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Inverse Association Between Cyclin D1 Overexpression and Retinoblastoma Gene Mutation in Thyroid Carcinomas

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Cyclin D1 plays a key role in the regulation of the G1/S transition through the cell cycle. Deregulation of cyclin D1, most often leading to overexpression of the gene, has been reported in many tumor types. It has been suggested that cyclin D1 overexpression could be an alternative mechanism for pRb inactivation. We have previously found Rb gene mutations in 55% of malignant thyroid tumors. In the present study, we examined the cyclin D1 gene expression and amplification in 24 tumor samples (two of them are benign goiters) randomly selected from the same series of thyroid tumors, to see whether cyclin D1 overexpression is present in those specimens without Rb gene mutations. We found a four- to fivefold increase in cyclin D1 expression in 7 of 22 thyroid carcinomas as compared with that in benign nodular goiters. Six of them were found in carcinomas without Rb gene mutations. Among the remaining 15 thyroid carcinoma samples, 11 were found previously to have Rb gene mutations. The association between increased cyclin D1 expression and absence of Rb mutation is statistically significant ($p < 0.05$). We found no evidence of the cyclin D1 gene amplification or rearrangement to account for such an increase in cyclin D1 expression. We conclude that cyclin D1 overexpression may be relevant to thyroid carcinogenesis. Two mechanisms may be involved in the inactivation of pRb: one is through Rb gene mutations, and the other is by cyclin D1 overexpression.

Key Words: Cyclin D1; Rb; gene overexpression; cell cycle; thyroid neoplasm.

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Introduction

Cell-cycle deregulation is the hallmark of many, if not all, human malignancies *(1)*. Progression of eukaryotic cells through the cell cycle is regulated by a family of proteins called cyclins and their associated catalytic subunits, the cyclin-dependent kinases (Cdks) as well as a group of small cyclin-Cdk inhibitor proteins *(2,3)*. Cyclins complex with Cdks to govern key transitions through the cell cycle and are thought to be essential proteins in cell cycle regulation owing to their specific and periodic expression during cell cycle progression *(4)*. The eukaryotic cell cycle is believed to be regulated at two major decision points: a point in G1, at which cells become committed to DNA synthesis, and the G2/M boundary, at which cells become committed to mitosis *(5)*. Accordingly, cyclins have been categorized into two groups: the G1 cyclins, regulating the G1/S transition, and the mitotic cyclins (cyclins A and B), regulating G2/M transition *(5)*.

G1 is a major control point for cell proliferation in mammalian cells. Several human G1 cyclins have been identified: cyclins C, D1, D2, D3, and E *(5)*. It has been proposed that G1 cyclins are protooncogenes whose inappropriate expression may lead to cell-cycle deregulation and tumorigenesis. Cyclin D1 dysfunction is perhaps the most frequent event among the members of the G1 cyclin family. Aberrant expression of cyclin D1 is a common feature of a variety of human tumors, including parathyroid adenomas, B-cell lymphomas, and carcinomas of lung, esophagus, breast, the head and neck, and larynx *(6–13)*. Recently, transfection and transgenic mice studies have provided more direct evidence that overexpression of cyclin D1 can play a role in tumorigenesis *(14–16)*.

The cyclin D1 candidate oncogene is located on chromosome 11q13 and encodes a 34-kda protein of 295 amino acids *(8,17)*. Several studies have indicated that excessive cyclin D1 is able to overcome the retinoblastoma tumor suppressor protein-(pRB) mediated G1 arrest, presumably

through phosphorylation and functional inactivation of pRB *(18,19)*. It is thus conceivable that cyclin D1 overexpression could be an alternative mechanism of pRB inactivation in tumor specimens without RB gene mutations.

We have previously examined Rb gene mutations in thyroid carcinomas *(20,21)*. In the present study, we investigated cyclin D1 overexpression in the same series of thyroid tumor samples to see whether cyclin D1 overexpression is present in those samples without Rb gene mutations.

