Stromal Lactic Acid Transporter: MCT4 expression as an indicator of Prognosis in Papillary Thyroid Cancer

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Directed by Professor Lee, Jandee

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ABSTRACT

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Purpose: ^{18}F-FDG-PET/CT is often used to detect tumors by taking advantage of malignant cells' characteristic of high glucose uptake. However, because intra-tumoral heterogeneity or cancer-associated fibroblast (CAF) metabolism can affect glucose uptake, tissue or cells showing high ^{18}F-FDG, uptake may be heterogenous. Many studies focusing on the prognostic impacts of ^{18}F-FDG-PET/CT did not consider the specific cell or tissue types' tumor specificity. In this study, papillary thyroid cancer (PTC) showing high FDG uptake on ^{18}F-FDG-PET/CT was classified into subgroups according to the expression pattern of monocarboxylate transporter 4 (MCT4; SLC16A3). The clinical properties of each subgroups were observed to be quite different depending on the cell type or tumor type specificity for glucose and lactate metabolism.

Methods: The expression status of MCT1 and MCT4 in thyroid cancer was evaluated using public repositories including THCA data from TCGA, NCBI Gene Expression Omnibus (GEO) profiles, and my own RNA-sequence data. The mRNA expression status of several core components of glycolytic pathways in THCA such as

hexokinases (HK), glucose transporter (GLUT), lactic dehydrogenase (LDH), and pyruvate kinases (PK) was also investigated according to the status of MCT4 mRNA expression. Immunohistochemical (IHC) staining analyses of MCT1 and MCT4 were performed using human thyroid cancer tissue, and the expression status of these transporters were compared with clinicopathological characteristics.

Results: MCT1 and MCT4 expression were remarkably increased in PTC compared to paired normal thyroid tissue in THCA, GSE33630, and my own RNA-sequence data. In addition, the expression of MCT4 mRNA in PTC showed strong positive correlations with HK3 (r=0.4911, P<0.0001), GLUT1 (r=0.2723, P=0.0002), LDHA (r=0.4813, P=0.0001), PKLR (r=0.0797, P=0.0028), and PKM2 (r=0.4895, P<0.0001). In the analyses of the clinicopathological parameters using THCA data, high MCT4 expression was correlated with BRAFV600E mutation, intermediate and high recurrence risk, advanced tumor (stage T3 and T4), lymph node stage (N1a and N1b), and the simultaneous presence of BRAFV600E mutation and TERT promoter mutation (P<0.0001). Supporting the results from the public repository, in this study, IHC staining indicated that high stromal MCT4 levels (grade 2) were specifically associated with aggressive tumor behavior including advanced tumor (stage T3 and T4), lymph node stage (N1a and N1b), and TNM stage (III and IV) (P<0.0001). However, the degree of epithelial MCT4 staining had no prognostic value.

Conclusion: These findings indicate the pivotal role of MCT4 in PTC progression, and support the use of stromal MCT4 as a biomarker and potential therapeutic target in aggressive PTC.

Key words: thyroid cancer, MCT4, Warburg effect, lactate transporter

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I. INTRODUCTION

Cancer is characterized by a constellation of hallmarks acquired during the multistep carcinogenesis process. Originally, the hallmarks of cancer were comprised of six characteristics that are mainly related to cell proliferation, migration, and evading apoptosis. New scientific findings related to cancer-associated fibroblasts (CAF) and heterotypic signaling between cancer cells and the tumor microenvironment[1,2] has seen the inclusion of two new hallmarks such as metabolic rewiring and the role of tumor microenvironment to the list of hallmarks.

In the Warburg era, cancer metabolism was defined by aerobic glycolysis.[3] But these days, an increasing body of research has revealed that actual cancer metabolism is complicated by a multiplicity of engaged factors and players.[4] For example, beside inter-tumor heterogeneity, intra-tumoral heterogeneity is operational in certain tumors. While one subpopulation of tumors is glucose-dependent and exhibit the Warburg effect by secreting lactate; another subpopulation preferentially utilizes the

lactate from neighboring tumor cells. Interestingly, this symbiotic relationship also works between tumor and CAF. CAF upregulate glycolytic pathways and secret lactate, which is imported by better oxygenated tumors. The reverse Warburg effect is another recently discovered metabolic reprogramming which exhibits this symbiotic relationship between tumor and CAF.[5]

Presently, the vast majority of clinical oncologic PET/CT studies performed utilize an analog of glucose, 18F-2-fluoro-2-deoxy-d-glucose (FDG) which takes advantage of malignant cells' higher rates of aerobic glycolysis.[6] However, tissue or cells exhibiting high ^{18}F-FDG uptake may not be cancer itself because intra-tumoral heterogeneity or CAF metabolism can affect glucose uptake.[6] If there is intra-tumoral heterogeneity, one subpopulation showing glucose-dependent can be detected using ^{18}F-FDG-PET/CT, but another subpopulation using lactate may not be detected. In the case of CAF exhibiting a reverse Warburg effect, ^{18}F-FDG-PET/CT detects the CAF rather than tumor itself. It can be noted that many published studies focusing on the prognostic impacts of ^{18}F-FDG-PET/CT did not take cell type or tissue type specificity into consideration.

In the current study, it was hypothesized that high expression of lactate transporters will be associated with a poor outcome in thyroid cancer. To prove this hypothesis, TCGA public data and Severance hospital data were gathered.[7] Many analyses of the clinicopathologic factors with expression of lactate transporters have been published. In this study, papillary thyroid cancer (PTC) showing high FDG uptake on ^{18}F-FDG-PET/CT was classified into subgroups according to the expression pattern of monocarboxylate transporters (MCT) 1 (Solute Carrier Family 16 Member 1; SLC16A1) and 4 (SLC16A3).[8,9] The molecular biological and clinical properties of the two subgroups were observed to be quite different for glucose and lactate metabolism depending on cell type or tumor type specificity.

