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Analysis of P16INK4A Protein Expression in Follicular Thyroid Tumors*

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P16INK4A (P16) protein expression was analyzed immunohistochemically in archival material derived from 12 cases of follicular thyroid carcinoma, 57 cases of follicular adenoma and 17 cases of nodular goiter. Among follicular carcinomas, 11 out of 12 examined cases (91.7%) were positive for P16INK4A protein. Among follicular adenomas the percentage of immunopositivity was 76.5% (45/57) and among nodular goiter cases it was 19.3% (13/17). Overexpression of P16INK4A protein was found in 66.7% (8/12) of follicular carcinomas and in 19.3% (11/57) of follicular adenomas; the values of this parameter were statistically significantly higher in the follicular carcinoma group ($p<0.005$). No P16INK4A protein overexpression was noted in nodular goiter cells. High immunohistochemically-detected expression of P16INK4A protein in follicular thyroid carcinoma cells suggests that the altered expression pattern of P16INK4A protein may disturb the regulatory mechanisms of thyreocyte cell cycle and plays a significant role in the formation of benign neoplasms and their malignant counterparts derived from follicular thyroid cell.

Introduction

Multiple genetic changes in the cellular genome are involved in the genesis and progression of human cancers [1, 48, 49]. Recent papers show that carcinogenesis is inevitably linked to disturbances in the cell cycle regulation [5, 34, 49]. Commitment of cellular division usually occurs at the end of G1 phase at the so-called internal (restriction) control point G1/S (cell-cycle checkpoint). The passage through G1/S checkpoint is regulated by several proteins, such as serine-threonine protein kinases (cdk2, cdk4, cdk6) known as cyclin-dependent kinases, cyclins D and E and members of the cyclin-dependent kinase inhibitors of the INK4 and Cip/Kip families [15, 22, 33]. The key roles in the regulation of cell cycle G1/S passage are also played by three of the so far best-studied suppressor genes i.e. *Rb, p16INK4A* and *p53* [5, 34, 49].

p16INK4A gene, also known as *CDKN2A* or *MTS1* encodes a 16kDa nuclear protein P16INK4A, localized in 9p21 chromosome region [4, 29, 36, 46]. P16INK4A protein belongs to the INK4 protein family and forms complexes with cdk4 and cdk6 kinases, the regulatory subunits of which are formed by cyclin D [5, 22]. The basic function of P16INK4A protein in the regulation of cell cycle G1/S passage is accomplished via competitive binding to cdk4 (cdk6), instead of cyclin D. In this way P16INK4A protein inhibits phosphorylation of Rb protein and blocks the transition to the S phase of the cell cycle [5, 34, 49].

Loss of genetic material in the 9p21 region, leading to the loss of *p16INK4A* gene suppressor function, was observed in cells of many primary neoplasms [8, 18, 24, 25,

* The study was supported by the Grant No. P05B 037 10 from the National Committee for Scientific Research

30, 40] and neoplastic cell lines [3, 28, 39]. Besides the point mutations or homozygous deletions in the *p16INK4A* gene, its inactivation results also from cytosine methylation in the CpG island-rich region, localized within the 5'end of the gene promoter [17, 20, 21, 27, 45].

Jones et al. [28] have identified homozygous deletions of the *p16INK4A* gene in two out of three investigated follicular thyroid cancer cell lines (R082, K5) and in two out of four papillary carcinoma cell lines (K1, K2). Ivan et al. [27] have identified a mutation (T/C transition) in the region flanking exon 1 of the *p16INK4A* gene in the papillary carcinoma cell line (NPA). Elisei et al. [7] have identified a mutation (GCC/GTC) in the codon 91 of the *p16INK4A* gene in one out of twelve papillary thyroid carcinomas.

Immunohistochemical detection of such suppressor proteins as P53 and Rb, as well as of P21/WAF1 and cyclins D and E, which take part in the regulation of cell cycle G1/S passage was a subject of numerous analyses in cases of follicular neoplasms of the thyroid gland [9, 16, 26, 32, 38, 51]. So far, there have been no reports dealing with immunohistochemical detection of *p16INK4A* gene expression product in the follicular carcinomas of the thyroid.

