

Mts1 Up-regulation is Associated With Aggressive Pathological Features in Thyroid Cancer

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Abstract. *Background/Aim:* Thyroid cancer is the most common type of endocrine cancer and its incidence and mortality are increasing. However, few studies on the molecular factors related to its poor prognosis have been performed. The aim of our study was to identify a poor prognostic factor for thyroid cancer to reduce its overtreatment, recurrence, and mortality. *Materials and Methods:* The present study is a retrospective study of 55 patients who were diagnosed with papillary thyroid cancer and operated in Korea from September 2013 to November 2015. *Results:* Mts1 is a member of the S100 protein family and is involved in tumor progression and metastasis. Mts1 was highly expressed in patients with thyroid cancer and high Mts1 levels were related to poor prognoses such as lymph node metastasis. *Conclusion:* Mts1 is associated with aggressive pathological features in thyroid cancer, and may be a poor prognostic factor for thyroid cancer.

Thyroid cancer is the most common endocrine-related cancer in the USA and the incidence of thyroid cancer has increased. According to SEER (Surveillance, Epidemiology, and End Results Program) data, rates for new thyroid cancer cases have been rising by an average of 3.1% each year over the last decade (1). In Korea, thyroid cancer is the third most common carcinoma, among all kinds of cancer, with an

incidence rate of 11.7% in 2015 (2). The first choice of treatment for thyroid cancer is surgery, followed by radioactive iodine therapy. In early thyroid cancer, hemithyroidectomy is suitable. The prognosis for thyroid cancer is excellent and the 5-year survival rate is over 98% in USA and Korea (1, 2). This good prognosis has been reported to be due to overtreatment of papillary thyroid microcarcinoma (PTMC), which is less than 1 cm. Diagnosis of small-sized cancers among all thyroid cancers has increased, and about 70% of the cancers now appear as 1-cm or smaller cancers. However, the incidence of advanced thyroid cancer, as well as PTMC, has been increasing, and the mortality rate from thyroid cancer has increased by an average of 0.7% each year from 2006 to 2015. In the case of distant metastasis, the 5-year survival rate decreases to 55.5% in SEER data and 71.0% in Korea (1, 2). Furthermore, in thyroid cancer, recurrence is a more important problem than mortality. The recurrence rate is high, ranging from 1.4% to 35% (3-5), and re-operation or systemic therapy with radioactive iodine must be performed in these cases. Even PTMC has been reported to recur, form distant metastasis, and cause death in some cases (6, 7). Although recurrence and mortality also occurred in thyroid cancer patients, the molecular biological factors associated with poor prognosis are not clear. Preoperative knowledge of molecular biological factors for poor prognosis prevents repetitive operations and radioactive therapy and reduces recurrence rates and mortality. It may also reduce overtreatment of thyroid cancer.

Mts1, also known as S100A4, is a polypeptide of 101 amino acids belonging to the S100 protein family. S100 proteins are Ca²⁺-binding proteins with a low molecular mass (10 to 12 kDa) (8-10). Mts1 has been shown to be associated with tumor progression and metastasis (8, 10, 11), by activating and integrating both intracellular and extracellular pathways to induce cancer metastasis (9, 11). In thyroid cancer, Mts1 has been related to poor prognosis, invasion, and metastasis in several studies (12-18). However, research using clinical and pathological data of thyroid cancer patients has not been performed yet. Therefore, Mts1 expression in thyroid cancer and

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the association between Mts1 and clinicopathological features of thyroid cancer were evaluated. Our results demonstrated that high levels of Mts1 in patients with thyroid cancer were associated with poor prognoses.

Materials and Methods

Protein-protein interaction (PPI) network analysis. Search Tool for the Retrieval of Interacting Genes (STRING 10.5, <https://string-db.org/>) and Gene Multiple Association Network Integration Algorithm (GeneMANIA, <http://genemania.org/>) database tools were used to analyze gene-gene interactions and gene functions associated with Mts1. PPIs of STRING analysis was based on experimental and text mining only, and an interaction score of ≥ 0.7 was used as the cut-off value (highest ≥ 0.9 , high ≥ 0.7 , medium ≥ 0.4 , low confidence ≥ 0.15).

