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Increased Expression of CD44 Variants in Differentiated Thyroid Cancers

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Expression of CD44 variants in thyroid tumors was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) with a fluorescent image analyzer. Increased expression of CD44 variants compared with normal thyroid tissues was observed in most thyroid follicular tumors, especially in follicular carcinomas, poorly differentiated papillary carcinomas and some follicular adenomas. However, variants were hardly detectable in an anaplastic carcinoma. Analysis with restriction enzymes revealed that the major PCR product, consisting of variant bands, was derived from CD44E. Therefore, the expression of CD44E may be associated with the proliferation of differentiated thyroid cells.

Key words: Thyroid tumor — CD44 — Reverse transcription-polymerase chain reaction

CD44 is a cell surface transmembrane glycoprotein encoded by a 50-kb gene located on the short arm of human chromosome 11 (11p13). The protein is present in many types of cells as several isoforms resulting from alternative exon splicing.[1] An almost ubiquitously expressed isoform without the insertion of variant exons is one of the smallest CD44 molecules, called CD44H.[2] Some of the splice variants of CD44 (CD44v), which can be detected by reverse transcription-polymerase chain reaction (RT-PCR) as larger products than CD44H, may play important roles in cancer progression and metastasis.[3]

Thyroid tumors of epithelial descent are classified on the basis of histopathological criteria into three types, follicular adenoma, differentiated carcinoma (papillary carcinoma and follicular carcinoma) and undifferentiated anaplastic carcinoma.[4] Each type of tumor cell differs in biological characters such as growth rate and ability to show invasion or metastasis. As in other cancers in colon or breast, CD44 may also be important in the expression of these characters of thyroid cancer cells. There are some reports on the expression of CD44 in thyroid tissues. Fukazawa *et al.* proved the expression of CD44 molecule on the surface of thyroid cells from patients with Graves' disease.[5] Figge *et al.* reported the strong expression of CD44 in papillary carcinomas.[6] Further, they found papillary carcinomas were stained with antibodies that recognized CD44v6. No detailed analysis, however, has been done to investigate if alternative splicing of the CD44 gene has a role in the progression of thyroid cancer.

Recently, the use of a fluorescent image analyzer has enabled us to measure relative expression levels of mRNA by competitive PCR with a shorter internal control RNA.[7,8] Expression of both CD44H and CD44 variants can be detected by RT-PCR using the same primer set, as previously described by Matsumura and Tarin.[9] In the present study, by using CD44H mRNA as an internal control, we calculated the relative expression level of CD44 variants in each type of tumor and in metastatic lymph nodes to investigate the relation between expression of CD44 variants and tumor progression in thyroid neoplasm.

MATERIALS AND METHODS

Materials M-MLV reverse transcriptase was purchased from GIBCO BRL (Gaithersburg, MD), Taq polymerase from Roche Molecular Systems (Branchburg, NJ), and oligonucleotides from Funakoshi (Tokyo). Other materials and chemicals were obtained from Wako (Osaka).

Tumor samples Fifteen differentiated thyroid carcinomas (thirteen papillary and two follicular carcinomas), their surrounding normal tissues, six lymph node metastases (four papillary and two follicular carcinomas), one anaplastic carcinoma, five follicular adenomas and four thyroid tissues from patients with Graves' disease were obtained at surgery. Tissue samples were frozen rapidly in liquid nitrogen and were kept frozen at −70°C until use.

PCR method RT-PCR was performed as previously described.[9] In brief, total cellular RNA was extracted using the method of Chomczynski and Sacchi.[10] One microgram of total RNA was prepared and reverse transcription was performed in a volume of 20 μl. cDNA synthesis was primed with oligo-dT. One microliter of first-strand cDNA was used as the template for PCR reaction in a total volume of 20 μl. The oligonucleotides used as primers are P1 (5′-GACACATATTGCTTCAA-

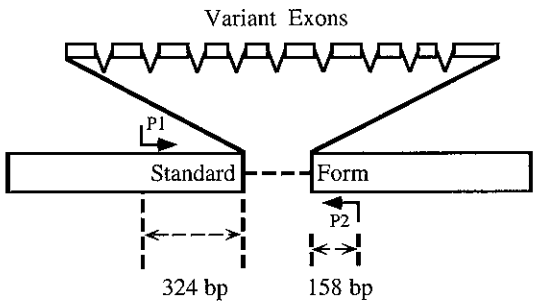

Fig. 1. Map of CD44 gene products showing primers.