Material and Methods

Material

Paraffin-embedded archival tissues of 12 cases of follicular thyroid carcinoma, 57 cases of follicular thyroid adenoma and 17 cases of nodular goiter were studied. All the sections were examined by two pathologists (J.S. and S.S.), using a conference microscope and were histopathologically classified, as suggested by the WHO Committee [19].

Immunohistochemical staining

Representative paraffin blocks, containing tumor material from each case, were sectioned at 4μm, affixed to silanized slides and dried overnight at 56.7°C. The sections for immunohistochemical study were stained using the avidin-biotin (ABC) method, according to Hsu et al. [23]. Deparaffinized sections were treated with 3% hydrogen peroxide (H_2O_2) in methanol for 5min to block endogenous peroxidase activity. Non-specific antibody binding was reduced by incubation of the sections for 20min with normal goat serum. The slides were incubated with a 1:100 dilution of a primary mouse anti-human P16INK4 monoclonal antibody (clone G175-405, PharMingen, San Diego, CA, USA). In the negative control reaction the primary antibody was omitted. The reaction products were demonstrated using the Novostain Super ABC kit (NCL/ABCm) from Novocastra. 3, 3'-diaminobenzidine (DAB) was used as chromogen and the sections were counterstained with Mayer's hematoxylin, dehydrated and mounted. The paraffin-embedded sections from ductal breast carcinoma were used as a positive control. The immunohistochemical staining of cells was estimated by means of the semiquantitative method, using Hogg's net. The results were expressed as the percentage of positive cells per 1000 follicular thyroid cells, counted in 10HPF (objective magnification x40). To score the P16INK4A staining pattern, we used the criteria proposed by Geradts and Wilson [14]. Only cells with evidence of nuclear staining were considered positive. The relative number of immunoreactive cells was graded as follows: (-) – less than 10% of tumor cells stained positively; (+) – 10–50% of tumor cells stained positively; (++) >50% of tumor cells stained positively [31]. Lesions scored as (++) were considered as showing high expression (overexpression) of the P16INK4A protein [11].

Statistical procedure

All parameters, represented as means were compared using Mann-Whitney's test, where $p<0.05$ was considered significant. Associations between categorical variables with P16INK4A expression were assessed using Fisher's exact test.

Results

The percentage of cells positive for P16INK4A was highest in the follicular carcinoma group (62.1±34.2) (Fig. 1) followed by the follicular adenoma group (32.0±26.0) and nodular goiters (19.6±10.8). Within the group of follicular adenomas the percentage of positive cells was 36.2±27.0 in microfollicular ones, 24.2±23.4 in normo-/macrofollicular and 33.1±27.1 for adenomas derived from oxyphilic cells (Table 1). The average percentage values of cells positive for P16INK4A protein were statistically higher in the group of follicular carcinomas, compared to follicular adenomas and nodular goiters ($p<0.005$). A comparison of the average percentage values of cells positive for P16INK4A protein in follicular adenoma and nodular goiter groups did not reveal any significant difference ($p=0.061$). The average percentage values of cells positive for P16INK4A protein in micro-, normo-, macrofollicular and oxyphilic cell-derived adenomas were not statistically different, when compared to each other.