Results

The cyclin D1 gene expression was analyzed by Northern blot hybridization in 24 thyroid tumor specimans, consisting of 2 benign goiters, 12 carcinomas with Rb mutations, and 10 without Rb mutations. As shown in Fig. 1 and Table 1, a four- to fivefold increase in cyclin D1 gene expression was observed in 7 out of 22 (32%) thyroid carcinoma samples as compared with that in benign goiters. Six of them were found in carcinomas without Rb mutations. The remaining 15 thyroid carcinoma samples exhibited either a decrease or no change in cyclin D1 expression as compared with nodular goiters. Among the 15 thyroid carcinoma samples, 11 were found previously to have Rb mutations (Table 1). The association between increased cyclin D1 expression and absence of Rb mutation in thyroid carcinoma was statistically significant (chi-square, 4.69; $P < 0.05$). Two follicular and two anaplastic carcinomas in this series were not found to have cyclin D1 overexpression. However, these two follicular cancer specimens have Rb mutations (Table 1). No correlation was found between cyclin D1 overexpression and the following parameters: tumor stage, tumor pathology, age, and sex of the patients. However, more samples need to be analyzed to draw a conclusion.

The cyclin D1 overexpression was often found to be associated with cyclin D1 gene amplification or rearrangement in many different kinds of tumors, including parathyroid adenomas, B-cell lymphomas, and carcinomas of lung, esophagus, and breast. We therefore investigated possible cyclin D1 gene amplification or rearrangement in this group of thyroid tumor specimens by Southern blot analysis. As shown in Fig. 2, no gene amplification or rearrangement was detected in this series of thyroid tumor samples. However, with the probe used in this study, we cannot rule out the presence of rearrangements involving more distant breakpoints as they occur in lymphoid neoplasms.

Discussion

We have shown in this study that cyclin D1 transcripts were moderately increased in 39% (7/18) thyroid papillary carcinoma specimens and that this increase was present most often in those samples without Rb mutations. The increase in cyclin D1 expression was not, however, associated with gene amplification or rearrangement. The cyclin

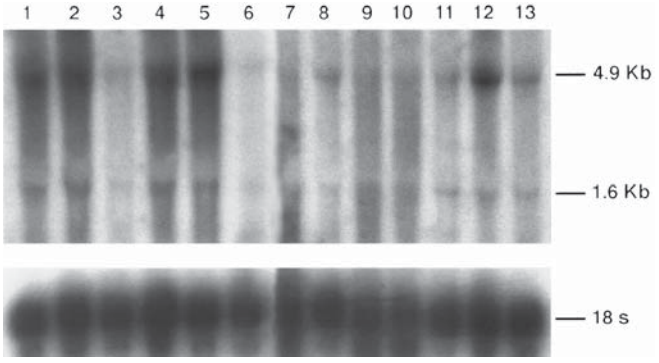

Fig. 1. Northern blot analysis of cyclin D1 gene expression in thyroid tumor specimens. Total RNA was electrophoresed on agarose/formaldehyde gel and blotted onto a nylon membrane. Hybridization was carried out with a cyclin D1 cDNA probe (upper panel). Lower panel: The same blot was reprobed with an 18S oligonucleotide probe to monitor the actual RNA loading. Information on each tumor is shown in Table 1. Lanes 1–13 correspond to tumor nos. 5, 6, 1, 8, 13, 12, 4, 15, 21, 14, 2, 10, and 23, respectively, in Table 1.

D1 overexpression in the absence of gene amplification or rearrangement has also been found in several other tumors, including larynx *(13)*, cervix *(22)*, and breast *(23,24)*. Although the mechanisms involved are unknown, the data suggest that additional genetic or epigenetic factors may be involved in the deregulation of cyclin D1 expression.

