Comparison and Analysis of Expression of C-myc and p16 in Cervical Carcinoma

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Abstract **Objective** To investigate the correlation between the expression of c-myc and p16 and their roles in the genesis and development of the uterine cervical carcinoma and chemotherapy response. **Methods** Using in situ hybridization, 37 cases of cervical carcinoma (including 11 cases with chemotherapy), 21 cases of precancerous lesion and 5 cases of normal cervix were observed for C-myc and p16 mRNA with Dig-labeled probes. An image analytic system was used to detect the gray degree values of the positive signals. **Results** The positive expression rates of p16 in normal cervix,CIN(cervical intraepithelial neoplasia)and cervical carcinoma were 100%, 71.4%, and 21.6% respectively(p=0.0001), whereas the expression rates of c-myc were 0%, 42.9%, and 75.7%(p=0.0011). Statistically significant difference was found among the three groups for both p16 and c-myc. The expression of positive signals of c-myc increased with the increase of malignant degree, and the positive signals were also higher in CINⅢ than that in CINⅡ and CINⅠ. The expression rates of c-myc were decreased in cervical carcinoma after chemotherapy. There was a tendency of negative correlation between the expression of c-myc and p16. Expression of p16 and c-myc showed no significant difference between effectual and ineffectual chemotherapy groups. **Conclusion** Both over expression of c-myc and descended expression of p16 may play an important role in the genesis and development of uterine cervical carcinoma. The increased expression of c-myc in different grade CIN suggests that carcinogenesis of cervix be progressive.

Key Words Cervical carcinoma; c-myc; p16; In situ hybridization

To study the function of genes in the oncogenesis and development of tumor is very popular nowadays. The activation of proto-oncogene and inactivation of tumor suppressor genes plays an important role in occurrence and development of malignant tumor. The main function of p16 in normal cells is to restricte cells from G1 phase to enter into S phase by inhibiting CDK4, which possess inhibitory effects on proliferation of cells. Mutation or loss of p16 leads to lose the proliferation inhibition effect and induce uncontrolled proliferation of cells. c-myc is one of the key genes in carcinogenesis. It promotes sensitive of cell to growth factor and co-operates with bcl-2 to accelerate cells transformation. c-myc was known both to induce cell proliferation and activate apoptosis, which depend on the influencing from the cellular signal received (the growth factor, for example)[1]. About 95% cervical carcinomas are squamous cell carcinoma (SCC). In this study the expression of C-myc and p16 were detected in 37 cases of cervical carcinoma, 21 cases of precancerous lesion and 5 cases of normal cervix using in situ hybridization, the positive signals was evaluated by image analytic system, in order to explore the correlation between the expression of c-myc mRNA and p16 mRNA and their roles in the genesis, histological grade, pathologic types and clinical stages in cervical squamous carcinoma.

MATERIALS AND METHODS

Tissue specimens

Tissue specimens embedded in paraffin were obtained from the Department of Pathology of Kunming Medical College and Cancer Hospital from January 1998 to may 2002. 37 cases of cervical carcinoma (Among them, 26 cases was no chemotherapy and 11 cases was treated 1-2 cycles

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with DDP combination chemotherapy regimen, there were 14 cases of Low-differentiation SCC; 18 cases of Mid-differentiation SCC and 5 cases of High-differentiation SCC), 21 cases of CIN (CIN Ⅰ, CIN Ⅱ and CIN Ⅲ were 8, 8 and 5 cases respectively) and 5 cases of normal cervix (were collected from the cervix without epithelial proliferation by autopsy) were detected using in situ hybridization. Among 37 specimens of cervical carcinoma, 26 cases biopsy with hematoxylin-eosin stain were obtained after 1-2 cycles treated by epigastrica inferior arterial intubations and used to evaluate chemotherapeutic response. 11 cases show significant effectual chemotherapy response and 15 cases show ineffectual chemotherapy response.

In situ hybridization

C-myc DNA and p16 cRNA of Dig-labeled probes and ISH kits were provided by Department of Pathology of Beijing University. Tissue specimens were fixed in 10% formaldehyde solution, and paraffin embedded tissue blocks were cut into 5μm sections. Experiments were performed following the manufacturer´s instructions. Briefly, sections were treated with 0.1% HCL and proteinase K(1μg/ml). 20μl hybridized solution (Digoxin labeled probe with prehybridized solution, 2:18, denatured at 80℃) was added to each section, then incubated at 42℃ for 20h. Washing of sections was done with ssc and Buffer1. The slides were incubated in anti-Dig-AP (1:500) at 37℃ for 1h, then stained with NBT/BCIP and sealed with glycerogelatin. All dilute and consumer goods were compounded or treated with DEPC. Gloves and gauze mask were used to avoid RNase pollution. Control sections were used.