Gene expression profile data. Mts1 expression profile data of various normal tissues (GDS3113) and normal versus anaplastic thyroid cancer (GDS5362) were obtained from Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>).

Patients & methods. The present study is a retrospective study of patients with thyroid cancer. The study population consisted of 55 subjects who had been diagnosed with papillary thyroid cancer (PTC) and operated on from September 2013 to November 2015 at Gachon University Gil Medical Center in Korea. Medical records, pathology reports, and operation records suggestive of prognostic factors were retrospectively evaluated. The staging of these thyroid cancers was classified according to the American Joint Committee on Cancer classification (AJCC, 8th edition). For this study, patients who had been experienced recurrence of thyroid cancer were excluded. This study was approved by the Institutional Ethical Committee and Review Board of the Gachon University Gil Medical Center (IRB No. GAIRB2015-122).

Western blot analysis. Tissue samples were lysed in lysis buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and 5 mM EDTA) supplemented with protease inhibitor cocktail (GeneDEPOT, Katy, TX, USA) on ice for 15 min. Cell lysates were centrifuged at 12,000 rpm at 4°C for 15 min. Protein concentration in the supernatants was measured by the Bradford assay (Bio-Rad, Hercules, CA, USA). The lysates were separated on 10% SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane (Millipore, Burlington, MA, USA). The antibodies used for Western blots were as follow: Mts1 (A5114, Dako, Via Real Carpinteria, CA, USA), GAPDH (sc-47724, Santa Cruz, Dallas, TX, USA).

Immunohistochemistry. The expression of Mts1 in human thyroid cancer tissues and matching adjacent normal thyroid tissues was examined by immunohistochemistry. After embedding in paraffin, tissue sections were de-paraffinized in xylene and dehydrated in a progressively decreasing ethanol series and incubated in 10 mM sodium citrate buffer, pH 6.0 in a microwave oven for 20 min. After inactivation of the endogenous peroxidase activity with 3% hydrogen peroxide at room temperature for 5 min, the sections were blocked with 1% normal horse serum for 30 min, and incubated with primary Mts1 antibody (1:200) overnight at 4°C. The sections

were washed with phosphate buffered saline (PBS) three times, and incubated with biotinylated secondary antibody at room temperature for 30 min, and visualized with 3,3'-diaminobenzidine hydrochloride (DAB) (VECTOR laboratories, Burlingame, CA, USA). Finally, the sections were counterstained with hematoxylin (VECTOR laboratories), and photographs were taken using a microscope with a digital camera.

Statistical analysis. Statistical analyses were performed using IBM SPSS version 23.0. Pearson's χ^2 test, Fisher's exact test, and independent t tests were used to evaluate the significance of the relationship between Mts1 and thyroid cancer. Logistic regression analysis was performed for multivariate analysis. The *p*-values less than 0.05 were considered statistically significant.

Results

Mts1 associated with a variety of cancer-related pathways. Since Mts1 is closely related to tumor progression and metastasis, database tools were used to investigate cancer-related signaling pathways associated with Mts1 in various cancers. The protein-protein interaction (PPI) network associated with the Mts1 protein was analyzed using Gene Multiple Association Network Integration Algorithm (GeneMANIA) and Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database tools. Mts1 is involved in tumorigenesis through regulation of apoptosis, cell cycle, DNA damage checkpoint and focal adhesion signaling pathway by physical interactions or co-expression etc., with TP53, AKT1, Raf family and MAPKs (Figure 1A left). GeneMANIA analysis also indicated that Mts1 is associated with a variety of growth factors, such as EGF, EGFR, and TGF β and that it is also involved in EMT, cell growth and proliferation (Figure 1A right). Similarly, the STRING database revealed that Mts1 forms three cluster networks, according to functional classification through 28 nodes (genes) and 84 edges (interactions) (PPI enrichment *p* < 0.001). The first red cluster includes genes involved in apoptosis signaling pathways, cell cycle progression, and tumorigenesis. The second green cluster is associated with snRNP assembly pathway and the blue is undefined. Among the clusters, genes with the highest degree of association as functional partners with Mts1 are summarized in the Figure 1B right. CDH1 and TP53 are well-known genes that are closely related to tumorigenesis (19-22). CDH1, a key component of EMT, was negatively associated with Mts1 at the transcription level (interaction score: 0.777) (23, 24). Moreover, the stability of tumor protein p53 was regulated through interaction with Mts1 (interaction score: 0.787) (Figure 1B) (25, 26). These data suggest that Mts1 could regulate tumor progression, metastasis, and poor prognosis through a variety of cancer-related pathways, as mentioned in previous studies. However, it is unclear whether Mts1 promotes tumor progression in patients with cancer because few studies