TGCTTCAGC) and P2 (5′-CCTGAAGAAGATTGTACATCAGTCACAGAC), as shown in Fig. 1. The PCR was carried out for 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The PCR product (5 μl) was electrophoresed on 2% agarose. The gel was stained with SYBR GREEN I (Molecular Probes, Eugene, OR) for 2 h, then the fluorescent image of each band was quantified with a Fluor Imager (Molecular Dynamics, Sunnyvale, CA).[11]

PCR cloning and nucleotide sequencing The PCR product was extracted and purified from the gel by SUPREC-01 (Takara, Otsu) and was cloned into pMOS*Blue* vector (Amersham, Buckinghamshire, UK) according to the

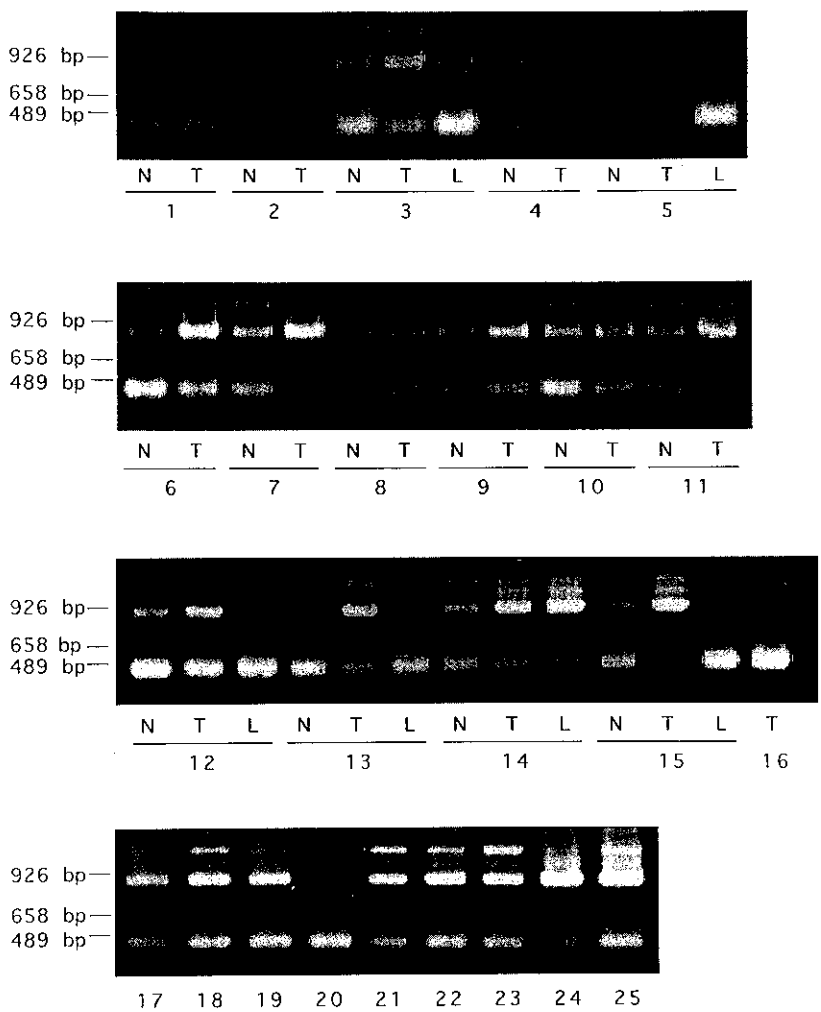

Fig. 2. Gel images of reverse transcription-PCR products using RNA from thyroid tumors. PCR products from papillary carcinomas (1T–13T), follicular carcinomas (14T, 15T), an anaplastic carcinoma (16), thyroid tissues from patients with Graves' disease (17–20) and follicular adenomas (21–25) were resolved on 2% agarose, stained with SYBR GREEN I, and scanned with a Fluor Imager. The normal thyroid tissue (N) and a lymph node metastasis (L) from the same patient were analyzed simultaneously.

