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博 士 論 文

CD44v8-10 and CD44s Are Age-dependently Expressed in Primary Cultured Papillary Thyroid Carcinoma Cells and Are Associated with Cell Proliferation

(甲状腺乳頭がん初代培養細胞において、CD44v8-10 および CD44s が
年齢依存的に発現し、細胞増殖能とも関連する)

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CD44v8-10 and CD44s Are Age-dependently Expressed in Primary Cultured Papillary Thyroid Carcinoma Cells and Are Associated with Cell Proliferation

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Papillary thyroid carcinoma (PTC) is the most common endocrine malignancy, arising from follicular cells, and accounts for more than 80% of all thyroid malignant tumors. Although age is the strongest prognostic factor of PTC, and various cut-off ages (40-55 years) were suggested in previous studies, the molecular mechanisms causing age-related changes of PTC cell proliferation remain unclear. CD44 is a major cell surface receptor for hyaluronate and is known as a cancer stem cell marker. However, the association between CD44 and PTC is still unknown. Therefore, we determined the proliferation of primary cultured cells obtained from patients with PTC, and the CD44 mRNA expression profile to elucidate age-related association of CD44 with PTC. The results showed that cell proliferation was significantly decreased according to age. We also found that CD44v8-10 and CD44 splice variants were expressed dominantly in patients with PTC. Moreover, the CD44v8-10/CD44s mRNA expression ratio was significantly increased according to age, and there was a significant negative correlation between this expression ratio and cell proliferation. Our findings suggest that the CD44v8-10/CD44s expression ratio in PTC cells is useful for screening for aggressive PTC and may provide clinically valuable information.

INTRODUCTION

Papillary thyroid carcinoma (PTC) is the most common endocrine malignancy arising from follicular cells and accounts for more than 80% of all thyroid malignant tumors. PTC generally has indolent characteristics and grows very slowly. However, PTC often spreads to cervical lymph nodes and distant organs such as the lung, bone, and brain (16,19,24,44).

Previous studies identified several prognostic factors of PTC such as age, sex, tumor size, extra-thyroidal extension, node metastasis, and distant metastasis (6,13,17). Especially, age was the strongest prognostic factor and various classifications of cut-off ages (40-55 years) were suggested (3,12,15,38,41). Miyauchi *et al.* reported that thyroglobulin-doubling time was a very strong prognostic predictor and had a strong correlation with patients' age (28,29). On the other hand, Mazzaferri *et al.* reported that lymph node recurrences were most frequent at the extremes of age (< 20 and > 59 years) (23), and the same trend was reported by Ito *et al.* (18). Nevertheless, the exact reason for this trend is still unclear.

Recent studies suggest that CD44, one of the most common cancer stem cell (CSC) markers, and its variants (CD44v) by alternative splicing, are related to the progression of several cancers (9,20). CD44 has been implicated in various roles including leukocyte homing and activation, wound healing and cell migration, as well as tumor cell invasion and metastasis (11,31,34). On the other hand, CSCs have the ability to self-renew and to differentiate (14). These facts suggest that CD44 might have some cancer-specific role. Therefore, we determined the expression of CD44 mRNA and cell proliferation activity with primary cultured cells derived from PTC tumors, and analyzed the association with patients' age.

MATERIAL AND METHODS

Patients and tissue samples

The patient cohort consisted of 27 females and 13 males, aged from 16 to 79 years (44.2 ± 16.8 ; median = 43). Clinical data are shown in Table 1. All PTC tissues were removed surgically and provided by Kuma Hospital (Kobe, Japan) from April 2014 to July 2017. The study was approved by the institutional ethical committee of the Graduate School of Health Sciences, Kobe University, and was carried out according to the Declaration of Helsinki principles (Permission Number: 275-1).

