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# $\gamma$ -tocotrienol regulates gastric cancer by targeting notch signaling pathway

Ling Xie<sup>1</sup> and Juan Yan<sup>2\*</sup>

## Abstract

**Background** Gastric cancer is a common cause of death from cancer and an important global health care issue. Consequently, there is an urgent need to find new drugs and therapeutic targets for the treatment of gastric cancer. Recent studies have shown that tocotrienols (T3) have significant anticancer ability in cancer cell lines. Our previous study found that  $\gamma$ -tocotrienol ( $\gamma$ -T3) induced apoptosis in gastric cancer cells. We further explored the possible mechanisms of  $\gamma$ -T3 therapy for gastric cancer.

**Methods** In this study, we treated gastric cancer cells with  $\gamma$ -T3, collect and deposit the cells.  $\gamma$ -T3-treated gastric cancer cells group and untreated group were subjected to RNA-seq assay, and analysis of sequencing results.

**Results** Consistent with our previous findings, the results suggest that  $\gamma$ -T3 can inhibit mitochondrial complexes and oxidative phosphorylation. Analysis reveals that  $\gamma$ -T3 has altered mRNA and ncRNA in gastric cancer cells. Significantly altered signaling pathways after  $\gamma$ -T3 treatment were enriched for human papillomavirus infection (HPV) pathway and notch signaling pathway. The same significantly down-regulated genes notch1 and notch2 were present in both pathways in  $\gamma$ -T3-treated gastric cancer cells compared to controls.

**Conclusions** It is indicated that  $\gamma$ -T3 may cure gastric cancer by inhibiting the notch signaling pathway. To provide a new and powerful basis for the clinical treatment of gastric cancer.

**Keywords**  $\gamma$ -tocotrienol, Gastric cancer, Mitochondria, Oxidative phosphorylation, Notch signaling pathway

## Introduction

Cancer is a major public health problem worldwide. In countries around the world, cancer is a leading cause of death and poses a significant barrier to increasing life expectancy [12]. According to World Health Organization (WHO) estimates, cancer is the top four cause of death by age 70 [55]. Collectively, this reflects the rapidly

increasing burden of cancer morbidity and mortality worldwide [40]. Gastric cancer is the second most common cause of cancer deaths worldwide [26]. Gastric cancer is caused by many factors, such as smoking and a diet high in nitrates and nitrites or infection with helicobacter pylori. It also includes some non-modifiable factors, such as age, gender and race [26]. The treatment of early gastric cancer is mainly endoscopic resection. Patients with advanced gastric cancer require chemotherapy. Some of the currently approved targeted therapies for the treatment of gastric cancer include trastuzumab, ramilumab and nivolumab or pembrolizumab [53]. However, the treatment methods for gastric cancer need to be advanced so as to bring benefits to patients.

More and more studies are focusing on finding more effective anticancer drugs from plant reservoirs [2, 15, 36]. T3 are naturally occurring compounds of the vitamin

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E family and have been reported to be present in the seeds of plants [51]. Studies have reported the role of T3 in various types of cancer. T3 exert their anticancer effects in multiple ways, including inhibition of angiogenesis, promotion of apoptosis and metastasis [16, 37, 47]. T3 is divided into four isomers, including  $\alpha$ -T3;  $\beta$ -T3;  $\gamma$ -T3; and  $\delta$ -T3 [4]. Of which,  $\gamma$ -T3 and  $\delta$ -T3 exhibited stronger anticancer activity [5].

Studies have confirmed that T3 exerts anticancer activity mainly by inhibiting the transcription factors NF- $\kappa$ B and STAT3 and their regulatory gene products [34]. Furthermore, it was found that in pancreatic cancer cell lines,  $\gamma$ -T3 and  $\delta$ -T3 effectively inhibited the activation of Akt to induce apoptosis in pancreatic cancer cells [52]. Studies have reported that  $\gamma$ -T3 can increase the production of mitochondrial reactive oxygen species (ROS) to induce apoptosis in human T-cell lymphoma Jurkat cells [62]. In addition, one study reported that  $\gamma$ -T3 can activate both ER stress-mediated apoptosis and autophagy to enhance cell death in breast cancer cells [57]. Therefore,  $\gamma$ -T3 has great research value in the treatment of gastric cancer.

