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Analysis of Cyclin D1 and Retinoblastoma Protein Immunoreactivity in Follicular Thyroid Tumors*

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Protein products of cyclin D1 and retinoblastoma (*Rb*) genes play crucial roles in regulation of G1/S transition in the cell cycle. In this study we analyzed, using immunohistochemical methods, the expression of cyclin D1 and Rb proteins in material from medical archives (12 cases of follicular thyroid carcinoma, 57 cases of follicular adenoma and 17 nodular goiter cases). A positive nuclear reaction for cyclin D1 was observed in 83.3% (10/12) of the follicular carcinomas, in 96.5% (55/57) of the follicular adenomas and in 23.5% (4/17) of nodular goiters. Overexpression of cyclin D1 (more than 50% of positively staining cells) was noted in 25% (3/12) of the follicular carcinomas and in 22.8% (13/57) of the follicular adenomas. No overexpression of cyclin D1 was noted among nodular goiters. The number of carcinoma cases with cyclin D1 overexpression did not differ statistically in any significant way from the follicular adenoma group (p=1.000). A positive nuclear reaction for Rb protein was noted in 100% of the follicular carcinomas (12/12), in 96.5% of the follicular adenomas (55/57) and in 47.1% of the cases (8/17) of nodular goiter. Rb protein overexpression (more than 50% of positively staining cells) was found in 83.3% (10/12) of the follicular carcinomas, in 68.4% (39/57) of the follicular adenomas and in 11.8% (2/17) of the nodular goiters. The number of cases with Rb protein overexpression in the follicular carcinoma group did not differ significantly from that in the follicular adenoma group (p=0.486). A positive correlation was found in the groups studied between the expressions of Rb protein and cyclin D1. However, the correlation was statistically significant only in the nodular goiter group (Rs=0.567; p=0.018). In the follicular carcinoma group, that correlation was borderline (Rp=0.437; p=0.072) and, in the follicular adenoma group, it was statistically insignificant (Rs=0.217; p=0.105). Our results confirm the existence of mutual regulation mechanisms of Rb and cyclin D1 protein expressions, which are observed in cells from various carcinomas.

Introduction

Recent studies suggest that cancerogenesis is inseparably linked with disturbances of the cell cycle process. Cell commitment to division usually occurs at the end of G1 cycle phase, at the, so-called, G1/S checkpoint [7, 16, 23, 34, 36, 39, 52, 57]. Cell transition through G1/S checkpoint is regulated by several proteins including serine-threonine protein kinases (cdk2, cdk4, cdk6) [23, 40, 41] and cyclins D and E [22, 42, 43]. Also involved are products of some suppressor genes, such as *Rb* [18, 50, 52, 55], *p16INK4A* [11, 15, 29, 45, 51] and *p53* [7, 9, 49].

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The *Rb* gene is located in chromosomal region 13q14 and comprises 27 exons. This gene encodes nuclear phosphoprotein (pRb) of 105–110 kDa, which has suppressor activity [18]. Inside the cell, Rb protein is hypophosphorylated during G0 phase and early G1 phase of the cycle, while in late G1 phase and during S, G2 and M phases, it becomes hyperphosphorylated. Dephosphorylation of Rb protein starts at the end of mitosis (anaphase) [18, 23, 50, 52, 55].

Molecular mechanisms, by which Rb protein regulates cell proliferation by influencing the G1/S phase of the cell cycle, involve binding of an unphosphorylated form of Rb protein to a transcription factor from the E2F family; then, gradual phosphorylation of Rb protein proceeds at first, following the formation of a complex, involving cyclin D1 and either cdk4 or cdk 6 kinase and then, in late G1 phase of the cycle, a complex formed by cyclin E and cdk2 kinase. The phosphorylated Rb protein releases the E2F transcription factor, which activates the transcription of several genes, including cyclin E gene and *E2F* gene itself (self-regulation), responsible for G1/S transition in the cell cycle. It also activates the transcription of genes initiating DNA synthesis [4, 16, 23, 36, 39, 40, 50, 52].