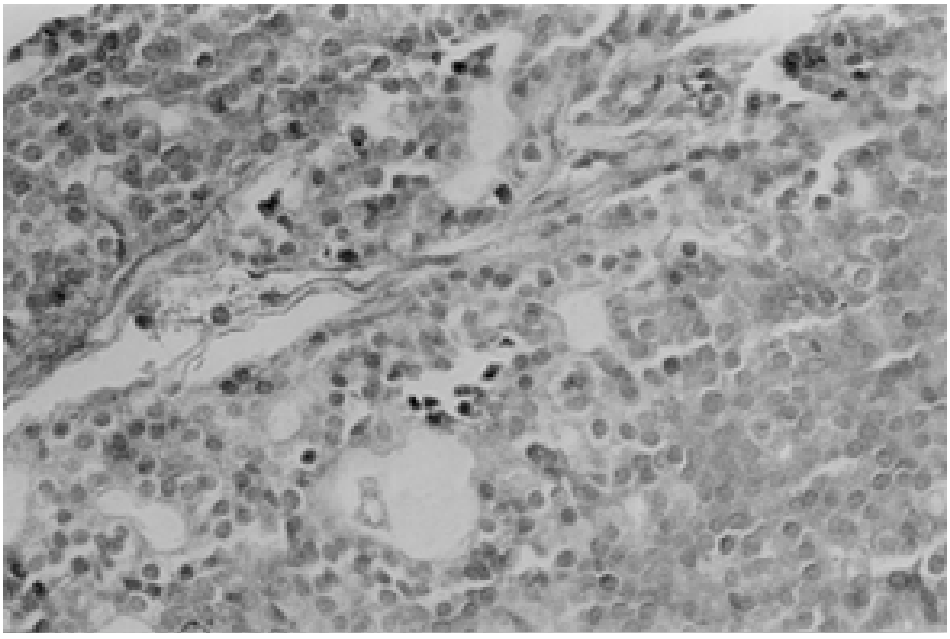

Fig. 1. P16INK4A positivity in thyroid follicular carcinoma – distinct nuclear and weak cytoplasmic reaction.

TABLE 1

The average per cent of P16INK4A protein-positive cells in the examined groups

Group	N	M	SD	Min	Max
Follicular carcinoma	12	62.1	34.2	7.4	99.8
Follicular adenoma including:	57	32.0	26.0	0.0	96.5
– microfollicular	31	36.2	27.0	0.0	96.5
– normo- and macrofollicular	18	24.2	23.4	4.1	87.2
– from oxyphilic (Hürthle) cells	8	33.1	27.1	5.6	86.1
Nodular goiter	17	19.6	10.8	3.1	42.3

N – number of cases; M – arithmetic mean; SD – standard deviation; Min – minimum value; Max – maximum value.

TABLE 2

The number of P16INK4A protein-positive and negative cases in the examined groups

Group	N	n (%)		
		++	+	–
Follicular carcinoma	12	8 (66.7)	3 (25.0)	1 (8.3)
Follicular adenoma including:	57	11 (19.3)	34 (59.6)	12 (21.1)
– microfollicular	31	7 (22.6)	18 (58.1)	6 (19.3)
– normo- and macrofollicular	18	2 (11.1)	11 (61.1)	5 (27.8)
– from oxyphilic (Hürthle) cells	8	2 (25.0)	5 (62.5)	1 (12.5)
Nodular goiter	17	0 (0.0)	13 (76.5)	4 (23.5)

(++) – >50%; (+) – 10–50%; (–) – <10% positive cells; N – number of cases studied; n (%) – number (per cent) of positive or negative cases

The noted number of P16INK4A protein-positive cases was 11 (91.7%) in the group of follicular carcinomas, 45 (78.9%) in the follicular adenoma group and 13 (76.5%) in the nodular goiter group. Within the group of follicular adenomas the number of positive cases was 25 (80.7%) for microfollicular ones, 13 (72.2%) for normo-/macrofollicular and 7 (87.5%) for adenomas derived from oxyphilic cells (Table 2). The number of cases positive for P16INK4A did not differ significantly when examined groups were compared with one another.

A high expression (overexpression) of P16INK4A protein was noted in 8 out of 12 (66.7%) follicular carci-

nomas and in 11 out of 57 (19.3%) follicular adenomas, mainly microfollicular ones. In the nodular goiter group no cases with overexpression of P16INK4A protein were noted. The values of this parameter in the follicular carcinoma group were statistically significantly higher compared to both the follicular adenoma and the nodular goiter groups ($p<0.005$ and $p<0.0001$, respectively).

Discussion

Recent years have brought numerous reports, analyzing P16INK4A protein expression in various types of primary human tumors, using immunohistochemical methods [6, 11–14, 37, 42, 46, 50]. So far, no papers were published dealing with evaluation of P16INK4A protein expression in follicular thyroid cancers using this method. We cannot, therefore, juxtapose our results yet with those of other research.

