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STUDY OF DELETION OF P16 GENE IN THE PROGRESSION OF BRAIN ASTROCYTOMAS

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Objective: To study the relationship between deletion of P16 gene and occurrence and progression of astrocytomas. Methods: The techniques of polymerase chain reaction (PCR) and immunohistochemistry were used to detect the deletion of exon2 of P16 gene and expression of P16 gene in 52 cases of Brain astrocytoma. Results: The deletion rate of exon2 of P16 gene in the tumors analyzed was 34.6%. Most of them with deletion of exon2 of p16 gene were high grade astrocytomas (grade III 42%, grade IV 50%). 61.5% of the tumors were absent from expression of p16 and the deletion rate of p16 protein increased with the grade of astrocytoma (X^2=10.83, P<0.005). Conclusion: Deletion of p16 gene and protein may correlate with the malignant progression of astrocytoma.

Key words: Brain, Astrocytoma, p16, Gene

P16 gene is a new tumor suppressor gene demonstrated by kamb, et al[1] firstly in 1994. It is also named multiple tumor suppressor gene 1 (MTS1) because it's found abnormal in various cancer cell lines and tumors. The protein encoded by this gene, $p16^{ink}$, was identified as a inhibitor of the cyclin-dependent kinase CDK4/cyclin, which is involved in regulating the progression of cell cycle. High frequent homozygous deletion of p16 gene in glioma cell lines suggested that p16 gene may exert an important role in the occurrence of glioma, but the deletion rates of primary gliomas were far lower than that of glioma cell lines and different from individual experiemnt.[3-5] To study the relationship between deletion of p16 gene and occurrence and progression of astrocytoma, we detected the frequency of deletion of p16 gene and protein in 52 cases of primary Brain astrocytomas by PCR and immunohistochemistry in the present study.

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MATERIALS AND METHODS

Specimens

Fifty-two tumor specimens were obtained from patients undergoing operation from January 1997 to June 1998 at the First and Second Affiliated Hospital, Hubei Medical University, Wuhan, China. Tumor Samples were snap frozen in liquid nitrogen and stored at-70°C after sufficient material was taken to be formalin foxed and embedded in paraffin. Histological diagnosis was done by a neuropathologist. The tumors analyzed included 5 grade I, 8 grade II, 19 grade III and 20 grade IV astrocytomas. Tumors were graded according to the WHO classification. Five normal Brain tissues were used as control.

DNA Extraction and Polymerase Chain Reaction

Frozen tumor samples were crushed to a fine powder. DNA was isolated by proteinase k digestion, followed by extraction with phenol: chloroform: isoamyl alcohol.

PCR Amplification

The primer set used for amplifying exon2 of p16 was: 5'-TGGCTCTGACCATTCTGT-3' (Sense) and 5'-AGCTTTGGAAGCTCTCAG-3' (antisense). The primer set of glyceraldehyde phosphate dehydrogenase (GAPDH) gene served as an internal control was: 5'-GTGAAGGTCGGAGTCAAC-3' (Sense), and 5'-GAGATGATGACCCTTTTGGC-3' (antisense). PCR was performed in a final volume of 50μl containing 0.2μg of genomic DNA, 1.5mM Mgc12, 50mM Kcl, 10mM Tris-Hcl (PH8.3), 0.2mM deoxyribonucleotide triphosphates, 20pmol of each primer. Cycling was performed under the following conditions: Initial denatural at 95°C for 5min and adding 1.25 unit of Taq polymerase in the reaction system were followed by 35 cycles of PCR: 94°C 60", 49°C 60", 72°C 60". At last cycle, another extension of 5 min at 72°C was added. The products of PCR reactions were electrophoresed on 2% agarose gels for 1 hour at 80 volt voltage and stained with ethidium bromide. Then the gels were either observed or photographed under ultraviolate light.

Immunohistochemistry

Five-μm paraffin sections of tumors were deparaffinated, rehydrated, blocked with goat serum and reaction with p16 antiserum (Santa Cruz Corporation, America) at 1:50 dilution at 37°C for 2 hours or 4°C overnight. Subsequent steps were performed with SABC kits according to manufacturer's instruction (bo shi de biologic corporation, Wuhan, China), then diaminabenzidine method was used to visualized the sections. Hematoxylin was used as counterstain. The completed slides were then examined microscopically, p16-positive tumors cells were stained yellow or yellow-brown.

RESULTS

Deletion of Exon2 of P16 Gene

The p16 gene in DNAs from 18 tumors of the 52 astrocytomas (34.6%) could not be amplified by PCR, while GAPDH gene amplified in DNAs from all tumor samples. Each of the five normal Brain tissues had positive amplification of p16 gene. The tumors with deletion of exon2 of p16 gene are all high grade astrocytomas (Table 1 and Figure 1,2)

Table 1. Amplification of p16 exon2 of 52 astrocytomas

Grade	Number	Positive	Negative
I	5	5	0
II	8	8	0
III	19	11	8
IV	20	10	11

X^2-test comparison between grade I, II and grade III, IV, $P<0.005$, comparison between grade III and grade IV, $P>0.05$.