II. MATERIALS AND METHODS

Subjects and clinical analyses

Study participants were patients with newly diagnosed PTC who underwent preoperative ^{18}F-FDG-PET/CT from March 2007 to February 2014 at Severance Hospital, Seoul, South Korea. Of these 231 patients, 4 with no visible uptake of tumor site on ^{18}F-FDG-PET/CT were excluded. Eight patients who showed diffuse hyper-metabolism at the thyroid gland on preoperative ^{18}F-FDG-PET/CT were also excluded because the primary tumor sites were difficult to determine. Therefore, a total of 219 PTC patients (66 male and 153 female) with visible primary tumor sites on ^{18}F-FDG-PET/CT prior to operation were enrolled in this study. The study patients underwent total thyroidectomy with or without neck node dissection. The sample size was calculated by Web-based Sample Size/Power Calculations (http://www.stat.ubc.ca). Patient information and clinicopathological parameters were analyzed retrospectively. The overall median follow-up time was 8.2 ±5.1 years. All protocols were approved by the institutional review board of Severance Hospital. The relationships between lactate transporter and other clinicopathologic factors were analyzed using data from mRNA to cellular and the tissue levels using different methods. The experiments of each level are as below.

Public data: TCGA & GEO

Public data and statistical analysis public repository microarray data from The Cancer Genome Atlas (TCGA, https://tcga-data.nci.nih.gov/ tcga/) and the Gene Expression Omnibus (GEO) of NCBI (Gene expression data available at www.ncbi.nlm.nih.gov/ projects/geo; accession no. GSE33630) were subjected to GSEA. Data from the GeneNetwork (a free scientific web-based resource, http://www.genenetwork.org/) were also subjected to analysis. A total of 505 patients' data was obtained. Clinicopathologic data including age, gender, tumor size, MACIS score, histologic subtype, extrathyroidal extension, multifocality, TNM staging, mRNA cluster

number, miRNA cluster number, Ras driver mutation, BRAF driver mutation, TERT promoter mutation, RAS/RAF score, ERK score, differentiation score, residual disease, and tumor status were obtained.

lab: RNA sequencing data

Laboratory specimens were collected from 7 patients' fresh frozen thyroid tissue harvested after thyroid surgery from March 2014 to August 2014. Seven PTC tumor samples and 7 paired normal tissue and metastatic lymph node samples and matching tumor tissue samples were collected and the diagnosis of each sample was confirmed via pathology report. Extraction of RNA from frozen tissue was performed using QIA cube and RNeasey. RNA was assessed for quality and concentration measurement using an RNA 6000 Nano LabChip on a 2100 Bioanalyzer (Agilent Inc., Palo Alto, CA). The sequencing libraries were sequenced on a HiSeq 2000 platform (Illumina, SanDiego, CA).

Western blot analyses

Western blot analyses were performed on the different cell lines to investigate the basal expression levels of MCT1 and MCT4 in thyroid cell lines. The following cell lines were used: normal thyroid cell line (H-Tori3 and Nthy-ori3) [10], PTC cell line (BCPAP, K1 and TPC1) [7,8,11], follicular thyroid cancer cell line (FTC133 and ML-1)[12], and anaplastic cancer cell line (8505C, SW1736, CAL62, HTH83 and C64)[13]. Western blot analysis was performed according to standard methods with commercially available antibodies: MCT1(*SLC16A1*) rabbit polyclonal antibody (sc50324, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), MCT4(*SLC16A3*) rabbit polyclonal antibody (HPA021451, Sigma, St Louis, MO Cell Signaling, USA), and anti-b-Actin Antibody (#4967, Cell Signaling).

Immunohistochemical staining method and scoring

Immunohistochemical staining (IHC) for MCT1 and MCT4 was performed in the 219

cases of PTC and matched normal tissue. Briefly, 4 mm tissue sections were heated to 608℃, deparaffinized in xylene, and hydrated in a graded series of alcohol. Antigen retrieval was performed by microwaving in citrate buffer for 10 min. Endogenous peroxidase activity was inactivated by incubation in 3% hydrogen peroxide for 10 min. Nonspecific binding sites were blocked by incubating in 10% normal goat serum diluted with phosphate-buffered saline. Tissue sections were then incubated with primary antibodies: MCT1 (sc50324) or MCT4 rabbit polyclonal antibody (HPA021451) for 60 min at room temperature. All sections were sequentially treated with biotinylated anti-rabbit immunoglobulin for 30 min, peroxidase-labeled streptavidin for 30 min, and diaminobenzidine in the presence of hydrogen peroxide. Controls were incubated with PBS in place of a primary antibody, and no positive staining was observed in any cases in the control group. Staining was scored as follows:

(1) Stromal scoring: Stromal MCT1 or MCT4 expression was scored semi-quantitatively as 0 (negative, no staining), 1 (weak, either diffuse weak staining or strong staining in less than 30% of stromal cells per core) or 2 (strong, defined as strong staining of 30% or more of the stromal cells).

(2) Epithelial scoring: MCT1 or MCT4 expression in tumor epithelial cells were evaluated using a previously developed scoring system.[14] Sections were scored semi-quantitatively as follows: 0, (0% immune-reactive cells), 1 (<5% immune-reactive cells), 2 (5-50% immune-reactive cells), and 3 (>50% immune-reactive cells). Similarly, the intensity of staining was evaluated semi-quantitatively on a scale 0-3, with 0 representing negative; 1 (weak staining), 2 (moderate staining), and 3 (strong staining). Then, the final score (0-2) was calculated to reflect both the percent of immune-reactive cells and staining intensity.[14,15]

Two different individuals performed the review of IHC stains using the above scoring system. Mismatched data was labeled “undetermined data” and grouped separately.

Statistical analysis

Statistical analysis was carried out using SPSS version 20.0 for Windows (IBM Corporation, Armonk, New York, USA) or GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). Data are presented as the mean ± SD. All P values are 2-sided.

III. RESULTS

Relationship of mRNA expressions between MCT1 and MCT4

The relationship between MCT1 and MCT4 is represented in Figure 1 (Fig. 1). In the TCGA dataset, the thyroid cancer group is labeled "THCA dataset." There was no relationship between MCT1 mRNA and MCT4 mRNA. This was the same for the GSE33630 public dataset, GSE76039 (public dataset of poorly differentiated thyroid cancer), GSE76039 (anaplastic thyroid cancer dataset), and ONCONT.