The notch signaling pathway plays an important role in immune cell generation [17, 39]. Dysregulation of the notch signaling pathway leads to multiple pathophysiologies in cancer diseases. Notch signaling pathway was found to be involved in cell proliferation and apoptosis [44], and dysfunctional notch signaling was reported to induce gastric cancer [63]. It was found that the expression levels of notch1/2/3 were higher in gastric cancer tissues compared to normal tissues. It is believed that notch1/2/3/4 may be a potential target for precision treatment of gastric cancer [23].

Our previous study showed that  $\gamma$ -T3 was able to induce apoptosis in gastric cancer cells [59]. To further investigate the mechanism of  $\gamma$ -T3-induced apoptosis in gastric cancer, we performed RNA-seq assay on  $\gamma$ -T3-treated MKN-45 cells. Firstly, analysis of RNA-seq results from  $\gamma$ -T3-treated gastric cancer cells and untreated cells revealed significant differences in mRNA and ncRNA between the two groups. There were 669 up-regulated genes and 320 down-regulated genes in mRNA in the  $\gamma$ -T3-treated group. Additionally, there were 95 up-regulated genes and 42 down-regulated genes in ncRNA in the  $\gamma$ -T3-treated group. We analyzed mRNAs with down-regulated gene expression using kyoto encyclopedia of genes and genomes (KEGG) and found common genes notch1 and notch2 in the gene-enriched human HPV and notch signaling pathway. These results suggest that  $\gamma$ -T3 induces apoptosis in gastric cancer cells by downregulating notch1 and notch2 gene expression in the notch signaling pathway. To establish a basis for the use of  $\gamma$ -T3 as a clinical target for the treatment of gastric cancer.

## Materials and methods

### Reagents

$\gamma$ -T3 ( $\geq 97\%$ ) was purchased from Hygeia Industries Inc (USA).  $\gamma$ -T3 was dissolved in ethanol (95%) to make a 100 mM solution and stored at  $-20^\circ\text{C}$ . RPMI-1640 medium, fetal bovine serum, 0.25% EDTA, and penicillin/streptomycin were purchased from Gibco (Grand Island, NY, USA).

### Cell culture

The human gastric cancer cell line MKN-45 (obtained from the Cancer Institute of the Chinese Academy of Medical Science) were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin/streptomycin at  $37^\circ\text{C}$  in a humidified incubator with 5%  $\text{CO}_2$ . MKN-45 cells were seeded into 96-well plates at 5000 cells per well and allowed to adhere overnight. MKN-45 cells were treated with  $\gamma$ -T3 (30  $\mu\text{mol/L}$ ) or not for 24 h. Cells were collected and total cellular RNA was isolated using TRIzol reagent (Molecular Research Center, USA).

### RNA-seq

RNA extraction and quality control and RNA-seq library construction and quality control experiments were performed by Shanghai Rongxiang Biotechnology Co., Ltd. The constructed RNA-seq libraries were sequenced by illumina sequencer. Quality control and analysis of the sequenced data were performed by Shanghai Rongxiang Biotechnology Co., Ltd. Gene expression was expressed by Fragments Per Kilobase per Million (FPKM), and differential gene expression analysis was performed using Deseq2 to compare the treatment and control groups, and genes with  $|\log_2\text{FoldChange}| \geq \log_2 21.5$  and  $\text{padj} < 0.05$  were selected as differentially expressed genes screening criteria to obtain up down-regulated genes [35]. Differences between the treatment and vehicle groups were assessed using multivariate linear models. The Bonferroni multiple correction method was used to correct for false discovery errors.  $p$ -value  $< 0.05$  was set as the threshold of significance for screening out differentially expressed genes (DEGs). DEGs were then used for volcano plot, heat map and functional analysis. ClusterProfiler was used to evaluate biological pathways [65] and we performed gene ontology (GO) [9], KEGG pathway [30] and gene set enrichment analysis (GSEA) [29].

### Statistical analysis

Sequencing data were processed using DEseq2 software. mRNAs and ncRNAs were defined as differentially expressed when  $|\log_2\text{FoldChange}| \geq \log_2 21.5$  and

$p_{adj} < 0.05$ .  $p$  values  $< 0.05$  were considered statistically significant.

