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Low Level of Cyclin D1 Protein Expression in Thyroid Microcarcinomas from an Autopsy Series

Gabor Laszlo Kovacs,[1] Eva Stelkovics,[2] Laszlo Krenacs,[2] Gabor Gonda,[3] Miklos Goth,[1] Laszlo Kovacs,[1] Zoltan Gorombey,[1] Erika Hubina,[1] and Istvan Szabolcs[1]

[1]National Medical Center, Department of Internal Medicine, Budapest; [2]Biomedical Department, Bay Zoltan Foundation for Applied Research, Institute for Biotechnology, Szeged; and [3]Department of Pathology, National Medical Center, Budapest, Hungary

In a recent epidemiological screening study in an autopsy series, we found a high prevalence of microcarcinomas (MCs) (21/443 = 4.74%). We found no iodine intake-, gender-, or age-dependent differences in the prevalence of MCs. The results suggest a different and benign behavior of MCs compared to clinical cancer. The role of cyclin D1 overexpression in the pathogenesis of thyroid tumors is not known clearly; however, overexpression of this protein was reported in well-differentiated papillary cancers and in incidentally found metastasizing MCs. To date, cyclin D1 expression has not been investigated in autopsy-derived thyroid MCs. Eight MCs were available for immunostaining and comparison with 15 clinically detected papillary thyroid cancers. Fourteen out of 15 clinical carcinomas expressed cyclin D1 (93.3%), while in the MCs this ratio was 1 out of 8 (12.5%) ($p = 0.0001$). The only cyclin D1–positive MC was multifocal (both lobes of the gland were affected). We concluded that the benign behavior of most autopsy-derived MCs may be associated with the lack of cyclin D1 overexpression.

Key Words: Thyroid microcarcinoma; cyclin D1 expression; autopsy; immunohistochemistry.

Introduction

Thyroid cancers are the most frequent endocrine malignancies and account for approx 0.5–1.5% of all malignant tumors *(1)*. Thyroid microcarcinomas (MCs) are defined as small papillary carcinomas measuring 1 cm or less than 1 cm in diameter. They are common findings in histopathology following thyroid surgery *(2)*. MCs of the gland were found in series of autopsies with a frequency of 4.0–35.6%, which is 100–1000 times higher than the incidence of thyroid cancers with clinical manifestation *(3)*. Recently, we investigated the epidemiological and histological characteristics of thyroid microcarcinomas obtained from a consecutive autopsy series in two geographical areas of different iodine intake. Twenty-one microcarcinomas were found in 443 samples (4.74%), but one of them was larger than 1 cm, so it can be termed as occult thyroid carcinoma.

The frequency of MCs was not related either to iodine intake or to gender or nodularity. Both in the iodine-deficient and in the iodine-sufficient areas, the highest rate of microcarcinoma prevalence was found in the 40–49 yr age group. Based on these data we suppose that most microcarcinomas remain silent with minimal growth, or some tumors even disappear spontaneously, thus keeping the occurrence of microcarcinomas at a relatively constant level *(4)*.

It is still not clear whether autopsy-derived MCs are neoplastic disorders of different pathomechanism or if they are equivalent to clinical cancer but are small enough not to have clinical manifestation. It is possible that one or several gene mutations or overexpressions could set off neoplastic formation, inducing the oncogenetic step from MC to clinical cancer.

Cyclin D1 protein is an important regulatory subunit of a holoenzyme in mammalian cell cycle, and its gene is located on chromosome 11q23. *(5,6)*. The 35-kDa cyclin D1 protein is encoded by five exons, and this protein shares structural homology with the other cyclins. The amino terminus of cyclin D1 contains a motif leucine-X-cysteine-X-glutamic acid (where X means any amino acid) and is involved in binding the tumor-suppressor retinoblastoma protein (pRb). Cyclin D1 and cyclin-dependent kinase (Cdk) 4 and 6 complexes phosphorylate pRb. The phosphorylation of pRb and relief of transcriptional repression by Rb protein induces genes involved in the induction of cell-cycle S-phase (DNA replication) entry. In quiescent cells, cyclin D1 protein levels are low. Nuclear abundance increases as the cells progress through the G1 phase. As the cells pass into the S phase, cyclin D1 moves from the cell nucleus to the cytoplasm. Cyclin D1 is described as an endocrine tumor oncogen in human parathyroid adenomas *(5)*.