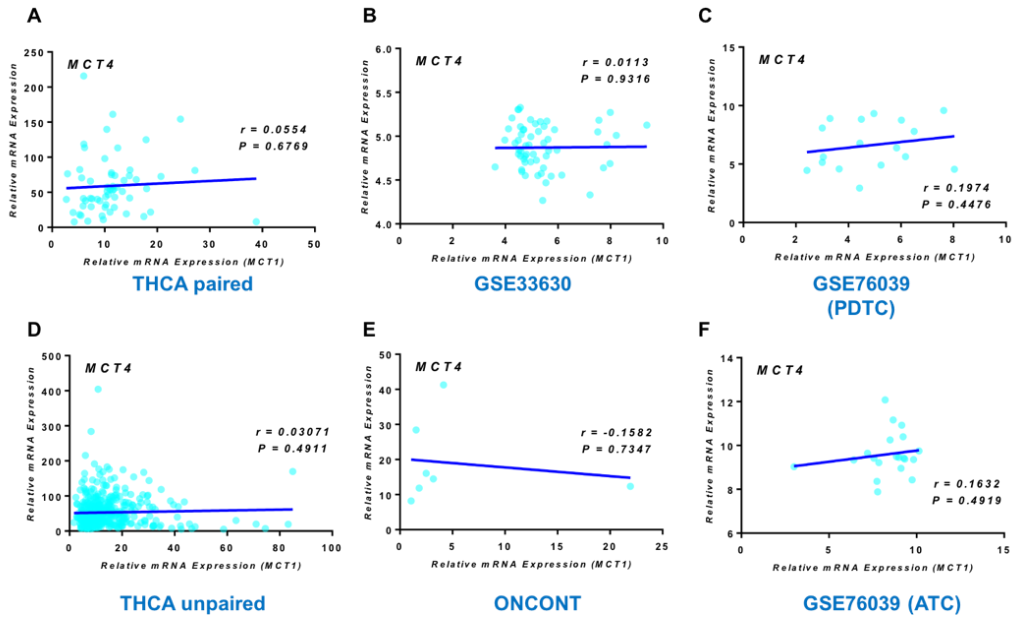

Figure 1. No statistically significant correlation between MCT1 and MCT4 mRNA expressions in thyroid cancer.

Increased mRNA expression of MCT1 and MCT4 in thyroid cancer and clinical impact

The expression status of the MCT1 and MCT4 lactate transporters in thyroid cancer were evaluated using public repository and my own RNA-sequence data. Both MCT1 and MCT4 expression was remarkably increased in PTC compared to the paired or unpaired normal thyroid tissue in THCA data from the TCGA (Fig. 2). In addition, MCT1 and MCT4 mRNA was also increased in thyroid cancer from the other public repository data from the Chernobyl tissue bank (GSE33630, Fig. 2). Supporting the results from both public repositories, my own RNA-sequence data also indicated MCT1 and MCT4 overexpression in PTC compared to matched normal thyroid tissue (Fig. 2). In an analysis of clinicopathological parameters using the THCA data, high MCT1 expression was significantly associated with the simultaneous presence of BRAFV600E mutation and TERT promoter mutation (P=0.019, Table 1). Interestingly, high MCT4 expression was correlated with BRAFV600E mutation, intermediate and high recurrence risk, advanced tumor (pathological T3 and T4) and lymph node (pathological N1a and N1b) stages, and simultaneous presence of BRAFV600E mutation and TERT promoter mutation (P<0.0001, Table 2).

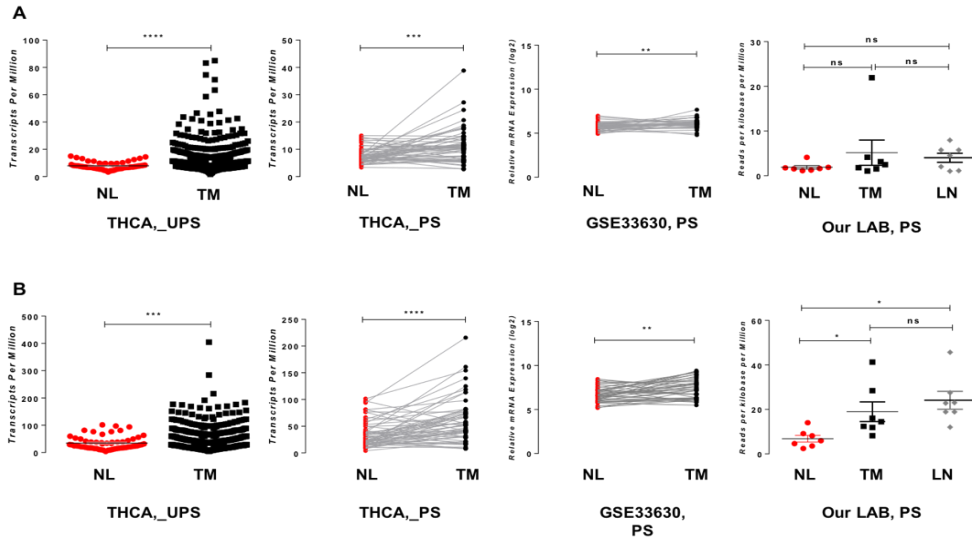

Figure 2. Increased mRNA expressions of MCT1 and MCT4 in thyroid cancer compared to normal tissue.

Table 1. Clinicopathological characteristics of patients with papillary thyroid cancer according to *MCT1* mRNA expression from the TCGA thyroid cancer database (n=505)

	MCT1 mRNA expression		*P* value
	Lowest (< 7.4) (n=122) (%)	Highest (>15.3) (n=122) (%)	
Age (years)	47.2 ± 15.5	47.9 ± 17.3	0.724*
Gender (Female)	73	72.9	0.978†
Tumor size (cm)	2.7 ± 1.6	3.1± 1.5	0.14*
MACIS score	5.2 ± 1.5	5.7± 1.8	0.063*
Histologic subtype			
Conventional	63.5	66.1	0.698†
Follicular variant	28.6	22.9	
Tall cell variant	6.1	9.4	
Others§	1.8	1.6	
Extrathyroidal extension			
No	71.7	63.9	0.153†
Minimal	25.8	28.7	
Gross	2.5	7.6	
Multifocality			
No	51.7	57	0.404†
Yes	48.3	43	
T stage			
T1	32.2	21.4	0.008†
T2	37.4	33.3	
T3	27.8	37.6	
T4	2.6	7.7	
N stage			
N0	68.2	51.6	0.015†
N1a	19.3	24.2	
N1b	12.5	24.2	
M stage			
M0	95.2	96.7	0.675†
M1	4.8	3.3	
TNM stage group			
I/II	69.1	65	0.498†
III/IV	30.9	35	