Table 1. Clinical data of recruited patients with PTC

No.	Sex	Age	TNM	pT	pN	pEx	DM
1	F	79	2	3	1a	1	-
2	M	55	4	3	1b	1	Lung
3	F	54	1	2	1b	0	-
4	F	44	1	3	1b	1	-
5	M	26	2	3	1b	1	Lung
6	F	72	2	*	1b	0	-
7	M	34	1	3	1b	0	-
8	M	65	2	3	1b	1	-
9	F	16	1	3	1b	1	-
10	M	72	2	3	1b	0	-
11	M	56	1	3	1b	1	-
12	F	43	1	3	1a	1	-
13	F	21	1	2	1a	0	-
14	F	22	1	2	1a	0	-
15	F	28	1	2	1b	0	-
16	F	70	2	3	1b	1	-
17	F	54	1	3	1b	1	-
18	F	52	1	3	1b	0	-
19	F	27	1	3	1b	1	-
20	F	23	1	3	1b	1	-
21	F	58	1	3	1b	1	-
22	M	33	1	3	1b	0	-
23	F	47	2	2	1b	0	Lung
24	M	43	1	3	1a	1	-
25	F	62	2	3	1b	1	-
26	M	35	1	3	1b	1	-
27	F	46	1	3	1b	0	-
28	F	33	1	3	1b	1	-
29	M	31	1	3	1b	1	-
30	M	31	2	3	1b	1	Lung
31	M	62	2	3	1b	1	-
32	M	21	1	3	1b	1	-
33	F	66	2	3	1b	1	-
34	F	60	2	3	1a	1	-
35	F	23	1	2	1a	0	-
36	F	45	1	2	1a	0	-
37	F	41	1	3	1a	1	-
38	F	36	1	2	1a	0	-
39	F	39	1	3	1b	1	-
40	F	43	1	3	1a	1	-

F, Female; M, Male; TNM, AJCC TNM staging; pT, pathological tumor status; pN, pathological nodal status; pEx, pathological extrathyroidal extension; DM, distant metastasis; *, Meta LN recurrence

Cell culturing

The basic culture medium was Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Company, Tokyo, Japan) supplemented with penicillin (100 IU/mL), streptomycin (100 µg/mL), L-glutamine (2 mM), sodium bicarbonate (0.195%) (all from Thermo Fisher Scientific, Waltham, MA, USA), and 10% fetal bovine serum (Biowest, Miami, FL, USA).

The primary culture method was based on the procedure described by Miller *et al.* (25). PTC tissues (0.6 g wet weight) were placed immediately in DMEM at 4°C. Within 24 h, the tissues were minced into 1 mm³ pieces in DMEM, followed by incubation at 37°C with shaking for 2 h in phosphate-buffered saline containing 0.3 U/mL type II collagenase (Worthington Diagnostic Systems, Freehold, NJ, USA). To remove cell aggregates, the resulting suspensions were filtered through a 100 µm cell strainer (Greiner Bio-One, Monroe, NC, USA) and the cells were washed two times with DMEM. The cell suspensions were put into 75 cm² culture flasks with vented caps (Iwaki Glass, Tokyo, Japan) and placed in an incubator at 37°C with 5% CO₂.

RNA extraction and the real-time polymerase chain reaction (RT-PCR) for CD44

Total RNA was extracted from primary cultured PTC cells using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). cDNAs were reverse-transcribed using the PrimeScript II First Strand cDNA Synthesis Kit (Takara Bio, Kusatsu, Japan) following the manufacturer's protocol. The 35 cycles of PCR amplification were performed at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min by using TaKaRa Ex Taq Polymerase (Takara Bio). Primer pairs were purchased from Sigma-Aldrich (St. Louis, MO, USA) and sequences were as follows: forward, 5' -GAGCAGCACTTCAGGAGGT-3' (hCD44-992F); reverse, 5' -GGGTGGAATGTGTCTTGGTC-3' (hCD44-2247R). The PCR products were electrophoresed on 1.0% agarose gels and sequenced by Eurofins Genomics (Tokyo, Japan).

Quantitative (q)RT-PCR of CD44

CD44v8-10 and CD44s mRNA expression levels were measured quantitatively by the qRT-PCR using Taqman® Gene Expression Assays (IDs: Hs01081475_m1, Hs01081473_m1) and Taqman® Universal PCR Master Mix (Thermo Fischer) following the manufacturer's protocols (Patient ID: 10, 11, 13, 14, 15, 17, 19, 20, 21, 23, 24, 26, 27, 28, 29, 31, 32 and 33 as shown in Table 1.). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs02758991_g1) was used as the reference gene. The relative expression ratio of CD44/GAPDH mRNAs was determined.