## Results

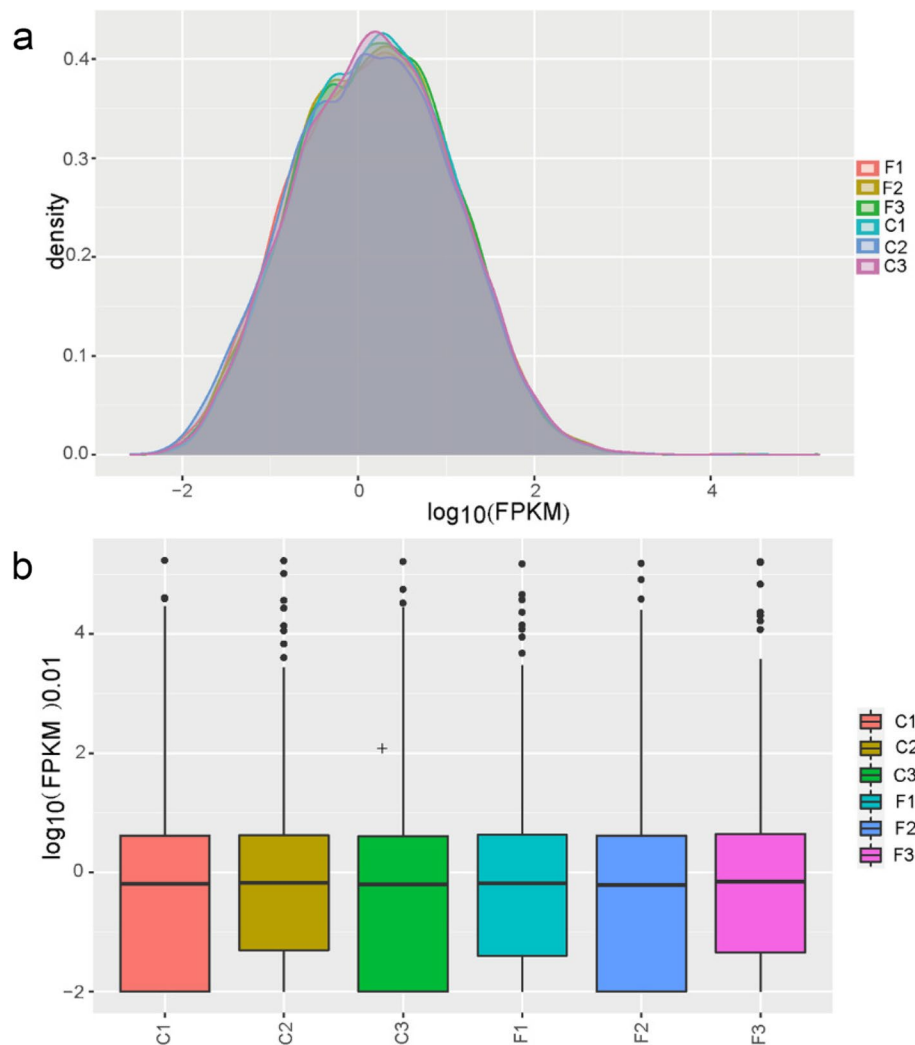
### RNA-seq assay of $\gamma$ -T3-treated gastric cancer cells

To investigate the mechanism of the role of  $\gamma$ -T3 in regulating gastric cancer. We performed RNA-seq assay on  $\gamma$ -T3-treated MKN-45 cells and untreated group cells, and analyzed the data for both groups of results. Based on the gene expression of all samples, an expression density plot of the samples was obtained (Fig. 1a), and the overall distribution trend of the expression of the samples was viewed using the distribution boxplot of the expression of all samples (Fig. 1b). The results showed differentially

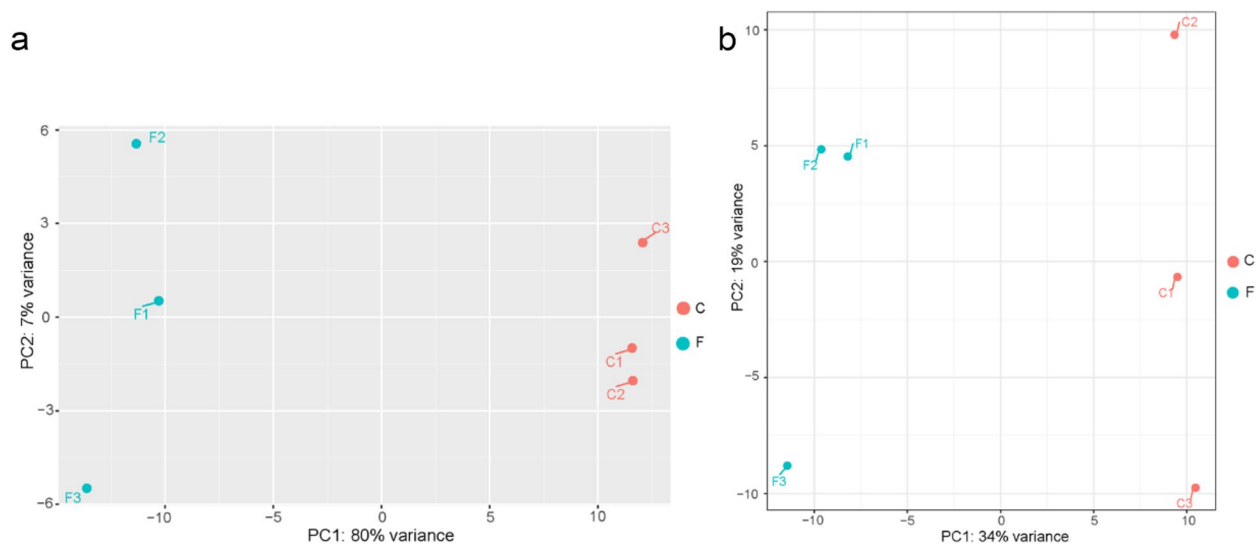
altered genes in the  $\gamma$ -T3-treated group compared to the control group.

