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# Expression of glutamine metabolism-related proteins in Hürthle cell neoplasm of thyroid: Comparison with follicular neoplasm

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**Summary.** Purpose. We evaluated the expression of glutaminolysis-related proteins in Hurthle cell neoplasms (HCN) and follicular neoplasms (FN) of the thyroid, and investigated its clinical implication.

Methods. Tissue microarrays were constructed from 264 FNs (112 follicular carcinomas [FCs] and 152 follicular adenomas [FAs]) and 108 HCNs (27 Hurthle cell carcinomas [HCCs] and 81 Hurthle cell adenomas [HCAs]. The immunohistochemical staining result of 3 glutaminolysis-related proteins (Glutaminase 1 [GLS1], glutaminate dehydrogenase [GDH] and alanine- serine, cysteine-preferring transporter 2 [ASCT2]) was analyzed.

Results. GLS1 and GDH showed significantly higher expression rates in HCN compared to FN (P<0.001). More HCN cases showed co-positivity of multiple glutaminolysis-related proteins than those of FN cases (P<0.001). In silico analysis, both GLUD1 and GLUD2 showed higher expression rate in HCA compared to FA (P=0.027 and P=0.018, respectively). SLC1A5 expression was highest in HCA, followed by FC and FA (HCA vs FC, P=0.023; FC vs FA, P=0.002).

Conclusion. FN and HCN exhibit a different expression pattern for glutaminolysis-related proteins, and GLS1 and GDH have higher expression rates in HCN and FN.

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**Key words:** Metabolism, Glutamine, Hurthle cell, Thyroid

## Introduction

The metabolism of malignant tumors is partly explained by the Warburg effect theory, which describes the metabolic shift from oxidative phosphorylation (OXPHOS) to glycolysis in tumoral mitochondria (Warburg, 1956). Although aerobic glycolysis is the major metabolic source of cancer cells, other metabolic systems are also present. This flexibility in the metabolic system of cancer cells is the main obstacle in cancer therapy using cell metabolism. Glutamine metabolism is another important metabolic pathway in cancer cells (DeBerardinis and Cheng, 2010). Previous studies using cell cultures and tumor implantations showed that cancer cells highly prefer the utilization of glutamine over any other amino acids. This suggests that glutamine metabolism is one of the essential metabolic phenotypes in proliferating tumor cells (Collins et al., 1997; Friday et al., 2011). It serves two key roles in proliferation: producing ATP and supplying intermediates for macromolecular synthesis (DeBerardinis and Cheng, 2010). During the glutamine metabolic pathway, alanine-, serine-, cysteine-preferring transporter 2 (ASCT2) is involved in the cellular transportation of glutamine into tumor cells (McGivan and Bungard, 2007). Glutaminase 1 (GLS1) converts glutamine into glutamate (Curthoys and Watford, 1995). Glutamate is converted into α-ketoglutarate by glutaminate dehydrogenase (GDH) and enters the tricarboxylic acid cycle (Dang, 2010).

Hürthle cell neoplasm (HCN) of the thyroid is a variant of follicular neoplasm (FN). Hürthle cell adenoma (HCA) comprises 10-15% of follicular adenoma (FA), and Hürthle cell carcinoma (HCC) accounts for 20-25% of follicular carcinoma (FC). Hürthle cell originates from the follicular epithelial cell, and is characterized by an ample granular cytoplasm and a prominent nucleolus. Although HCN is classified as a variant of FN in current WHO classification, HCN appears to be a distinct disease entity from FN, since it demonstrates nodal metastasis, higher rate of recurrence and disease related mortality. Furthermore, TERT C228T promoter mutation is frequently found as a molecular alteration of HCN (Chindris et al., 2015). Based on these tumor biology characteristics, we hypothesized that HCN and FN may have different tumor metabolic characteristics. Moreover, it is reported that HCA has a higher [$^{18}$F]-2-fluoro-2-deoxy-D-glucose (FDG) uptake and maximum standardized uptake value ($SUV_{max}$) than FA (Pathak et al., 2016). This supports the possibility of distinct metabolic features between HCN and FN. The objective of this study is to investigate the expression of glutamine metabolism-related proteins of HCN and FN, and to determine its clinical implication.