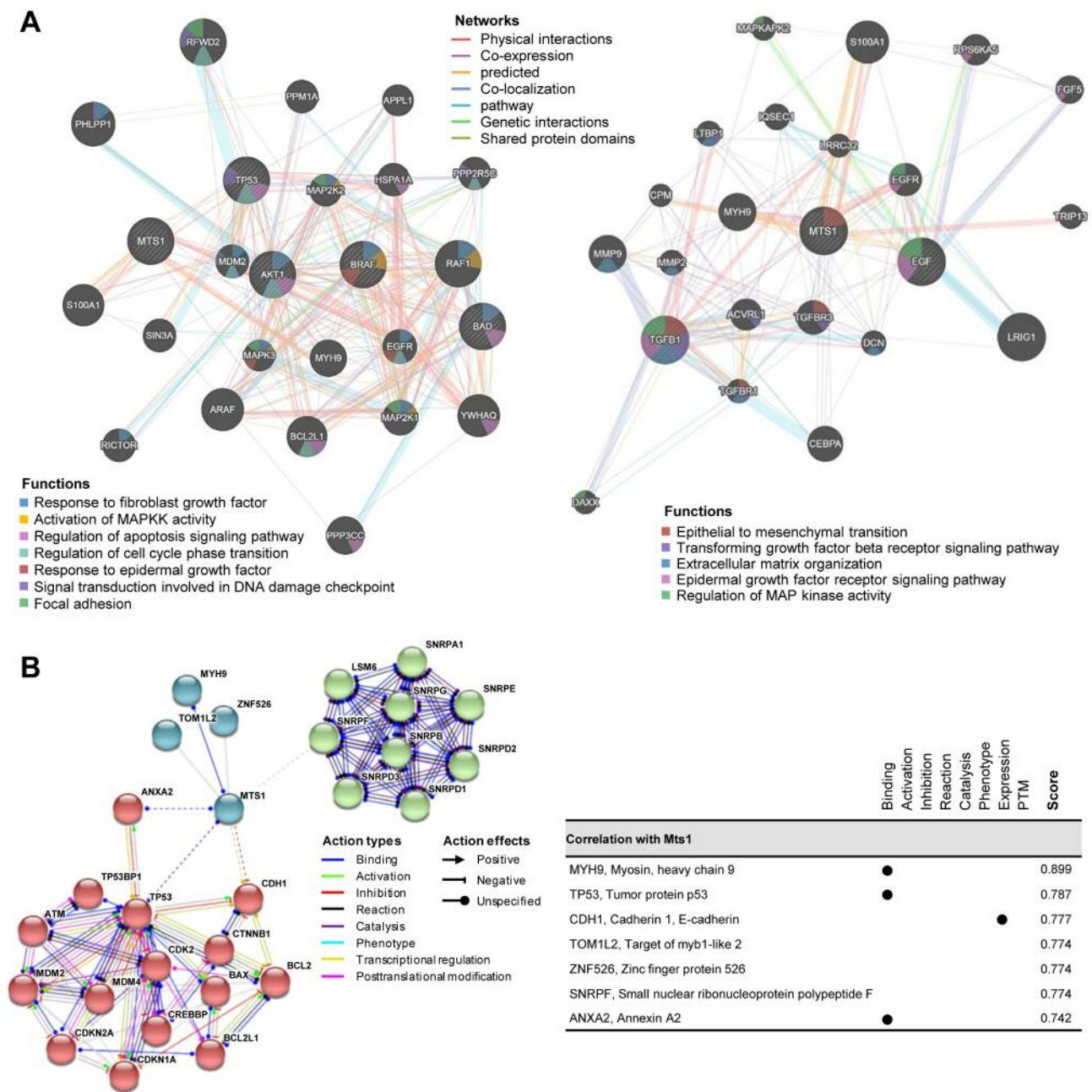


Figure 1. Analysis of protein–protein interaction (PPI) networks associated with Mts1. PPI networks analysis related to Mts1 by GeneMANIA (A) and String 10.5 (interaction score; highest ≥ 0.9 , high ≥ 0.7 , medium ≥ 0.4 , low confidence ≥ 0.15) (B) database tools.

have been performed in patients using clinical and pathological data, especially in patients with thyroid cancer. In addition, there have been some reports that up-regulation of Mts1 expression is associated with tumorigenesis. Thus, the expression of Mts1 in human tissues, including thyroid cancer was examined to determine the function of Mts1 in patients with cancers.