mRNA cluster number			
1	39.5	25.6	0.687†
2	10.1	8.3	
3	10.9	28.1	
4	12.6	31.4	
5	26.9	6.6	
miRNA cluster number			
1	1.6	2.4	0.371†
2	19.7	39	
3	20.5	10.6	
4	33.6	20.3	
5	18.9	9.8	
6	5.7	17.9	
Ras driver mutation			
Absent	78	91.1	0.005†
Present	22	8.9	
BRAF driver mutation			
Absent	56.9	43.9	0.041†
Present	43.1	56.1	
TERT promoter mutation			
Absent	92.8	84.2	0.058†
Present	7.2	15.8	
RAS/RAF score	-0.5 ± 0.8	-0.3± 0.7	0.01*
ERK score	-2.8 ± 21.6	10.2± 21.3	< 0.001*
Differentiation score	0.2 ± 1.2	-0.3± 1.1	< 0.001*
Residual disease			
Absent	92	84.5	0.086†
Present	8	15.5	
Tumor status			
Free	92.1	88.3	0.335†
With tumor	7.9	11.7	

* *P* values calculated by Student's t-test. Data are mean ± SD.
† *P* values calculated by χ^2 test or linear-by-linear association.
ᶜOthers: columnar cell variant, diffuse sclerosing variant, cribriform-morular variant, etc.

Table 2. Clinicopathological characteristics of patients with papillary thyroid cancer according to *MCT4* mRNA expression from the TCGA thyroid cancer database (n=505)

	MCT4 mRNA expression		*P* value
	Lowest (< 25.9) % (n=122)	Highest (>69.7) (n=122) (%)	
Age (years)	46.5 ± 14.9	47.1 ± 16.7	0.731*
Gender (Female)	79.5	68.9	0.087†
Tumor size (cm)	2.9 ± 1.5	3.2± 1.6	0.188*
MACIS score	5.1 ± 1.3	5.6± 1.6	0.017*
Histologic subtype			
Conventional	47.9	82.5	<0.001†
Follicular variant	47.9	5.3	
Tall cell variant	2.6	9.6	
Others§	1.7	2.6	
Extrathyroidal extension			
No	79.7	56.9	<0.001†
Minimal	20.3	36.2	
Gross	0	6.9	
Multifocality			
No	51.7	57	0.404†
Yes	48.3	43	
T stage			
T1	31	22.1	0.003†
T2	39.7	26.5	
T3	27.6	46	
T4	1.7	5.3	
N stage			
N0	73.4	45.5	<0.001†
N1a	12.8	31.8	
N1b	13.8	22.7	
M stage			
M0	98.2	98.6	0.906†
M1	1.8	1.4	
TNM stage group			
I/II	78.3	62	0.006†
III/IV	21.7	38	

mRNA cluster number			
1	64.5	13.2	<0.001†
2	5	8.8	
3	7.4	21.1	
4	20.7	6.1	
5	2.5	50.9	
miRNA cluster number			
1	50.4	13.1	<0.001†
2	9.9	7.4	
3	1.7	3.3	
4	20.7	7.4	
5	15.7	5.7	
6	1.7	63.1	
Ras driver mutation			
Absent	66.9	98.4	<0.001†
Present	33.1	1.1	
BRAF driver mutation			
Absent	71.9	38.5	<0.001†
Present	28.1	61.5	
TERT promoter mutation			
Absent	92.9	90.6	0.558†
Present	7.1	9.4	
RAS/RAF score	0.3 ± 0.7	-0.6± 0.5	< 0.001*
ERK score	-6.2 ± 16.7	16.1± 19.6	< 0.001*
Differentiation score	0.6 ± 1	-0.5± 1	< 0.001*
Residual disease			
Absent	94.2	82.1	0.007†
Present	5.8	17.9	
Tumor status			
Free	93.6	86.2	0.072
With tumor	6.4	13.8	

* *P* values calculated by Student's t-test. Data are mean ± SD.
† *P* values calculated by χ^2 test or linear-by-linear association.
ᶜOthers: columnar cell variant, diffuse sclerosing variant, cribriform-morular variant, etc.

Classification of thyroid cancer cell lines according to MCT1 and MCT4 expression

Next, western blot analyses were performed to investigate the basal expression level of MCT1 and MCT4 in thyroid cell lines. Interestingly, MCT1 protein expression was uniformly detected across every kind of cell line (Fig. 3A). On the other hand, MCT4 expression differed notably between specific cell types (Fig.3A). For example, MCT4 was barely detected in PTC cell lines such as BCPAP and TPC1, whereas it was highly expressed in K1 cells. In the case of anaplastic thyroid cancer (ATC) cell lines, SW1736 showed strong expression of MCT4 but CAL62 did not. Observation of heterogeneous expression pattern of MCT4 in various thyroid cell lines, new western blot analyses using human thyroid tissue samples showed something more. In fact, because increased MCT1 and MCT4 mRNA expression had already been observed in THCA data, these new blottings confirmed the statistical analyses using public depository. As shown in Fig. 3B, the thyroid cancer tissue and contralateral normal thyroid tissue clearly showed increased expression of MCT1 and MCT4 in human thyroid cancers. Additionally, another 9 cases of PTC slides were made. Most cases showed stronger activity of MCT4 on tumor tissue than normal tissue (Fig. 3C).