## Materials and methods

### *Patient selection*

Patients who underwent thyroid resection for FN and/or HCN in Severance Hospital between January, 2000 and December, 2013 were included. This study was approved by the Institutional Review Board of Yonsei University Severance Hospital. Patients who received neoadjuvant chemotherapy were excluded. A histologic review was performed by a thyroid pathologist (Koo JS) with hematoxylin and eosin-stained slides. All available slides were retrospectively reviewed. Clinicopathologic information was obtained from the patients' medical records and included age at diagnosis, disease recurrence, metastasis, current status, and length of follow up. Information on the tumor size, location (right or left lobe), extent (confined to the thyroid parenchyma or extrathyroidal spread) and number of metastatic lymph nodes were obtained from the slides and the surgical pathology reports.

### *Tissue microarray*

Representative areas were selected from the hematoxylin and eosin-stained slides and the corresponding spot was marked on the surface of the matched paraffin block. A five-mm tissue core was punched out from the selected areas and placed into a 5x4 recipient block. More than 2 tissue cores were extracted from each case to minimize extraction bias. Each tissue core was assigned a unique tissue microarray location number that allowed access to its corresponding clinicopathologic data in the database.

### *Immunohistochemistry*

Antibodies used for immunohistochemistry are listed in Table 1. All immunohistochemistry was performed with formalin-fixed, paraffin-embedded tissue sections using an automatic immunohistochemistry staining device (Benchmark XT, Ventana Medical System, Tucson, AZ, USA). Briefly, 5-$\mu$m-thick formaldehyde fixed paraffin-embedded tissue sections were transferred onto adhesive slides and dried at 62°C for 30 minutes. Standard heat epitope retrieval was performed for 30 minutes in ethylene diamine tetraacetic acid, pH 8.0, in the autostainer. The samples were then incubated with primary antibodies. Afterwards, the sections were subsequently incubated with biotinylated anti-mouse immunoglobulins, peroxidase-labeled streptavidin (LSAB kit, DakoCytomation), and 3,30-diamino-benzidine. Negative control samples were processed without the primary antibody. Slides were counterstained with Harris hematoxylin. Positive control tissue was used as per the manufacturer's recommendation. Slides were counterstained with Harris hematoxylin. Optimal primary antibody incubation times and concentrations were determined by serial dilution for each immunohistochemical assay using a tissue block fixed and embedded exactly for the experiments.

### *Interpretation of immunohistochemical staining*

Immunohistochemical markers were accessed by light microscopy in a semi-quantitative manner as described previously (Henry et al., 2007). Tumor cell staining was assessed as followed: 0, negative or weak immunostaining in <1% of the tumor/stroma; 1, focal expression in 1-10% of tumor; 2, positive in 11-50% of tumor; 3, positive in 51-100% of tumor. The whole tumor area was evaluated and the immunohistochemical staining results were defined as negative for score 0 and 1, low positive for score 2, and high positive for score 3. Positive staining in the stroma was assigned when 10% or more stroma were positive.

### *In silico analysis*

Microarray data from the E-GEOD-29315 ArrayExpress dataset was analyzed for variable gene

**Table 1.** Source, clone, and dilution of used antibodies.

| Antibody | Clone | Dilution | Company |
|---|---|---|---|
| GLS1 | Polyclonal | 1:50 | Abcam, Cambridge, UK |
| GDH | Polyclonal | 1:100 | Abcam, Cambridge, UK |
| ASCT2 | Polyclonal | 1:100 | Abcam, Cambridge, UK |

GLS1, glutaminase 1; GDH, glutamate dehydrogenase; ASCT2, amino acid transporter-2.

expression levels in GLS, GLUD1 (glutamate dehydrogenase 1), GLUD2 (glutamate dehydrogenase 2), and SLC1A5 (ASCT2) in distinct thyroid neoplasm. 17 FA samples, 9 FC samples and 9 HCA samples were included in the original dataset. Since the dataset we used did not include any HCC samples, HCC analysis results were unavailable. CEL files were background corrected and normalized with RMA (robust multi-array average expression measure) using the affy Bioconductor package. The annotated gene expression data for the specific genes were plotted into separate grouped scatter plots.