Cell proliferation assay

Cell proliferation was measured using the Premix WST-1 Cell Proliferation Assay System (Takara Bio). After 7–10 days of incubation, the culture medium was removed, and PTC cells (Patient ID: 1-40 as shown in Table 1.) were harvested using a trypsin/EDTA solution (Clonetics Lonza, Walkersville, MD, USA). The cells were then plated in triplicate in 96-well plates at a density of 1×10^4 cells/well, followed by incubation for another 48 h after which 10 µL of WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium) reagent were added to each well. After 2 h at 37°C, the absorbance of each well was measured at 450 nm (reference: 655 nm).

Statistical analyses

Age-related differences in PTC cell proliferation were determined by a one-way analysis of variance, Welch's t-test, and Pearson's correlation analysis. Correlations between CD44v8-10 or CD44s mRNA expression levels and patient's age or cell proliferation activity were determined by Pearson's correlation analysis using 'EZ R' (Easy R) statistical software. Data are expressed as means ± standard deviation. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

Cell proliferation was significantly decreased with age

To elucidate the correlation between PTC cell proliferation and age, the proliferation of primary cultured PTC cells was measured using the WST-1 assay. The proliferation of PTC cells after 48 h of culturing from the young (under 40 years of age), middle-aged (40-59 years of age), and elderly (over 60 years of age) groups were 1.12 ± 0.52 , 0.859 ± 0.463 , and 0.631 ± 0.269 (absorbance at 450 nm), respectively. Significant differences were observed in cell proliferation among the three age groups; cell proliferation of the young group was significantly

higher than that of the elderly group ($P < 0.05$) (Fig. 1A). Additionally, cell proliferation activity was significantly decreased according to age ($P < 0.05$) (Fig. 1B).

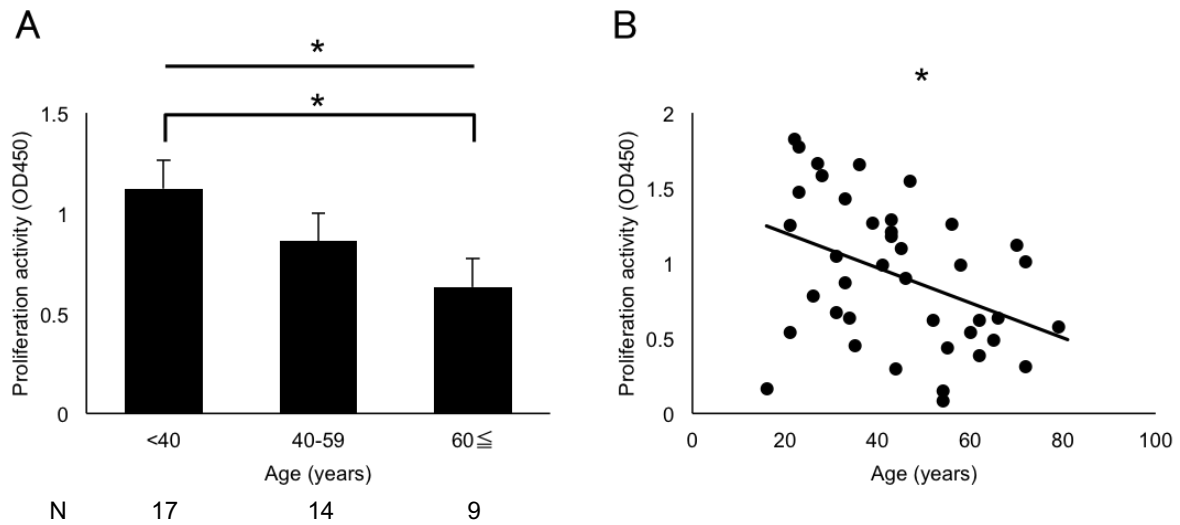


Figure 1. Proliferation of papillary thyroid carcinoma cells.