Mts1 is highly expressed in thyroid cancer. Microarray-based RNA profiling analysis using the Gene Expression Omnibus (GEO) datasets showed that the expression levels of Mts1 in the thyroid were relatively low compared to other normal human tissues (Figure 2A). However, the expression levels of Mts1 were largely increased in anaplastic thyroid cancer compared to normal thyroid tissue (Figure 2B). These results

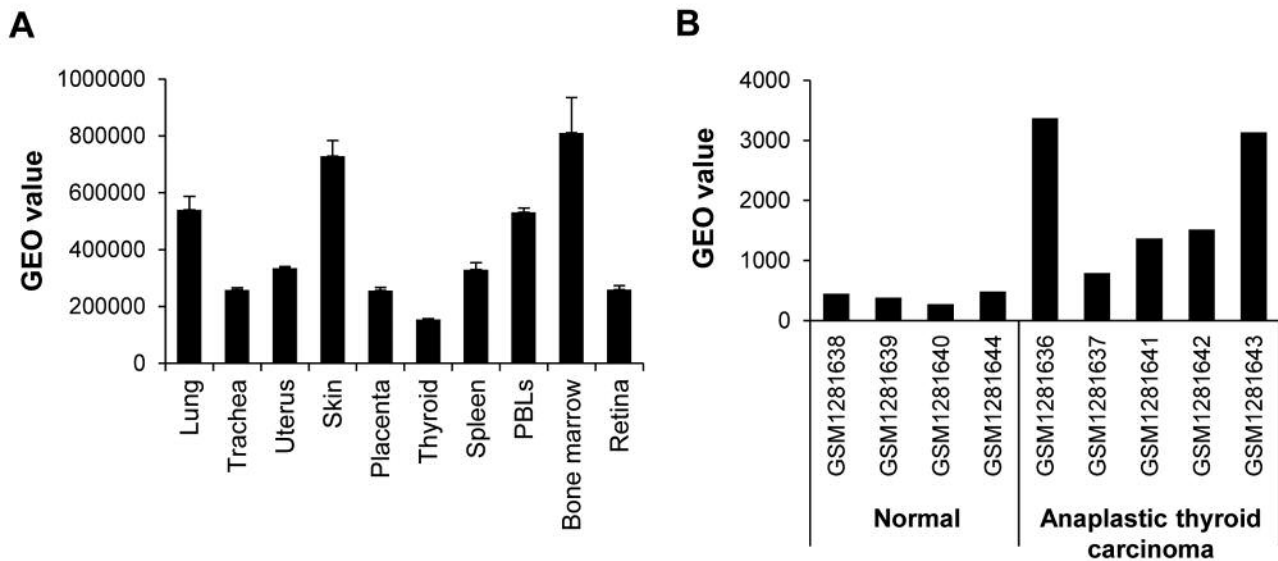


Figure 2. *Mts1* expression using GEO databases. The NCBI Gene Expression Omnibus (GEO) data sets were utilized for profiling the expression of *Mts1* in various human tissues (A), and in normal thyroid and anaplastic thyroid tissues (B).

prompted us to investigate the clinical and pathological effects of *Mts1* in the tumorigenesis of thyroid cancer.