### $\gamma$ -T3 affects gastric cancer cells

By performing principal component analysis (PCA) on the mRNA data of each sample detected by RNA-seq in the  $\gamma$ -T3-treated and untreated groups, the results showed the feasibility of three replicates for both experimental groups and differences between the two experimental groups (Fig. 2a). Meanwhile, we performed PCA on the ncRNA data of each sample from the RNA-seq assay of the  $\gamma$ -T3-treated and untreated groups, and the results similarly showed the feasibility of three replicates of the two experimental groups and the difference between the two experimental groups (Fig. 2b). The



**Fig. 1** RNA-seq assay of  $\gamma$ -T3-treated gastric cancer cells. MKN-45 cells were incubated for 24 h with  $\gamma$ -T3 (30  $\mu\text{mol/L}$ ). **a** Expression density plots of  $\gamma$ -T3-treated and control samples. **b** Expression distribution boxplot of  $\gamma$ -T3-treated and control samples. F:  $\gamma$ -T3-treated group; C: control group.  $n=3$



**Fig. 2** Changes in  $\gamma$ -T3-treated gastric cancer cells. **a** Principal component analysis of mRNA. **b** Principal component analysis of ncRNA. F:  $\gamma$ -T3-treated group; C: control group.  $n=3$

results showed that there was a significant difference between the  $\gamma$ -T3-treated group and the untreated group, and the treatment of  $\gamma$ -T3 had an effect on gastric cancer cells.

### $\gamma$ -T3 alters gene expression in gastric cancer cells

Next, we examined the expression of genes in gastric cancer cells by RNA-seq assay. The results showed that  $\gamma$ -T3 treatment altered the expression of genes in gastric cancer cells. We used Deseq2 for differential gene expression analysis to compare the treatment and control groups, and selected genes with  $|\log_2\text{FoldChange}| \geq \log_2 1.5$  and  $\text{padj} < 0.05$  as differentially expressed genes screening criteria to obtain up- and down-regulated genes. There were 669 up-regulated genes and 320 down-regulated genes in mRNA in the  $\gamma$ -T3-treated group compared to the control group (Fig. 3a-c). In addition, there were 95 up-regulated genes and 42 down-regulated genes in ncRNA in the  $\gamma$ -T3-treated group compared to the control group (Fig. 3a, d-e). The findings suggest that  $\gamma$ -T3 affected gastric cancer cells and significantly altered the mRNA and ncRNA expression levels.