(A) Cell proliferation according to age groups, analyzed by using a one-way analysis of variance and Welch's t-test. N= 17 (<40), 14 (40-59), 9 (60≤)

(B) Correlation between age and cell proliferation analyzed using Pearson's correlation analysis. N=40

* $P < 0.05$

CD44v8-10 and CD44s mRNAs were expressed dominantly in PTC cells and the expression levels varied significantly among age groups

CD44 is the most common CSC marker and is widely expressed on the cell surface. Various isoforms of CD44 formed by alternative splicing are associated with the progression of many cancers (32,33,45). Therefore, we investigated mRNA levels of the CD44 variant isoforms using the qRT-PCR. The results showed that the CD44v8-10 and CD44s variants were expressed dominantly in patients with PTC (Fig. 2A).

We next investigated the correlation between CD44v8-10 mRNA expression and patient's age. Relative to GAPDH, significantly different expression levels of CD44v8-10 were found between the young (36.6 ± 13.5), middle-aged (57.4 ± 23.4), and elderly (27.3 ± 17.3) groups. The expression level of CD44v8-10 mRNA in the middle-aged group was significantly higher than that of the elderly group (Fig. 2B). There was no correlation between the CD44v8-10 mRNA expression level and patient's age (Fig. 2C).

We also examined the expression level of CD44s mRNA. Unlike the tendency with CD44v8-10, no significant changes of CD44s mRNA were found between age groups (young: 11.3 ± 8.62 , middle-aged: 5.38 ± 3.28 , elderly: 4.39 ± 4.87) (Fig. 2D). However, the CD44s mRNA expression level was significantly decreased according to age (Fig. 2E).