To explore the relationship between the expression levels of *Mts1* and tumorigenesis of thyroid cancer, we examined the expression levels of *Mts1* in 55 pairs of Korean thyroid cancer patients (tumor vs. adjacent non-tumors) from 2013 to 2015. Expression levels of *Mts1* in 16 thyroid cancer patients were analyzed by immunoblotting (Figure 3A). Quantification of *Mts1* expression levels in adjacent non-tumor and thyroid cancer tissues revealed that the average expression of *Mts1* in thyroid cancer was significantly higher than in adjacent non-tumor (12229 ± 1195 vs. 4471 ± 615.5 ; $p < 0.0001$) (Figure 3B). As described above, *Mts1* showed high expression in thyroid cancer. However, to determine the relative expression of *Mts1* in tumor tissue vs. adjacent non-tumor tissue in more detail, the tissue pairs were analyzed again. Expression of *Mts1* was 76.4% higher in tumor tissues than in adjacent non-tumor tissues. The highest level of *Mts1* in thyroid cancer tissue was about 12 times higher than that of non-tumors. In contrast, the ratio of high *Mts1* in adjacent non-tumor tissues was only 23.6% compared to tumor tissues (Figure 3C). Histological analysis showed that normal thyroid tissue displays follicles lined by epithelium, containing homogenous, acidophilic materials called colloid (upper, left, H&E $\times 10$), while a case of PTC displayed papillary structure and nuclei changes such as nuclear elongation, chromatin clearing, and longitudinal grooves (lower, left, H&E $\times 10$). Also, weak cytoplasmic staining of thyroid follicular cells was observed in normal thyroid tissue (upper, middle and right,

IHC $\times 10$) and cytoplasmic membrane and the nuclei in some tumor cells were strongly immunoreactive for *Mts1* (lower, middle and right, IHC $\times 10$) consistent with the immunoblotting data of *Mts1* (Figure 3D). Overall, these results showed that *Mts1* was up-regulated in thyroid cancer. These findings indicated that high levels of *Mts1* are associated with thyroid cancer.

Up-regulation of Mts1 expression correlates with clinicopathologic features of thyroid cancer. Next, to investigate the implication of *Mts1* expression in patients with thyroid cancer, the clinical characteristics of 55 patients were analyzed. Analysis of the expression of *Mts1* and clinicopathologic factors showed that patients' age at diagnosis, extrathyroidal extension (ETE), and lymph node metastasis (LNM) were correlated with high expression of *Mts1*. Patients with high expression of *Mts1* were younger than the other group and there were more patients of less than 55 years. They showed more ETE, LNM, and advanced N stage. In multivariate analysis, younger age, and LNM were significantly associated with high expression of *Mts1*. Consequently, high levels of *Mts1* were associated with poor prognosis in patients with thyroid cancer (Table I). These results suggested that overexpression of *Mts1* in patients with thyroid cancer leads more aggressive pathological features such as LNM.

Altogether, our clinical and pathological data and online database results suggest that *Mts1* may be a poor prognostic factor and a promising molecular biological factor that may help reduce recurrence rates and mortality.