### *Statistical analysis*

Data were analyzed using SPSS for Windows, Version 23.0 (SPSS Inc., Chicago, IL, USA). For determination of statistical significance, Student's t test and Fisher's exact tests were used for continuous variables and categorical variables, respectively. In the case of analyzing data with multiple comparisons, a corrected p-value with the application of the Bonferroni multiple comparison procedure was used. Statistical significance was set to $P<0.05$. Kaplan-Meier survival curves and log-rank statistics were employed to evaluate time to tumor recurrence and overall survival. Multivariate regression analysis was performed using the Cox proportional hazards model.

## Results

### *Basal characteristics of follicular neoplasms and Hurthle cell neoplasms*

A total of 265 FNs, including 153 FAs and 112 FCs (99 minimally invasive and 13 widely invasive types), were examined. Clinicopathologic features of the patients with FN are shown in Table 2. Additionally, a total of 108 HCNs, including 81 HCAs and 27 HCCs (25 minimally invasive and 2 widely invasive types), were examined. Clinicopathologic features of patients with HCN are shown in Table 3.

### *Expression of glutaminolysis-related proteins in follicular neoplasms and Hurthle cell neoplasms*

GLS1 and GDH showed higher expression rates in HCN compared to FN ($P<0.001$, Table 4 and Fig. 1). HCC and HCA showed no difference in expression rates of glutaminolysis-related proteins but FC had higher expression rates of glutaminolysis-related proteins than FA ($P<0.001$, Table 5). HCN had more positive glutaminolysis-related proteins than FN ($P<0.001$, Fig. 2).

**Table 2.** Basal characteristics of thyroid follicular carcinomas.

| Parameters | Total N=112 (%) | FC, minimally invasive type N=99 (%) | FC, widely invasive type N=13 (%) | P-value |
|---|---|---|---|---|
| Age (years) | | | | 0.255 |
| <45 | 51 (45.5) | 47 (47.5) | 4 (30.8) | |
| ≥45 | 61 (54.5) | 52 (52.5) | 9 (69.2) | |
| Sex | | | | 0.233 |
| Male | 28 (25.0) | 23 (23.2) | 5 (38.5) | |
| Female | 84 (75.0) | 76 (76.8) | 8 (61.5) | |
| Tumor size (cm) | | | | 0.040 |
| ≤2.0 | 34 (30.4) | 34 (34.3) | 0 (0.0) | |
| >2.0, ≤4.0 | 49 (43.8) | 41 (41.4) | 8 (61.5) | |
| >4.0 | 29 (25.9) | 24 (24.2) | 5 (38.5) | |
| Capsular invasion | | | | 0.147 |
| No | 14 (12.5) | 14 (14.1) | 0 (0.0) | |
| Yes | 98 (87.5) | 85 (85.9) | 13 (100.0) | |
| Vascular invasion | | | | 0.028 |
| No | 66 (58.9) | 62 (62.6) | 4 (30.8) | |
| Yes | 46 (41.1) | 37 (37.4) | 9 (69.2) | |
| Tumor extension | | | | <0.001 |
| Intrathyroidal | 95 (84.8) | 89 (89.9) | 6 (46.2) | |
| Extrathyroidal | 17 (15.2) | 10 (10.1) | 7 (53.8) | |
| LN metastasis | | | | 0.220 |
| No | 110 (98.2) | 98 (99.0) | 12 (92.3) | |
| Yes | 2 (1.8) | 1 (1.0) | 1 (7.7) | |
| Distant metastasis | | | | 0.003 |
| No | 101 (90.2) | 93 (93.9) | 8 (61.5) | |
| Yes | 11 (9.8) | 6 (6.1) | 5 (38.5) | |

FC, follicular carcinoma; LN, lymph node.