Figure 1

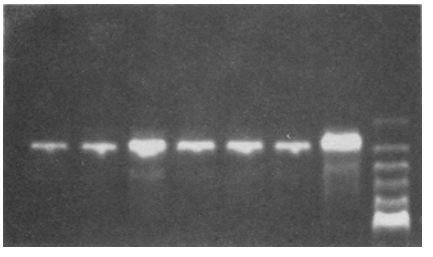

Figure 2

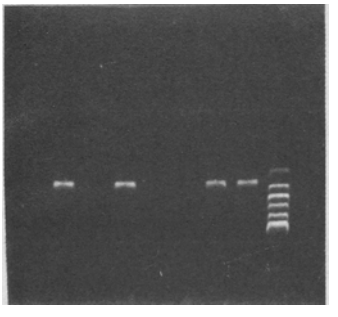

Figure 1: M represented PCR marker. Lane 1 was amplification of p16 exon2. Lane 2~7 were amplification of GAPDH gene.

Figure 2: Lane 1,2,5,7 had positive p16 exon2 amplification while lane 3,4,6 had no amplification of p16 exon2.

Immunohistochemical Deletion of P16 Expression

Thirty-two cases of 52 astrocytomas (61.5%) were absent of p16 expression. The negative rate of p16 protein of high grade astrocytomas was significantly higher than that of low grade ones ($P<0.005$) (Table 2). None of the 18 cases of

astrocytomas with p16 gene deletion were found p16 expression. All of the positive staining samples showed diffuse nuclear staining.(Figure 3,4)

Table 2. P16 expression of 52 astrocytomas

Grade	Number	Positive	Negative
I	5	4	1
II	8	6	2
III	19	6	13
IV	20	4	16

X^2 –test comparison between grade I, II and grade III, IV, $P<0.005$, comparison between grade III and IV, $P>0.05$.

Figure 3

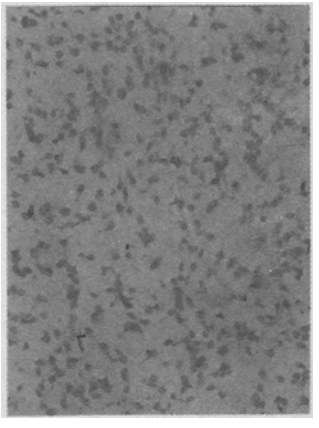

Figure 4.

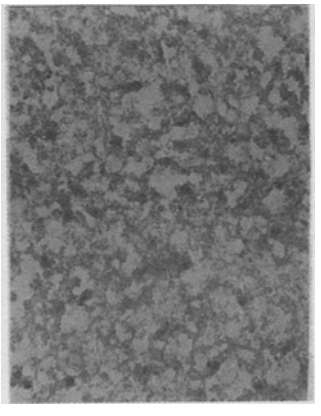

Figure 3: negative expression of p16 200×

Figure 4: positive expression of p16 400×

DISCUSSION

P16/MSTI gene is located on human chromosome 9p21, which consists of 3 exons and 2 introns. The product of p16 gene can specifically inhibit the function of cyclin-dependent kinase CDK_4 which is involved in regulating progression of cell cycle, so it indirectly prevent cells from entering into s phase through G_1 phase.[9] Cells grow uncontrolly if p16 gene is abnormal. Kamb, et al.[1] and Nobori, et al.[2] had reported the high frequent deletion of p16 gene in glioma cell lines (with deletion rate 82% and 87.5% respectively). Gomi, et al.[3] reported their results in 1995 that 7 of 9 high grade gliomas has p16 gene deletion, while only one case of 5 low grade gliomas was found deletion of p16 gene. Walker's result[4] also demonstrated that deletion of P16 gene was more frequent in high grade gliomas, but the rate of deletion of p16 gene (grade III 13/25, grade IV 27/46) was lower than that reported by Gomi, et al. In the present experiment, exon2 of p16 gene in DNAs from 52 cases of astrocytomas were amplified by PCR. Eighteen cases from the 52 astrocytomas, which were all high grade astrocytomas, were found deletion of p16 exon2 (grad III 8/19, grade IV 10/20). The results were almost consistant with that of Walker, so we suggested that deletion of p16 gene correlate with the malignant progression of astrocytoma.

Immunohistochemistry demonstrated a higher deletion frequency of p16 protein than that of p16 gene. Although most of the tumors with deletion of p16 protein were high grade astrocytomas, we also found deletion of p16 protein in low grade astrocytomas. The deletion rate of p16 protein in low grade astrocytoma is far lower than that found in high grade tumor ($X^2=10.83$, $P<0.005$). These results also provide evidence for a role for p16 gene deletion in the progression of low-grade astrocytomas to high-grade tumors, but there is a question why the deletion rates of p16 gene and protein are different. The explains may be the following: (1) Contamination of normal cells lead to false-positive amplification. (2) There may be other mechanisms of p16 gene inaction, such as mutation and methylation of 5'CPG island of p16 gene which also silence the transcription of p16 gene. Recent research has demonstrated the existence of mutation and 5'CPG island methylation of p16 gene.[6,7,8] Based on these and our observation, we concluded that immunohistochemical technique is a simple rapid method to detect abnormality of p16 gene.

Although it can't tell the type of p16 gene inaction, it is more useful in astrocytoma for judgement of malignant progression, estimation of prognosis and instruction of treatment.

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