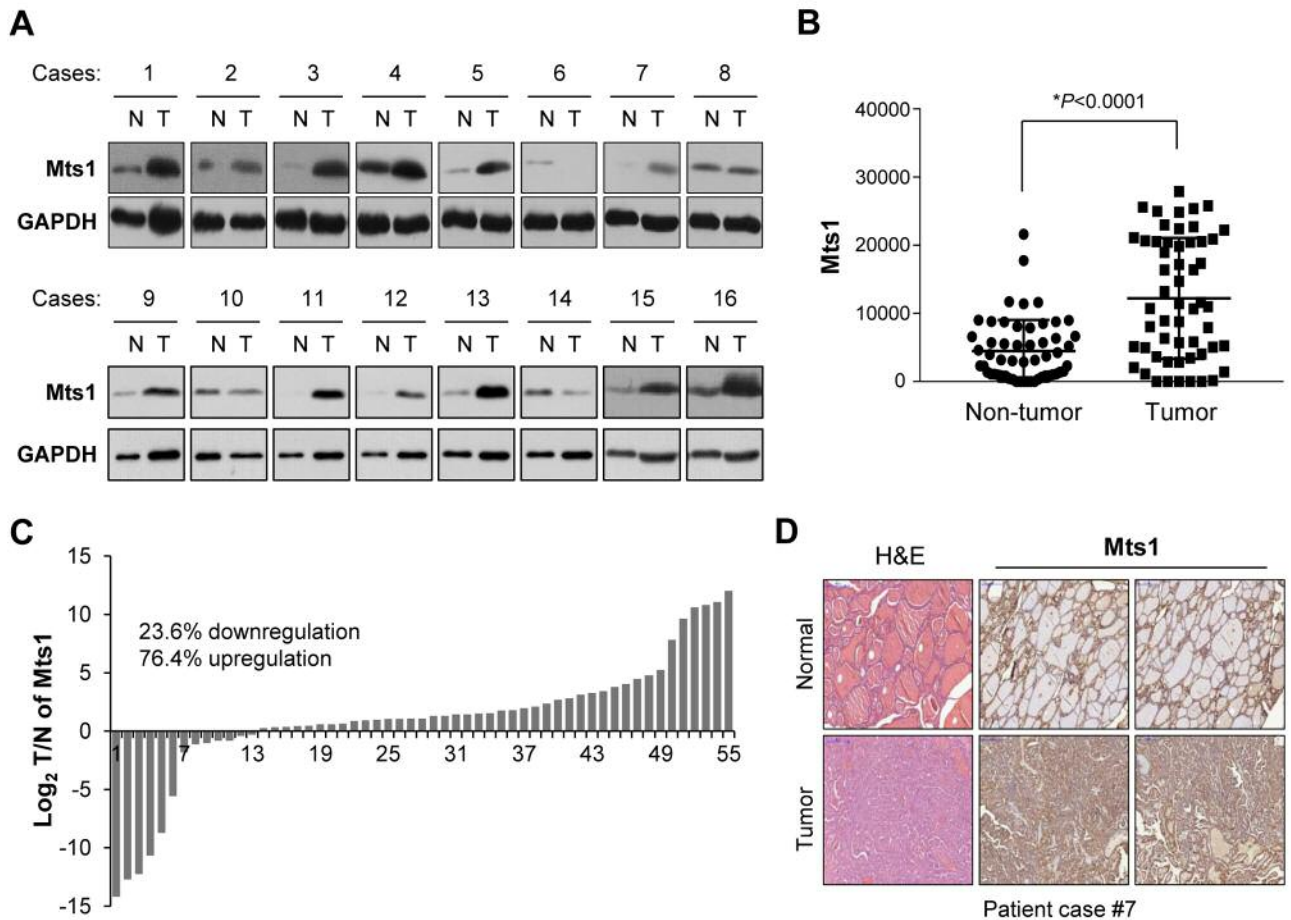


Figure 3. *Mts1* expression in patients with thyroid cancer. (A) Western blot analysis of *Mts1* expression in adjacent non-tumor (N) and tumor (T) tissues from patients with thyroid cancer. (B) *Mts1* average expression measured in 55 pairs of thyroid cancer tissues. (* $p<0.0001$). (C) Relative expression of *Mts1* in tumors normalized to adjacent non-tumor tissues (T/N fold change). (D) Representative immunohistochemistry images of *Mts1* in a thyroid cancer patient (#7).

Discussion

ETE and LNM are associated with disease recurrence and persistence of thyroid cancer. N stage, a part of AJCC TNM cancer staging manual, is correlated with disease-related survival (27). Since high expression of *Mts1* was correlated with ETE, LNM, and N stage, *Mts1* could be a factor indicating disease recurrence, persistence, and disease-related survival. Furthermore, in multivariate analysis, patients with high expression of *Mts1* showed significantly more LNM. In a study of a murine model of thyroid cancer, *Mts1* was related to advanced cases and LNM. Analysis of primary tumors and their matched LNM in humans demonstrated that significantly higher *Mts1* transcripts were present in metastatic tumors compared to the primary tumors. This suggested that overexpression of *Mts1* is associated with thyroid tumor invasion and metastasis (13, 16). Interestingly,

Mts1 was detected even in PTMC, less than 1cm sized PTC. This suggested that *Mts1* plays a constitutive role in PTC and it may be a useful marker for early detection of PTC (28). In another study of PTMC, *Mts1* was related to LNM. On multivariate analysis, expression of *Mts1* was found to be an independent predictive factor of LNM, macro-metastases, and lateral node metastasis (17). In our study, tumor size was smaller and the proportion of microcarcinoma was higher in the high expression group, though not statistically significant. PTMC usually has an excellent long-term prognosis, but it can metastasize to neck lymph nodes and distant organs (29). *Mts1* might be associated with tumor invasion and metastasis in small thyroid cancer.