The second important role of Rb protein is the regulation of the level of RNA polymerases (I, II, III) inside cells, which allows controlling of transcription and translation processes [50, 56]. Currently, it is assumed that Rb protein plays a substantial role in the process of cell differentiation [52, 58].

Inactivation of the suppressor function of *Rb* gene product follows deletion, point mutation or viral oncoprotein attachment, including large antigen T of SV40 virus, human E1A adenoviruses and papilloma E7 viruses. Biological consequences of this fact include deregulation of cell cycle, which may lead to uncontrolled proliferation [3, 18, 35, 55].

Cyclin D1 is a protein, encoded by *CCND1/PRAD1* gene, located in chromosomal region 11q13 [22, 30, 42]. In mammalian cells, cyclin D1 and cyclin E belong to the G1 phase family of cyclins. The main role of D1 cyclin as a regulator subunit is to activate cdk4 and cdk6 serine-threonine protein kinases. There exists also a functional link in the G1 phase of cell cycle between cyclin D1 and Rb protein, as well as p21WAF1 and P53 proteins [1, 5-8, 40, 42, 43, 50]. The overexpression of cyclin D1 accompanying various neoplasms, most often as a result of gene amplification or translocation, disturbs the above relations [12, 22, 30].

In this study we analyzed the expressions of cyclin D1 and Rb protein in carcinoma, three types of follicular adenoma and in nodular goiter, using immunohistochemical methods. Relationships were also assessed between the expressions of cyclin D1 and Rb protein.

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, 20].

Immunohistochemical screening

Representative paraffin blocks, containing tumor material from each case were sectioned at 4 μm, affixed to silanized slides and dried overnight at 56.7°C. Antigen retrieval for Rb1 was performed using citrate buffer (0.01 M, pH 6.0) and, for cyclin D1, EDTA buffer (0.001 M, pH 8.0) in a standard microwave unit. The sections for immunohistochemistry were stained using the avidin-biotin (ABC) method, according to Hsu et al. [26]. Deparaffinized sections were treated with 3% hydrogen peroxide (H_2O_2) in methanol for 5min to block the endogenous peroxidase activity. The non-specific antibody binding was reduced by incubation of the sections with normal goat serum for 20 min. The slides were incubated with a 1:50 dilution of a primary mouse monoclonal anti-Rb1 antibody (clone 84-B3-1, Novocastra, UK) and a 1:50 dilution of a primary mouse monoclonal anti-cyclin D1 antibody (clone P2D11F11, Novocastra, UK). 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) (Sigma, USA) was used as a 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 the cells was estimated by means of the semiquantitative method, using Hogg's net. The results were expressed as a percentage of positive cells per 1000 follicular thyroid cells, counted in 10 microscopic fields (high magnification of objective lens, 40x). Specific staining for each protein was categorized as either positive or negative, based on the presence of brown-colored staining. Only the cells with the evidence of nuclear staining were considered positive; cytoplasmic staining was disregarded. Using the criteria proposed by Kyomoto et al. [31], 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. The lesions scored as (++) were considered as those with high expression (overexpression) of Rb and cyclin D1 proteins.

Statistical procedure

All the parameters, represented as means, were compared using Mann-Whitney's test, where $p<0.05$ was considered significant. Associations between categorical variables and cyclin D1 or Rb expression were assessed using Fisher's exact test. The relationship (Spearman's correlations, Rs) between Rb and cyclin D1 expression levels were analyzed.

Results

Rb protein

The average percentage of cells positively stained for Rb protein was 68.9% (SD=19.6) in the group of follicular carcinomas, 58.8% (SD=23.2) in the group of follicular adenomas and 20.2% (SD=21.6) in the nodular goiter group. Within the follicular adenoma group, the percentage of cells stained positively for Rb protein was 62.9% (SD=23.8) for microfollicular adenomas, 57.1% (SD=19.2) for normo- and macrofollicular adenomas and 46.5% (SD=26.7) for adenomas derived from oxyphilic cells (Table 1). A comparison of the average percentage values for Rb protein-positive cells in follicular carcinomas and adenomas did not reveal any significant difference (p=0.241). No significant difference was observed between average percentage values for Rb protein-positive cells in particular types of follicular adenomas. The noted average percentage values of cells, positively stained for Rb protein in the group of follicular carcinomas and adenomas, were significantly higher than those in the nodular goiter group (p=0.0001 and p=0.0001, respectively).