**Table 3.** Basal characteristics of Hurthle cell neoplasms.

| Parameters | Total N=108 (%) | Hurthle cell adenoma N=81 (%) | Hurthle cell carcinoma N=27 (%) | P-value |
|---|---|---|---|---|
| Age (years) | | | | 0.310 |
| <45 | 45 (41.7) | 36 (44.4) | 9 (33.3) | |
| ≥45 | 63 (58.3) | 45 (55.6) | 18 (66.7) | |
| Sex | | | | 0.136 |
| Male | 18 (16.7) | 16 (19.8) | 2 (7.4) | |
| Female | 90 (83.3) | 65 (80.2) | 25 (92.6) | |
| Tumor size (cm) | | | | 0.005 |
| ≤2.0 | 76 (70.4) | 63 (77.8) | 13 (48.1) | |
| >2.0, ≤4.0 | 20 (18.5) | 13 (16.0) | 7 (25.6) | |
| >4.0 | 12 (11.1) | 5 (6.2) | 7 (25.9) | |
| Capsular invasion | | | | <0.001 |
| No | 84 (77.8) | 81 (100.0) | 3 (11.1) | |
| Yes | 24 (22.2) | 0 (0.0) | 24 (88.9) | |
| Vascular invasion | | | | 0.002 |
| No | 105 (97.2) | 81 (100.0) | 24 (88.9) | |
| Yes | 3 (2.8) | 0 (0.0) | 3 (11.1) | |
| Tumor extension | | | | <0.001 |
| Intrathyroidal | 102 (94.4) | 81 (100.0) | 21 (77.8) | |
| Extrathyroidal | 6 (5.6) | 0 (0.0) | 6 (22.2) | |
| LN metastasis | | | | n/a |
| No | 108 (100.0) | 81 (100.0) | 27 (100.0) | |
| Yes | 0 (0.0) | 0 (0.0) | 0 (0.0) | |
| Distant metastasis | | | | n/a |
| No | 108 (100.0) | 81 (100.0) | 27 (100.0) | |
| Yes | 0 (0.0) | 0 (0.0) | 0 (0.0) | |

LN, lymph node.

### *Relationship between the expression of glutaminolysis-related proteins and prognosis*

No significant association was found between the expression of glutaminolysis-related proteins and the prognosis of FC and HCC (Table 6).

### *Expression of glutaminolysis-related genes in FA, FC, and HA by in silico analysis*

In silico analysis revealed that GLUD1, GLUD2, and SLC1A5 were differently expressed between FA, FC, and HCA. Both GLUD1 and GLUD2 showed higher expression rate in HCA compared to FA (P=0.027 and P=0.018, respectively). SLC1A5 expression was highest in HCA, followed by FC and FA, and the differences between HCA, FC, and FA were significant (HCA vs FC, P=0.023; FC vs FA, P=0.002, Fig. 3).

**Table 4.** Expression of glutaminolysis-related proteins in follicular neoplasms and Hurthle cell neoplasms.

| Parameters | Total N=371 (%) | Follicular neoplasm N=264 (%) | Hurthle cell neoplasm N=107 (%) | P-value |
|---|---|---|---|---|
| GLS1 | | | | <0.001 |
| Negative | 202 (54.4) | 185 (70.1) | 17 (15.9) | |
| Positive | 169 (45.6) | 79 (29.9) | 90 (84.1) | |
| GDH | | | | <0.001 |
| Negative | 170 (45.8) | 144 (54.5) | 26 (24.3) | |
| Positive | 201 (54.2) | 120 (45.5) | 81 (75.7) | |
| ASCT2 | | | | 0.804 |
| Negative | 351 (94.6) | 249 (94.3) | 102 (95.3) | |
| Positive | 20 (5.4) | 15 (5.7) | 5 (4.7) | |

GLS1, glutaminase 1; GDH, glutaminate dehydrogenase; ASCT2, alanine- serine, cysteine-preferring transporter 2.

