IN SITU PCR AND IMMUNOHISTOCHEMICAL STUDIES ON p16 GENE IN PITUITARY ADENOMAS

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ABSTRACT

Objective: To examine the occurrence of p16 gene deletion and to analyze p16 expression on paraffin-embedded human pituitary adenoma specimens. Efforts were made to optimize the technical conditions for *in situ* PCR. Methods: *In situ* PCR techniques and inimuno-histochemistry were used. Results: Immunohistochemically, p16-positive tumor cells were observed in all cases with various proportions. The majority of the stromal cells and part of tumor cells was devoid of p16 immunostaining, but signal of *in situ* PCR for p16 gene, exon 2, was displayed in these cells. Conclusion: The results implied that p16 gene might not be deleted in these pituitary adenomas. It also indicated that *in situ* PCR, both direct and indirect methods, proved feasible and informative to the aim of DNA detection. It is critical to overcome non-specific amplification in direct *in situ* PCR by means of higher annealing temperature, fewer cycle, lower magnesium concentration and stringent washing. A target DNA-deleted sample as the negative control is extremely necessary. For the indirect method, the way to improve the sensitivity is to loosen the conditions for amplification and washing, so that more amplification products are subject to hybridization, and signal detection is facilitated.

Key words: *In situ* PCR, Immunohistochemistry, p16 gene, Pituitary adenoma

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The alteration of p16, a member of cyclin-dependent kinase inhibitor family, has been intensively investigated by the approaches of molecular biology. The gene encoding p16 protein, named Multiple Tumor Suppressor 1 (MTS 1), was reported to be homozygously deleted in a large number of tumor cell lines.[1,2] While these data suggested possible wide and essential involvement of p16 deletion in multiple types of human tumors, p16 gene deletion in primary tumors has been found much less predominant than in cultured cell lines.[3-5] This disparity was attributed, at least to some extent, to a impropriety of methodology: When a homogenate was used for gene-deletion detection with molecular biological techniques, non-neoplastic tissue admixed in specimens of primary tumor will contaminate the tumor DNA.[6,7]

In situ gene amplification techniques, i.e. *in situ* polymerase chain reaction (PCR) techniques, are the alternation and development of PCR techniques in fluid phase. They allow a specific DNA fragment amplified within the original cell, then detected *in situ*.[8] Since the cells are morphologically identified, one could know if a specific gene or its fragment exists in a certain population of cells. It seems reasonable for such kind of techniques to help determining the deletion of gene like p16, albeit the application of *in situ* PCR in this aspect has not been documented.

In the present study, we examined the occurrence of deletion of p16 gene, specifying on its exon 2 which was reported to be lost most frequently, with *in situ* PCR techniques on paraffin-embedded human pituitary adenoma specimens. The status of p16 expression was also analyzed by immunohistochemistry (IHC). Efforts were made to optimize the technical conditions for *in situ* PCR of both direct and indirect methods, as well as to validate the results of *in situ* PCR and immuno-histochemistry. Comparison of direct and indirect PCR approaches was discussed.

MATERIALS AND METHODS

Tumor Specimens

Thirty-one specimens were freshly collected from pituitary adenomas surgically resected in Rui Jin Hospital and Rui Jin Hospital, and were uniformly prepared. After a four-hour fixation in 4% paraformaldehyde buffered in phosphate saline (PBS), tissues were dehydrated through graded ethanol and xylene, and embedded in paraffin. Six μm-thick sections were cut and mounted on 1.22 mm-thick silane-coated glass slides. The adjacent sections were used respectively for *in situ* PCR and immunohistochemistry. The slides bearing the sections were heated on a 60°C hot plate in order to strengthen the adhesion of the tissue to the slides. Before testing, sections were dewaxed by xylene and rehydrated through graded ethanol.

In Situ PCR

In Situ Gene Amplification

The sections, prior to *in situ* PCR, underwent a digestion with 20 μg/ml proteinase K (Merck; Damastadt, Germany) for 8 minutes at 37°C followed by a heating at 95°C for the purpose of inactivating the proteinase. The tissue was subsequently digested with 20 μg/ml RNase A (SABC; Luoyang, China) at 37°C for 1 hour. After rinsing in PBS, sections were dehydrated using 50% and 100% ethanol. The sections could be kept in 100% ethanol before PCR reaction mixtures were prepared, then finally taken out and allowed to air dry.