A positive nuclear reaction for Rb protein was noted in 100% of the follicular carcinomas (12/12) (Fig. 1), in 96.5% (55/57) of the follicular adenomas and in 47.1% (8/17) of the nodular goiters. In the follicular adenoma group positive

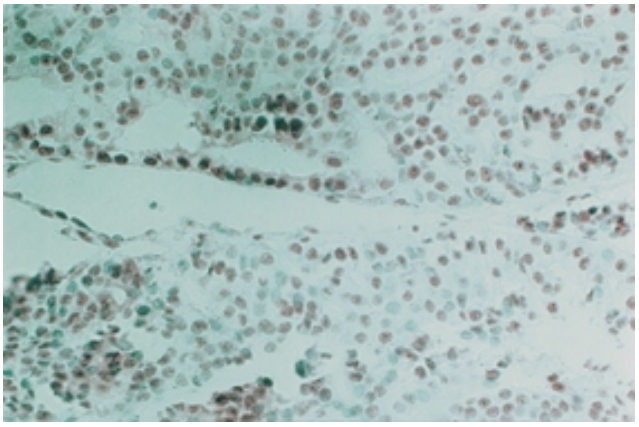

Fig. 1. Expression of Rb protein in follicular carcinoma.

TABLE 1

The average per cent of Rb protein-positive cells in the study groups

Group	N	M	SD	Min.	Max.
Follicular carcinoma	12	68.9	19.6	33.8	97.2
Follicular adenoma including:	57	58.8	23.2	2.1	94.7
– microfollicular	31	62.9	23.8	2.1	94.7
– normo- and macrofollicular	18	57.1	19.2	12.3	87.6
– from oxyphilic (Hürthle) cells	8	46.5	26.7	12.5	79.3
Nodular goiter	17	20.2	21.6	2.3	78.2

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

TABLE 2

The number of Rb protein-positive and negative cases in the study groups

Group	N	n (%)		
		++	+	-
Follicular carcinoma	12	10 (83.3)	2 (16.7)	0 (0.0)
Follicular adenoma including:	57	39 (68.4)	16 (28.1)	2 (3.5)
– microfollicular	31	24 (77.4)	5 (16.1)	2 (6.5)
– normo- and macrofollicular	18	12 (66.7)	6 (33.3)	0 (0.0)
– from oxyphilic (Hürthle) cells	8	3 (37.5)	5 (62.5)	0 (0.0)
Nodular goiter	17	2 (11.8)	6 (35.3)	9 (52.9)

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

reaction for Rb protein was observed in 93.5% (29/31) of microfollicular adenomas, in 100% (18/18) of normo- and macrofollicular adenomas and in 100% (8/8) of adenomas derived from oxyphilic cells. No significant differences were noted in the number of cases positive for Rb protein comparing particular types of follicular adenomas. The number of Rb protein-positive cases in the follicular carcinoma group did not differ significantly from the follicular adenoma group (p=1.000). The number of Rb-positive cases, observed in either the follicular adenoma or the carcinoma group, was statistically significantly higher, compared to respective values in the nodular goiter group (p=0.003 and p=0.0001, respectively).

Overexpression of Rb protein was noted in 83.3% (10/12) of follicular carcinomas, in 68.4% (39/57) of follicular adenomas and in 11.8% (2/17) of nodular goiters (Table 2). The number of cases with Rb protein overexpression in the group of follicular carcinomas did not differ significantly from that in the follicular adenoma group (p=0.486). The number of cases with Rb protein overexpression was statistically higher in the follicular carcinoma and adenoma groups, compared to the values in the nodular goiter group (p=0.0001 and p=0.0001, respectively).