Similar to the p53-MDM2 autoregulatory feedback loop, in which p53 activates the transcription of MDM2 gene and the MDM2 protein, in turn, can complex with p53 to inactivate its activity *(25)*, there also exists a pRb–cyclin D1 regulatory feedback loop *(26)*. The pRb stimulates the transcription of cyclin D1 gene by activation of its promotor, whereas the cyclin D1, by associating with its catalytic partner cdk4, can functionally inactivate pRb through phosphorylation of its serine and threonine residues. Any factors that can disrupt the loop would likely to cause altered cyclin D1 expression, leading to cell-cycle deregulation and tumor development. Indeed, activated *ras* and *myc* oncogenes have been shown to induce cyclin D1 overexpression *(27,28)* and both of them have been implicated in thyroid carcinogenesis *(21,29,30)* and many other tumors. These suggest that cyclin D1 may function as one of the mediators in thyroid oncogenic transformation.

Most of the samples with cyclin D1 overexpression (five out of six) were found to have normal Rb genes, and those with Rb gene mutations often did not have increased cyclin D1 expression. The data indicate an inverse association between cyclin D1 overexpression and Rb mutation in thyroid cancer. A more extended study is needed to confirm the existence of such an association. Jiang et al. *(10)* demonstrated the presence of an inverse association between Rb mutation and cyclin D1 overexpression in esophageal tumors. They found that the two events were mutually exclusive, and tumors usually did not have both defective pRb and increased cyclin D1 expression. It is probably

Table 1

Rb Mutation and Cyclin D1 Expression in Thyroid Carcinoma

Tumor no.	Type	Stage	Age	Sex	Cyclin D1 overexpression	Rb mutation
(1)	Goiter		31	F		–
(2)	Goiter		39	F		–
(3)	Papillary Ca	VI	70	F		+
(4)	Papillary Ca	I	21	F	Fourfold	–
(5)	Papillary Ca	III	24	F	Fivefold	–
(6)	Papillary Ca	II	46	M	Fourfold	+
(7)	Papillary Ca	II	35	F	Fourfold	–
(8)	Papillary Ca	I	32	F		–
(9)	Papillary Ca	II	45	F	Fourfold	–
(10)	Papillary Ca	II	40	F	Fourfold	–
(11)	Papillary Ca	IV	76	F		+
(12)	Papillary Ca	III	63	F	Fivefold	–
(13)	Papillary Ca	I	23	F		–
(14)	Papillary Ca	IV	47	F		+
(15)	Papillary Ca	III	59	F		+
(16)	Papillary Ca	III	25	F		+
(17)	Papillary Ca	III	60	M		+
(18)	Papillary Ca	I	70	F		+
(19)	Papillary Ca	IV	24	F		+
(20)	Papillary Ca	III	48	M		+
(21)	Follicular Ca	II	48	F		+
(22)	Follicular Ca	II	55	F		+
(23)	Anaplastic Ca	IV	81	M		–
(24)	Anaplastic Ca	IV	43	F		–

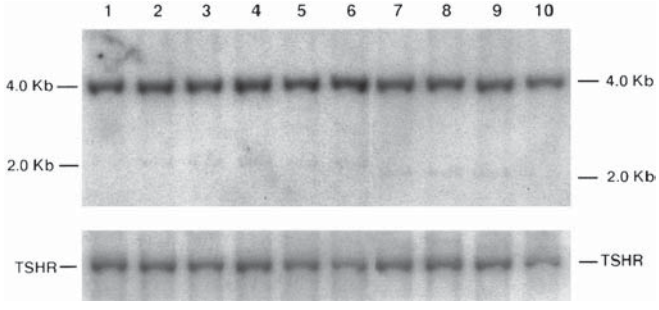

Fig. 2. Southern blot analysis of cyclin D1 gene amplification in thyroid tumor specimens. Ten micrograms of genomic DNA digested with *Eco*RI were fractionated on 1% agarose gel and blotted onto nylon membranes. Hybridization was carried out with a cyclin D1 cDNA probe (upper panel). The same blot was reprobed with a human TSH receptor cDNA probe (lower panel) to monitor the actual DNA loading and cyclin D1 gene amplification. Information on each tumor is shown in Table 1. Lanes 1–10 correspond to tumor nos. 1, 2, 3, 5, 6, 7, 10, 13, 16, and 17, respectively, in Table 1.

redundant and has no selective advantage to have both defective pRb and cyclin D1 present in the same tumor cell. This inverse association was also found in lung cancers *(9)* and may be relevant to many other tumor types resulting from deregulation in the cyclin D1–pRb regulatory loop.