CD44 VARIANTS ARE AGE-DEPENDENTLY EXPRESSED IN PTC CELLS

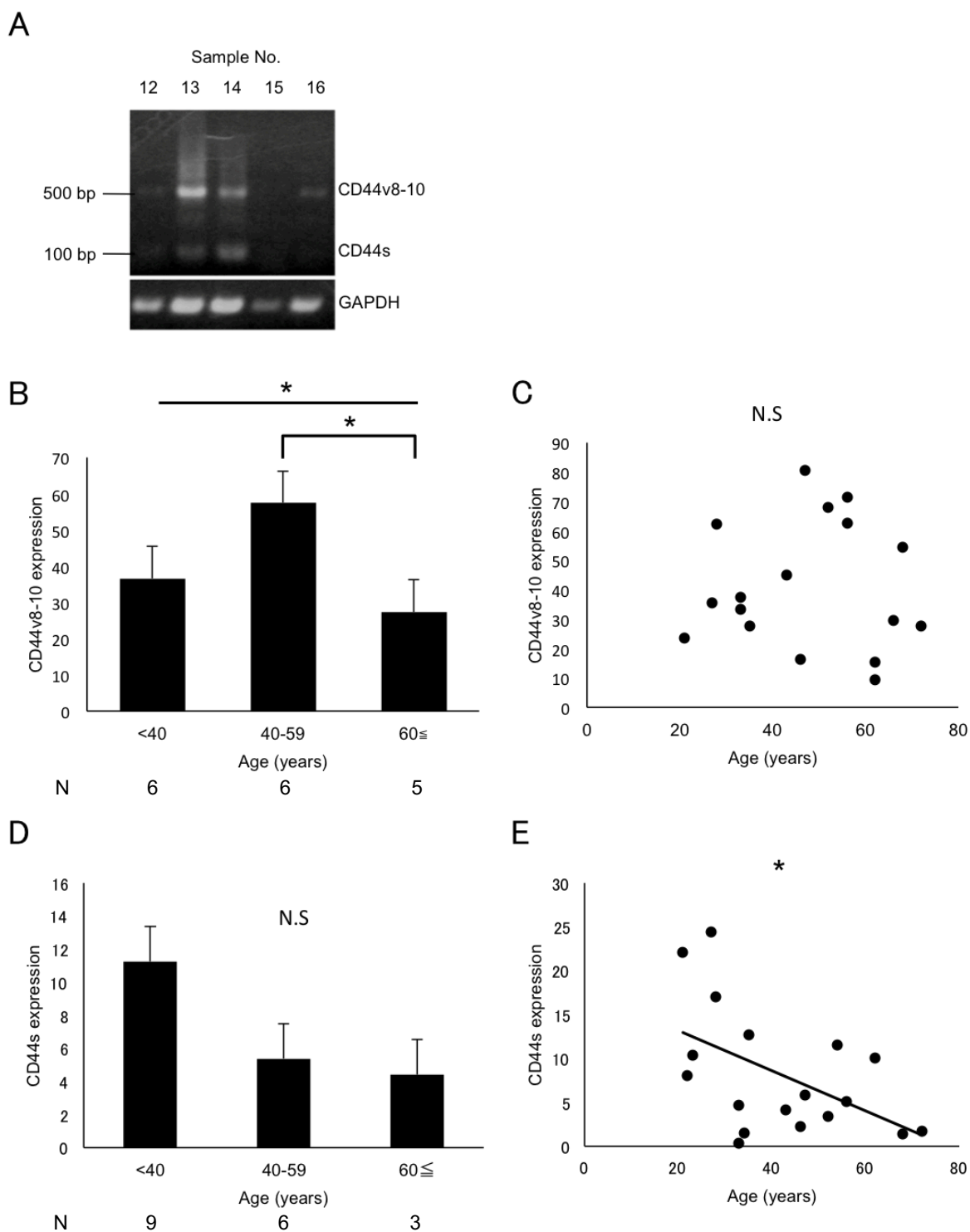


Figure 2. Identification and quantification of CD44s and CD44v8-10 mRNAs in papillary thyroid carcinoma cells.

Expression levels were calculated as the relative expression ratio of CD44/GAPDH.

(A) Typical results of the RT-PCR for CD44 mRNA

(B) CD44v8-10 mRNA expression levels according to age groups analyzed by a one-way analysis of variance and Welch's t-test. Patient ID: 10, 11, 13, 15, 18, 19, 20, 23, 24, 25, 26, 27, 28, 29, 30, 32 and 33, as shown in Table 1. N= 6 (<40), 6 (40-59), 5 (60≤)

(C) CD44v8-10 mRNA expression levels according to age analyzed by Pearson's correlation analysis.

(D) CD44s mRNA expression levels according to age groups analyzed by a one-way analysis of variance and Welch's t-test. Patient ID: 10, 11, 13, 14, 15, 17, 19, 20, 21, 23, 24, 26, 27, 28, 29, 31, 32 and 33, as shown in Table 1. N= 9 (<40), 6 (40-59), 3 (60≤)

(E) CD44s mRNA expression levels according to age analyzed by Pearson's correlation analysis.

* $P < 0.05$

The CD44v8-10/CD44s expression ratio was significantly increased according to age, and there was a significant correlation between this ratio and cell proliferation

The CD44v8-10/CD44s mRNA expression ratio has been suggested as a novel prognostic predictor in patients with urothelial cancer (26). Therefore, we compared this ratio in PTC cells between age groups. The results showed significant differences among the three age groups (young: 3.04 ± 2.44 , middle-aged: 13.20 ± 4.72 , elderly: 26.9 ± 15.4). The CD44v8-10/CD44s expression ratio of the young group was significantly lower than that of the middle-aged group (Fig. 3A). Additionally, the CD44v8-10/CD44s expression ratio was significantly increased according to age (Fig. 3B). We also observed a correlation between the CD44v8-10/CD44s expression ratio and cell proliferation; the ratio was significantly decreased according to cell proliferation activity (Fig. 3C).