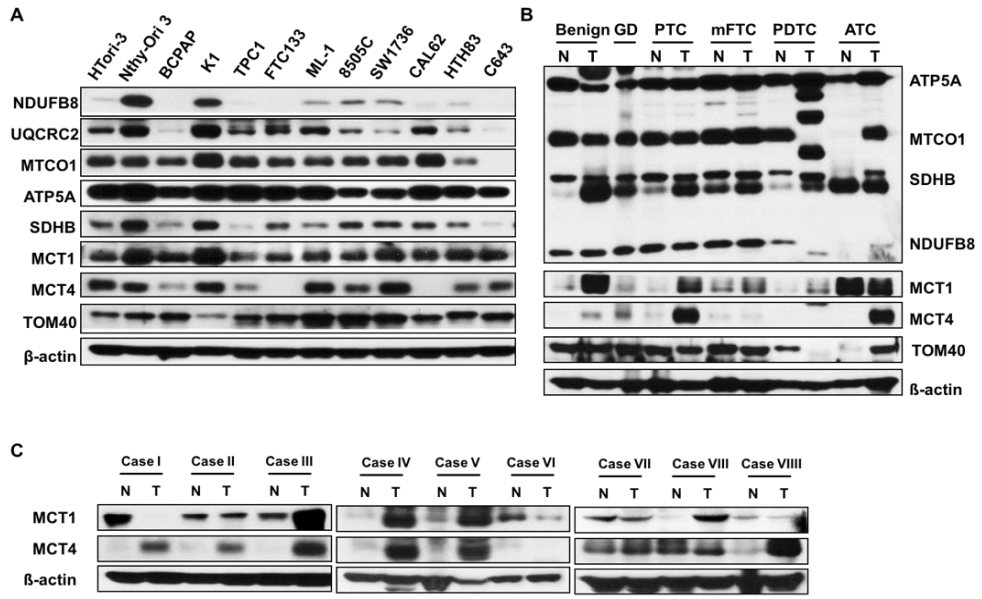

Figure 3. Western blot analysis of MCT1 and MCT4 in thyroid cancer cell lines and tissue samples.

MCT4 as a marker of core components of glycolytic pathways

As MCT4 functions as a mediator of lactate efflux, it was postulated that high MCT4 expression may reflect high glycolytic activity in cells. To verify this hypothesis, the mRNA expression status of the core components of glycolytic pathways such as hexokinases (HK), glucose transporter (GLUT), lactic dehydrogenase (LDH), and pyruvate kinase (PK) in THCA was investigated according to the status of MCT4 mRNA expression (Fig. 4 and Fig.5).[16-19] The expression of MCT4 mRNA in PTC showed strong positive correlation with HK3 (r=0.4911, P<0.0001), GLUT1 (r=0.2723, P=0.0002), LDHA (r=0.4813, P=0.0001), PKLR (r=0.0797, P=0.0028), and PKM2 (r=0.4895, P<0.0001).

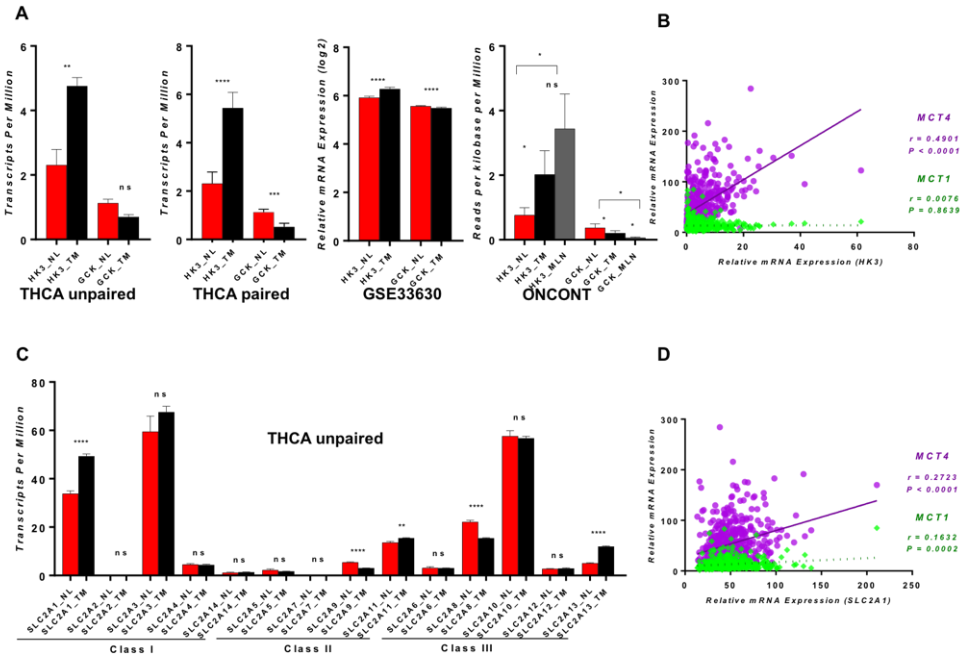

Figure 4. Analysis of mRNA expression for glycolytic pathways (A) hexokinase (HK) and glucokinase (GCK) expression in normal and thyroid cancer samples, (B) correlation of HK3 with MCT4 and MCT1 in RNA levels, (C) glucose transporter (GLUT) expression in normal and thyroid cancer, and (D) correlation of GLUT1 with MCT4 and MCT1 in RNA levels.

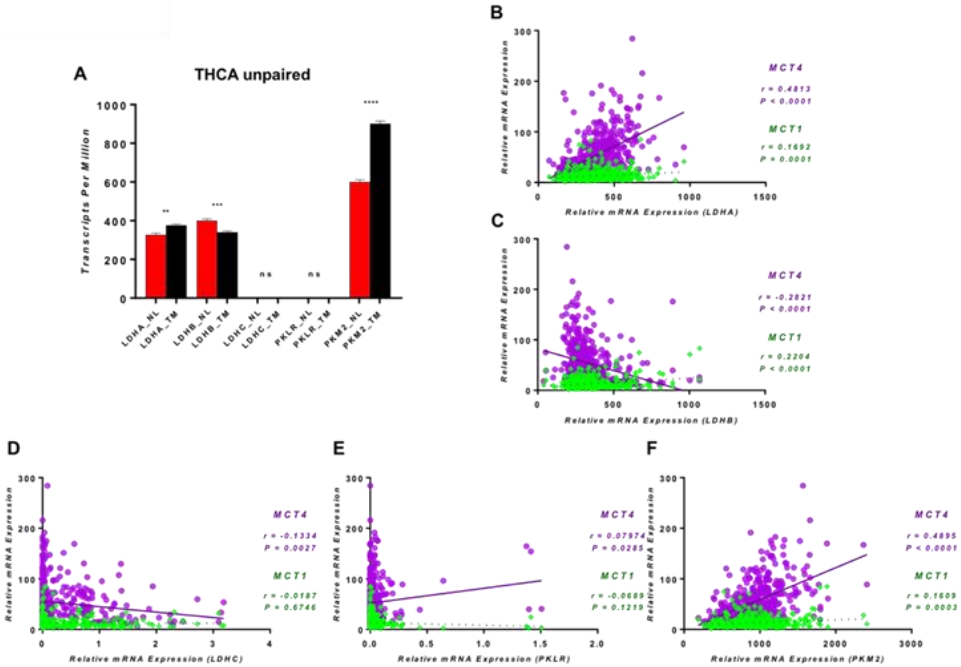

Figure 5. Analysis of mRNA expression for lactate dehydrogenase (LDH) and pyruvate kinase isoenzyme (PKM). (A) LDH and PKM expression in normal and thyroid cancer samples, (B) correlation of LDHA with MCT4 and MCT1 in RNA levels, (C) correlation of LDHB with MCT4 and MCT1 in RNA levels, (D) correlation of LDHC with MCT4 and MCT1 in RNA levels, (E) correlation of PKLR with MCT4 and MCT1 in RNA levels, and (F) correlation of PKM2 with MCT4 and MCT1 in RNA levels.