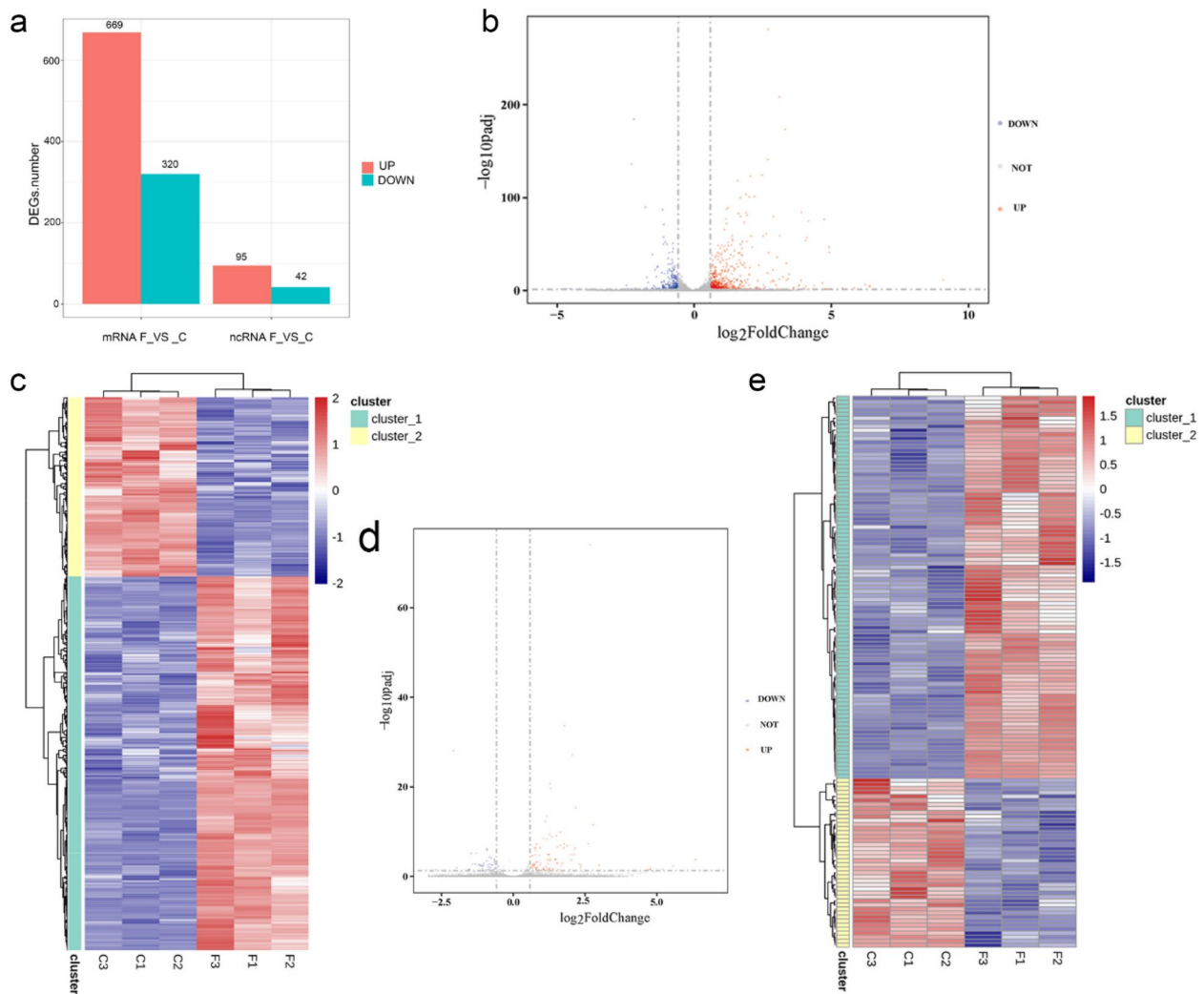
### $\gamma$ -T3 effects gene function in gastric cancer cells

We selected mRNAs with decreased gene expression after  $\gamma$ -T3 treatment of gastric cancer cells compared to control group for GO analysis. The biological process (BP), cellular component (CC), and molecular function (MF) of the genes that are down-regulated are summarized. The analysis revealed that  $\gamma$ -T3 treatment affected several gene MF of gastric cancer cells, including embryonic organ morphogenesis, negative regulation of

nervous system development, pattern specification process, etc. (Fig. 4a, b). In addition,  $\gamma$ -T3 also significantly affected the CC of gastric cancer cells through synaptic membrane, neuron to neuron synapse, basement membrane, and extracellular matrix component (Fig. 4c, d). Furthermore, the analysis of BP revealed that  $\gamma$ -T3 significantly affected acetylglucosaminyltransferase activity, transferase activity, transferring glycosyl groups, unfolded protein binding, etc. in gastric cancer cells (Fig. 4e, f). These data indicate that  $\gamma$ -T3 plays a significant role in the molecular function, cellular component, and biological process of gastric cancer cell genes.