Other authors take note of P16INK4A protein distribution within cell [13, 14, 46], since a large percentage of examined neoplastic tissues shows, besides immunohistochemical reaction for this protein in the nucleus, also its cytoplasmic localization. This indicates that P16INK4A protein may take part in so far little known mechanisms of intra- and intercellular interactions [13]. According to Dong et al. [6] the observed effect of immunohistochemical detection of P16INK4A protein in both nucleus and cytoplasm may reflect simply a penetration of this protein into cytoplasmic region in case of large accumulation within the nucleus. In turn, according to Geradts et al. [13], it is not known to what degree the observed cytoplasmic P16INK4A protein reaction is unspecific and to what degree it actually reflects extranuclear accumulation of this protein. In our study, besides positive nuclear immunohistochemical reaction, we also observed a cytoplasmic reaction for this protein within the follicular thyroid carcinoma cells, follicular adenomas and nodular goiters.

Transition of cells through G1/S restriction checkpoint is regulated by product of *Rb, p53* and *p16INK4A* suppressor genes [5, 15, 22, 34, 49]. It has been known that the anti-pRb antibody is able to detect immunohistochemically both unphosphorylated and phosphorylated forms of pRb protein. In turn, various clones of anti-p53 antibodies can detect both mutated and wild forms of P53 protein [47]. Unfortunately, we do not know the antigenic determinant (epitope) responsible for detection of anti-P16INK4 antibody used in our study. This makes an exact interpretation of P16INK4A protein expression and distribution within follicular thyroid carcinoma cells difficult, when only the immunohistochemical detection method is applied, as it was the case in our study.

Talve et al. [46] have observed a correlation between P16 protein expression and the proliferative activity of skin malignant melanoma cells, as assessed by anti-Ki-67 antibody. These authors have observed that tumor tissues, in which cells showed negative immunohistochemical reaction for P16 protein, had also statistically higher number of cells showing positive reaction for Ki-67 antigen, as compared to tumor tissues, where cells were also positive for P16 protein. According to these authors, the majority of examined P16 protein-negative malignant melanoma cells have proceeded beyond both G1/S checkpoint and early S phase of the cell cycle. On the other hand, cells derived from such neoplasms beyond G2/M restriction checkpoint show little positive immunohistochemical reaction for P16 protein.

The two most common mechanisms of *p16* inactivation are homozygous deletion or hypermethylation of the gene. Immunohistochemically, these changes are manifested as absence of nuclear staining in neoplastic cells [14]. Milde-Langosch et al. [37] analyzed P16 protein expression by immunohistochemistry in paraffin sections of 94 primary ovarian carcinomas of various histological subtypes. A loss of expression was detected in 19 (20%) primary tumors, mainly mucinous and endometrial carcinomas. A loss of or weak *p16* expression was caused by hypermethylation of the 5'-CpG islands in the promoter/exon 1 of the *p16/MTS1* gene in 12 out of 19 immunohistochemically negative cases, by somatic mutation in 10 cases and homozygous deletion in 1 case. Fujita et al. [10] have concluded, that rather than gene deletion or point mutation it is an inactivation of *p16* gene that may play an important role in the genesis of human ovarian epithelial tumors. As a consequence of 5'-CpG island hypermethylation, the inactivation is followed by loss of p16 mRNA and protein expression. Dong et al. [6] analyzed immunohistochemically expression of P16INK4A in paraffin sections from 159 cases of malignant and 31 benign epithelial ovarian tumors. Most of benign ovarian epithelial tumors (65%) lacked immunodetectable P16INK4A protein, compared to only 11% of malignant tumors. Talve et al. [46] reported that 46 out of 79 (58%) skin malignant melanomas and all benign melanocytic nevi (100%) were found to be P16-positive.

In our study, P16INK4A-positive cases were as follows: 12 out of 13 (91.7%) follicular thyroid carcinomas, 45 out of 57 (78.9%) follicular adenomas and 13 out of 17 (76.5%) nodular goiters. Next, P16INK4A overexpression was noted in 8 out of 12 cases (66.7%) of follicular carcinoma and 11 out of 57 cases (19.3%) of

follicular adenoma. In the cells derived from nodular goiters no P16INK4A protein overexpression was found. Values of this parameter noted for follicular carcinomas were statistically higher, compared to both follicular adenoma and nodular goiter groups ($p<0.005$ and $p<0.0001$, respectively).