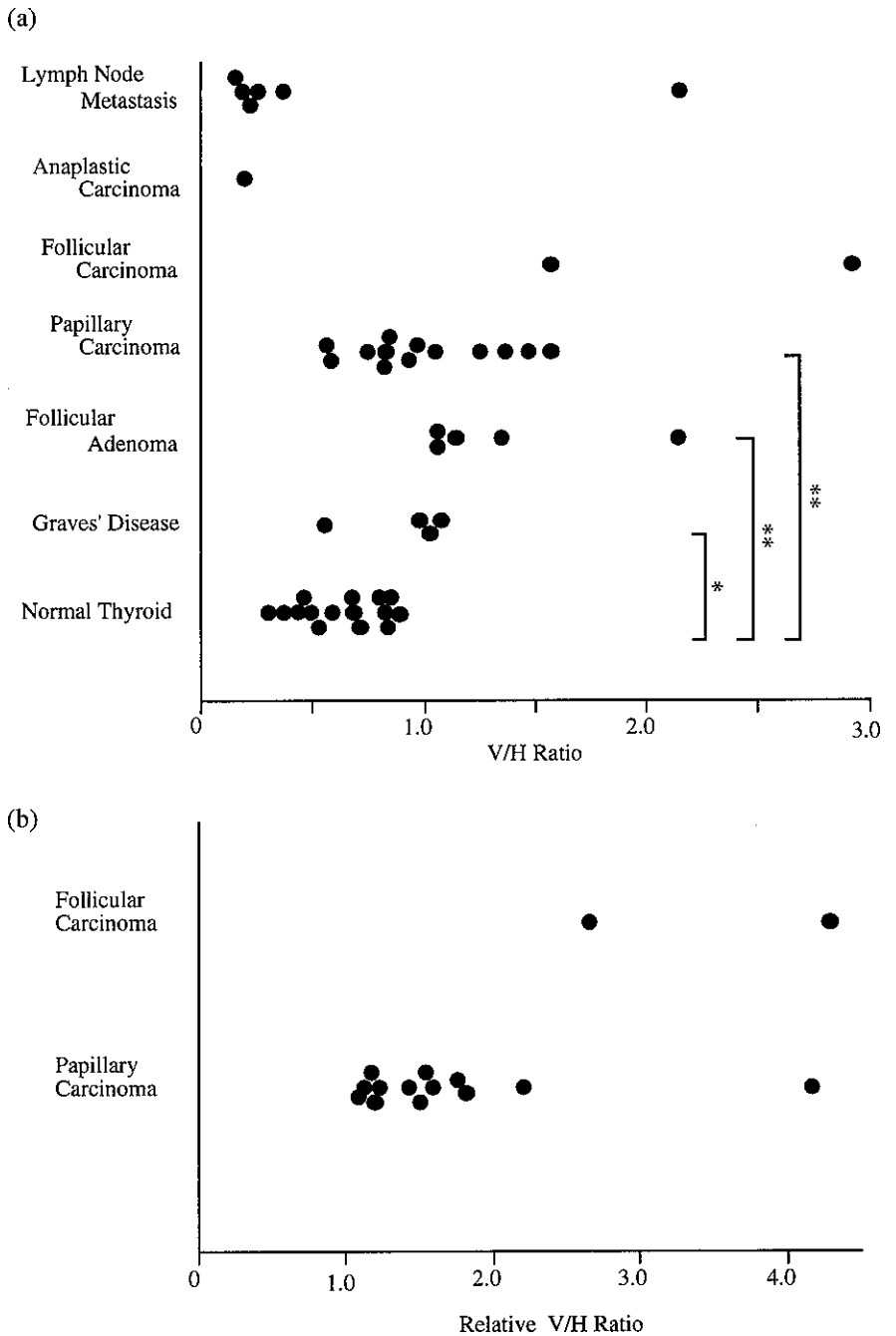

Fig. 3. The V/H ratio (a) and relative V/H ratio (b) in thyroid tumors. After reverse transcription-PCR with CD44 specific primers and gel electrophoresis, fluorescence from the bands at approximately 490 bp (H) and 890 bp (V) was quantified. The ratio of V and H was calculated as V/H ratio (a) (** $P<0.01$, * $P<0.05$). The relative V/H ratio of differentiated thyroid carcinomas compared with their surrounding normal thyroid tissues is shown in (b).

manufacturer's protocol. The cloned product was sequenced using Taq Dye Primer Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA).

Digestion with restriction enzymes The PCR product was extracted and purified from the gel by SUPREC-01 according to the manufacturer's protocol, then it was

digested with 10 U of either *Rsa* I or *Pst* I (Wako, Osaka) for 2 h at 37°C.

Statistical analysis Statistical analysis of differences between the groups was carried out using Student's *t* test. *P* values of less than 0.05 were considered significant.

RESULTS

In most of the samples, two major bands of PCR products derived from CD44H and CD44 variants, at approximately 490 bp (H) and 890 bp (V) respectively, were observed by the Fluor Imager (Fig. 2). The fluorescent image of each band was quantified by the Fluor Imager then the ratio of V and H was calculated (V/H ratio).

The V/H ratio in each thyroid tissue is shown in Fig. 3a. The V/H ratio in most of the tissues with proliferative diseases, including Graves' disease, was higher than that in normal thyroid tissues. It was greatly increased in two follicular carcinomas (V/H ratio=1.57 and 2.93) and one of four follicular adenomas (V/H ratio=2.93). The PCR products derived from CD44 variants were only weakly detectable in five of six lymph node metastases and one anaplastic carcinoma, so the V/H ratio in these tissues showed low values.

