Low expression of ryanodine receptor 2 is associated with poor prognosis in thyroid carcinoma

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Abstract. Genetic alterations are vital in the progression of thyroid carcinoma. Ryanodine receptor 2 (RyR2) is reported to serve an important role in several types of human carcinoma. However, the expression and effect of RyR2 in thyroid carcinoma remain unknown. Therefore, the present study analyzed the status of RyR2 in thyroid carcinoma using bioinformatics tools. The mRNA profiles of thyroid carcinoma were downloaded from The Cancer Genome Atlas. RyR2 was distinguished as a differentially expressed gene that has not been reported in thyroid carcinoma. Further analysis indicated that there was selective downregulation of RyR2 expression in thyroid carcinoma tissues compared with that in normal thyroid tissues. Survival analysis showed that RyR2 expression was associated with poorer disease-free survival (DFS) for all patients with thyroid carcinoma. Univariate analysis revealed that a low expression of RyR2 was significantly associated with lymphatic metastasis, extracapsular extension, and the Tumor-Node-Metastasis stage. Cox analysis demonstrated that RyR2 was an independent prognostic factor in thyroid carcinoma for DFS. The biological processes and signaling pathways of RyR2 were reviewed with Gene Set Enrichment Analysis. In conclusion, the present study has revealed that RyR2 is downregulated in thyroid carcinoma, and that low expression of RyR2 is associated with poor prognosis in patients with thyroid carcinoma. RyR2 may therefore serve as a promising tumor suppressor gene in thyroid carcinoma.

Introduction

Thyroid carcinoma is the most common malignancy of the endocrine system, and its incidence rate has been increasing worldwide over the past 10 years. The global incidence rate is 10.2 cases per 100,000 in women and 3.1 cases per 100,000 in men (1-5). In the United States (6,7), thyroid carcinoma is now the fifth most common cancer in women and will replace colon cancer as the fourth leading cancer by 2030. In Korea (8), thyroid carcinoma has rapidly increased by 24.2% per year in women between 2000 and 2010, and the incidence rate of 18.1 cases per 100,000 is the highest in the world, while in China (9), thyroid carcinoma is the third most common cancer in women, and has become a common disease harmful to human health. This increase may partly be explained by environmental pollution, ionizing radiation, sex hormone levels and occupational stress (10-14). Concurrent with the developing economy, there has been an increase in the concentration of pollutants in the environment. This includes air, water, soil and noise pollution as well as an increase in contaminant garbage waste (15-18). It is well-established that ionizing radiation is a risk factor for thyroid carcinoma (19). Major sources of ionizing radiation includes radioactive material from nuclear waste and medical examinations (for example, computerized tomography and x-rays), and there is an increased probability of exposure to ionizing radiation. Additionally, as society and societal roles have developed, there are now more women in the workforce which may result in additional work-related stress and thus changes to hormone levels (20).

Thyroid carcinoma is generally considered a neoplastic disease, and most patients usually have a favorable prognosis with a 10-year survival rate >90 percent when treated by conventional surgery and adjuvant radioiodine. However, thyroid carcinoma cells can metastasize in the early stages of the disease to lymph nodes through the blood and lymphatic systems, which may lead to recurrence and distant metastasis in 10-20% of patients (21,22). Some cases have a poor response to conventional treatment resulting in a low 10-year survival rate of 20-30% (23,24). Therefore, a better understanding of thyroid carcinoma progression and prognosis prediction is urgently required to improve the outcome of patients with thyroid carcinoma.

Ryanodine receptors (RyRs) are the largest of the ion channels. Ryanodine receptor type 2 (RyR2), a member of the RyR family, is a homotetrameric protein complex that regulates Ca^{2+} release from sarcoplasmic reticulum into the cytosol (25). Gene transcription, vesicle secretion, and cell proliferation are controlled by intracellular Ca^{2+} levels (26), and imbalances in Ca^{2+} homeostasis and abnormal increases in cytosolic Ca^{2+}

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induce apoptosis (27). There is increasing evidence to suggest that intracellular Ca^{2+} homeostasis is altered in cancer cells, and that these alterations are involved in tumor angiogenesis, genetic mutations, and cellular migration (28). Previous studies have confirmed that RyR2 is associated with several types of cancers, including melanoma (29), breast cancer (30), lymphoma (31), and prostate cancer (32). However, few studies to date have assessed the expression and biological function of RyR2 in thyroid carcinoma.