The study of Sakaguchi et al. [42] revealed a positive nuclear and cytoplasmatic immunohistochemical reaction for P16 protein in half (31/61) of the investigated cases of non-small cell lung carcinoma. This study reported an amplification of *p16/CDKN2* gene in 37 cases, including 13 with negative immunohistochemical reaction for P16 protein. It is interesting whether the overexpression of P16INK4A protein in follicular thyroid carcinoma cells, as observed in our study, could have been the result of *p16INK4A* gene amplification or it would rather have reflected the proliferative potential of follicular cells from these tumors. Next, these authors claim that the amplification of cyclin D1 gene, which also plays a key role in cell cycle G1/S transition, is related to the immunohistochemically-detected overexpression of this protein in various kinds of tumors, including those affecting thyroid [2, 12, 32, 36, 43].

Resnick et al. [41] examined the P27/kip1 protein expression with the immunohistochemical method in 87 benign and malignant thyroid neoplasms. All the examined thyroid tumors exhibited a significantly lower P27/kip1 expression than did normal thyroid tissue. Poorly differentiated carcinomas had the lowest P27/kip1 staining frequency of all the thyroid carcinomas examined. The P27/kip1 staining frequency among papillary carcinomas was significantly lower than that among follicular carcinomas.

The high expression of P16INK4A protein observed in follicular carcinomas of the thyroid suggests that an altered expression of P16INK4A protein may disturb the thyreocyte cell cycle and plays a significant role in the formation of benign neoplasms and their malignant counterparts derived from follicular thyroid cell.

References

1. *Akhurst RJ, Balmain A:* Genetics events and the role of TGFb in epithelial tumour progression. J Pathol 1999, 187, 82–90.
2. *Barbareschi M, Pelosio P, Caffo O et al:* Cyclin D1 gene amplification and expression in breast carcinoma: relation with clinicopathologic characteristics and with retinoblastoma gene product, p53 and p21WAF1 immunohistochemical expression. Int J Cancer 1997, 74, 171–174.
3. *Bartkowa J, Lukas J, Guldberg P et al:* The p16-cyclin D/Cdk4-pRB pathway as a functional unit altered in melanoma pathogenesis. Cancer Res 1996, 56, 5475–5483.
4. *Clurman BE, Groudine M:* The CDKN2A tumor-suppressor locus – a tale of two proteins. N Engl J Med 1998, 338, 910–912.
5. *Cordon-Cardo C:* Mutation of cell cycle regulators: biological and clinical implications for human neoplasia. Am J Pathol 1995, 147, 545–560.
6. *Dong Y, Walsh MD, McGluckin MA et al:* Increased expression of cyclin-dependent kinase inhibitor 2 (CDKN2A) gene product $p16^{INK4A}$ in ovarian cancers is associated with progression and unfavourable prognosis. Int J Cancer 1997, 74, 57–63.
7. Elisei R, Shiohara M, Koeffler HP et al: Genetic and epigenetic alterations of the cyclin-dependent kinase inhibitors p15INK4b and $p16^{INK4a}$ in human thyroid carcinoma cell lines and primary thyroid carcinomas. Cancer 1998, 83, 2185–2193.