The V/H ratio in differentiated thyroid cancers was divided by that of the surrounding normal thyroid tissues dissected simultaneously and the resultant value was designated as relative V/H ratio. The relative V/H ratio in each differentiated thyroid cancer is shown in Fig. 3b. Although increase of the V/H ratio in papillary carcinomas was not clear in Fig. 3a because of the varied values of the V/H ratio in the normal tissues, relative V/H ratio showed a clear increase of relative expression level of CD44 variants in all papillary carcinomas compared with their adjacent normal tissues (relative V/H ratio > 1.0). The relative V/H ratio showed extremely high values in two follicular carcinomas (relative V/H ratio=2.67 and 6.25). Interestingly, although the expression of CD44 variants was only weakly increased in most of the papillary carcinomas, it was greatly increased in two poorly differentiated papillary carcinomas (relative V/H ratio= 2.20 and 1.77) and two well-differentiated carcinomas from young patients (thirty- and thirty-two-year-old; relative V/H ratio=4.16 and 1.55, respectively).

cDNA from a papillary carcinoma was amplified by PCR with primers P1 and P2, then the PCR product at approximately 890bp was extracted from the gel and cloned into pMOS*Blue* vector for nucleotide sequencing. Sequencing analysis revealed the insertion of variant exons, exons 12–14, which was consistent with the sequence of CD44E (data not shown).[12] The extracted PCR product was digested with *Rsa* I and *Pst* I. Three bands at approximately 80 bp, 210 bp and 530 bp were observed after *Rsa* I digestion, and two bands at approximately 210 bp and 670 bp after *Pst* I digestion. These results are consistent with the digestion sites of the CD44E gene (Fig. 4). We obtained similar results using cDNA from several other tissues, including normal thyroid tissues, follicular adenomas and follicular carcinomas. These results indicated that the major part of the PCR products consisting of the band of CD44 variants was derived from CD44E.