### $\gamma$ -T3 induces apoptosis in gastric cancer cells

The sequencing data were analyzed for the treatment group compared to the control group. It was found that tumor necrosis factor (TNF) was able to kill cancer cells in mice, and the TNFR signaling pathway was able to induce apoptosis [10]. We performed GSEA analysis of the BP in GO and found that the pathway enriched to negatively regulate the TNF superfamily cytokines was activated (Fig. 5a). It is suggested that  $\gamma$ -T3 may induce apoptosis in gastric cancer cells through TNF signaling pathway. Calcium signaling functions extensively in many cellular processes and calcium signaling promotes the progression of several cancer types such as glioma [14, 33], prostate cancer [21] and breast cancer [32] by activating STAT3, an important transcription factor in cancer. Blocking calcium signaling may be a strategy to improve the anti-tumor immune response. The calcium-regulated extracellular secretory pathway was inhibited compared to the control group (Fig. 5b), suggesting that



**Fig. 3**  $\gamma$ -T3 modifies the gene expression of mRNA and ncRNA in gastric cancer cells. **a** Differential expression analysis of mRNA and ncRNA genes. **b, c** Volcano and heat map analysis of mRNA in  $\gamma$ -T3-treated group and control group. **d, e** Volcano and heat map analysis of ncRNA in  $\gamma$ -T3-treated group and control group. Genes with  $|\log_2\text{FoldChange}| \geq 1.5$  and  $\text{padj} < 0.05$  as differentially expressed genes screening criteria. F\_VS\_C:  $\gamma$ -T3-treated group VS control group. F:  $\gamma$ -T3-treated group; C: control group.  $n = 3$

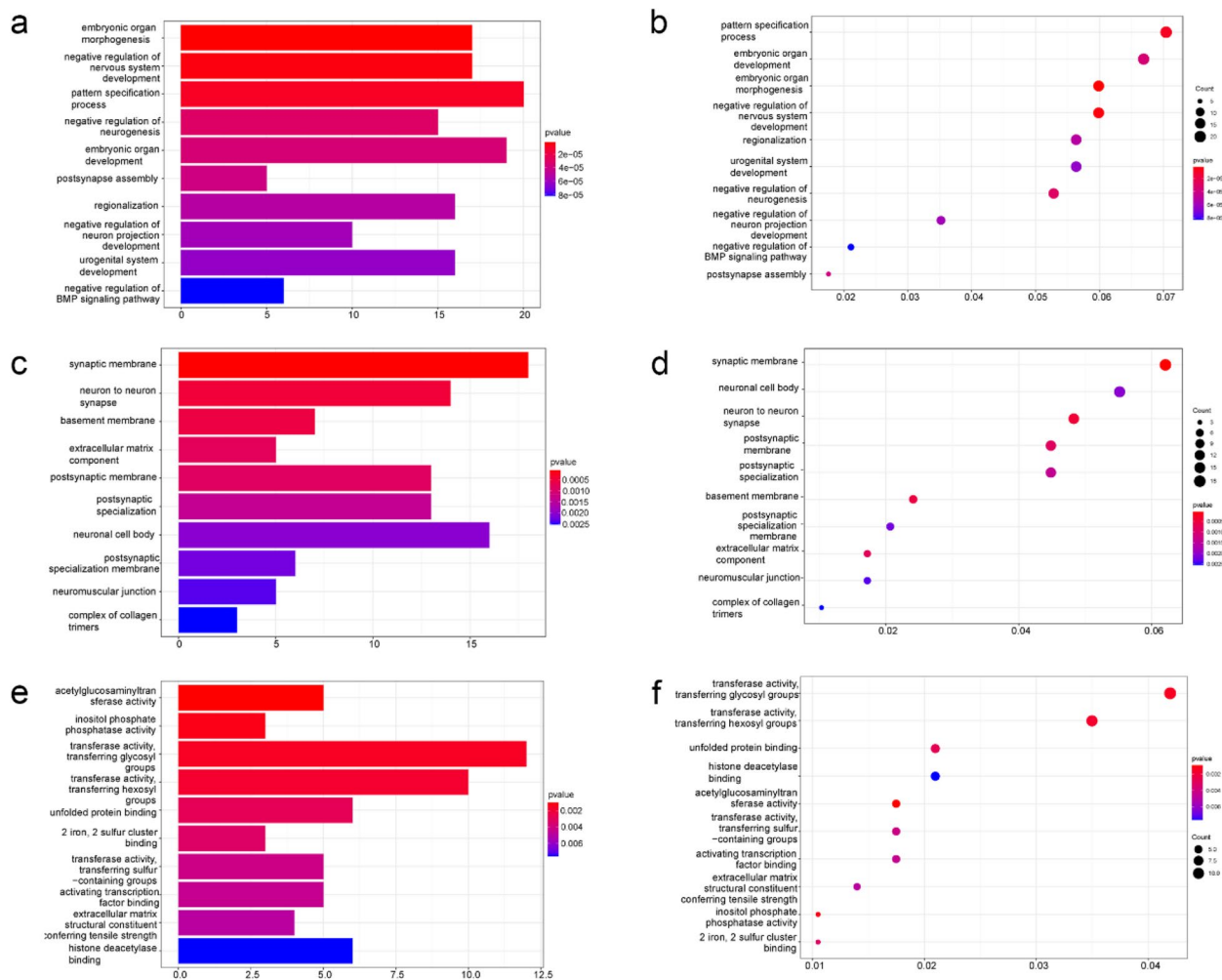
$\gamma$ -T3 could act by downregulating the calcium signaling pathway.