Cyclin D1

The average percentage of cells stained positively for cyclin D1 was 34.9% (SD=23.4) in the follicular carcinoma group, 34.3% (SD=16.5) in the follicular adenoma group and 6.3% (SD=8.4) in the nodular goiter group. Within the follicular adenoma group, the percentage of cells positive for cyclin D1 was 39.1% (SD=17.0) for microfollicular adenomas, 29.6% (SD=11.9) for normo- and macrofollicular adenomas and 26.2% (SD=19.0) for adenomas derived from oxyphilic cells (Table 3).

A comparison of the average percentage values of cells stained positively for cyclin D1 in the follicular carcinoma and adenoma groups did not reveal any significant differences (p=0.918). Within the follicular adenoma group the average values of the percentage of cyclin D1-positive cells were significantly higher for microfollicular adenomas than for normo- and macrofollicular adenomas (p=0.029). The average values of the percentage of cells stained positively for cyclin D1 observed in the follicular carcinoma and adenoma groups were statistically higher than in the nodular goiter group (p=0.0001 and p=0.0001, respectively).

A positive nuclear staining for cyclin D1 was noted in 83.3% (10/12) of the follicular carcinomas and in 96.55 (55/57) of the follicular adenomas, and in 23.5% (4/17) of the nodular goiters. Within the follicular adenoma group a positive reaction for cyclin D1 was seen in 93.6% (29/31) of microfollicular adenomas, in 100% (18/18) of normo- and macrofollicular adenomas and in 100% (8/8) of adenomas derived from oxyphilic cells. No statistical difference was noted in the number of cyclin D1-positive cases, when particular types of follicular adenoma were compared. The number of cyclin D1-positive cases, noted either in the follicular carcinoma or adenoma group, was significantly higher compared to the nodular goiter group (p=0.003 and p=0.0001, respectively).

An overexpression of cyclin D1 was observed in 25% (3/12) of follicular carcinomas and in 22.8% (13/57) of follicular adenomas. In cells of nodular goiter no overexpression of cyclin D1 was observed (Table 4). The number of cases with cyclin D1 overexpression in the group of follicular carcinomas did not differ statistically from that in follicular adenomas (p=1.000). The number of cases with cyclin D1 overexpression in the group of follicular adenomas was significantly higher than that in the group of nodular goiters (p=0.031) and in the group of follicular carcinomas it was borderline significant (p=0.06).

A positive correlation was observed between the expression of Rb protein and that of cyclin D1 in the study groups. However, the correlation was statistically significant only in the nodular goiter group (Rs=0.567; p=0.018). In the group of follicular carcinomas that correlation was borderline significant (Rp=0.437; p=0.072) and, in the group of follicular adenomas, it was statistically insignificant (Rs=0.217; p=0.105).

TABLE 3
The average per cent of cyclin D1-positive cells in the study groups

Group	N	M	SD	Min.	Max.
Follicular carcinoma	12	34.9	23.4	2.3	79.3
Follicular adenoma including:	57	34.3	16.5	1.6	63.7
– microfollicular	31	39.1	17.0	1.6	63.7
– normo- and macrofollicular	18	29.6	11.9	11.8	58.2
– from oxyphilic (Hürthle) cells	8	26.2	19.0	10.7	62.7
Nodular goiter	17	6.3	8.4	0.0	27.6

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

TABLE 4
The number of cyclin D1-positive and negative cases in the study groups

Group	N	n (%)		
		++	+	-
Follicular carcinoma	12	3 (25.0)	7 (58.3)	2 (16.7)
Follicular adenoma including:	57	13 (22.8)	42 (73.7)	2 (3.5)
– microfollicular	31	11 (35.5)	18 (58.1)	2 (6.4)
– normo- and macrofollicular	18	1 (5.6)	17 (94.4)	0 (0.0)
– from oxyphilic (Hürthle) cells	8	1 (12.5)	7 (87.5)	0 (0.0)
Nodular goiter	17	0 (0.0)	4 (23.5)	13 (76.5)

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

Discussion

Correct protein products of *Rb* and cyclin D1 genes play a key role in the regulation of G1/S cell cycle transition. Rb protein is particularly active when unphosphorylated or weakly phosphorylated. It is then capable of binding the transcription factor E2F, which becomes inactive when complexed to Rb protein [18, 52, 55]. Inactivation of the Rb protein product suppressor function as a result of gene deletion or point mutation or due to binding to viral oncoproteins (large T antigen of the SV40 virus, human E1A adenoviruses, papilloma E7 viruses) interferes with the above-described properties of Rb protein. The biological consequences involve deregulation of the cell cycle that may lead to uncontrolled cell proliferation [7, 35, 40, 52].