The primers specific for the exon 2 of p16 gene were synthesized according to a sequence[9] as follows: sense 5'-TGGCTCTGACCATTCTGT; antisense 5'-AGCTTT-GGAAGCTCTCAG. The target amplificants was 400 base-pair long which was checked by electrophoresis. *In situ* PCR reaction mixture was comprised of 10 mmol/L Tris-HCl, pH 9.0 (at 25°C), 50 mmol/L KCl, 1.5 or 3.5 mol/L $MgCl_2$, 0.1%Triton X-100, 200 μmol/L dATP, dCTP, dGTP and dTTP each, 0.4 μmol/L specific primer pair, of 3' end and 5' end each, 0.15 U/μl Taq DNA polymerase (Promega; Madison, WI). In direct *in situ* PCR method, 200 μmol/L dTTP were replaced by 190 μmol/L dTTP and 10 μmol/L digoxigenm-11-dUTP (Boebringer; Mannheim, Germany) thus to make the amplification products labeled, and the concentration of $MgCl_2$ was 1.5 mmol/L. In indirect method, 3.5 mmol/L $MgCl_2$ was selected. For each sample, 20 μl of reaction mixture was added within the wells made by gene frames (Hybaid, Teddington, UK) which surrounded the sections on slides before being covered and sealed with the films Suresea1 (Hybaid). *In situ* PCR was carried out on TouchDown thermal cycling system with *in situ* blocks (Hybaid). It was programmed for direct *in situ* PCR at 94°C for 5 minutes; 10 cycles of 94°C for 1 minute and then at 52°C for 2 minutes followed by 72°C for 2 minutes. For indirect method, annealing temperature was changed to 45°C and number of cycles became 30. After cycles were completed, samples were incubated for additional 5 minutes at 72°C.

While optimizing the direct *in situ* PCR conditions, we tested the variables including cycle number of 15, annealing temperature at 48°C or the concentration of $MgCl_2$ at 2.5 and 3.5 mmol/L. For indirect method, annealing temperature at 52°C and the concentration of $MgCl_2$ of 1.5 or 2.5 mmol/L were tried.

Post-PCR Wash

The sections were washed, after direct *in situ* PCR, in a higher stringency condition, i.e. 2 × standard saline citrate (SSC) at 37°C, 1 × SSC at 45°C, then 0.5 × SSC at 45°C, each for 15 minutes. In indirect method, i.e. PCR *in situ* hybridization, the post-PCR wash was briefly in 2×SSC at room temperature (RT) for 5 minutes.

Hybridization and Detection

For indirect PCR method, the post-PCR sections underwent *in situ* hybridization with the specific probe in hybridization mixture at 42°C for overnight in a humidifying chamber after a denaturation at 95°C for 10 minutes. Probe for p16 mRNA was a 0.8 kilobase-long cDNA molecule (provided by Department of Pathology, Beijing Medical University) labeled with digoxigenin (using a kit purchased from Boebringer Mannheim). Hybridization mixture contained, in addition to the probe, 50% deionized formamide, 10% dextran sulfate, 1× Denhardt, 10 mmol/L Tris-HC1 pH 8.0, 1 mmol/L EDTA, 0.25 mg/ml salmon sperm DNA and 0.3 mol/L NaC1. Sections were then rinsed in 2 × and 1× SSC each for 10 minutes at room temperature before immunohisto-chemical detection.

Detection of labeled hybrids or amplificants was performed by incubation with a monoclonal anti-digoxigenin antibody conjugated with alkaline phos-(AKP) (Boebringer Mannheim), diluted in 1:1000, for 2 hours at RT. The signal was finally visualized through the substrates of AKP (NBT and BCIP, Boebringer Maimheim). Some of the sections were counterstained with eosin.

Negative Controls

Specificity of the amplification and hybridization was confirmed by omitting either the p16 primers or probe. EC-8712, an esophageal carcinoma cell line known to be defective of p16 gene[10] was used as negative control.

Cultured EC-8712 cells were trypsin-digested and spinned down before being prepared into paraffin-embedded sections similar to that of pituitary tumors.

IHC

Sections were treated in 3% H_2O_2 to inhibit endogenous peroxidase activity, then in 1% bovine serum albumin (Sigma; St. Louis, MO) to block nonspecific protein binding sites. The p16 expression was detected by polyclonal anti-p16 antibody C-20 (Santa Cruz; Santa Cruz, CA) in a dilution of 1:60. After an incubation of primary antibodies overnight at 4°C, visualization was achieved by the biotin-strepavidin-peroxidase method (LSAB kit; DAKO; Glostrup, Denmark) with 3, 3' diaminobenzidine (DAB) as a chromogene. Sections were finally counterstained with hematoxylin.