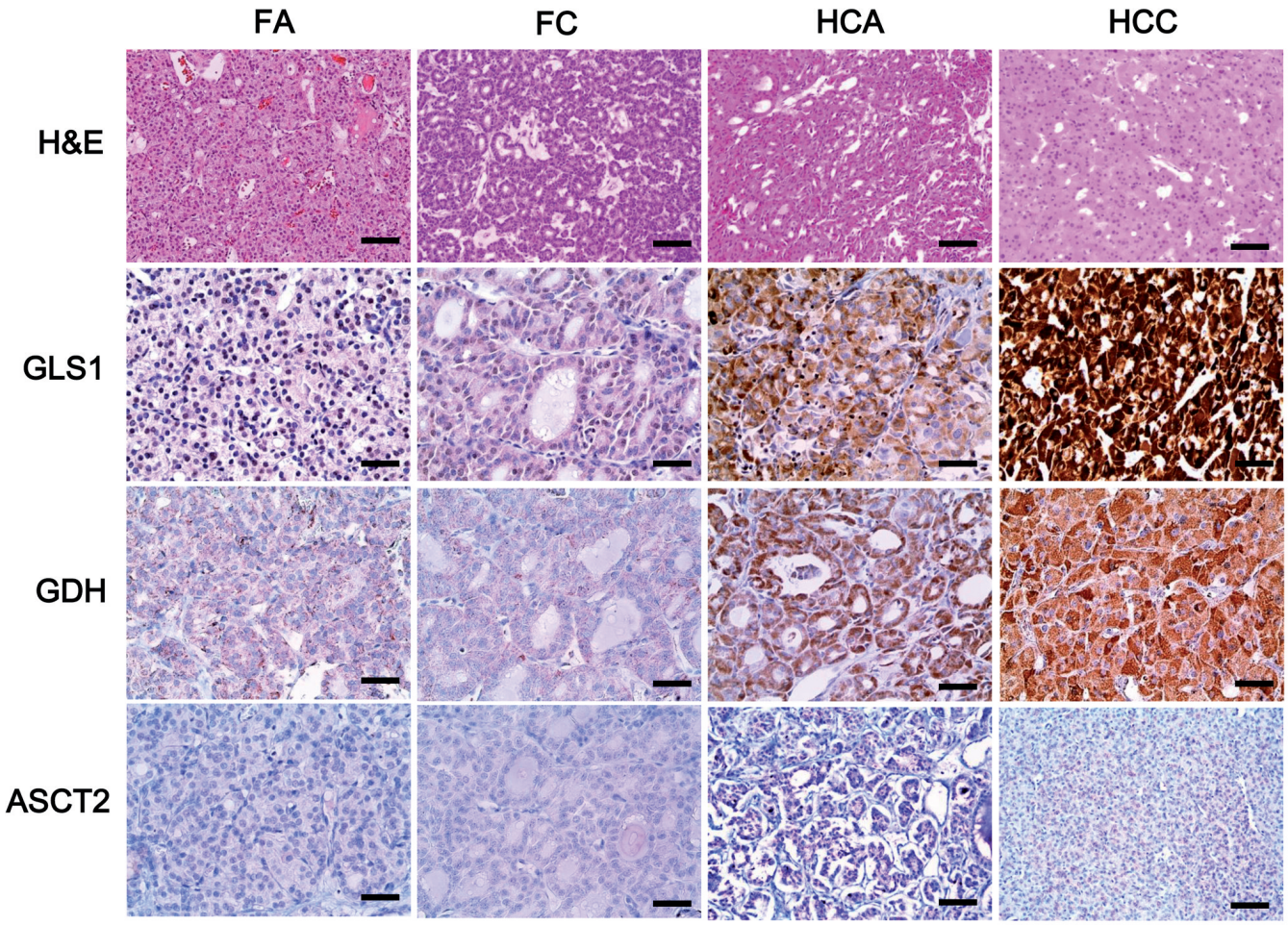


**Fig. 1.** Expression of glutaminolysis-related proteins in follicular neoplasm (FN) and Hurthle cell neoplasm (HCN). GLS1 and GDH show higher expression rates in HCN compared to FN. FC has higher expression rates of glycolysis-related proteins than FA. Scale bars: first row, HCA-ASCT2 and HCC-ASCT2, 50 $\mu$m; others, 100 $\mu$m.