8. Farrell WE, Simpson DJ, Bicknell JE et al: Chromosome 9p deletions in invasive and noninvasive nonfunctional pituitary adenomas: The deleted region involves markers of the MTS1 and MTS2 genes. Cancer Res 1997, 57, 2703–2709.
9. *Figge J, Bakst G, Weisheit D et al:* Image analysis quantitation of immunoreactive retinoblastoma protein in human thyroid neoplasms with a streptavidin-biotin-peroxidase staining technique. Am J Pathol 1991, 139, 1213–1219.
10. *Fujita M, Enomoto T, Haba T et al:* Alteration of p16 and p15 genes in common epithelial ovarian tumors. Int J Cancer 1997, 74, 148–155.
11. *Gallo O, Santucci, Franchi A:* Cumulative prognostic value of p16/CDKN2 and p53 oncoprotein expression in premalignant laryngeal lesions. J Nat Cancer Inst 1997, 89, 1161–1163.
12. *Gansauge S, Gansauge F, Ramadani M et al:* Overexpression of cyclin D1 in human pancreatic carcinoma is associated with poor prognosis. Cancer Res 1997, 75, 1634–1637.
13. *Geradts J, Kratzke RA, Niehans GA, Lincoln CE:* Immunohistochemical detection of the cyclin-dependent kinase inhibitor 2/multiple tumor suppressor gene 1 (CDKN2/MTS1) product $p16^{INKA4A}$ in archival human solid tumors: correlation with retinoblastoma protein expression. Cancer Res 1995, 55, 6006–6011.
14. *Geradts J, Wilson PA:* High frequency of aberrant $p16^{INK4A}$ expression in human breast cancer. Am J Pathol 1996, 149, 15–20.
15. *Gillett CE, Barnes DM:* Cell cycle. J Clin Pathol Mol Pathol 1998, 51, 310–316.
16. *Godballe C, Asschenfeldt P, Jorgensen KE et al:* Prognostic factors in papillary and follicular thyroid carcinomas: p53 expression is a significant indicator of prognosis. Laryngoscope 1998, 108, 243–249.
17. *Gonzalez MV, Pello MF, Lopez-Larrea C et al:* Deletion and methylation of the tumor suppressor gene p16/CDKN2 in primary head and neck squamous cell carcinoma. J Clin Pathol 1997, 50, 509–512.
18. *Hangaishi A, Ogawa S, Imamura N et al:* Inactivation of multiple tumor-suppressor genes involved in negative regulation of the cell cycle, MTS1/$p16^{INK4A}$/CDKN2, MTS2/p15INK4B, p53, and Rb genes in primary lymphoid malignancies. Blood 1996, 87, 4949–4958.
19. *Hedinger CE, Williams ED, Sobin LH:* Histological Typing of Thyroid Tumours. The WHO International Histological Classification of Tumours. 2nd rev ed. Springer-Verlag, Berlin 1988.
20. *Herman JG, Civin CI, Issa J-PJ et al:* Distinct patterns of inactivation of $p15^{INK4B}$ and $p16^{INK4A}$ characterise the major types of hematological malignancies. Cancer Res 1997, 57, 837–841.
21. *Hirabayashi H, Fujii Y, Sakaguchi M et al:* $p16^{INK4}$, pRb, p53 and cyclin D1 expression and hypermethylation of CDKN2 gene in thymoma and thymic carcinoma. Int J Cancer 1997, 73, 639–644.
22. *Hirama T, Koeffler HP:* Role of the cyclin-dependent kinase inhibitors in the development of cancer. Blood 1995, 86, 841–154.
23. *Hsu SM, Raine L, Fanger H:* Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC

and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981, 29, 577–580.
24. *Huang DP, Lo K-W, Van Hasselt CA et al:* A region of homozygous deletion on chromosome 9p21-22 in primary nasopharyngeal carcinoma. Cancer Res 1994, 54, 4003–4005.
25. *Iolascon A, Faienza MF, Coppola B et al:* Analysis of cyclin-dependent kinase inhibitor genes (CDKN2A, CDKN2B, and CDKN2C) in childhood rhabdomyosarcoma. Genes Chromosome Cancer 1996, 15, 217–222.
26. *Ito Y, Kobayashi T, Takeda T et al:* Expression of p21 (WAF1/CIP1) protein in clinical thyroid tissues. Br J Cancer 1996, 74, 1269–1274.
27. *Ivan M, Wynford-Thomas D, Jones CJ:* Abnormalities of the P16INK4A gene in thyroid cancer cell lines. Eur J Cancer 1996, 32A, 2369–2370.
28. *Jones CJ, Shaw JJ, Wyllie FS et al:* High frequency deletion of the tumour gene $P16^{INK4a}$ (MTS1) in human thyroid cancer cell lines. Mol Cell Endocrinol 1996, 116, 115–119.
29. *Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q et al:* A cell cycle regulator potentially involved in genesis of many tumor types. Science 1994, 264, 436–440.
30. *Kim SK, Ro J, Kemp BL et al:* Identification of three distinct tumor suppressor loci on the short arm of chromosome 9 in small cell lung cancer. Cancer Res 1997, 57, 400–403.
31. *Kyomoto R, Kumazawa H, Toda Y et al:* Cyclin D1 gene amplification is a more potent prognostic factor than its protein overexpression in human head-and-neck squamous cell carcinoma. Int J Cancer 1997, 74, 576–581.
32. *Lazzereschi D, Sambuco L, Cornovale Scalzo C et al:* Cyclin D1 and cyclin E expression in malignant cells and human thyroid carcinomas. Int J Cancer 1998, 76, 806–811.
33. *Lloyd RV, Ericson LA, Jin L et al:* $p27^{kip1}$: A multifunctional cyclin-dependent kinase inhibitor with prognostic significance in human cancers. Am J Pathol 1999, 154, 313–323.
34. *Lundberg AS, Weinberg RA:* Control of the cell cycle and apoptosis. Eur J Cancer 1999, 35, 531–539.
35. *Mao L, Merlo A, Bedi G et al:* A novel p16INK4A transcript. Cancer Res 1995, 55, 2995-2997.
36. *Marchetti A, Doglioni C, Barbareschi M et al:* Cyclin D1 and retinoblastoma susceptibility gene alterations in non-small cell lung cancer. Int J Cancer 1998, 75, 187–192.
37. *Milde-Langosch K, Ocon E, Becker G, Löning T:* p16/MTS1 inactivation in ovarian carcinomas: high frequency of reduced protein expression associated with hypermethylation or mutation in endometrioid and mucinous tumors. Int J Cancer 1998, 79, 61–65.
38. *Moore D, Ohene-Fianko D, Garcia B et al:* Apoptosis in thyroid neoplasms: relationship with p53 and bcl-2 expression. Histopathology 1998, 32, 35–42.
39. *Nabori T, Miura K, Wu DJ et al:* Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature 1994, 368, 753–756.
40. *Piccinin S, Doglioni C, Maestro R et al:* p16/CDKN2 and CDK4 gene mutations in sporadic melanoma development and progression. In J Cancer 1997, 74, 26–30.
41. *Resnick MB, Schacter P, Finkelstein Y et al:* Immunohistochemical analysis of p27/kip1 expression in thyroid carcinoma. Mod Pathol 1998, 11, 735–739.
42. *Sakaguchi M, Fuii Y, Hirabayashi H et al:* Inversely correlated expression of p16 and Rb protein in non-small cell lung cancers: an immunohistochemical study. Int J Cancer 1996, 65, 442–445.
43. *Sheyn I, Noffsinger AE, Heffelfinger S et al:* Amplification and expression of the cyclin D1 gene in anal and esophageal squamous cell carcinoma. Hum Pathol 1997, 28, 270–276.
44. *Simpson DJ, Bicknell JE, McNicol AM et al:* Hypermethylation of p16/CDKN2A/MTS1 gene and loss of protein expression is associated with nonfunctional pituitary adenomas but not somatotropinomas. Genes Chromosome Cancer 1999, 24, 328–336.
45. *Stone S, Jiang P, Dayananth P et al:* Complex structure and regulation of the p16 (MTS1) locus. Cancer Res 1995, 55, 2988–2994.
46. *Talve L, Sauroja I, Collan Y et al:* Loss of expression of the p16INK4/CDKN2 gene in cutaneous melanoma correlates with tumor cell proliferation and invasive stage. Int J Cancer 1997, 74, 255–259.
47. *Tanum G, Holm R:* Anal carcinoma: a clinical approach to p53 and Rb gene proteins. Oncology 1996, 53, 369–373.
48. *Weiner T, Cance WG:* Molecular mechanisms involved in tumorigenesis and their surgical implications. Am J Surg 1994, 176, 428–434.
49. *Wynford-Thomas D:* Cellular senescence and cancer. J Pathol 1999, 187, 100–111.
50. *Yang G, Zhang Z, Liao J et al:* Immunohistochemical studies on Wafp21, p16, pRb and p53 in human esophageal carcinomas and neighboring epithelia from a high-risk area in northern China. Int J Cancer 1997, 72, 746–751.
51. *Zedenius J, Larsson C, Wallin G et al:* Alterations of p53 and expression of WAF/p21 in human thyroid tumors. Thyroid 1996, 6, 1–9.

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