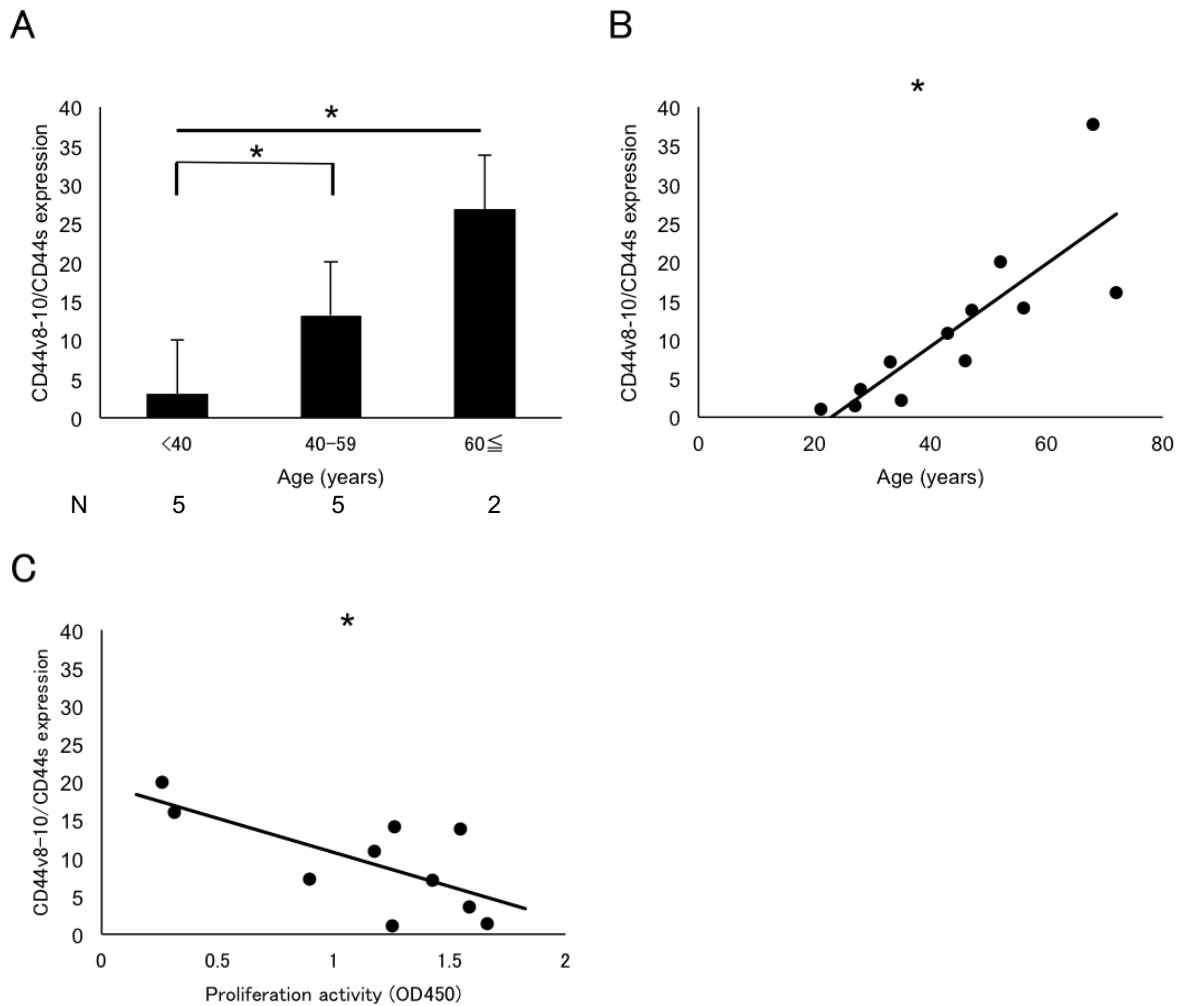


Figure 3. Correlations between the CD44v8-10/CD44s mRNA expression ratio and age, and papillary thyroid carcinoma cell proliferation.

(A) CD44v8-10/CD44s expression ratios according to age groups analyzed by a one-way analysis of variance and Welch's t-test. Patient ID: 10, 11, 13, 15, 19, 20, 23, 24, 26, 27, 29 and 32, as shown in Table 1. N= 5 (<40), 5 (40-59), 2 (60≤)

(B) CD44v8-10/CD44s expression ratios according to age analyzed by Pearson's correlation analysis.

(C) CD44v8-10/CD44s expression ratios according to cell proliferation analyzed by Pearson's correlation analysis. Patient ID: 10, 11, 13, 15, 19, 20, 23, 24, 27 and 32, as shown in Table 1.