**Table 5.** Expression of glutaminolysis-related proteins in follicular adenomas, follicular carcinomas, Hurthle cell adenomas and Hurthle cell carcinomas.

| Parameters | Follicular neoplasms N=264 (%) | | Hurthle cell neoplasms N=107 (%) | | P-value |
|---|---|---|---|---|---|
| | FA N=152 (%) | FC N=112 (%) | HCA N=81 (%) | HCC N=26 (%) | |
| GLS1 | | | | | <0.001 |
| Negative | 115 (75.7) | 70 (62.5) | 12 (14.8) | 5 (19.2) | |
| Positive | 37 (24.3) | 42 (37.5) | 69 (85.2) | 21 (80.8) | |
| GDH | | | | | <0.001 |
| Negative | 96 (63.2) | 48 (42.9) | 20 (24.7) | 6 (23.1) | |
| Positive | 56 (36.8) | 64 (57.1) | 61 (75.3) | 20 (76.9) | |
| ASCT2 | | | | | 0.291 |
| Negative | 146 (96.1) | 103 (92.0) | 76 (93.8) | 26 (100.0) | |
| Positive | 6 (3.9) | 9 (8.0) | 5 (6.2) | 0 (0.0) | |

FA, follicular adenoma; FC, follicular carcinoma; HCA, Hurthle cell adenoma; HCC, Hurthle cell carcinoma; GLS1, glutaminase 1; GDH, glutaminate dehydrogenase; ASCT2, alanine- serine, cysteine-preferring transporter 2.

**Table 6.** Univariate analysis of glutaminolysis-related protein expression in follicular carcinomas and Hurthle cell carcinomas on disease-free survival and overall survival.

| Parameter | Number of patients | Disease-free survival | | Overall survival | |
|---|---|---|---|---|---|
| | (N=139) /recurrence/death | Mean survival, months (95% CI) | P-value | Mean survival, months (95% CI) | P-value |
| GLS1 | | | 0.474 | | 0.838 |
| Negative | 76/8/3 | 112 (103-121) | | 118 (110-127) | |
| Positive | 63/4/2 | 100 (93-107) | | 104 (99-109) | |
| GDH | | | 0.173 | | 0.336 |
| Negative | 55/7/3 | 109 (97-120) | | 118 (110-126) | |
| Positive | 84/5/2 | 101 (95-107) | | 104 (99-109) | |
| ASCT2 | | | N/A | | N/A |
| Negative | 130/12/5 | N/A | | N/A | |
| Positive | 9/0/0 | N/A | | N/A | |

GLS1, glutaminase 1; GDH, glutaminate dehydrogenase; ASCT2, alanine- serine, cysteine-preferring transporter 2.