Mitochondria are the main site of oxidative respiration in cells. The oxidative respiratory chain is composed of four protease complexes located on the inner mitochondrial membrane, called complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (ubiquinone-cytochrome chromogranin c reductase) and complex IV (cytochrome c oxidase). Inhibition of tumor cell mitochondria may be an effective strategy for cancer therapy [61]. Furthermore, it was found that inhibition of NADH production in the cytoplasm significantly inhibited tumor growth [42]. Our previous study showed that  $\gamma$ -T3 induces apoptosis in gastric cancer

cells through inhibition of mitochondrial complex I [59]. Analysis of the CC in GO revealed that the mitochondrial protein containing complex and NADH\_dehydrogenase complex pathways were inhibited (Fig. 5c, d), which is consistent with our previous findings that  $\gamma$ -T3 inhibits the mitochondrial complex to induce apoptosis in gastric cancer cells.

In addition, MF analysis in GO also confirmed that  $\gamma$ -T3 inhibits NADH dehydrogenase activity and oxidation-reduction-driven activity of active transmembrane transporters pathways acting in gastric cancer cells. (Fig. 5e, f). In summary, the analysis showed that  $\gamma$ -T3 induced apoptosis in gastric cancer cells through multiple pathways.





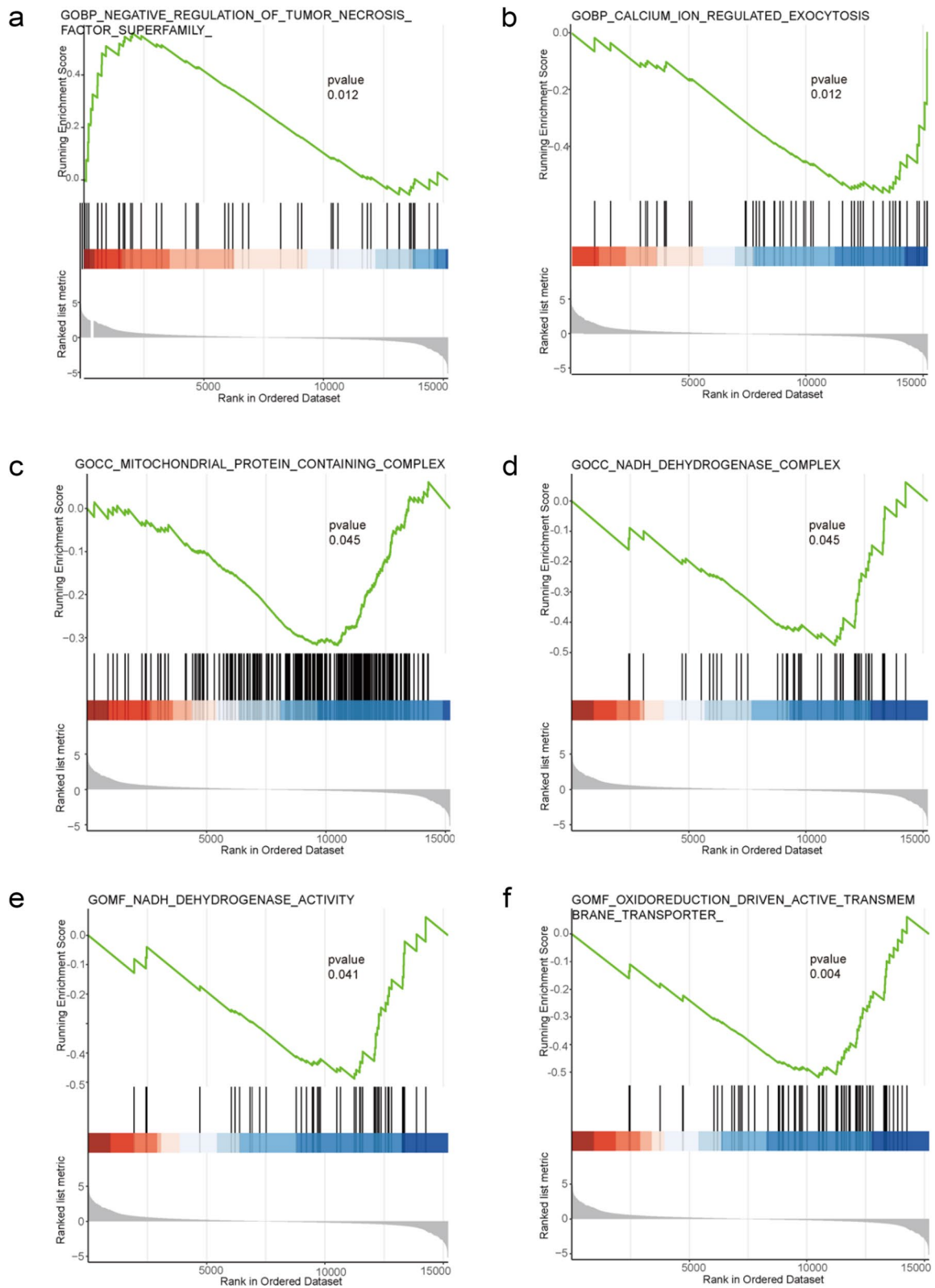
**Fig. 4**  $\gamma$ -T3 influences the gene function of gastric cancer cell. **a, b** BP involved in the decreased mRNA expression of genes in the  $\gamma$ -T3-treated group compared to the control group. **c, d** CC involved in the decreased mRNA expression of genes in the  $\gamma$ -T3-treated group compared to the control group. **e, f** MF involved in the decreased mRNA expression of genes in the  $\gamma$ -T3-treated group compared to the control group.  $p < 0.05$ .  $n = 3$