The present study analyzed the relationship between RyR2 mRNA expression and thyroid carcinoma. A bioinformatics analyses revealed that RyR2 was downregulated in thyroid carcinoma tissues, and low expression levels of RyR2 were closely associated with poor prognosis in thyroid carcinoma patients.

Materials and methods

Data source. Gene expression data and clinical information on patients with thyroid carcinoma were obtained from the Genomic Data Commons Data Portal within The Cancer Genomes Atlas (TCGA) (https://portal.gdc.cancer.gov/) using TCGAbiolinks R/Bioconductor package (in October 2018). First, the RNA-seq data files were merged into a matrix file using the merge script of the Perl language (http://www.perl.org/). Then, the gene name was converted from the Ensembl ID to the matrix of the gene symbol through the Ensembl database version 84 (asia.ensembl.org/index.html). These downloaded data included a total of 506 thyroid carcinoma samples and 57 normal thyroid samples. The data of RyR2 were extracted and analyzed. The differentially expressed genes (DEGs) analysis with RNAseq data was performed using R (version 3.6.0) (33) package edgeR version 3.27.6 (r-project.org/). False discovery rate <0.05, $\log_2$ counts per million >1 and $|\log_2$ fold change$|$>2 were set as inclusion criteria for the DEGs selection. The gene expression level based on microarray data was calculated using R package limma (version 3.40.2; bioconductor.org/packages/release/bioc/html/limma.html) with robust multiarray average (RMA) correction.

Gene information acquisition and clinicopathological features. Gene information for normal thyroid and thyroid carcinoma expressing RyR2 were obtained from the downloaded data. Cases missing relevant clinicopathological parameters (including age, sex, race, lymphatic metastasis, extracapsular extension and Tumor-Node-Metastasis (TNM) stage (34) and prognostic follow-up data were eliminated. A total of 555 samples (498 tumors and 57 normal samples) were analyzed in the present study. The median expression level of 134 for RyR2 in thyroid carcinoma was used as the cutoff. Low RyR2 expression in each of the 249 patients was defined as a value below the 50th percentile. High RyR2 expression in each of the 249 patients was defined as a value above the 50th percentile. Significance was first evaluated using the Kruskal-Wallis test. A Mann-Whitney test was used to evaluate the differences between any two groups, followed Bonferroni's correction with a cut-off of P=0.0167 was used to correct for multiple comparisons. The χ^2 and Fisher exact tests were used to evaluate the association between clinicopathological characteristics and RyR2 expression.

Prognostic analysis. Using the median value of RyR2 expression as mentioned above, patients with thyroid carcinoma were classified into RyR2 high group and RyR2 low group. Kaplan-Meier analysis was used to generate disease-free survival (DFS) curves, and log-rank tests were performed to assess DFS differences between RyR2 high and low expression groups. Univariate and multivariate analyses with Cox proportional hazards regression for DFS were performed on individual clinical risk factors with and without the RyR2 expression. Hazard ratios and 95% confidence intervals were determined.

Functional enrichment analysis. Enrolled patients were divided into high and low expression groups based on their RyR2 expression as described above. In the present study, the c2.cp.kegg.v6.0.symbols.gmt data set was downloaded from the Molecular Signatures Database from the Gene Set Enrichment Analysis (GSEA) website (version 3.0; software.broadinstitute.org/gsea/index.jsp). Then, enrichment analysis was performed by the default weighted enrichment method, and the number of random combinations was set at 1,000.

