s exact test.

Expression of $p21^{WAF-1}$ protein in fetal esophageal epithelia

No positive immunoreaction of $p21^{WAF-1}$ was detected in all the fetal esophageal epithelia. However, $p21^{WAF-1}$ positive immunostaining cells were observed in adult esophagus with 39 % (11/28) in normal, 38 % (14/37) in BCH, 27 % (3/11) in DYS and 14 % (1/7) in SCC.

DISCUSSION

To our knowledge, this is the first report about the role of PCNA as an indicator of proliferation status of fetal esophageal epithelium. In our study, we observed that most PCNA immunostaining positive cells were located in the basal layer

of fetal esophageal epithelium. PCNA is an important index of cell proliferation kinetics. Qian *et al*[18] found that according to the proliferation status, most cell nuclei were in the G1 phase to S phase of cell cycle in the cell clones with high proliferative activity. And the positive rate of PCNA expression increased gradually in cell nuclei with the progress of G1 phase and reached a peak when entering S phase. Our research found that all fetal esophageal epithelia showed a positive immunoreaction of PCNA and possessed high level of positive immunostaining cells per mm^2, which was much higher than that in normal adult esophageal epithelium and BCH. This result is concordant with previous theory and suggests that PCNA may act as a good marker for fetal esophageal epithelium proliferation status.

In our study, we also observed that malignant adult esophageal epithelia possessed much more PCNA positive immunostaining cells than fetal esophageal epithelia. This phenomenon is plausible. It was reported that PCNA played a role in DNA damage repair (DDR). With the presence of nucleotide excision, PCNA binds replication protein A (RPA) and constitutes a subunit of DNA polymerase δ[19,20]. Kieczkowaske *et al*[21] also supposed that PCNA could combine with hMSH6 and hMSH3, the subunits of hMutSalpha and hMutSbeta that acted as cofactors in DNA mismatch repair system. Malignant tissue was characterized by high frequencies of DNA mismatch, breakages and mutations, which would in turn induce more expression of PCNA for its repair function. As for fetal esophageal epithelia, there were few opportunities of contacting external environmental carcinogens and incurring much DNA damages and mutations. As a result, adult malignant esophageal epithelia had much more PCNA positive immunostaining cells, which implied that PCNA could potentially act as an indicator of malignant proliferation.

For the expression of p53, the positive immunostaining rate was 10 % in fetal esophageal epithelia, which was much lower than that in adult esophageal epithelia of each histopathological subtype. These differences were due to lack of induced p53 expression by external environmental factors in fetal esophageal epithelia. But in our study, there were some cases that were observed with expression of p53. Guo *et al*[11] reported that no expression of p53 was detected in fetal esophageal epithelia that had been cultured by NMBzA for up to 3 weeks. p53 supervised cell cycle through G1 phase checkpoint[21]. Then we supposed that stable expression of p53 might be required for normal cell cycle in the highly proliferative fetal esophageal epithelia. Lowe *et al*[21] found that disfigurement was not detected during development of rat fetus, which showed strong p53 expression. It was suggested that p53 possessed a protective function in fetal tissue development. Although this mechanism should be studied further, our study confirmed its existence in fetal esophageal epithelium development and maybe this function of p53 worked in a special stage of fetal esophageal epithelium proliferation and differentiation.

No positive expression of p21^{WAF-1} was observed in fetal esophageal epithelia. This protein has been reported to be trans-activated by p53 and could repair genomic DNA damages[7, 8]. Fetal esophageal epithelium encountered few outer carcinogens and few DNA damages occurred in this tissue. We hypothesize that there may not be a large number of p21^{WAF-1} required for DNA damage repair in development of fetal esophageal epithelium.

In conclusion, PCNA can reflect the proliferation status of fetal esophageal epithelium, and p53 may contribute to its development. But p21^{WAF-1} may not play a role in the process of its development. Further studies to explore the molecular mechanisms of these proteins in esophageal development should be performed to provide more pronounced evidences.

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