MCT1 & MCT4 in Immunohistochemical stain

As mentioned earlier, high MCT4 expression correlates with advanced thyroid cancers' clinicopathological factors. MCT4 may be expressed in tumor cells themselves (epithelial cells) and Cancer-Associated-Fibroblast (CAF). In order to specify where MCT4 expression occurred, immunohistocheminal stain slides were made from pathologic slides obtained from ONCONT. Epithelial area and stromal area were scored (Table 3, Table 4, and Fig. 6) and categorized into 4 groups using this data. Group A was negative for both tumor and stroma (Fig. 7A), Group B was positive for tumor but negative for stroma (Fig. 7B), Group C was negative for tumor, but stroma positive (Fig. 7C), Group D was positive for both tumor and stroma (Fig. 7D). Clinicopathological characteristics according to MCT4 epithelial expression are

displayed in Table 5. Although no meaningful relationship was noted, MCT4 stromal expression showed quite interesting features. The factors which are known to lead to poor prognosis including extrathyroidal extension, advanced TNM staging, and tumor recurrence showed significant differences between low and high expression (Table 6)[20,21] and these significant differences remained after a multivariate analysis (Table 7).

Table 3. MCT1 grouping (n=219)

Score	0 (Lower)	1	2 (Higher)	Undetermined*
Stromal	47	108	59	5
Epithelial	67	110	39	3

Table 4. MCT4 grouping (n=219)

Score	0 (Lower)	1	2 (Higher)	Undetermined*
Stromal	44	107	62	5
Epithelial	51	111	55	2

*Undetermined represents mismatch of scoring results from two different individuals

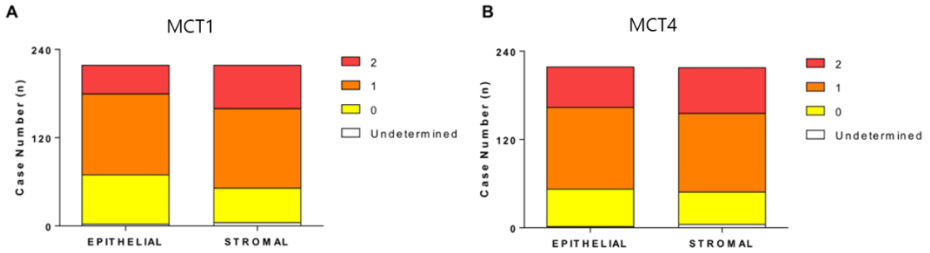

Figure 6. Population of IHC-P study.

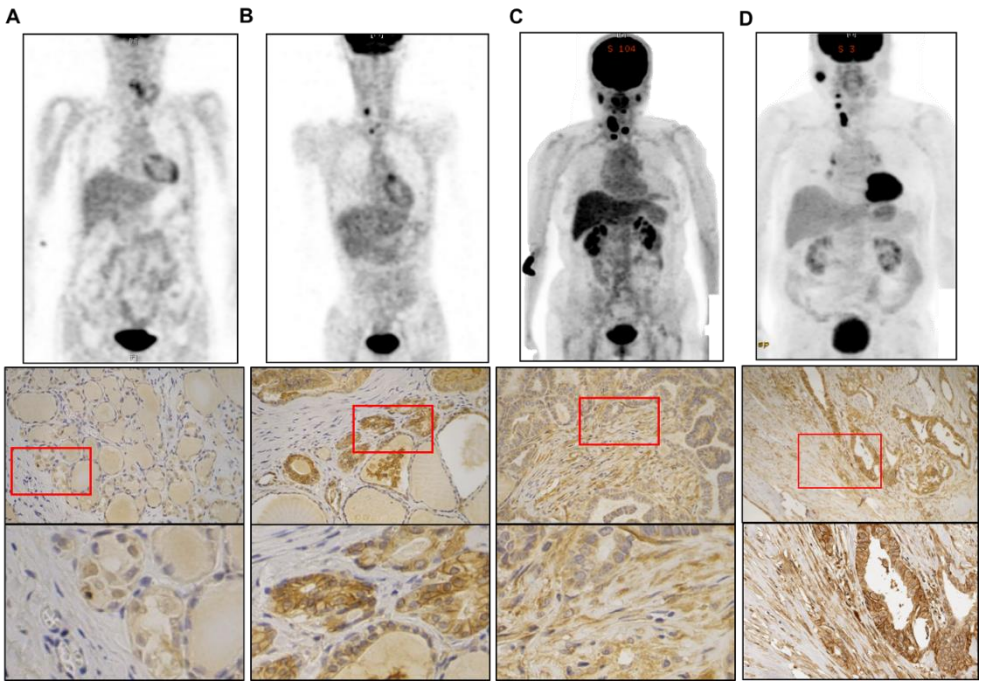

Figure 7. Representative 18F-FDG-PET/CT images according to MCT4 expression pattern.

Table 5. Clinicopathological characteristics of patients with papillary thyroid cancer according to *MCT4* epithelial expression status in study group.