Statistical analysis. Statistical analyses were performed using SPSS v21.0 software (IBM, Corp.). The edger function was used to analyze mRNA profiles between normal and thyroid carcinoma tissues. The different expression of RyR2 mRNA in normal, high and low RyR2 expression groups were evaluated using the Kruskal-Wallis test, following which the Mann-Whitney test was used to evaluate the differences between any two groups, and the Bonferroni's correction was performed to account for multiple corrections. X^2 and Fisher exact tests were used to evaluate the association between clinicopathological characteristics and RyR2 expression. In addition, the association between RyR2 and the prognosis of patients with thyroid carcinoma was evaluated with the Kaplan-Meier method and log-rank test. Univariate and multivariate analyses were performed using the Cox regression model. P<0.05 were considered to indicate a statistically significant difference.

Results

RyR2 is significantly downregulated in thyroid carcinoma. The expression of RyR2 mRNA in normal and thyroid carcinoma tissue was investigated using gene expression profiles downloaded from TCGA database. The results from TCGA cohort indicated that RyR2 expression was significantly lower in both high RyR2 expressing and low RyR2 expressing thyroid carcinoma groups compared with the normal thyroid group (P<0.01) (Fig. 1).

Relationship between RyR2 expression and clinicopathological characteristics. To explore whether RyR2 expression is associated with clinicopathological features of thyroid carcinoma, the clinicopathological characteristics of 498 patients with thyroid carcinoma from TCGA database were analyzed. According to the median value of RyR2 expression, patients were divided into either high RyR2 expression (n=249) or low RyR2 expression (n=249) groups. In the present study, RyR2 expression was significantly associated with lymph node metastasis (P<0.001), extracapsular extension (P<0.001), and TNM stage (27) (P<0.001) (Table I). However, there was no association with other characteristics including age, sex, or ethnic background (P>0.05).