Cyclins belong to the family of nuclear proteins. The level of their synthesis depends on the cell cycle phase [42, 43, 50]. High expression (overexpression) of the cyclin D1 gene, characterized by an increased level of mRNA and/or protein product of the *CCND1* gene, is most often a result of either amplification or translocation of this gene into a different locus. Biological consequences of high cyclin D1 level and increased activities of the cyclin-dependent cdk4 and cdk6 kinases involve faster and more effective phosphorylation of Rb suppressor protein, leading to the shortening of G1 cell cycle phase [22, 30, 42, 43]. On the other hand, it has been pointed out that high levels of cyclin D1 may stop the cell cycle at G1 phase, while also inducing cell differentiation. The basic role in the regulation of this mechanism is played by p21WAF1 protein, the expression of which, along that of P53 protein, may induce high levels of cyclin D1 expression [1, 5, 8].

Both *Rb* gene and *CCND1* gene expressions at the mRNA level and, using immunohistochemical methods, at the protein level, has been the goal of numerous studies performed on human neoplasms [1, 2, 6, 10, 17, 21, 27, 28, 37, 54]. Cyclin D1 was a subject, reviewed in this respect by Donnellan and Chetty [12]. Until now, only few works have appeared concerning the expression of these proteins in follicular thyroid carcinomas. It makes any more detailed comparison with the results obtained here rather difficult.

In our immunohistochemical studies, the number of Rb protein-positive cases was 100% (12/12) for the follicular carcinoma group, 95.5% (55/57) for the follicular adenoma group and 47.1% (8/17) for the nodular goiter group. The number of cyclin D1-positive cases was 83.3% (10/12) for the follicular carcinoma group, 95.5% (55/57) for the follicular adenoma group and 23.5% (4/17) for the nodular goiter group. The studies of Zou et al. [59] demonstrated a deletion of *Rb* gene exon 21 from cells in 55% of cases of malignant thyroid tumors. Only in 1/3 of these cases were both *Rb* gene alleles lost. According to these investigators, among the remaining thyroid tumors the mutations studied must have affected either other exons of the second allele or regulatory sequences, since in all the concluded cases of *Rb* gene mutation they also observed reduced amounts of Rb protein. Studies of Figge et al. [14] estimated the percentage of cells positive for Rb protein to be 27.7% of follicular adenomas and 9.5% of papillary thyroid carcinomas, respectively. In our study, the average percentage of Rb protein-positive cells was 68.9% (SD=19.6) for the follicular carcinoma cases and 58.8% (SD=23.2) for the follicular adenomas, and 20.2% (SD=21.6) in the nodular goiter group.

Worth noting are the results of Rb protein expression analysis in chosen human neoplasms. Dong et al. [10] reported high Rb expression (overexpression; exceeding 50% of the cell nuclei), in 71% (89/125) of ovarian epithelial cancers. The high expression of Rb protein correlated positively with non-mucinous subtype of ovarian carcinoma. The above authors studied the degree of Rb protein phosphorylation in 9 cases of ovarian cancer, using the Western blot technique. They noticed, that tumors containing primarily cells with hypophosphorylated Rb protein presented

better prognosis than tumors containing cells with either equal amounts of hypo- and hyperphosphorylated Rb protein or with poorly detectable Rb protein. In our studies, overexpression of Rb protein was observed in 83.3% (10/12) of the follicular thyroid carcinomas, in 68.4% (39/57) of the follicular adenomas and in 11.8% (2/17) of the nodular goiters. The number of cases with Rb protein overexpression, noted for follicular carcinomas and adenomas of the thyroid was significantly higher, compared to that in the nodular goiter group ($p<0.0001$ and $p<0.0001$, respectively).