Age at diagnosis of thyroid cancer is an independent predictor of disease-specific survival in the staging system. Most of the other clinicopathological staging systems use an age cut-off between 40 and 55 years, or age as a continuous

Table I. Association between *Mts1* expression and clinical characteristics of patients with thyroid cancer.

| Variable | Mts1 (%) | | Univariate analysis <i>p</i> -Value | Multivariate analysis | |
|----------|-------------|-------------|--|-------------------------|-----------------|
| | Low n=12 | High n=43 | | HR (95%CI) | <i>p</i> -Value |
| Gender | | | | | |
| Female | 9 (75.0%) | 32 (74.4%) | n.s | | |
| Male | 3 (25.0%) | 11 (25.6%) | | | |
| AGE | 57.17±15.33 | 46.67±12.10 | 0.015 | 0.902 (0.822-0.989) | 0.029 |
| <55 | 4 (33.3%) | 33 (76.7%) | 0.012 | | n.s |
| ≥55 | 8 (66.7%) | 10 (23.3%) | | | |
| SIZE | 2.68±1.98 | 1.54±0.83 | n.s | | |
| ≤1.00 | 3 (25.0%) | 13 (30.2%) | n.s | | |
| >1.00 | 9 (75.0%) | 30 (69.8%) | | | |
| ETE | | | | | |
| No | 8 (66.7%) | 11 (25.6%) | 0.015 | | n.s |
| Yes | 4 (33.3%) | 32 (74.4%) | | | |
| LVI | | | | | |
| No | 10 (83.3%) | 31 (72.1%) | n.s | | |
| Yes | 2 (16.7%) | 12 (27.9%) | | | |
| LNM | | | | | |
| No | 10 (83.3%) | 20 (46.5%) | 0.046 | 36.843 (1.283-1058.007) | 0.035 |
| Yes | 2 (16.7%) | 23 (53.5%) | | | |
| T stage | | | | | |
| 1 | 6 (50.0%) | 31 (72.1%) | n.s | | |
| 2 | 3 (25.0%) | 6 (14.0%) | | | |
| 3 | 3 (25.0%) | 4 (9.3%) | | | |
| 4 | 0 (0.0%) | 2 (4.7%) | | | |
| N stage | | | | | |
| 0 | 10 (83.3%) | 20 (46.5%) | 0.041 | | n.s |
| 1a | 1 (8.3%) | 20 (46.5%) | | | |
| 1b | 1 (8.3%) | 3 (7.0%) | | | |
| M | | | | | |
| 0 | 12 (100.0%) | 42 (97.7%) | n.s | | |
| 1 | 0 (0.0%) | 1 (2.3%) | | | |
| Stage | | | | | |
| I | 9 (75.0%) | 36 (83.7%) | n.s | | |
| II | 3 (25.0%) | 5 (11.6%) | | | |
| III | 0 (0.0%) | 1 (2.3%) | | | |
| IV | 0 (0.0%) | 1 (2.3%) | | | |

ETE: Extrathyroidal extension; LVI: lymphovascular invasion; LNM: lymph node metastasis.

variable. The AJCC 8th TNM cancer staging manual uses 55 years as a cut-off. Disease specific survival is better in younger patients. Old age has also been reported to be an adverse factor in disease-free survival (30). However, advanced age was not an independent predictor of disease-free survival (31). Thyroid cancer in children and adolescents frequently showed more extensive disease, such as large tumor, more LNM, and distant metastasis (32). In our study, the patients with high expression of *Mts1* were younger than the other group. *Mts1* may be expressed and involved, particularly in young age of thyroid cancer. Overall, the higher expression of *Mts1* in thyroid cancer than in normal tissue was associated with younger age and LNM. This suggests that *Mts1* is associated with invasion, metastasis, and tumorigenesis of thyroid cancer.

Conflicts of Interest

There are no conflicts of interest to declare regarding this study.

Authors' Contributions

Conceptualization, M.G. Cheon, H.H. Jang and Y.S. Chung; investigation, M.G. Cheon and Y.W. Son; resources, J.H. Lee and Y.S. Chung; writing – original draft preparation, M.G. Cheon and Y.S. Chung; writing – review and editing, H.H. Jang; visualization, M.G. Cheon; supervision, H.H. Jang; funding acquisition, M.G. Cheon and Y.S. Chung.

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