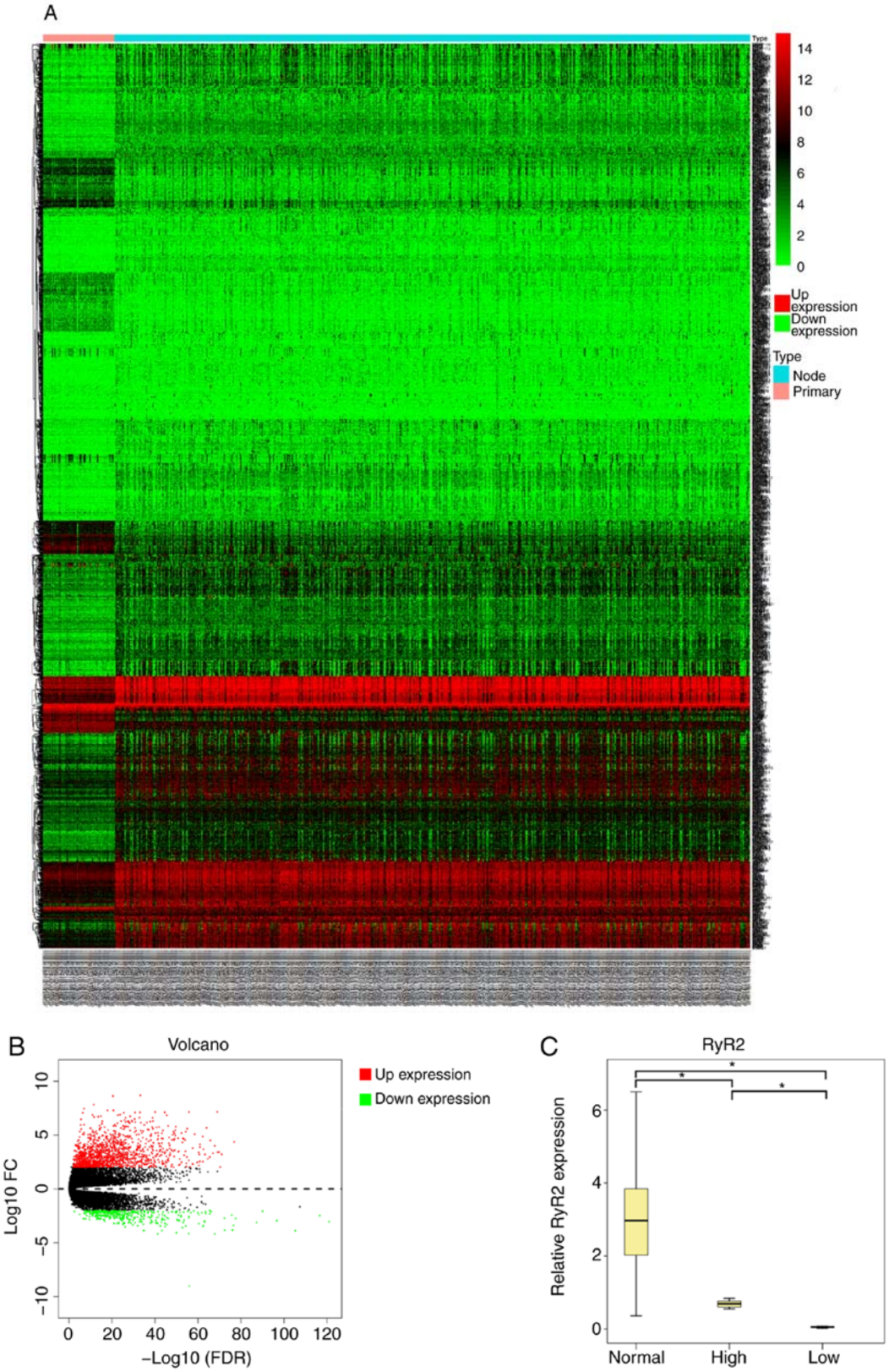

Figure 1. Expression of RyR2 in thyroid carcinoma from the TCGA database. (A) heatmap and (B) volcano plot of mRNA expression in thyroid cancer. (C) RyR2 mRNA expression levels. Thyroid cancer with high, low or normal RyR2 expression. *P<0.01.

Association between RyR2 expression and prognosis of thyroid carcinoma. The prognostic role of RyR2 in thyroid carcinoma was investigated using Kaplan-Meier and Cox regression analyses. The results indicated that patients with low RyR2 mRNA levels had a shorter disease-free survival (DFS) compared with that in patients with high RyR2 mRNA levels (P=0.00382; Fig. 2). Furthermore, univariate analysis showed that lymphatic metastasis, extracapsular extension,

Table I. Relationship between the RyR2 expression and clinicopathological characteristics in patients with thyroid carcinoma.

Clinicopathological characteristics	RyR2 expression		χ^2 value	P-value[a]
	Low, n=249	High, n=249		
Age, years			1.168	0.28
<45	106	118		
≥45	143	131		
Sex			0.01	0.919
Male	67	66		
Female	182	183		
Race			2.781	0.249
White	202	200		
Black or African American	20	13		
Asian	27	36		
Lymphatic metastasis			11.600	P<0.001
No	107	145		
Yes	142	104		
Extracapsular extension			10.364	P<0.001
No	135	170		
Yes	114	79		
TNM stage			11.036	P<0.001
I and II	148	183		
III and IV	101	66		

[a]Chi-square test.

TNM stage, and RyR2 expression were associated with DFS (P<0.05). Multivariate analysis demonstrated that RyR2 expression was an independent prognostic factor in thyroid carcinoma for DFS (Table II). Moreover, RyR2 expression was negatively associated with lymphatic metastasis, extracapsular extension, and TNM stage (Table I). The results of Tables I and II suggest that there is an association between RyR2 and the prognosis of thyroid carcinoma patients.

Function analysis of RyR2. Thyroid carcinoma patients were divided into high and low expression groups according to their RyR2 expression. The median expression level of RyR2 was used as the cutoff. To clarify the function of RyR2 in thyroid carcinoma, Gene Set Enrichment Analysis (GSEA) was used for enrichment. RyR2 is associated with 'β-alanine metabolism' (Fig. 3A; P<0.001), 'ascorbate and aldarate metabolism' (Fig. 3B; P=0.004), 'fatty acid metabolism' (Fig. 3C; P=0.002), 'primary bile acid biosynthesis' (Fig. 3D; P=0.008), 'glycine serine and threonine metabolism' (Fig. 3E; P=0.008), 'lysine degradation' (Fig. 3F; P=0.008), 'calcium signaling' (Fig. 3G; P=0.006), and 'TGF-β signaling' (Fig. 3H; P=0.008).