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Author to whom all correspondence and reprint requests should be addressed: Gabor Laszlo Kovacs, MD, 1135 Budapest, Szabolcs u. 33-35, Hungary. E-mail: g.kovacs@freemail.hu

Cyclin D1 overexpression has been shown to induce thyroid tumors. Goto et al. showed that cyclin D1 positivity is frequent in well-differentiated thyroid carcinomas (39/122), but is rarely seen in follicular adenomas (1/33) ($p < 0.05$) *(7)*. In another investigation it was demonstrated that cyclin D1 is more overexpressed in aggressive thyroid carcinomas (tall cell variant, anaplastic, etc.) suggesting that the cyclin D1 expression may play a role in tumor progression and may have prognostic significance in thyroid cancer *(8)*. Basolo et al. investigated cyclin D1 expression in thyroid carcinomas. Cyclin D1 overexpression (detected by both immunochemistry and Northern blotting) was observed at a significantly higher level in neoplastic tissue than in matched normal parenchyma. In well-differentiated carcinomas there was no significant correlation with gender and proliferative activity, but protein overexpression was higher in tumors from patients under 40 yr of age *(9)*. Overexpression of cyclin D1 in papillary thyroid cancers was found to be between 31.9% and 82.3% *(6,7,9)*.

Khoo et al. found that cyclin D1 is highly overexpressed in metastasizing papillary MCs (90.9%) as compared to nonmetastasizing papillary MCs (8%) ($p < 0.001$). This association between cyclin D1 overexpression and lymph node metastases is in keeping with findings in other malignant tumors *(10)*.

Cyclin D1 expression has not yet been studied in autopsy-derived thyroid MCs reflecting—as we believe—either a first step in thyroid carcinogenesis or, on the contrary, representing a different pathogenetic way of carcinoma formation. Our working hypothesis was that cyclin D1 would not be expressed in autopsy-derived thyroid MCs or, if yes, then only in those with a malignant behavior. If this were true, the lack or presence of cyclin D1 overexpression could be one of the factors determining the growth and malignant potential of thyroid cancer.

Results

Fourteen out of 15 tumors were positive (93.3%) for cyclin D1 in the PC group. The only negative slide was found to be grade 1, because of the weak focal positivity of the cells. The other samples got grade 2 or 3 showing the strong expression of cyclin D1 (Fig. 1).

Only one of the eight tumors showed positive cyclin D1 expression in the MC group (12.5%). Most of the other slides showed no or only weak focal staining (Fig. 2).

The only cyclin D1–positive MC was multifocal (both lobes of the gland affected; the foci being 6 and 2 mm, respectively). The autopsy-derived occult thyroid carcinoma (larger than 1 cm) was also cyclin D1 positive (grade 3), but because of the size it was excluded from the MC group. Each cyclin D1 negative tumor from the MC group was unifocal. The difference of the expression rate for cyclin D1 between MCs and PCs groups was significant ($p = 0.0001$). The results of the immunohistochemical study are shown in Table 1.

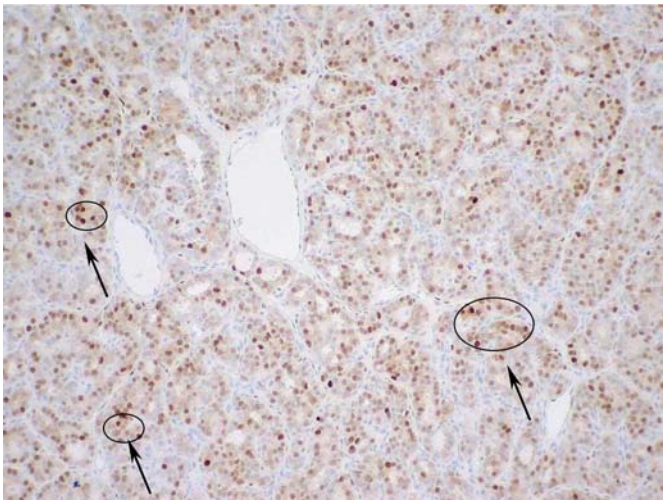

Fig. 1. Grade 3 cyclin D1 positive clinical papillary carcinoma (female, 53 yr). Arrows and rings show strong cyclin D1 positive areas of the slide. 40× magnification, hematoxylin counterstaining.