Negative control was set up by omitting the primary antibody incubation. EC-8712 cell line was also used as a negative control. An autoptic normal pituitary tissue underwent the similar IHC procedures.

RESULTS

p16 IHC

Part of tumor cells displayed positive p16 staining, showed by yellow-brown DAB deposits on the nuclei, with variation in intensity from cell to cell (Figure 1A). The proportions of the p16 positive cells also greatly differed on one patient's specimen from another. The majority of stromal cells in tissue showed negative staining which were seen as blue with hematoxylin counterstain. Only a few stromal cells were positive, but were usually weak. None of the cases showed complete absence of positive cells unless the specimens were of necrosis, which had been excluded from analysis. Cells in control with omitted p16 antibody incubation were absent in immunostaining. Samples of EC-8712 displayed no p16 immunostaining. All endocrine cells and stromal cells in normal postmortem pituitary specimen showed negative for p16.

p16 *in situ* PCR

After gene amplification through either direct or indirect methods, exon 2 of p16 gene was detected in the nuclei of all types of the cells in pituitary adenoma. Comparison of adjacent sections manifested that tumor cells as well as stromal cells and capillary epithelial cells that were absent for p16 immunostain all showed blue-purple gene-amplification signal (Figure 1B). Same location was devoid of signal in controls without specific p16 primers or probe. No color developed on sections of EC-8712.

In direct *in situ* PCR method, only a strict condition resulted in a true signal. This included simultaneously an annealing temperature high as 52°C, number of cycle not more than 10, concentration of magnesium ions equal to 1.5 mmol/L and a post-PCR wash in high stringency. False signal appeared when using lower annealing temperature as 48°C, increased cycle number as 15 or higher magnesium concentration as 2.5 mmol/L, in that EC-8712 cells developed color. Indirect method i.e. PCR *in situ* hybridization displayed the signal generally much weaker than in direct method (Figure 2A and 2B). A higher annealing temperature at 52°C or a lower magnesium concentration at 1.5 mmol/L made the signal undetectable. Samples omitting p16 probe showed no signal. Conservation of tissue morphology in indirect *in situ* PCR was poorer than in direct method.

DISCUSSION

Optimization and Validation of *in situ* PCR

In situ PCR is a novel technique for localization of low copy number of nucleotide sequences such as gene sequences or low-abundant RNA. There has been a large quantity of work referring to detecting low-abundant mRNA using RT-*in situ* PCR.[1] As for the purpose of localization of DNA sequences, this technique has been applied preferentially to virology,[8, 12] though detection of rearranged, translocated or mutated single-copy genomic DNA also proved possible.[13-15] In p16 study, RT-*in situ* PCR was used to evaluate the level of p16 expression in primary melanomas.[16] However, to our knowledge, detection of presence of p16 gene on clinical specimens has not been reported. In our findings on p16 immunohistochemistry, some of pituitary adenomatic cells were positive for p16 immunostaining, while others and most of stromal cells on the same section were negative. It thereby became interesting to examine the existence of p16 gene in these immunohistochemically p16 negative cells by *in situ* PCR and to determine if the absence of p16 immunostaining in part of tumor cells was a result from the loss of the gene.

The results of *in situ* PCR demonstrated that exon 2 of pl6 gene was detectable in both stromal cells and tumor cells. This specific DNA fragment existed not only in cells positive for p16 immunostaining, but also in those negative ones (including tumor cells and stromal cells). The results indicated that the absence of p16 immunostaining in some tumor cells was not due to the deletion of exon 2 of p16 gene. While getting information about the presence of gene, we tested optimized conditions for both direct and indirect methods of *in situ* PCR.