	MCT4 Epithelial expression		*P* value
	Lower (n = 51) n (%)	Higher (n = 55) n (%)	
Age (years)	54.5 ± 14.1	51.2 ± 15	0.251*
Gender (F/M)	14/37	19/36	0.431†
Tumor size (cm)	2.7 ± 1.4	3.1 ± 3.4	0.428*
Histologic subtype			
Follicular variant	7 (13.7)	4 (7.3)	0.539†
Conventional	40 (78.4)	45 (81.8)	
Tall cell variant	0	4 (7.3)	
Others§	4 (7.9)	2 (3.6)	
Extrathyroidal extension			
No	12 (23.5)	7 (12.7)	0.147†
Yes	39 (76.5)	48 (87.3)	
Multifocality			
No	22 (43.1)	26 (47.3)	0.137†
Yes	29 (56.9)	29 (52.7)	
T stage			
T1	6 (11.8)	4 (7.3)	0.263†
T2	4 (7.8)	0	
T3	30 (58.8)	40 (72.7)	
T4a	10 (19.6)	8 (14.5)	
T4b	1 (2)	3 (5.5)	
N stage			
N0	19 (37.3)	10 (18.2)	0.057†
N1a	8 (15.6)	12 (21.8)	
N1b	24 (47.1)	33 (60)	
M stage			
M0	38 (74.5)	48 (87.3)	0.093†
M1	13 (25.5)	7 (12.7)	
TNM stage group			
I/II	19 (37.3)	17 (30.9)	0.491†
III/IV	32 (62.7)	38 (69.1)	

Tumor recurrence			
No	47 (92.2)	51 (2.7)	0.912†
Yes	4 (7.8)	4 (7.3)	

* *P* values calculated by Student's t-test. Data are mean ± SD.
† *P* values calculated by χ^2 test or linear-by-linear association.
ᶜOthers: columnar cell variant, diffuse sclerosing variant, cribriform-morular variant, etc.

Table 6. Clinicopathological characteristics of patients with papillary thyroid cancer according to *MCT4* stromal expression status in study group

	MCT4 Stromal expression		*P* value
	Lower (n = 44) n (%)	Higher (n = 62) n (%)	
Age (years)	50.4 ± 15.4	54.4 ± 13.9	0.398*
Gender (F/M)	16/28	17/45	0.327†
Tumor size (cm)	2.5 ± 1.2	3.2 ± 3.2	0.115*
Histologic subtype			
Follicular variant	6 (13.6)	5 (8.1)	0.402†
Conventional	35 (79.5)	50 (80.6)	
Tall cell variant	0	4 (6.5)	
Others‡	3 (6.9)	3 (4.8)	
Extrathyroidal extension			
No	15 (34.1)	4 (6.5)	<0.001†
Yes	29 (65.9)	58 (93.5)	
Multifocality			
No	21 (47.7)	27 (43.5)	0.654†
Yes	23 (52.3)	35 (56.5)	
T stage			
T1	10 (22.7)	0	<0.001†
T2	3 (6.8)	1 (1.5)	
T3	26 (59.1)	44 (71)	
T4a	5 (11.4)	13 (21)	
T4b	0	4 (6.5)	
N stage			
N0	24 (54.5)	5 (8.1)	<0.001†
N1a	5 (11.4)	15 (24.1)	
N1b	15 (34.1)	42 (67.8)	
M stage			
M0	32 (72.7)	54 (87.1)	0.062†
M1	12 (27.3)	8 (12.9)	
TNM stage group			
I/II	22 (50)	14 (22.6)	0.003†
III/IV	22 (50)	48 (77.4)	

Tumor recurrence			
No	43 (97.7)	55 (88.7)	0.083[†]
Yes	1 (80.0)	7 (11.3)	

[*] *P* values calculated by Student's t-test. Data are mean ± SD.
[†] *P* values calculated by χ^2 test or linear-by-linear association.
[ς]Others: columnar cell variant, diffuse sclerosing variant, cribriform-morular variant, etc.

Table 7. Multivariate analysis of the association of highest *MCT4* expression with the high-risk clinicopathological parameters of study patients

	Highest *MCT4* expression		
	Odds ratio	95% CI	*P*-value
Extrathyroidal invasion			
Model A	8.603	2.459–30.098	0.001
Model B	4.635	2.253–28.038	0.001
Model C	3.398	1.309–29.187	0.022
Lymph node metastasis			
Model A	9.741	3.127–39.214	<0.001
Model B	7.912	2.127–32.517	<0.001
Model D	6.287	1.992–37.147	0.002
Tumor stage III/IV			
Model A	5.153	1.514–17.544	0.009
Model B	4.72	1.381–16.131	0.013
Model E	4.414	0.920–21.191	0.064

Model A. Adjusted for age at diagnosis, and gender
Model B. Adjusted for age at diagnosis, gender, tumor size, and multifocality
Model C. Adjusted for age at diagnosis, gender, tumor size, multifocality, lymph node metastasis, and tumor stage III/IV
Model D. Adjusted for age at diagnosis, gender, tumor size, multifocality, extrathyroidal invasion, and tumor stage III/IV
Model E. Adjusted for age at diagnosis, gender, tumor size, multifocality, extrathyroidal invasion, and lymph node metastasis
Abbreviation: CI, confidence interval

IV. DISCUSSION

This study focused on aerobic glycolysis which an important of hallmark of cancer. Since the first reports of the Warburg effect in the 1920, lactate transporters' important relationship with carcinogenesis and cancer metastasis has been well documented.[22-24] Considering intra-tumoral heterogeneity and CAF metabolism, the exact location where MCT4 acts affects the metabolism of thyroid cancer. Witkiewicsz et al. previously reported that stromal MCT4 is a poor prognostic marker in triple negative breast cancer. Nevertheless, as yet, no thyroid cancer research has published similar results. In the current study, it was demonstrated that high expression of stromal

MCT4 is correlated with poor prognosis in thyroid cancer.

Because no correlation between the lactate transporters (MCT1 and MCT4) has been noted, it can be assumed that MCT1 and MCT4 work independently from each other as lactate transporters. The expression status of these transporters in thyroid cancer was evaluated. The lactate transporters were found to be more highly expressed in the mRNA levels of thyroid cancer than in normal thyroid tissue (Fig. 2) in public repository data. The high expression of lactate transporter correlated to BRAF and TERT mutations which are known poor prognostic factors[25-27] that have been confirmed in a previous thyroid cancer study.[28] It was postulated that a high expression of lactate transporters may have a role on the carcinogenesis of thyroid cancer. Western blot analysis confirmed the statistical results of TCGA data. The tumors showed a higher expression of lactate transporters than the contralateral normal tissue exhibited. An additional case study of PTC tissue showed stronger activity of MCT4 on tumor tissue than normal tissue. The heterogeneity of MCT4 is interesting topic to discuss. In Fig.2, the heterogeneity of MCT4 expression on tumor group is displayed. Also, on western blot analysis, different levels of expression on MCT4 were noted. While MCT1 showed uniformly equal levels of expression across cell lines, MCT4 had distinctly different levels between each cell line (Fig. 3B), which leads to the reasonable conjecture that MCT4 may have more potential for distinguishing poor prognosis than MCT1.