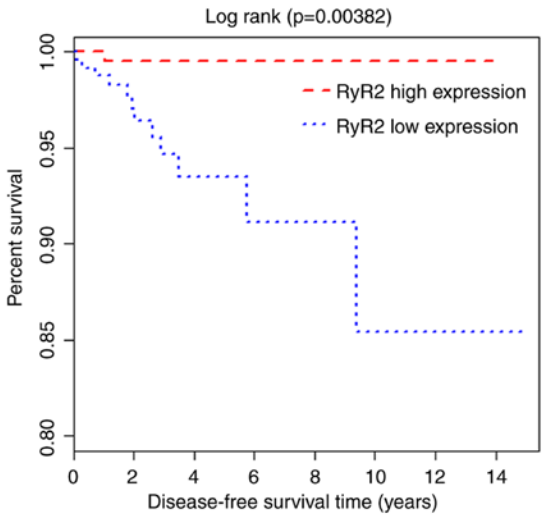

Figure 2. Association between RyR2 expression and disease-free survival in patients with thyroid carcinoma.

Discussion

Thyroid carcinoma is one of the most common malignant tumors in China. It is generally considered to be a neoplastic disease with a good prognosis. However, thyroid carcinoma has a high recurrence rate after surgery, and there are individual differences in patients (35,36). Thus, novel factors that could more effectively predict the prognosis of thyroid carcinoma require identification. In the present study, the expression of RyR2 in thyroid carcinoma and its function in predicting the prognosis of patients with thyroid carcinoma was investigated. Low RyR2 expression was associated with poor prognosis of patients with thyroid carcinoma.

Table II. Cox regression analysis of RyR2 expression and clinicopathological characteristics for disease-free survival in patients with thyroid carcinoma.

	Univariate analysis		Multivariate analysis	
Clinicopathological characteristics	HR (95% CI)	P-value	HR (95% CI)	P-value
Age, years				
<45 vs. ≥45	1.183 (0.437-3.325)	0.619	1.351 (0.572-2.968)	0.714
Sex				
Male vs. female	1.057 (0.266-2.639)	0.894	1.315 (0.402-3.347)	0.629
Race				
White vs. Black or African American vs. Asian	1.258 (0.714-1.893)	0.376	1.421 (0.503-4.624)	0.481
Lymphatic metastasis				
No vs. Yes	2.417 (1.611-3.690)	0.027[a]	2.762 (1.783-4.176)	0.034[a]
Extracapsular extension				
No vs. Yes	1.682 (1.012-2.656)	0.031[a]	1.538 (1.124-2.537)	0.042[a]
TNM stage				
I and II vs. III and IV	3.531 (2.219-5.647)	0.015[a]	3.726 (2.401-5.893)	0.019[a]
RyR2 expression				
Low vs. high[a]	5.293 (3.505-8.044)	0.007[a]	5.341 (3.547-8.128)	0.012[a]

[a]The median expression level of RyR2 was used as the cutoff. HR, hazard ratio; CI, confidence interval.

Intracellular calcium ions (Ca^{2+}) have important roles in fundamental cellular physiology (37). Disruption of intracellular Ca^{2+} homeostasis contributes to tumor cell proliferation and reduced apoptosis (38,39). Additionally, accumulating evidence has demonstrated that severe and persistent endoplasmic reticulum (ER) stress or suppression of ER stress results in tumor cell death (40). RyR is an ER cation channel that releases ER Ca^{2+} into the cytosol (41). RyRs form large protein complexes comprising calmodulin, protein kinases, and protein phosphatases. In previous research, it has been reported that RyR can upregulate the activity of mitogenic pathways, including RAS/mitogen-associated protein kinase (42), and promote T cell activation (43). Furthermore, Abdul *et al* (44) found that there was a strong negative association between RyR levels and breast tumor grade. However, the association between the expression of RyR2 and clinicopathological characteristics in thyroid carcinoma remained unclear.

