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P16 GENE EXPRESSION IN OVARIAN EPITHELIAL CYSTADENOCARCINOMA

Ni Xinghao 倪型灏 Xu Shenhua 许沈华 Wu Xiongwei 吴雄伟
Zhang Gu 张谷 Qian Lijuan 钱丽娟 Gao Yongliang 高永良

Zhejiang Cancer Hospital, Hangzhou 310022

P16 gene expression was measured by immnohistochemical method in poor differentiated serous cystadenocarcinoma cell line, xenograft of highly metastasizing human ovarian carcinoma in nude mice and paraffin embedded tissues from 69 patients with ovarian carcinoma. The result showed that P16 gene was positive expression in HO-8910 cell of mother line, HO-8910PM cell line and xenograft of highly meatstasizing human ovarian carcinoma in nude mice. However, P16 gene in the metastatic cell had a weaker expression. P16 gene positive expression were also found in 51 cases of 69 cases (73.9%) in the ovarian epithelial carcinoma paraffin embedded tissues. Comparative studies showed that the positive rate of P16 gene expression markedly reduced with the increase of pathologic grade and clinical stage, metastasis in the lymph node and decrease of 5-year survival ($P<0.05$, $P<0.01$).

P16 gene is not only a controller of cytokerastic cycle, but also a key member of tumorigenic suppresser; its absence and expression degree are also correlated with the ovarian carcinoma genesis and development, especially with the metastasis of the ovarian cancer.

Key words: Ovarian carcinoma, Multiple tumor suppresser gene, Gene absence, Immunohistochemistry.

It is well known that c-oncogene overexpression as well as antioncogene absence or inactivation play a very important role in carcinogenesis. P16 gene (or MTS1, Multiple tumor suppresser 1) has been widely analyzed in various primary tumors, such as melanoma, neuroblastoma and tumors in kidney, esophagus, stomach, bladder, breast and pancreas. The results from different research showed a high-frequent P16 gene mutation and absence.[1-4] To explain the relationship between P16 gene and ovarian cancer, we had analyzed P16 expression in human ovarian poorly differentiated cystadenocarcinoma cell line, a model of highly metastasizing human ovarian cancer transplanted into subcutis of the nude mice and 69 cases of ovarian epithelial carcinoma paraffin tissue.

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MATERIALS AND METHODS

Cell Line

The cell of the 80th subcultures of human poorly differentiated ovarian serous cystadenocarcinoma cell line HO-8910[5] were cultured in vials with coverslip at 37 ℃ for 4 days, then the coverslip were fixed in 95% alcohol, at last immunostaining was performed.

The tumor tissues used were obtained from the 7th generation of a model of highly metastasizing human ovarian cancer transplanted into subcutis of the nude mice for cell culture. A cell line was successfully established *in vitro*. This cell line was named HO-8910PM (to be reported). The cell of the 81th subcultures of HO-8910PM were cultured in vials with coverslip at 37 ℃ for 4 days, then the coverslip were fixed in 95% alcohol, at last immunostaining was

performed.

A Model of Highly Metastasizing Ovarian Cancer Transplanted into of the Nude Mice[6]

Fresh tissue of tumor explant, pulmonary metastases and left axially lymph node metastases of the 18th generations (in No. 498 mice) were fixed in 10% buffered formalin, paraffin embedded, cut (serial sections, 4 μm thickness) and stained (HE and immunostaining).

Cases of Ovarian Epithelial Carcinoma

Tissues were obtained form 69 cases of ovarian epithelial cystadenocarcinoma. All cases were categorized into different groups according to Liu histological classification criteria and FIGO staging criteria.[7] The specimens were fixed in 10% buffered formalin, paraffin embedded, cut and stained (HE and immunostaining). Their ages ranged from 21 to 71 years (mean 48 years). 55 of them received postoperative chemotherapy and 11 patients received both postoperative chemotherapy and radiation. Six cases of benign cystadenoma were selected randomly to be a parallel control.

Immunostaining

1:100 diluted multicolonal rabbit antihuman P16 antibody (Santa Crug Co., American) and S-P kit (Zymed Co., American) were purchase from Peking Zhongshang bio-tech Co. S-P method was adopted. Before staining the sections were treated in a microwave ovem for antigen retrieval. Sections of breast cancer renown to be P16 positive was included as a positive control, and a negative controls obtained by omitting the primary antibody.

Results Assessment

P16 positive staining showed a diffused or random yellow granules. The staining intensity and the proportion of cells staining positive were classified as fellow:

" – " no staining or as weak as background;

"+" weakly staining or a little stronger than background positive cells proportion <15%;

"++" moderate staining, intensity and proportion were between "+" and "+++";

"+++" strong staining, positive cells >50%;

The results were assessed with x^2 analysis.