Results Analysis

Both c-myc and p16 mRNA positive expressed were showed dark purple in cytoplasm of cells, not in nuclear areas. Sections stain was assigned to positive or negative categories only. Negative staining (-) means no staining or positive cells were equal or less then 5%; Positive staining (+) means positive cells were more then 5%.

An image analytic system (HPIAS-1000) was used to detect the gray degree values of the positive signals: Each section were obtained 6 fields of vision under microscope (×400), and detected 50 cells. The cell was drawn an outline with mouse, then the gray degree values of the positive signals were detected automatically by computer. The higher the positive signals were, the less the gray degree values were. Hematoxylin and eosin(HE) stain was used to evaluate chemotherapeutic response. Compared with the cases of no chemotherapy, the cases after chemotherapy was considered effectual chemotherapy if the following was observed: cancer cells showed spotty or piecemeal necrosis; nuclear was swelling, karyorrhexis karyolysis or out of shape; cytoplasm was coagulate and stained red or showed fatty degeneration and balloon degeneration; showed inflammatory infiltration, proliferation of fibers and formation of scar in stroma. If the cases no such change, they were considered ineffectual chemotherapy

Statistical analyses

The results analysis was performed using ANOV test, χ^2 test and rank correlation. $P<0.05$ was considered statistically significant.

RESULTS

Expression of c-myc and p16 mRNA in Cervical Carcinoma and CIN

Both positive expression of c-myc and p16 mRNA Showed dark purple in cytoplasm, not in nuclear areas. The positive expression carcinoma cells of c-myc were dispersive in the groups of carcinoma cells. The stroma was negative expression (Fig.1). Positive expression of c-myc mRNA was only showing atypical hyperplasia cells in CIN. The normal epithelia and stroma were negative

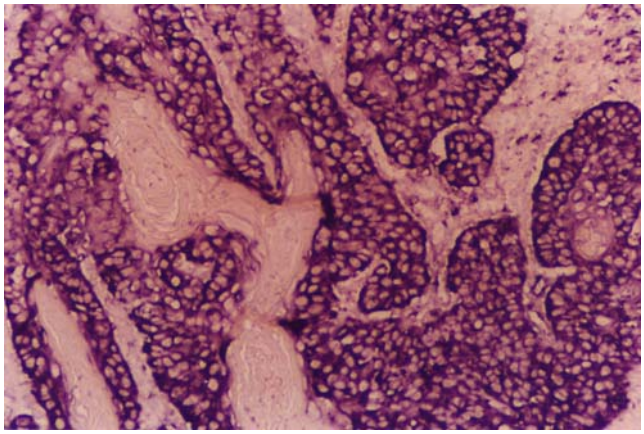

Fig.1 Expression of c-myc mRNA in cervical squamous carcinoma.

Positive expression carcinoma cells showing dark purple in cytoplasm, not in nuclear areas, the stroma was negative expression. ISH NBT/BCIP Staining, ×100

(Fig.2). The expression rates of c-myc were increased gradually, it was higher in cervical carcinoma than in CIN and eventually lost expression in the normal cervix. There were statistically significant among the three groups (χ^2=13.68, P=0.0011, table 1). In 37 cases of squamous cell carcinoma (SCC), the expression rates of c-myc were 40.0% (2/5)in high-differentiation SCC and 85.7% (12/14)in low-differentiation SCC, respectively. A significant difference existed between the two groups (P<0.01); whereas mid-differentiation SCC whose expression rates were 77.8% (14/18), there were no significant differences between mid-differentiation groups and high-differentiation groups or mid-differentiation groups and low-differentiation groups.

Positive expression cells of p16 mRNA were mainly in the epithelia of normal cervix (Fig.3) and normal cells of stroma. Most of the groups of carcinoma cells and atypical hyperplasia cells in CIN were negative expression. The expression rates of p16 were increased gradually from cervical carcinoma and CIN to the normal cervical epithelia, which showed high significantly different (X^2=20.27, P=0.0001, see tab.1). The expression rates of p16 were 60.0%(3/5)in high-differentiation SCC 27.8% (5/18)in mid-differentiation SCC and 0 (0/14)in low-differentiation SCC. A significant difference existed in three groups(P=0.0135).