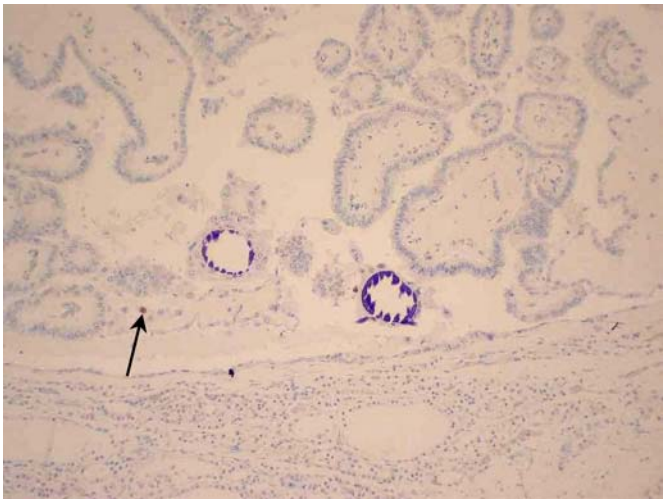

Fig. 2. Grade 1 (focal) cyclin D1 staining from a microcarcinoma (female, 64 yr). Arrow shows the only positive cell. 30× magnification, hematoxylin counterstaining.

Discussion

We investigated cyclin D1 expression in clinical papillary thyroid cancer versus autopsy-derived MCs to find a possible explanation for the relatively benign behavior of the latter. The rate of cyclin D1 overexpression in our series of clinically detected cancer (93.3%) was substantially higher than the 35% found by Basolo et al. *(9)* but nearer to the 63.2% reported by Khoo et al. *(6)* and the 82.3% described by Wang et al *(8)*, indicating possible differences in the immunohistochemistry methodology or evaluation criteria.

Khoo et al. also investigated cyclin D1 expression in papillary thyroid MCs found in the course of thyroid surgery because of benign goiter (incidentalomas). The expression

Table 1
Age, Gender, and Cyclin D1 Staining Distribution of the Samples

	Age (yr)	m: male, f: female	Diameter (mm)	Extrathyroid extension, lymph-node metastases, multifocality	Grade of cyclin D1 positivity
Microcarcinomas					
1	57	m	1,7		0
2	57	f	3		0
3	40	m	2		0
4	47	m	5		1
5	85	f	4		1
6	61	f	2		1
7	64	f	4		1
8	78	f	6 and 2		3
	Age (yr)	m: male f: female	Diameter (mm)	Extrathyroid extension, lymph-node metastases, multifocality	Grade of cyclin D1 positivity
Clinically Detected Papillary Carcinomas					
1	36	f	11	capsule infiltration	1
2	47	f	12	capsule infiltration	3
3	58	f		Multifocal cancer with several 3-11 mm foci	3
4	25	f	18	lymph-node metastasis	3
5	75	m	35	capsule infiltration	3
6*	54	f	13	lymph-node metastasis	3
7	57	f	17	capsule infiltration	2
8**	27	f	40	lymph-node metastasis	2
9**	69	f	40	lymph-node metastasis	3
10	47	f	28 and 6	distant metastasis	2
11	53	f	11	capsule infiltration	3
12	62	m	14	capsule infiltration	2
13	85	m	30	capsule infiltration	3
14	52	f	11	capsule infiltration	3
15	32	f	20 and 2	multifocal	2

*Poorly differentiated variant; **follicular variant.

rate was 8.8%. Contrary to this finding, the cyclin D1 expression rate was 90.9% in MCs detected clinically by their sentinel lymph-node metastasis. Unfortunately, neither the age of the patients nor the diameter of the microcarcinomas nor the rate of metastasizing microcarcinomas found in nongoitrous thyroid glands are given in this report.