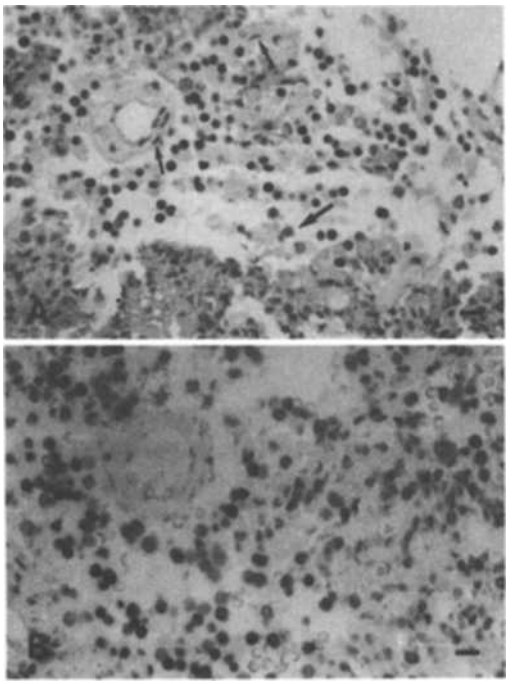

Fig. 1. Immunohistochemistry (A, hematoxylin- counter-stained) and direct *in situ* PCR; (B) for p16 protein and gene in pituitary adenoma. Positive immunostaining (brown- yellow) was predominantly in the nuclei of tumor cells. Most of stromal cells (small arrows) and part of tumor cells (big arrow) were devoid of immunostaining (A), but p16 gene, exon 2, existed in these cells as demonstrated by *in situ* PCR signal (B, blue-purple). Bar = 18 μm

The results suggested that direct method got stronger signal and hence had higher sensitivity, but the attention should be focused on its lower specificity. In the direct PCR method, a labeled nucleotide is incorporated into the amplificants, then the signal is visualized by an immunihistochemical detection. Test in this work impressed us that a nonspecific amplification signal was very easy to occur in direct PCR. This is accordant with the findings of some investigators,[13,17,18] though some others did not emphasize false-positive risk in this technique.[14] Our results indicated that three variables on the procedure of direct *in situ* amplification were critical to suppress the non-specific DNA amplification, these being higher annealing temperature, fewer cycles and lower magnesium concentration. A magnesium concentration higher than 1.5 mmol/L should be used with great prudence. Post-PCR wash under higher stringent conditions was also important to help reduce non-specific signal, as we washed the sections in 2×SSC at 37°C, 1×SSC and 0.5×SSC at 45°C for longer time. More reliability was guaranteed by taking not simply a primer-minus sample, but rather, a gene-deleted cell sample as negative control. Some authors indicated an inevitable false positive signal when using direct *in situ* PCR on the tissue sections,[13,17,18] since nonspecific amplification was believed to link with DNA repair that frequently occurred in the embedded tissue sections, and was primer-independent. In the present work, we were able to avoid the occurrence of false positivity on various negative control samples and verify the truth of signal by means of monitoring the conditions for amplification and washing, as well as designing the proper controls.

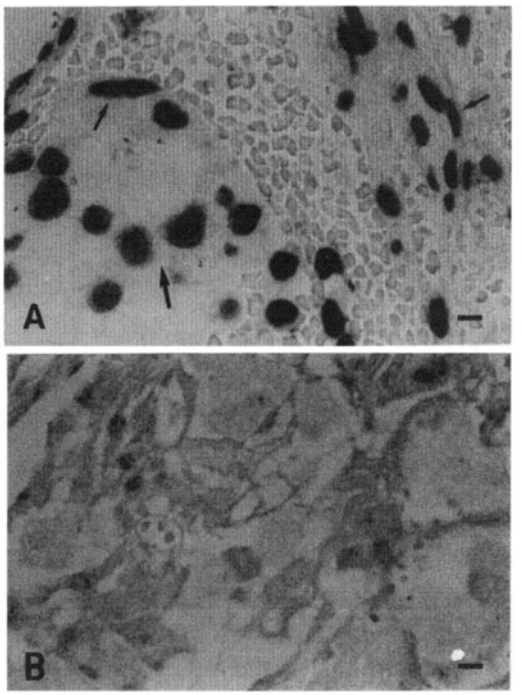

Fig. 2. *In situ* PCR for p16 gene exon 2 in pituitary adenoma with direct (A) and indirect (B, eosin-counterstain) methods. *In situ* PCR signal were displayed in both stromal cells (small arrows) and tumor cells (big arrow). Indirect method showed weaker signal and poorer morphological structures. Bar = 9 μm

In indirect PCR approach, the specific primers are not labeled, and a post-amplification hybridization allows a labeled probe bound with the amplificants before the immunohistochemical detection to visualize the signal. Contrary to the direct method, indirect *in situ* PCR in our work provided better specificity but compromised the signal intensity and morphology. Despite in PCR procedures of our experiments, a low annealing temperature, increased cycle number and magnesium concentration were used, which all together allowed producing more amplificants, the amount of specific amplificants that could be hybridized with the labeled probe was still limited, which made visualized signal weaker. The post-amplification hybridization served additionally as a screening to let only specific amplificants to be visualized, and thus accounted for

lower signal-intensity and higher specificity. The way to facilitate the signal detection in our experiments was to loosen the conditions for amplification and washing, so that more amplificants were maintained before hybridization, and sensitivity was improved. Morphological structure of the sections in indirect *in situ* PCR was damaged because of a long time exposure to the high temperature during the thermal cycle, but it did not usually prevent from cell identification or signal localization. Taken together, the techniques of *in situ* PCR, by either both direct and indirect methods, proved feasible and informative to our aim of pl6 gene detection.