The results from the image analysis were shown as follows: The expression of c-myc gradually decreased from cervical carcinoma, CIN to the normal cervix; the positive expression increased with the increase of malignant degree. The low-differentiation SCC was markedly higher than high-differentiation SCC; CIN Ⅲ was higher than CIN Ⅱ and CIN Ⅰ (P<0.05). The expressions of p-16 was negatively correlated with the expression of c-myc in each groups(rs=-0.907, P<0.05). The expression of p16 gradually increased from cervical carcinoma, CIN to the normal cervix; the positive expression decreased with the increase of malignant degree of SCC in cervix. The low-differentiation SCC was markedly lower than the mid-differentiation SCC and high-differentiation SCC; CIN Ⅲ was lower than CIN Ⅰ

Tab.1 Expression of c-myc and p16 mRNA in Cervical Carcinoma and Cervical Intraepithelial Neoplasia

Group	n	c-myc			p16		
		−	+	Positive Rate(%)	−	+	Positive Rate (%)
Cervical squamous carcinoma	37	9	28	75.7	29	8	21.6
CIN	21	12	9	42.9	6	15	71.4
Normal Cervix	5	5	0	0.0	0	5	100.0

CIN: Cervical intraepithelial neoplasia

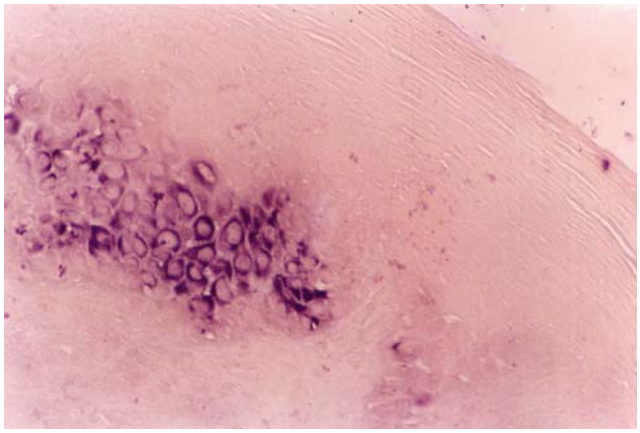

Fig.2 Expression of c-myc mRNA in Cervical intraepithelial neoplasia
Positive expression cells were in atypical hyperplasia of the 1/3 base epithelia. Showing purple only in cytoplasm, not in nuclear areas. The normal epithelia and stroma were negative expression. ISH NBT/BCIP Staining, ×200

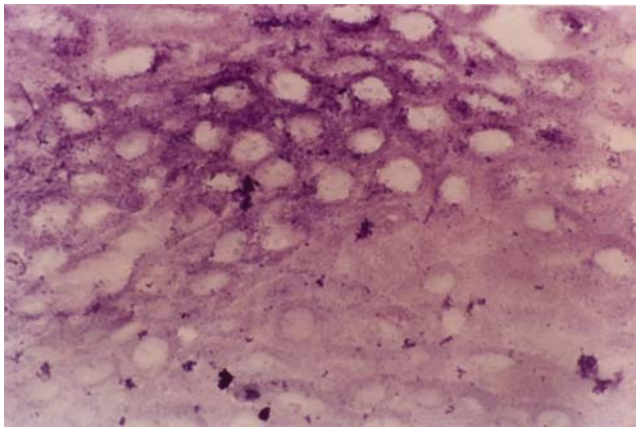

Fig.3 Expression of p16 mRNA in nomal cervix
Positive expression cells were the epithelia of nomal cervix. ISH NBT/BCIP Staining, ×200

(P<0.05). See tab.2.

The relationship between the expressions of c-myc and p16 and the chemotherapeutic response

The expressions rate of c-myc was 81.8%(9/11) in the effectual chemotherapy cases before chemotherapy and was 93.3%(14/15) in the ineffectual chemotherapy cases. There were no statistically significant difference between the two groups (P=0.3632); The expression rate of p16 was 27.3%(3/11)in effectual chemotherapy cases and was 20.0%(3/15) in ineffectual chemotherapy cases. There were no statistically significant difference between the two groups (P=0.9710). The expression both c-myc and p16 were observed in cervical carcinoma before and after chemotherapy. The expression rate of c-myc were 88.5% (23/26)before chemotherapy and 45.5% (5/11)after chemotherapy. The difference was significance in statistics (P = 0.0053); The expression rate of p16 were 23.1%(6/26)before chemotherapy and 18.2% (2/11)after chemotherapy. The difference was no statistical significance (P=0.7404).

The relationship between the expressions of c-myc and p16 and clinical stages of squamous cell carcinoma in cervix.