Our present report is the first one on cyclin D1 expression in thyroid MCs found in a consecutive series of autopsies from areas of different iodine intake. We believe that screening for autopsy-derived thyroid carcinoma is probably one of the best methods to investigate epidemiology, as well as the natural course of thyroid cancer and its pathogenesis. As indicated in the introduction, the rate of these microcarcinomas was not related to iodine intake, gender, and presence or absence of goiter and there seemed to be a weak correlation to age; the rate of MCs was more prevalent in the middle age group of investigated subjects.

Only 8 out of 21 autopsy-derived MCs and an occult carcinoma (all of the papillary type) could be investigated. The others were not suitable for semiquantification of cyclin D1 expression, as they contained less than 1000 cells. Seven MCs were negative for cyclin D1 overexpression. Overexpression could be detected only in the multifocal thyroid MC and the occult thyroid carcinoma.

Our data, i.e., the lack of cyclin D1 positivity in most of the autopsy-derived unifocal MCs versus 93.3% positivity found in clinical cancer, correlates rather well with the observations of Khoo et al. obtained on surgery-derived thyroid incidentalomas and metastasizing MCs *(10)*. Based on these data we presume that first, the different and benign behavior of most of the autopsy-derived MCs as compared to clinical cancer (high prevalence, small size, no iodine intake-, or gender-related differences in the prevalence) is may be associated with the lack of cyclin D1 overexpression; second, cyclin D1 overexpression in papillary thyroid cancer seems to be connected with the growth and the metastasizing activity of thyroid cancer, while the two processes are probably independent from each other.

Materials and Methods

We investigated cyclin D1 positivity in MCs found in an autopsy series. Thyroid glands were obtained from 443 consecutive autopsies performed in hospitals located in two Hungarian areas of different iodine supply: an area of iodine deficient- and one of -sufficient intake. Twenty-one papillary MCs were found with no iodine intake- or gender-related differences in the prevalence of MCs. One of the 21 microcarcinomas was found as 15 mm, therefore, it was not termed a MC but an occult thyroid carcinoma. The patients had no previous clinical data of thyroid diseases *(4)*. Eight out of 20 MCs (3 males aged 40–57 yr and five females aged 57–78 yr) and the only autopsy-derived occult carcinoma were suitable for further immunohistochemical investigation (i.e., they were larger than 1 mm).

Cyclin D1 positivity was also investigated in tissue samples of 15 patients (3 males and 12 females) with clinically detected papillary thyroid cancer (PC). The median age in the MC group was 59 yr, while in the PC group it was 53 yr.

Sections 3 μm thick from formalin-fixed, paraffin-embedded tissue blocks were used for immunohistochemical studies. The tissue sections were mounted on 3-aminopropylethoxysilane-coated slides (SuperFrost Plus, Menzel GmbH & Co KG, Braunschweig, Germany) and dried overnight at 65°C. Sections were deparaffinized in xylene and rehydrated in a descending ethanol series before immunostaining. The slides were treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. Heat-induced antigen retrieval was performed by heating in a pressure cooker (Tefal Clipso) in 0.5 *M*, pH 10.0, Tris base for 2 min at full pressure *(11–13)*. A rabbit monoclonal cyclin D1 antibody diluted 1:100 (clone SP4; Lab Vision Co. Westinghouse, CA, USA) was used as primary antibody. After 90 min incubation at room temperature EnVision+ (DakoCytomation Carpinteria, CA. USA) was used as secondary antibody in accordance with the manufacturer's instructions *(14, 15)*. The peroxidase reaction was developed using DAB (diaminobenzidine) Substrate-Chromogen System from DakoCytomation in keeping with the instructions provided. After 2 min hematoxylin counterstaining, the slides were mounted in glycerol–gelatine.

We graded the distribution and rate of positive cells in tumors that stained for cyclin D1 according to Khoo et al. *(10)*: grade 0 = no positive cell at all; grade 1 = focal staining, in less than 10% of tumor cells (this means only one or two positive tumor cells in the visual field in most cases); grade 2 = widespread staining in 10–50% of the tumor cells; and grade 3 = diffuse staining in more than 50% of the tumor cells.

The χ^2 test was used for statistical analysis to determine the difference in cyclin D1 immunostaining between MCs and PCs. Grade 0 or 1 was declared as negative, grade 2 and 3 were declared as positive immunostaining. Statistical significance was ascribed at $p < 0.05$.

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