In summary, we have shown cyclin D1 overexpression occurs predominantly in thyroid tumor samples with wild-type Rb gene. The increased expression is not the result of cyclin D1 gene amplification. The data suggest that two mechanisms may be involved in the inactivation of pRb: one is through Rb gene mutations, and the other is by cyclin D1 overexpression.

Materials and Methods

All thyroid tumor tissues were obtained at surgery, and were immediately frozen in liquid nitrogen and stored at –70°C until processed. The clinical staging of thyroid cancers was based on the TNM classification introduced in 1987 by the International Union Against Cancer *(31)*.

Twenty-four thyroid tumors were studied: 2 multinodular goiters (adenomatous goiters), 18 papillary, 2 follicular, and 2 anaplastic carcinomas. These tumors represent a randomly selected subset of 57 tumors previously studied for Rb gene mutations *(20)*.

Probes

Partial human cyclin D1 cDNA fragment (560 bp) was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) using the following oligonucleotide primers: 5'-ATGCGGAAGATCGTCGCCACC-3' and 5'-TCT

GGGTCACACTTGATCACT-3'. The primers were based on the published human cyclin D1 cDNA sequence *(17)*. The resulting PCR product was verified by DNA sequencing following subcloning into a TA cloning vector (Invitrogen Co., San Diego, CA).

The full-length human TSH receptor probe was kindly provided by E. Milgrom, Faculty of Medicine, University of Paris, France *(32)*.

The oligonucleotide probe for 18S ribosomal RNA was synthesized, and the sequence is as follows: 5'-GGTCAG CGCTCGTCGGCATGTAATAG-3'.

Northern Blot Hybridization for Cyclin D1 Gene Overexpression

Total RNA extraction and Northern blot analysis were performed as described previously *(34)*. Briefly, 20 µg of total RNA were fractionated on 1% agarose gel containing 2.2 *M* formaldehyde and blotted onto nylon membranes (Hybond-N, Amersham, Arlington Heights, IL) by capillary transfer. The accuracy of RNA loading was monitored by ethidium bromide staining and later by hybridization to an oligoprobe for 18S ribosomal RNA. The cyclin D1 probe was labeled with [α-^{32}P]dCTP to a specific activity of 10^9cpm/µg using Pharmacia's random primer labeling kit (Piscataway, NJ). Hybridization was performed at 42°C for 18 h in 6X SSPE, 10 m*M* EDTA, 5X Denhardt's solution, 0.5% SDS, 100 µg/mL denatured salmon testis DNA and 50% formamide. The membranes were then washed twice in 2X SSPE at 65°C and exposed to Kodak X-OMAT AR-5 film (Rochester, NY) at –70°C with intensifying screens.

Following autoradiography, band intensities were quantitated by a Bio-Rad scanning densitometer (Hercules, CA) and normalized by comparison with the 18S ribosomal band.

DNA Extraction and Southern Blot Hybridization

Genomic DNA extraction from tumor samples and Southern blot hybridization were performed as previously described *(34)*. Briefly, 10 µg of genomic DNA were digested with *Eco*RI, fractionated on 1% agarose gel, and blotted onto nylon membranes (Hybond-N, Amersham) by capillary transfer. The DNA on filters was then sequentially hybridized with probes for cyclin D1 and TSH receptor genes. Probe labeling and hybridization were performed as described above.

Following autoradiography, band intensities were quantitated by a Bio-Rad scanning densitometer and normalized by comparison with the TSH receptor band.

Statistical Analyses

Association between the absence of Rb mutation and increased cyclin D1 expression was sought using the chi-square test (with "Yates" correction for continuity).

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