The Expression of pl6 Gene in Pituitary Adenomas

In spite of the variations in criteria or experimental procedures, the relative content of a special protein in neoplastic cells is measurable by IHC as long as it is always compared with that in non-neoplastic cells on the same section. Immunohistochemical evaluation of p16 protein level in the tumor cells was achieved when the surrounding or admixed stromal cells were taken as internal control.[19–21]

p16 is believed to be expressed in very low level in normal tissue.[19, 21, 22] Being consistent with this idea, in our results, a majority of the stromal cells in pituitary adenoma and all cells in normal postmortem pituitary showed negative for p16 immunostaining. Comparatively, on the same specimen, when part of neoplastic cells, more or few, displayed positive p16-immunostaining, they should be considered to possess higher p16 content than the normal cells, and then defined as to overexpress p16 gene. Interestingly, in our previous work, the percentages of the p16-positive tumor cell, which might represent the degree of p16 overexpression, showed to link with clinico-pathological characteristics of the patients. A higher percentage of p16-positive tumor cells was paralleled with a bigger tumor size, easier invasion of the sphenoid or more recurrence.[23]

In p16 study, loss of immunostaining in tumor cells was usually considered as non-expression or inactivation of p16 protein,[24, 25] and could be deduced as to have p16 gene deletion in these cells. In our work, the stromal cells in adenomas and normal pituitary cells showed negative p16-immunostaining; they would by no means lose the gene. As we could demonstrate, through the comparison of results of IHC and *in situ* PCR, the absent p16 immunostaining in part of tumor cells was not due to a gene deletion, but rather, probably due to a lower or normal p16 level.

Recently, several authors reported the elevated p16 expression and its implications for more malignancy and poorer prognosis in transformed cells and a few types of primary tumors like ovarian tumors, bladder carcinomas and so on.[21, 22, 26–28] Together with these previous data, the present study supported the conclusion that the patterns of p16 gene alteration may differ in different types of tumors. Overexpression of p16 could be one of the events involved in development and progression of pituitary adenomas. The possibility for other molecular events accounting for the absent immunostaining in some tumor cells, like mutation and hypermethylation of p16 gene,[29,30] yet deserve further investigation. The precise mechanism about how the abnormal overexpression of a tumor suppressor gene may result in or from the occurrence of tumor is waiting for an elucidation.

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REFERENCRS

[1] Kamb A, Gruis NA, Weaver-Feldhaus J, et at.,. A cell cycle regulator potentially involved in genesis of many tumor types. Science 1994; 264:436.

[2] Nobori T., Miura K, Wu DJ, et al. Deletion of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature 1994; 368:753.

[3] Cairns P, Mao L, Merlo A, et at., Rates of pl6 (MTSL) mutation in primary tumors with 9p loss. Science 1994; 265:415.

[4] Marx J. A challenge to p16 gene as a major tumor suppressor. Science 1994; 264:1846.

[5] Spruck III CH, Gonzalez-Zulueta M, Shibata A, et at. P16 gene in uncultured tumours. Nature 1994; 370:183.

[6] Kamb A, Liu Q, Harsbman K, et at. Response to: Rates of pl6 (MTS1) mutation in primary tumors with 9p loss. Science 1994(B); 265:416.

[7] Walker DG, Duan W, Popovic EA, et at. Homozygous deletion of the multiple tumor suppressor gene 1 in the progression of human astrocytoma. Cancer Res 1995; 55:20.

[8] Nuovo GJ. PCR *in situ* Hybridization, Protocols and Applications, 3rd ed. New York: Lippincott-Raven, 1997; p1–454.

[9] Osuke W, Masaaki N, Hirotaka O, et at. *In vivo* occurrence of p16 (MTSL) and p15 (MTS2) alterations preferentially in non-small cell lung cancers. Cancer Res 1995; 55:514.