According to the 8th AJCC staging system (34), minimal/minor extrathyroidal extension (mETE) is no longer regarded as a criterion of advanced stage cancer. Our hypothesis is that this is currently published in different versions of mETE in 8th AJCC staging system for the following reasons: First, capsula glanduloe thyreoideae is imperfect, and normal thyroid tissue may contain adipose or skeletal muscle tissue. Thus, mETE cannot be defined clearly. Secondly, a number of studies have found that mETE is not an independent prognostic risk factor in patients with thyroid carcinoma. Following the publishing of the 8th AJCC staging system, it also caused widespread controversy. Many researchers have presented different views against the new staging system, as they consider that mETE can increase the risk of thyroid carcinoma recurrence (45). In the 8th AJCC staging system, any lymph node involvement in either the central or lateral neck defines a patient as stage group II (in the absence of gross extrathyroidal extension or distant metastases) in patients aged 55 years or older. While in a number of studies, the presence of lymph node metastasis was associated with a statistically significant decrease in survival rate (46,47). Furthermore, lateral lymph node metastasis is considered to be an independent prognostic risk factor in patients with thyroid carcinoma (48,49). In the present study, RyR2 mRNA was downregulated in thyroid carcinoma, and low expression of RyR2 was associated with lymph node metastasis, extracapsular extension, and high TNM staging, which suggested poor prognosis of patients with thyroid carcinoma. Moreover, patients with low RyR2 expression had a poorer DFS compared with that in patients with high RyR2 expression. Univariate and multivariate Cox regression analyses showed that RyR2 was an independent prognostic factor for DFS in thyroid carcinoma. Taken together, these results indicated that RyR2 is involved in the protection of thyroid carcinoma. In order to reveal the biological processes and signaling pathways of RyR2 in thyroid carcinoma, GSEA was used, which revealed that RyR2 was enriched in 'β-alanine metabolism', 'ascorbate and aldarate metabolism', 'fatty acid metabolism', 'primary bile acid biosynthesis', 'glycine serine and threonine metabolism', and 'lysine degradation', these signaling pathways were closely associated with 'cellular metabolism' and 'cell activity'. RyR2 was also enriched in 'calcium signaling pathway' and 'TGF-β signaling pathway', these two signaling pathway themselves were closely associated with cell signaling and tumor regression. Further investigation is required to verify these biological processes and signaling pathways modulated by RyR2 in thyroid carcinoma in future studies.