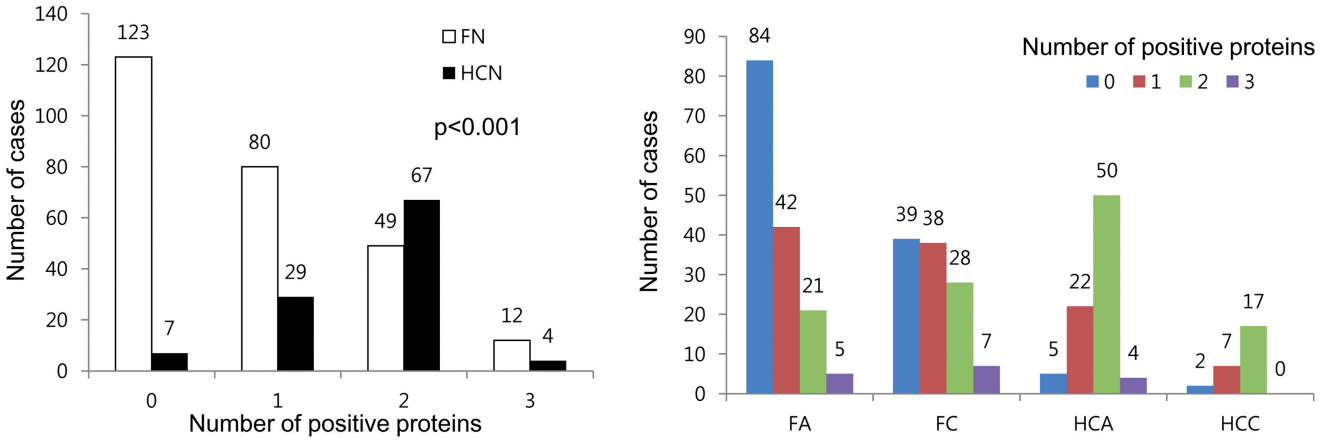


**Fig. 2.** The number of positive glutaminolysis-related proteins in follicular neoplasm (FN) and Hurthle cell neoplasm (HCN). Compared to FN, HCN showed a higher proportion of cases that co-expressed multiple glutaminolysis-related proteins.

## Discussion

In this study, two glutaminolysis-related proteins (GLS1 and GDH) showed significantly higher expression rates in HCN compared to FN. So far, the extent of glytaminolysis-related proteins in HCN has not been evaluated. A previous PET/computed tomography image study demonstrated higher $^{18}$F-FDG uptake in HCA compared to FA, which implies more active glycolysis of HCA (Pathak et al., 2016). The different expression profiles of glutaminolysis-related proteins could be associated with the PI3K-AKT-mTOR pathway, Wnt/β-catenin pathway, and hTERT. Firstly, the PI3K-AKT-mTOR pathway has been reported to be linked with glutaminolysis (Guo et al., 2016), which is also one of the metabolic alterations in cancer with mutant PI3K (Foster et al., 2012). HCN is a neoplasm with activated PI3K-AKT-mTOR pathway, which could affect glutaminolysis. Secondly, the Wnt/β-catenin pathway appears to have association with glutamine metabolism. The Wnt/β-catenin pathway is another activated signaling pathway in HCN, where glutamine uptake and metabolism is regulated by β-catenin signaling in astrocytes (Lutgen et al., 2016). Inhibition of β-catenin induces repression of the glutamine synthesis pathway in liver cells (Sekine et al., 2006). Lastly, hTERT is potentially associated with glutamine metabolism. HCN expresses the hTERT protein (Sugishita et al., 2014), and TERT C228T promoter mutation was detected frequently (Chindris et al., 2015). Genomic profiling of immortalized human mammary epithelial (hTERT-HME1) cells showed metabolic gene expression for proteins such as membrane glutamate transporter (SLC1A1), which also suggests the possible link between hTERT and glutamine transportation (Beaudin and Welsh, 2016). However, further in depth study is