[10] Zheng J, Hou P, Thou CN, et at. Loss of CDKN2 gene and amplification of cyclin Dl gene in human esophageal cancer. Chin J Onco 1996; 18:408.

[11] Simon S, Reipert B, Eibl MM, et al. Detection of

Pbosphatid-ylmositol glycan class A gene transcripts by RT *in situ* PCR. A comparative study on fluorescein, Texas Red, and digoxigenin-11 dUTP for color detection. J. Histochem. Cytochem 1997; 45:1659.

[12] Bagasra O, Hauplman SP, Lisgbner HW, et al. Detection of human immunodeficiency virus type 1 provirus in mononuclear cells by *in situ* polymerase chain reaction. New Eng J Med 1992; 326:1385.

[13] Long AA, Koniminoth P, Lee E, et at. Comparison of indirect and direct *in situ* polymerase chain reaction in cell preparations and tissue sections. Detection of viral DNA, gene rearrangements and chromosomal translocations. Histochemistry 1993; 99:151.

[14] Martinez A, Miller M, Quinn K, et at. Non-radioactive localization of nucleic acids by direct *in situ* PCR and in direct RT-PCR in paraffin-embedded sections. S Histochem Cytochem 1995; 43:739.

[15] Sagawa M, Jones JE, Ebina M, et at. Detection of K-ras point mutation by *in situ* PCR in cell suspensions: comparison of the indirect and direct methods. Lung Cancer 1997; 17:181.

[16] Keller MR, Schmidt R, Piepkom M. Expression of the tumor suppressor gene product pl6INK4 in benign and malignant melanocytic lesions. S Invest Dermatol 1998; 110:932.

[17] Sagawa M, Jones JE, Saito Y, et at. Specific primer design and exonuclease III treatment for the reduction of nonspecific staining in direct *in situ* PCR. Cell Vis 1998; 5:20.

[18] Nuovo GJ, Hohmanb RJ, Nardoneb GA, et al. *In situ* amplification using universal energy transfer-labeled primers. J Histochem Cytochem 1999; 47:273.

[19] Geradts J, Wilson PA. High frequency of aberrant $p16^{INK4A}$ expression in human breast cancer. Am J Pathol 1996; 149:15.

[20] McShea A., Harris PLR, Webster KR, et at. Abnormal expression of the cell cycle regulators p16 and CDK4 in Alzheinier's disease. Am S Pathol 1997; 150:1933.

[21] Dong Y, Walsh MD, McGuckin MA, et at. Increased expression of cyclin-dependent kinase inhibitor 2 (CDKN2A) gene product $p16^{INK4A}$ in ovarian cancer is associated with progression and unfavourable prognosis. Int J Cancer 1997; 74:57.

[22] Tam SW, Shay JW, Pagano M. 1994; Differential expression and cell cycle regulation of the cyclin-dependent kinase 4 inhibitor $p16^{INK4A}$. Cancer Res 1997; 54:5816.

[23] Yi J, Yu D, Chen Y, et al. p16 overexpression in pituitary adenomas studied by immunohistochemistry and *in situ* hybridization. Cbin Med J 2000; 113:162.

[24] Reed AL, Califano P, Cairnus W, et al. High frequency of p16 (CDK2/MTS-1/INK4A) inactivation in head and neck squamous cell carcinoma. Cancer Res 1996; 56:3630.

[25] Marsh KL, Varley JM. Frequent alterations of cell cycle regulators in early-stage breast lesions as detected by immunohistochemistry. Br J Cancer 1998; 77:1460.

[26] Hara E, Smith R, Parry D, et al. Regulation of p16CDKN2 expression and its implication for cell immortalization and senescence. Mol Cell BioL 1996; 16:859.

[27] Shigemasa K, Hu C, West CM, et al. P16 overexpression: a potential early indicator of transformation in ovarian carcinoma. J Soc Gynecol Invest 1997; 4:95.

[28] Asamoto M, Hori T, Baba Toriyama H, et al. P16 gene overexpression in mouse bladder carcinomas. Cancer Lett 1998; 127:9.

[29] Yoshimoto K, Tanaka C, Yamada S, et al. Infrequent mutations of $p16^{INK4A}$ and $p15^{ink4B}$ genes in human pituitary adenomas. Eur J Endocrinol 1997; 136:74.

[30] Woloschak M, Yu A, Post KD. Frequent inactivation of the p16 gene in human pituitary tumors by gene methylation. Mol Carcinogenesis 1997; 19:221.