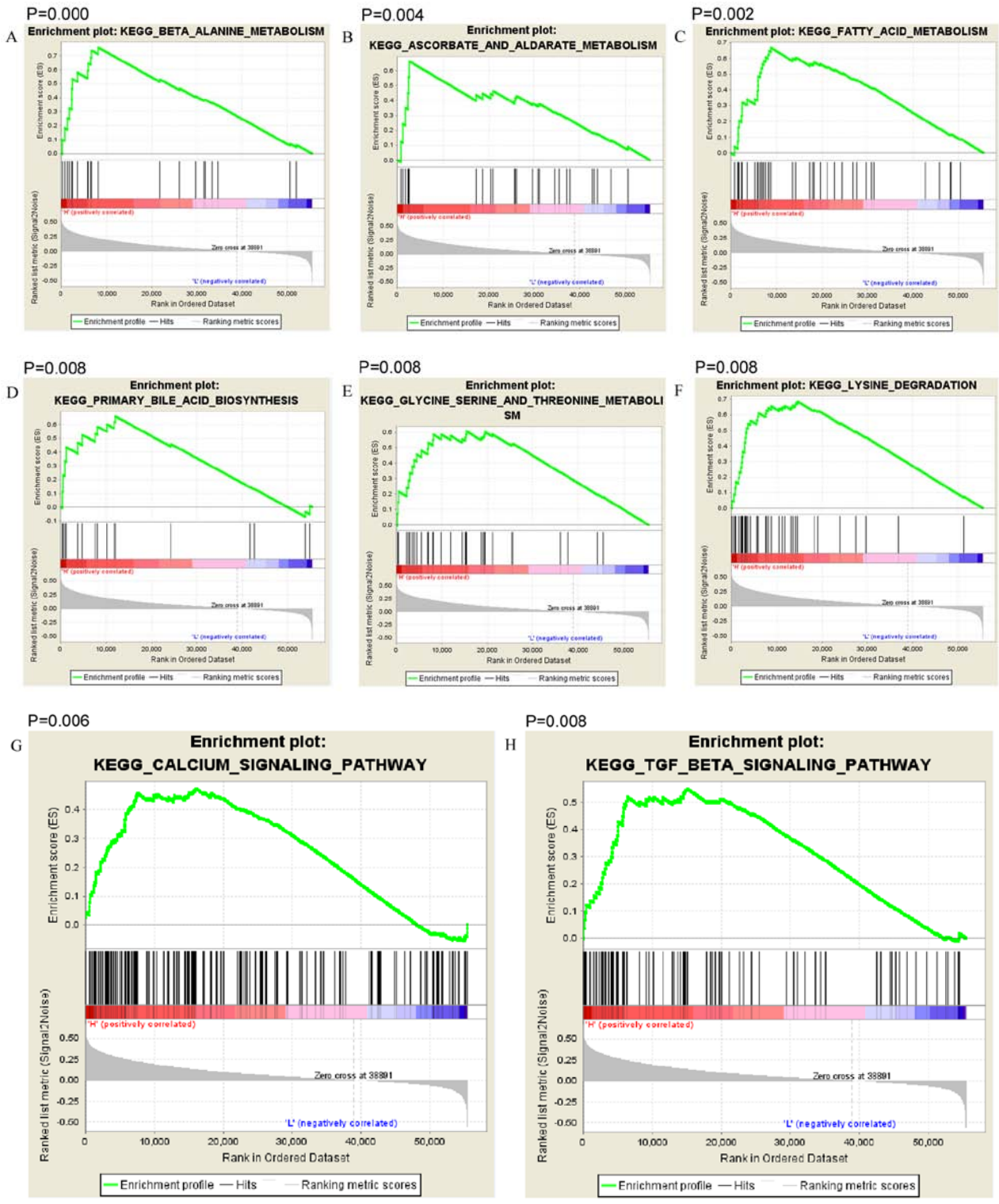

Figure 3. Biological process associated with RyR2 expression. (A) β-alanine metabolism. (B) Ascorbate and aldarate metabolism. (C) Fatty acid metabolism. (D) Primary bile acid biosynthesis. (E) Glycine serine and threonine metabolism. (F) Lysine degradation. (G) Calcium signaling. (H) TGF-β signaling.

Taken together, the present comprehensive analysis demonstrated the expression and prognostic value of RyR2 in thyroid carcinoma. We found that low expression of RyR2 is associated with the poor prognosis of patients with thyroid carcinoma. Therefore, RyR2 might be a potential tumor suppressor gene for thyroid carcinoma. The exact mechanisms of RyR2 in thyroid carcinoma remain unclear, and thus further studies investigating RyR2 and thyroid carcinoma pathogenesis are required. A total of 555 samples (498 tumors and 57 normal samples) were analyzed in our study. A larger cohort of tumor and normal samples is required in future studies to improve the statistical power. In the present study, the expression value of RyR2 in 498 patients with thyroid carcinoma were presented as abnormal distribution, but as the median level of RyR2 was used as the cutoff to divide patients into high and low RyR2 expression groups, this method may be inaccurate. Therefore, more accurate statistical

methods are required in future studies. In addition, the expression of RyR2 and its effects should be determined separately for the different subtypes of thyroid carcinoma, including papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), medullary thyroid carcinoma (MTC) and anaplastic thyroid carcinoma (ATC). PTC and FTC generally present with indolent behavior and has a favorable prognosis. MTC is an aggressive thyroid carcinoma, deriving from parafollicular cells. ATC is the most aggressive type of thyroid carcinoma and responsible for more than half of all thyroid cancer deaths. Different variants of thyroid carcinoma have distinct biological behaviors and prognosis, thus the expression of RyR2 may differ between different variants of thyroid carcinoma, and these will also require further investigation.

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Availability of data and materials

All the data collected and analyzed in the study are available from the corresponding author on reasonable request.

Authors' contributions

LG and NX designed the study. NX, DZ, JC and GH performed the data collection and analysis. All authors participated in the writing of the manuscript. All the authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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