RESULTS

Both the mother line HO-8910 cells and the high metastasis HO-8910PM cells showed P16 positive. The staining of yellow granules mainly located in the cytoplasm except a few others in the nuclear. Bigger and stronger staining granules were found in mother line HO-8910 cells (Figure 1, 2)

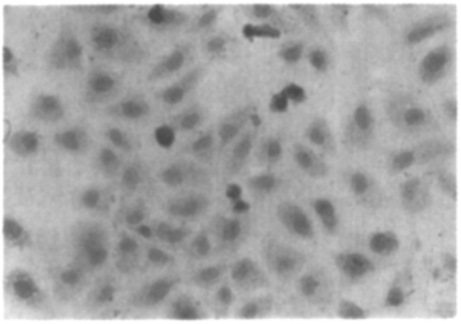

Fig. 1. Human ovarian cancer cell line HO-8910 of the 80th generation: P16 positive (S-P method ×300)

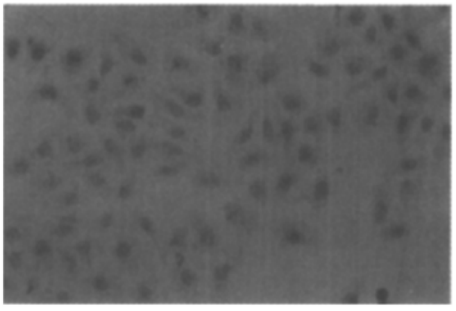

Fig. 2. HO-8910PM cell line of the 81th generation: P16 positive, its intensity is weaker than HO-8910 cell line (S-P method ×300)

In the transplanted tumor tissues of the 18th generations in the nude mice were found P16 strong positive (+++) (Figure 3), however, axially lymph node metastatic tumor in the nude mice P16 positive (+) and lugn metastatic tumor in the nude mice P16 negative (–).

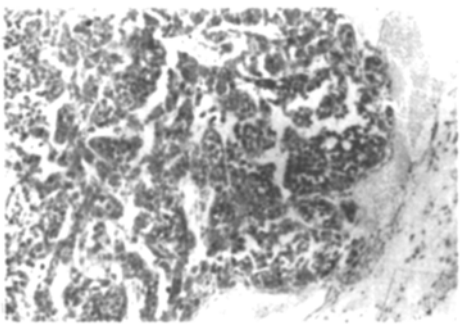

Fig. 3. Tumor tissues from the 18th generation of a model of NSMO transplanted into subcutis of the No. 498 nude mice: P16 strong positive (+++) (S-P method ×200)

In 69 cases of ovarian epithelial cystadenocarcinoma, 51 were P16 positive (73.9%). In the control group, 6 cases of mucous cystadenoma were all P16 positive with various staining intensity (100%, 6/6). Positive staining granules are mainly located in the cytoplasm, and a few in the nuclear (Figure 4, 5).

The relationship between P16 gene expression and pathohistological classification was analyzed (Table 1). Table 1 shows that P16 positive rate is statistically different bewteen each other subgroups ($P<0.05$, $P<0.01$), except between the group of borderline low grade mialignant cystadenoma and the group of cystadenocarcinoma grade 1 ($P>0.05$), and between grade II and grade III ($P>0.05$). It showed that P16 positive rate and staining intensity were lower and weaker when the tumor malignant degree was higher.

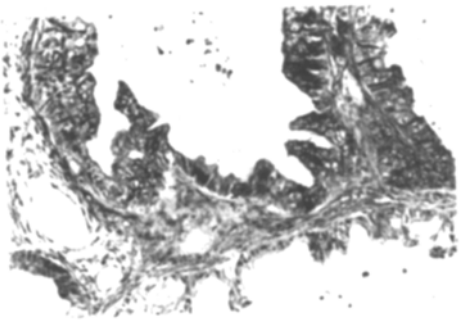

Fig. 4. Human ovarian epithelial cystadenocarcinoma (HE ×200)

Table 1. P16 gene expression in 69 cases of ovarian epithelial cystadenocarcinoma and pathological classification and differentiation

Pathohistological classification	Grade	Positive cases	Staining intensity			
			–	+	++	+++
Borderline low grade malignant epithelial tumor		11 (100%)	0	1	1	9
Epithelial cystadenocarcinoma	I	15 (100%)	0	1	6	8
	II	15 (68.2%)	7	5	6	4
	III	10 (47.6%)	11	5	4	1
Total		51 (73.9%)	18	12	17	22

Note: P value between the group of border line low grade malignant epithelial tumor and cystadenocarcinoma grade I ($x^2=3.084$, $P>0.05$), and grade II ($x^2=12.935$, $P<0.01$), and grade III ($x^2=18.769$, $P<0.01$); between grade I and grade II ($x^2=10.052$, $P<0.05$), and grade III ($x^2=19.025$, $P<0.01$); between grade II and grade III ($x^2=3.062$, $P>0.05$).