A high percentage (98.4%) of Rb protein-positive cases (92/97) was found in the study of rectal carcinomas, performed by Tanum and Holm [53]. Ishikawa et al. [27] observed 68.7% (55/80) of Rb protein-positive cases in esophageal carcinomas. In 76% (33/42) of non-small cell lung carcinomas, Marchetti et al. [37] found nuclear staining of Rb protein. Higashiyama et al. [21] observed, in turn, a lack of nuclear staining for Rb protein in 88% (7/8) of small cell lung carcinomas. For the non-small cell lung carcinomas, a total lack of expression was found in only 6% (6/100) of the cases and lowered expression in 17% (17/100) of the cases. Lai et al. [32] found 81% (17/21) of Rb-positive cases among neoplasms of the renal cortex (including 18 carcinomas and 3 oncocytomas). These authors analyzed the polymorphic region in intron 17 of the *Rb* gene in 12 informative carcinoma cases, using molecular techniques. In 50% of cases (6/12) they observed a loss of heterozygosity (LOH). Only in one out of six carcinoma cases with LOH, they found no Rb protein expression. Also, according to those investigators, the loss of heterozygosity in the 13q14 region was related to high histological malignancy of renal carcinomas and unfavorable prognosis.

An interaction of viral oncoproteins, including human papilloma E7 virus (HPV E7), with Rb protein may disturb its correct functioning [35]. Holm et al. [24] reported that HPV oncoproteins were found in 81% of tumors of the rectum diagnosed. According to Tanum and Holm [53], an incorrect expression of Rb protein, observed in 95% of rectal carcinoma cases, may result from Rb protein inactivation by a protein product, originating from the HPV E7 viral genome.

In our study, cyclin D1-positive cases were noted in 83.3% (10/12) of the cases in the follicular carcinoma group, in 96.5% (55/57) of the cases of follicular adenoma and in 23.5% (4/17) of the cases of nodular goiter. In studies of Lazzereschi et al. [33], cyclin D1-positive cytoplasmic immunohistochemical reaction was observed in 72.7% (8/11) of thyroid follicular adenomas. A lack of either nuclear or cytoplasmic cyclin D1-positive reaction was concluded in only one case of follicular thyroid carcinoma studied by those authors. In turn, 12 out of 19 (63.1%) cases of papillary thyroid carcinoma showed cyclin D1-positive cytoplasmic staining. Nuclear staining for cyclin D1 was found in 31.6% (6/19) of the cases of papillary carcinoma. In this type of thyroid carcinoma the authors observed also the occurrence of focal cell clusters showing cyclin D1 nuclear staining. In those cases, ca. 80% of cells showed positive cyclin D1 nuclear reaction.

In cells originating from various types of human neoplasms, investigators often observed overexpression of cyclin D1 [1, 8, 31, 37, 38, 47]. According to some authors, the overexpression of cyclin D1 gene is most often a result of gene amplification or translocation into a different chromosomal locus (for review see: Donnellan and Chetty [12]). As an example, in the study of Naitoh et al. [46] the amplification of cyclin D1 gene was observed in 42% (5/12) of the esophageal carcinomas and an overexpression of this gene protein product was found in 38% (21/55) of the cases. Kyomoto et al. [31] found the amplification of cyclin D1 gene in 22% (10/45) of the cases of head and neck carcinoma. Positive cyclin D1 nuclear staining was observed by those authors in 53% of the cases (24/45), including 9% (4/45) of cases, showing high cyclin D1 expression, exceeding 50% of cells. Positive nuclear staining for cyclin D1 was seen in 14 out of 35 (40%) carcinomas, without diagnosed amplification of cyclin D1 gene. However, cyclin D1 expression was statistically higher in those cancer cases, where amplification of *CCND1* gene was also present. Marchetti et al. [37] observed amplification of *CCND1* gene in 32% (18/57) of non-small cell lung carcinoma cases, while 44% (25/57) showed positive nuclear staining for cyclin D1. Amplification of *CCND1* gene coding for cyclin D1 was observed in ca. 15–20% of breast carcinomas, while overexpression of cyclin D1, at either the mRNA or protein level, was seen in 30–50% of the cases (for review see Barnes [2], Fernandez et al. [13]). Barbareschi et al. [1] found the amplification of cyclin D1 gene in 24% (13/65) of breast carcinomas. Positive nuclear staining for cyclin D1 was present in 92% (55/60) of the cases, including 40% (24/60) of the cases with overexpression of cyclin D1. Those authors found a significant correlation between cyclin D1 gene amplification and overexpression of this gene protein product. Michalides et al. [38] concluded overexpression of cyclin D1 in 34% of 248 surgical breast carcinoma cases (stage I and II). Jares et al. [28] noted overexpression of cyclin D1 mRNA in 41% (19/45) of breast carcinoma cases. Michalides et al. [38] and Jares et al. [28] observed a significant correlation between the presence of estrogen receptors and overexpression of cyclin D1 in breast cancer cells.