* $P < 0.05$

DISCUSSION

In this study, we used the WST-1 assay with primary cultured PTC cells and found significantly higher proliferation in cells from young patients as compared with other age groups. We also found a significant correlation between cell proliferation and age. These results are clinically plausible because young patients usually present with more aggressive disease characteristics than older patients (8,18). Ito *et al.* reported that the lymph node recurrence rate was high in young and elderly patients, although the carcinoma death rate was elevated only in elderly patients (18). Another study with patients younger than age 45 years reported that cervical lymph node metastasis should be recognized as a prognostic indicator (1). Our findings suggest that not only metastasis but also increased proliferation activity cause the unfavorable pathological condition of young patients as compared with other age groups. Further investigations are needed to determine the proliferation activity of cells from other major histological types of thyroid cancer and normal thyroid tissue according to age.

CD44 has a wide variety of splice variants. We found that the CD44v8-10 and CD44s variants were expressed dominantly in patients with PTC. Exons 1-5 and 16-20 are constitutive exons, and the standard isoform (known as CD44s or CD44E), without exon 6-15 (variant exon 1-10), is the most dominant isoform (34,35). CD44v have lower affinity for hyaluronate than that of CD44s (27,37). Furthermore, the ligand-binding specificity of CD44v can be changed, and cell migration and growth are also affected by the extracellular domain of CD44, including variant exons (39,42). It has been reported that dysregulation of mRNA alternative splicing can lead to aging or age-related diseases. Alternative splicing changes during physiological aging were recognized in multiple human tissues (22). How CD44 and its variant expression levels are changing with aging is not still elucidated. However, it has been reported that the MAPK-ERK pathway regulates alternative splicing of CD44 (43). As we found the age-related differences of the proliferation of PTC cells, it is suggested that cell proliferation change *via* the MAPK-ERK pathway could affect the splicing of CD44.

Previous studies reported that CD44 and its variants were found in several human malignancies and exhibited relationships with cancer progression and metastasis (20,31). It has been demonstrated that CD44v8-10 is expressed in patients with different human epithelial cancers, including breast, colorectal, urothelial, and gastric cancer, and its expression correlates with metastasis (26,45). A previous study also reported that overexpression of CD44v8-10 decreased its interaction with hyaluronate in bladder cancer cells, and it was suspected that these processes resulted in abnormal signal transduction from the extracellular domain and enhanced malignant potential (30). These findings suggest that an increased expression ratio of CD44v8-10/CD44s enhances malignant potential and is associated with tumorigenicity of cancer.

Previous studies using immunohistochemistry also showed that CD44 isoforms were found in patients with PTC (4,7,10,21). Furthermore, the expression ratio of CD44v/CD44s was significantly higher in PTC than normal thyroid cells (40). In this study, we found that CD44v8-10 was the dominant form of CD44v in PTC cells and the expression ratio of CD44v8-10/CD44s mRNA was significantly increased according to age. We also found that there was a negative correlation between the CD44v8-10/CD44s expression ratio and proliferation activity. We examined the associations of CD44v8-10/CD44s expression ratio and TNM classification, lymph node metastasis, and tumor invasion. As the result, while no associations were found between CD44v8-10/CD44s expression ratio and AJCC TNM staging or tumor invasion, CD44v8-10/CD44s expression ratio were 5.96 and 12.32, in cases with lymph node metastasis pN1a (n=2) and pN1b (n=10), respectively. However, there was no significant difference. Meanwhile, because it has been reported that CD44 variants are associated with EMT (epithelial-mesenchymal transition), we think further investigation of those association study is needed (5).

Böhm *et al.* also reported that low CD44s expression was an independent prognostic factor for the recurrence of differentiated thyroid carcinoma (2). Based on this report, our results suggest that, while the prognosis of young patients is mainly associated with increased cell proliferation rather than the decreased expression ratio of CD44v8-10/CD44s, the prognosis of elderly patients is mainly associated with the tumorigenicity according to the increased expression ratio of CD44v8-10/CD44s. Previous studies have used the TNM classification of the Union for International Cancer Control to propose cut-off ages as a risk factor based on the clinical characteristics of PTC (36). Although it is unknown the duration of morbidity in the recruited patients in this study, our results may provide evidence to set the clinical cut-off age of patients with PTC.

Overall, our findings suggest that the aggressive behavior of PTC cells and mortality have different mechanisms according to age. Our results also suggest that quantitative determination of the CD44v8-10/CD44s mRNA expression ratio of PTC cells could provide clinically valuable information, such as screening for aggressive PTC for pre-and post-operative follow up.

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