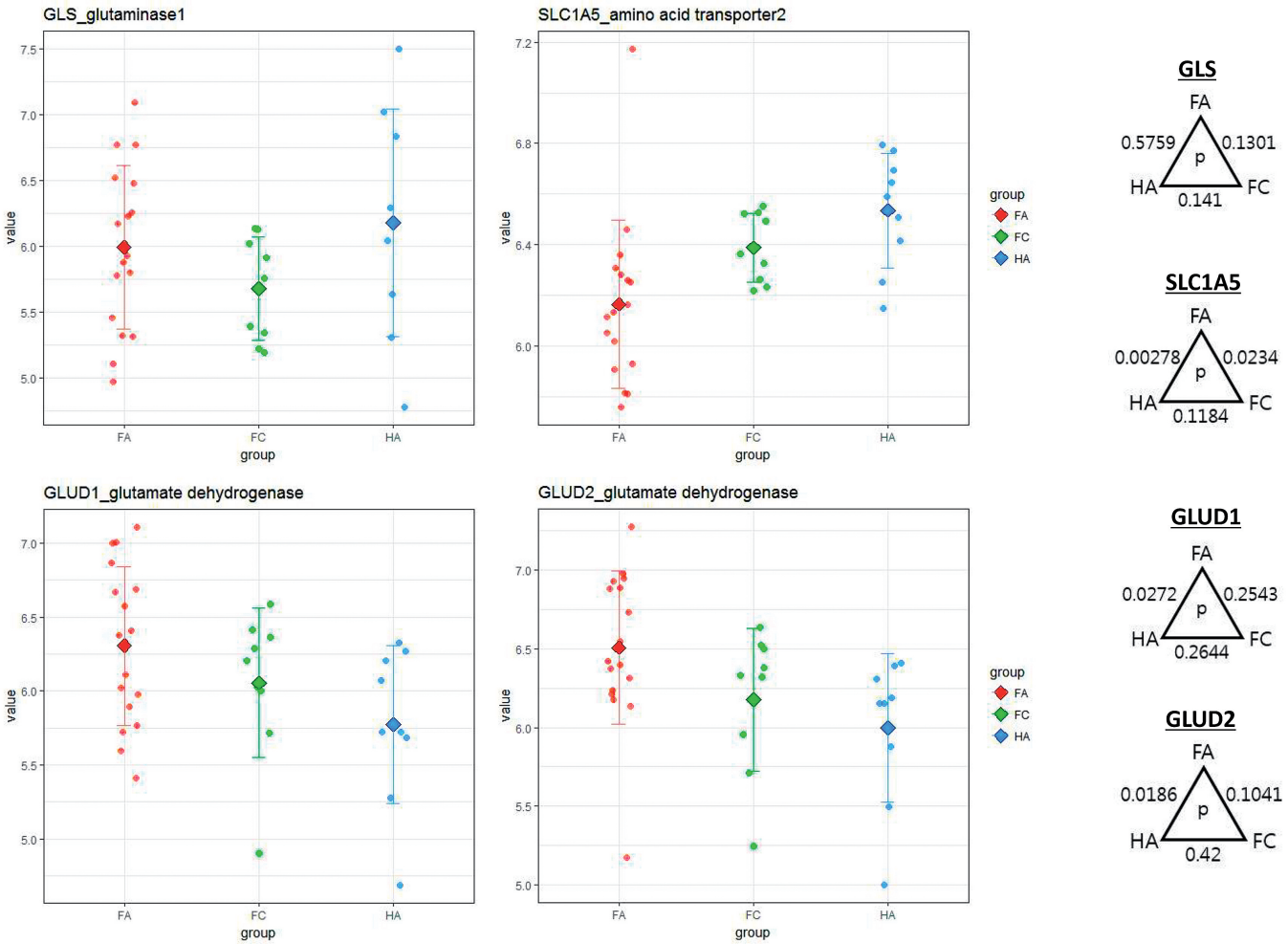


**Fig. 3.** Expression of glutaminolysis-related genes in follicular adenoma (FA), follicular carcinoma (FC), and Hurthle cell adenoma (HCA) by in silico analysis. Both GLUD1 and GLUD2 show higher expression rate in HCA compared to FA (P=0.027 and P=0.018, respectively). SLC1A5 expression is highest in HCA, followed by FC and FA (HCA vs FC, P=0.023; FC vs FA, P=0.002).

required to elucidate the evident association between glutamine metabolism and the PI3K-AKT-mTOR pathway, Wnt/β-catenin pathway, and hTERT in HCN.

In this study, there was no significant association between glutaminolysis-related proteins and prognosis. Since differentiated thyroid cancer takes an indolent clinical course, and the follow-up period of most patients was shorter than 5 years, only limited evaluation on survival was possible.

Differential expression of glutaminolysis-related proteins among the HCN and FN might be used as surrogate markers for future targeted therapy. The glutamine metabolism pathway is a potential therapeutic target, which can be controlled by the reduction of glutamine metabolic enzyme activity or glutamine uptake. Preclinial and clinical trials are in progress using GLS1 inhibitors in various tumors, such as BPTEP (Hartwich and Curthoys, 2012; Emadi et al., 2014; Xiang et al., 2015), 968 (Erickson and Cerione, 2010; Simpson et al., 2012; Katt et al., 2015), CB-839 (Gross et al., 2014; Jacque et al., 2015). BenSer, an ASCT2 inhibitor, is reported to suppress the proliferation of melanoma cells (Wang et al., 2014). Hence, further studies are required to investigate the effect of glutamine metabolic inhibition to HCC, which has a high glutamine metabolic activity.

In conclusion, the expression rates of glutaminolysis-related proteins differ between FN and HCN; HCN has a higher expression rate of GLS1 and GDH, compared to FN.

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