If lactate efflux demonstrates high glycolytic activity, mRNA expression of glycolytic pathway core components should be high. Very specific isozymes (but not all glycolytic components) were correlated with MCT4 expression. Hexokinase 3, Glucose Transporter 1, Lactate dehydrogenase A, and Pyruvate kinase isoenzymes showed positive correlations with MCT4 expression. Because HK3 is just one isoenzyme of three hexokinase groups, the specific isozymes seem to be involved in MCT4-related carcinogenesis. By a certain mechanism, these components of glycolytic enzymes are linked to each other including MCT4. Unlike MCT4, a high expression of MCT1 had no relationship with the glycolysis components. This

demonstrates the importance of MCT4 as a more promising prognostic factor than MCT1.

Because of the intra-tumoral heterogeneity of the reverse Warburg effect, PET positive patients could be classified into 4 categories. However, differences of prognoses between the 4 classifications were not evident, so they were re-classified into two categories: the epithelium and the stroma (Table 3 and Table 4). After statistic evaluation of MCT4 expression on cancer, stromal MCT4 expression was positively correlated with aggressive tumor behaviors, such as advanced tumor (stage T3 and T4), lymph node stage (N1a and N1b), and TNM stage (III and IV) (P<0.0001) (Table 6).[29,30] These factors showed a relationship after adjusting a few factors. However, epithelial MCT4 expression showed no correlation with any clinicopathologic factors.[15] The group with no expression of MCT4 on both epithelium and stroma is interesting (Group A). Although PET positive, MCT4 did not have expressed and this implies that there are lactate exporters other than MCT4 which may be linked to a reverse Warburg effect. Further studies are needed to identify this unknown lactate transporter and investigate its role.

V. CONCLUSION

Following a step-by-step process, the key roles of lactate transporter, from total mRNA to cell level, were determined, and the prognostic value of stromal MCT4 was established. The high expression of stromal MCT4 is related to poor prognosis. These findings establish the pivotal role of MCT4 in PTC progression, and support the use of stromal MCT4 as a potential biomarker and therapeutic target in aggressive PTC.

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ABSTRACT (IN KOREAN)

고위험 갑상선 암 환자에 있어서 Lactate Transporter의 역할

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목적: Warburg effect에 따라 종양의 높은 포도당 흡수율을 이용하여 ^{18}F-FDG-PET/CT를 사용하여 종양을 발견할 수 있다. 하지만 종양 내 이질성(Heterogeneity) 또는 암 관련 섬유 모세포(CAF) 대사가 포도당 섭취에 영향을 줄 수 있기 때문에 ^{18}F-FDG 섭취가 높은 세포가 종양이 아닐 가능성이 있다. 이전까지 알려진 ^{18}F-FDG-PET/CT의 예후에 초점을 맞춘 많은 연구에서는 종양 내 이질성이나, 조직 유형의 특이성을 고려하지 않았다. 본 연구에서는 Monocarboxylate transporter 1(MCT 1; SLC16A1)과 Monocarboxylate transporter 4(MCT 4; SLC16A3) 의 발현 패턴에 따라 ^{18}F-FDG-PET/CT에서 FDG 섭취가 높은 종양을 소그룹으로 분류 하였다. 이를 통하여 분자 생물학적 및 임상적 특성이 포도당과 젖산 대사에 대한 세포 유형, 종양 유형의 특이성에 따라 차이를 보인다는 것을 관찰했다.

방법: 공개 되어있는 TCGA, NCBI Gene Expression Omnibus (GEO) 프로파일 및 자체 RNA-sequence 데이터로부터 MCT1 및 MCT4의 발현 상태를 평가하였다. MCT4 mRNA 발현의 상태에 따라 THCA에서 hexokinases (HK), glucose transporter (GLUT), lactic dehydrogenase (LDH), pyruvate kinases (PK)와 같은 glycolytic pathway의 핵심 구성

요소의 mRNA 발현 상태 또한 조사 하였다. 인체 갑상선 암 조직을 이용하여 MCT1과 MCT4의 면역 조직 화학 염색(Immunohistochemical; IHC) 염색 분석을 실시하였고, 갑상선 암에서 이들 운반체의 발현 상태에 따라 임상병리인자와 어떤 관계를 보이는지에 대하여 비교 하였다.

결과: THCA, GSE33630 및 자체 RNA-sequence에서 MCT1과 MCT4의 발현률이 정상세포에서 보다 종양세포에서 현격히 증가하였다. 또한 PTC에서 MCT4 mRNA의 발현은 HK3 (r = 0.4911, p <0.0001), GLUT1 (r = 0.2723, p = 0.0002), LDHA (r = 0.4813, p = 0.0001), PKLR r = 0.0797, p = 0.0028), PKM2 (r = 0.4895, p <0.0001)로 양의 상관관계를 보였다. THCA 데이터를 이용한 임상병리인자분석에서 높은 MCT4 발현은 BRAFV600E 돌연변이, 중간 및 높은 재발 위험, 진행된 종양 (T3 및 T4 병기), 림프절 병기 (N1a 및 N1b) 및 BRAFV600E 변이 및 TERT promoter 변이 (p <0.0001)와 상관 관계가 있었다. 이 결과를 뒷받침하는 IHC 염색 결과는, 높은 간질 MCT4 발현률이 진행성 종양 (T3 및 T4 병기), 림프절 병기 (N1a 및 N1b) 및 TNM 병기 (III)와 관련되는 결과를 보였다.(P <0.0001). 그러나, 종양세포에서 MCT4 발현률은 예후에 영향을 미치지 않았다.

결론: 이 연구로 갑상선 암의 진행과정에서 MCT4가 중요한 역할을 함으로 밝혀졌으며, 공격적인 갑상선 암을 선별하는 표지자로서의 역할 및 잠재적 치료 표적으로서 결체조직의 MCT4 사용을 고려할 수 있다.

핵심 되는 말 : 갑상선 암, MCT4, Warburg 효과, 젖산 transporter