The relationship between P16 gene expression and clinical staging see Table 2. Table 2 shows that P16 positive rate between different clinical staging subgroups was statistically different ($P<0.05$, $P<0.01$), except between staging III group and IV group ($P>0.05$). The results also indicated that there is a

negative correlation between the clinical staging and P16 positive rate and staining intensity.P16 gene expression and ascetic, tumor dissemination, lymph node metastasis and 5-year survival see Table 3. The P16 positive rate in the group of ascetic (67.6%, 23/24) show a tendency to be lower than in the group of no ascetic (80%, 28/35), although this different was not statistically significant ($P>0.05$). P16 positive rate in the group of dissemination (62.5%, 25/40), lymph node metastasis (30%, 3/10), failure of 5-year survival (62.5%, 15/24) were obviously lower than the respondent group of no dissemination (89.7%, 26/29). No lymph node metastasis (81.4%, 48/59), success of 5-year survival (100%, 16/16). This discrepancy was statistically significant ($P<0.01$).

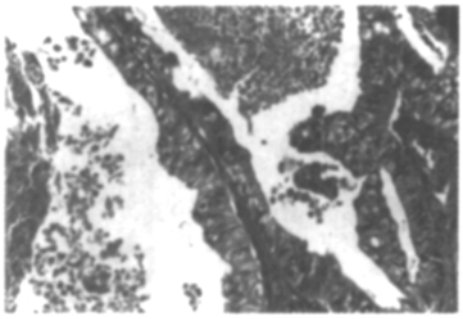

Fig. 5. Human ovarian epithelial cystadenocarcinoma show that P16 positive staining granules are mainly in the cytoplasm (S-P method ×200)

Table 2. P16 gene expression in 69 cases of ovarian epithelial cystadenocarcinoma and clinical staging

Staging	Positive cases	Positive staging intensity			
		-	+	++	+++
I	22 (88%)	3	0	8	14
II	11 (100%)	0	4	1	6
III	16 (55.2%)	13	6	8	2
IV	2 (50%)	2	2	0	0
Total	51 (73.9%)	18	12	17	22

Note: P value between staging I and II (x^2=12.648, $P<0.01$); between staging II and III (x^2=15.970, $P<0.01$); between staging III and IV (x^2=2.641, $P>0.05$).

Table 3. P16 gene expression in 69 cases of ovarian epithelial cystadenocarcinoma and the group of ascites, dissemination, lymph node metastasis, 5-year survival

Group/subgroup	Positive cases	Staging intensity				P
		-	+	++	+++	
Ascites						
yes	23/24 (67.6%)	11	7	10	6	x^2=6.282
no	28/35 (80%)	7	5	7	16	$P>0.05$
Cancer dissemination						
yes	25/40 (62.5%)	15	11	9	5	x^2=21.727
no	26/29 (89.7%)	3	1	8	17	$P<0.01$
Lymph node metastasis						
yes	3/10 (30%)	7	3	0	0	x^2=16.310
no	48/59 (81.4%)	11	9	17	22	$P<0.01$
5-year survival						
yes	16/16 (100%)	0	0	3	13	x^2=24.445
no	15/24 (62.5%)	9	7	6	2	$P<0.01$

DISCUSSION

It is well known that, in the cell cycle DNA synthesizes during G1/S transition and mitosis begins during G2/M transition. The transition depends on cycling and cyclin dependent kinase (CDK), which carry a self-control regulation to keep the balance between cell proliferation and anti-proliferation. Both overexpression of pro-proliferating factor and absence ordecrease of anti-proliferating factor can make cell proliferation out of control and carcinogenesis. P16 gene was first found by Kaub and Noborl in 1994,[1,2] since then, more and more researches of P16 gene were explored. There are still some disagreement about it between different research. However, a lot of experiments and researches in the various human tumors have proved that there are P16 gene absence or abnormal expression in these tumors. But little is known about P16 gene expression in the ovarian carcinoma. We believe that this important issue needs further exploration, and for this reason in the present paper, we investigated P16 gene expression in human ovarian poorly differentiated cystadenocarcinoma cell line, a highly metastasizing human ovarian cancer cell line, a model of highly metastasizing human ovarian cancer transplanted into subcutis of the nude mice and 69 cases of ovarian carcinoma paraffin tissues. Our founds indicated that both human ovarian carcinoma cell line and implanted tumor and metastases in the nude mice were P16 positive, but the staining intensity and positive cell proportion in metastases was weaker and lower. Therefore, we can postulate that P16 gene may play a role of anti-metastasis.

All 69 cases were categorized into various groups according to the pathohistological classification and differentiation, lymph node metastasis (yes/no), ascetic (y/n), cancer dissemination (y/n), and 5-year survival (y/n) P16 positive rate was compared with between different subgroups. The results showed that P16 was perfectly negative correlation with the tumor malignant degree, clinical staging and prognosis. P16 positive rate and its staning intensity were statistically lower and weaker when the tumor malignant degree and clinical staging increased, lymph node metastasis and dissemination occurred, and 5-year survival decreased ($P<0.05$, $P<0.01$). Our research proved that P16 gene was an important antioncogene. Its absence or abnormal expression in cells can influent the genesis, development and especially, metastasis of the ovarian carcinoma. We also fund that P16 gene expression could provide some accurate prognostic information. Further research of P16 gene will be valuable.

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