The expression rates of c-myc were 75.0%(3/4)、77.8%(14/18) and 73.3%(11/15)in stage Ⅰ、Ⅱ、and Ⅲ-Ⅳ of squamous cell carcinoma in cervix, respectively, no significant difference in the three groups (P=0.9565). The expression rates of p16 were 25.0%(1/4)、27.8%(5/18)、13.3%(2/15)in stage Ⅰ、Ⅱ、and Ⅲ-Ⅳ respectively, no significant difference in the three groups either (P=0.5953).

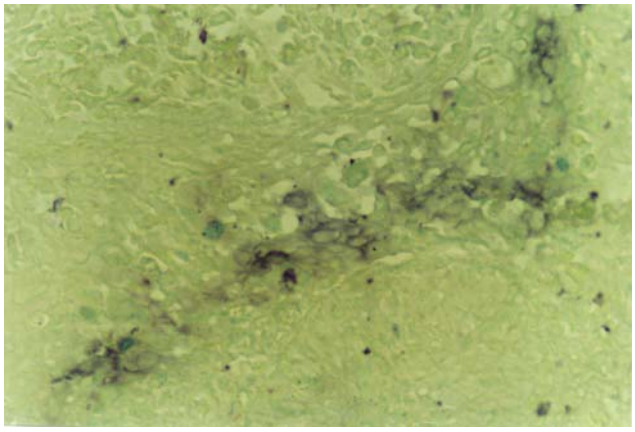

Fig.4 Expression of p16 mRNA in cervical squamous carcinoma

Positive expression in normal cells of stroma.Showing purple in cytoplasm, not in nuclear areas. The groups of carcinoma cells were negative expression. ISH NBT/BCIP Staining ×200

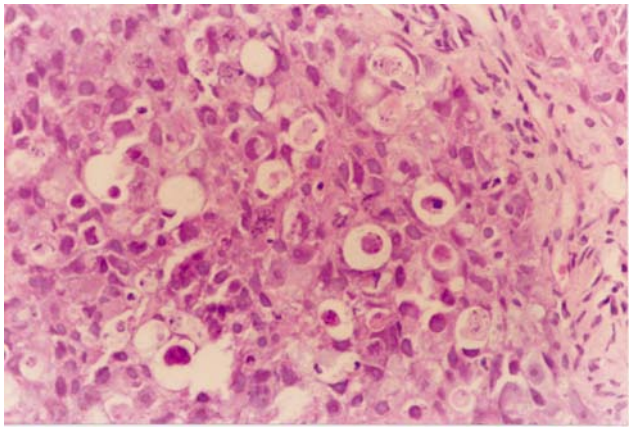

Fig.5 Cervical carcinoma after chemotherapy.

The effectual chemotherapy was observed in cervical carcinoma after chemotherapy. Showing nuclear swelling karyorrhexis or karyolysis and inflammatory infiltration in stroma. HE staining ×200.

Tab.2 The Gray Degree Values of Expression of c-myc and p16 with ISH in Groups of Cervical Carcinoma and Cervical Intraepithelial Neoplasia

Group	n	c-myc	p16
		Gray Degree Values($\bar{X}$±S)	Gray Degree Values($\bar{X}$±S
Low-differentiation SCC	14	62.59±12.88	130.88±29.19
Mid-differentiation SCC	18	67.22±24.43	93.74±26.20
High-differentiation SCC	5	84.47±6.60	88.04±7.86
CIN Ⅲ	5	96.99±8.08	61.71±7.09
CIN Ⅱ	8	117.79±19.57	54.00±11.39
CIN Ⅰ	8	140.08±24.19	30.55±12.03
Normal Cervix	5	209.53±23.63	12.58±9.67

CC:Squamous cell carcinoma; CIN: Cervical intraepithelial neoplasia

DISCUSSION

Proceeding into cell cycle is regulated by both negative and positive regulator in every strategy pass. One of the important strategy passes is from G1 phase into S phase. The eregulation of G1-S transition is a characteristic of all tumor cells[2]. p16 gene, located on chromosome 9p21, identified by Kamb[3] in1994, was one of the tumor suppressor genes which directly participate in cell-cycle regulation. The p16 protein could functionally inhibit cdks activity specifically and make Rb unphosphorylated, thus preventing the cell cycle progression from G1 phase into S phase[4] and leading to check of cell proliferation .The loss, mutation and inactivated of p16 gene may play an important role in the pathogenesis and progression of most malignant tumor[5-9]. Our data showed that the expression rate of p16 mRNA was significantly lower in cervical carcinoma than in CIN and the normal cervix. The expression of p16 gradually decreased with the increase of malignant degree of SCC in cervix. The results indicated that the occurrence and progression of cervical carcinoma were correlated with the low expression of p16 mRNA that induced by loss of p16 gene. The reduced expression of p16 could weaken and lost its suppressing function to cyclin D/CDK4, which makes cells enter S phase and unlimited proliferate, thus related to the occurrence and progression of cervical carcinoma.