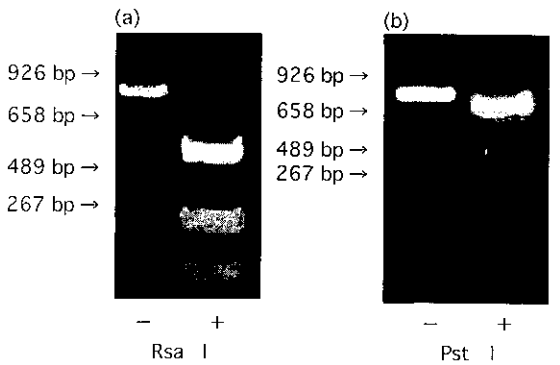

Fig. 4. Digestion with restriction enzymes of the PCR product derived from CD44 variants. The PCR product at approximately 890 bp was extracted from the gel, then digested with either *Rsa* I (a) or *Pst* I (b).

DISCUSSION

In this study, most of the thyroid cancer tissues showed increased relative expression of CD44 variants compared with the surrounding normal tissues. Also, increased values of V/H ratio were observed in benign tissues such as follicular adenomas and tissues from patients with Graves' disease. Thus, the increase in relative expression level of CD44 variants compared with that of CD44H may be a common phenomenon in proliferative thyroid diseases except anaplastic carcinoma.

The relative V/H ratio showed greatly increased values in follicular carcinomas and poorly differentiated papillary carcinomas. The increase in relative expression of CD44 variants may reflect the high growth rate of the tumor cells in differentiated thyroid cancers. However, some follicular adenomas also showed a large increase in V/H ratio. This does not invalidate the above assumption, because only relatively large follicular adenomas with rapid growth are usually taken as indicators for operation. Further studies on the expression of CD44 variants in microadenomas are needed.

The PCR products forming the major band of CD44 variants were mostly derived from CD44E, which is mainly expressed in epithelial cells.[12] CD44E is reported

to have a different function on the cell surface from that of CD44H and it may participate in the adhesion of carcinoma cells to laminin and type IV collagen.[13] Recently, increased expression of CD44 in breast and colon cancers was observed by RT-PCR using a similar technique to ours, so CD44E is suspected to be associated with the progression of these types of cancer.[14,15] As in these cancers, the increased expression of CD44E may be important in the cell proliferation and progression of differentiated thyroid tumors.

We further studied the expression of other variant exons, exon v6 and v7, by RT-PCR and Southern blot analysis as previously described by Wielenga *et al.*,[16] but we could not detect any clear differences in the expression of these variant exons among the tumor types or in lymph node metastases (data not shown). Therefore, the expression of v6 exon, which is often observed in advanced colon cancer,[16] might not be important in the progression of thyroid cancer.

Since white blood cells only express CD44H,[2] contamination of blood cells in tissue can affect the results. Such an effect may not be so important in thyroid tissues, because the tissues of Graves' disease, which are usually hypervascular, show a high value of V/H ratio. However, in the case of lymph node metastases, in most of which CD44 variants were hardly detectable, contamination with a large number of lymphocytes can occur, and might be a cause of this result. CD44E has three variant exons (v12–14), so that semi-quantitative detection of CD44E by immunohistochemistry is quite difficult and we could not establish that expression of CD44E changes in lymph node metastases.

Interestingly, although we could test only one sample, an aggressive anaplastic carcinoma did not express CD44 variants. Expression of CD44 variants may be one of the features of cell differentiation, like the expression of thyroglobulin, thyroid peroxidase and thyrotropin receptor mRNAs, which are not usually expressed in anaplastic carcinomas.[17]

CD44 variants can be easily detected by RT-PCR in only a small number of cells. We might be able to apply this technique to fine needle aspiration biopsy to examine the clinical features of thyroid tumors by extracting RNA from aspirated thyroid cells.

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