In studies of Nakashima et al. [47], overexpression of cyclin D1 mRNA was observed in 45% of follicular adenomas and 54% of papillary thyroid carcinomas. In our

study, overexpression of cyclin D1 (more than 50% of positively stained cells) was observed in 25% (3/12) of follicular carcinomas and in 22% (12/57) of follicular adenomas. In cells originating from nodular goiter no overexpression of cyclin D1 was observed. In turn, Hsi et al. [25] noted overexpression of cyclin D1 in 18% (12/65) of parathyroid adenomas.

The effect of *CCND1* gene amplification (or its lack) and/or cyclin D1 overexpression on the grade of histopathological malignancy, clinical staging and prognostic value was analyzed for various types of human neoplasms [27, 28, 31, 37, 38, 48, 54]. The highest consensus among researchers concerned the prognostic value of the above cited parameters. Generally, with cyclin D1 gene amplification and/or overexpression present, lowering of the 5-year percentage survival indicator was observed for many malignant neoplasms. This observation includes acute lymphoblastic leukemia in children [54], endometrial cancer [48], head and neck malignancies [31], esophageal cancer [27]. On the other hand, it has not been confirmed for either breast cancer [17, 28, 38] or non-small cell lung carcinoma [37].

The scheme of cyclin D1 and Rb protein synthesis regulation, proposed by Lukas et al. [35], takes into account an induction of cyclin D1 gene expression by Rb protein and the immobilizing effect of cyclin D1 overexpression upon functioning of Rb protein. According to Müller et al. [44], the level of cyclin D1 expression depends on the functional status of Rb protein. With relevance to neoplastic cells, Gillett et al. [17] observed a positive correlation between Rb protein expression and cyclin D1 expression in ductal breast cancer *in situ*. Barbareschi et al. [1] observed high expression of Rb protein in breast cancer cases with amplification of cyclin D1 gene. In turn, Marchetti et al. [37] found correct levels of mRNA and Rb protein in cells originating from non-small cell lung carcinoma, where amplification and overexpression of cyclin D1 were seen; conversely, low levels of mRNA or Rb protein were not accompanied by disturbances of cyclin D1 expression.

According to Jares et al. [28], considering only the impact of cyclin D1, two mechanisms may be responsible for disturbing the inhibitory role of Rb protein in the regulation of cell proliferation. The first one concerns the overexpression of cyclin D1 that accompanies correct Rb protein expression, while the second involves the lack of Rb protein expression and a simultaneous low expression level of cyclin D1. In our study we noted a positive correlation between Rb protein and cyclin D1 expression. However, the correlation was statistically significant only in the group of nodular goiters (Rs=0.567; p=0.018). In the group of follicular carcinomas, this correlation was borderline significant (Rp=0.437; p=0.072) and in the group of follicular adenomas, it was statistically insignificant (Rs=0.217; p=0.105).

Our results confirm an involvement of mutual regulation mechanisms in Rb and cyclin D1 protein expression that have been observed in cells originating from various neoplasms.

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