The c-myc gene localizes to human chromosome 8q24 and contains three exons[10]. The c-myc gene or its product c-myc mRNA has emerged as a central oncogenic switch in many human and animal cancers[11]. Several studies suggest an important role of c-myc in cellular differentiation and malignant proliferation. The c-myc gene amplified was the most frequently activation way. The expression of c-myc mRNA increase evidently in latter G1 phase and S phase of the cell cycle, which indicate that the overexpression of c-myc mRNA plays an important role in cells proliferation. C-myc gene encodes nuclear DNA binding proteins, which can bind regulator of target genes and regulate other genes transactivation directly. The cells were expressed c-myc immediately as soon as the cells were stimulated by growth-promoting factor, which enhance cellular disunite and growth. The c-myc target genes involved in cell growth, apoptosis, and metabolism [12]. The occurrence and progression of cervical carcinoma has explicit development steps: the epithelia of normal cervix→CIN Ⅰ→CIN Ⅱ→CIN Ⅲ (include carcinoma in situ) →early invasive carcinoma→invasive carcinoma. Our results show that the expression rate of c-myc significantly higher in cervical carcinoma than in CIN, and the expression of positive signals of c-myc increased with the increase of malignant degree. The results suggested that the c-myc proto-oncogene was activated in continuing progress from the epithelia of normal cervix, CIN to the cervical carcinoma. The expression and accumulate of gene product c-myc mRNA plays a role of the development of cancerization. The degree of its expression reflected the grade of tumor differentiation. Zhang Shu-hui [13] studies glioma and find that abnormal expression of c-myc mRNA may inhibit cellular differentiation and lead to cellular differentiation abnormalities, and the degree of c-myc gene amplification was positively correlated with the speed of tumor growth. Experimental observations indicate that the positive expression of c-myc mRNA are located in atypical hyperplasia cells of CIN, and the higher grade of CIN, the higher rate of positive expression cell, which illustrate cellular canceration advance gradually. Riou [14] raised that the overexpression of c-myc gene concerned with the development of invasive cervical carcinomas and more frequency appeared in high degree malignant cervical carcinomas, and could be used as an indicator of malignant degree and poor prognosis. Iwasaka and co-workers found that in 23 cases of cervical squamous carcinoma, the cases of c-myc over expression were 45% recur in 24 month and the cases of no c-myc over expression were not recur in the same time[15]. Our data also showed that the positive expression increased with the increase of malignant degree of SCC in cervix. Thereby detect the expression of c-myc gene could provide reference information about the malignant degree, growth speed and prognosis indicator of the tumor patients. However, if the positive expression of c-myc mRNA acted as a marker to latency malignant in every grad of CIN or not, which need accumulate more data of clinical information.

Activation of proto-oncogene and/or inactivation of tumor suppressor genes are related to the carcinogenesis of many tumors, to study the different gene expression profiling between tumor and normal cells could deepen recognition of the molecular mechanism in occurrence and development of malignant tumor [16]. The cervical carcinomas was re-

garded as one of the best molds to explore the multi step process in carcinogenesis. This study found that the expression of tumor suppressor gene p16 showed significantly lower in cervical carcinoma than in CIN and normal cervix, the result turned out contrary to oncogene c-myc, the expression of c-myc showed significantly higher in cervical carcinoma than in CIN, and negative in normal cervix. There was a tendency of negative correlation between the expression of c-myc and p16 in every stage of carcinogenesis of cervix, which suggested that both the overexpression of oncogene c-myc and descended expression of tumor suppressor gene p16 may play an important role in the genesis and development of uterine cervical carcinoma.

Over the past few decades, remarkable advances have been achieved in cancer therapy, including chemotherapeutic agents, their mode of application and more broader therapeutic strategies[17]. In recently years, using combination chemotherapy with DDP by epigastrica inferior arterial intubations were wildly used to treat cervical carcinoma, but the investigation about the correlation between the expression of oncogene and tumor suppressor gene and chemotherapy response rarely. This study compare and analyse the expression of c-myc and p16 before and after chemotherapy, the results showed that the expression rate of c-myc after chemotherapy was lower than before chemotherapy, which suggested that the uncontrolled cells proliferation of cervical carcinoma be inhibited, apoptosis and necrosis increase and the other unknown mechanism may participate in the low expression of c-myc after chemotherapy, there were no statistically significant difference of the expression rate of p16 between before and after chemotherapy groups, the reason needs further study.

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