### $\gamma$ -T3 inhibits oxidative phosphorylation

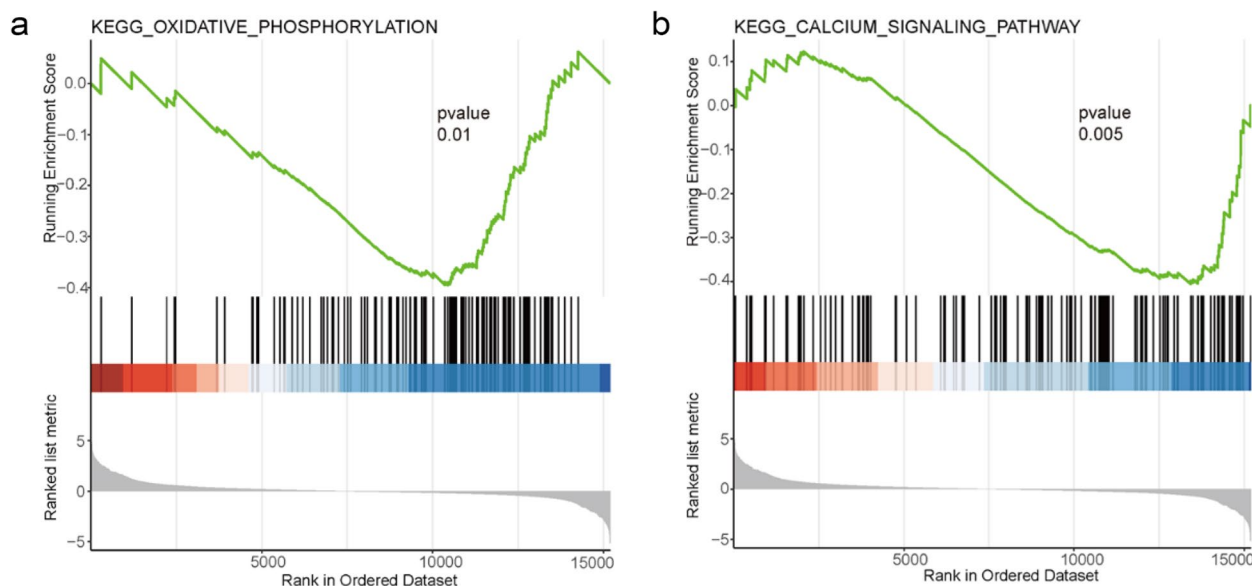
Then, we performed GSEA on gene sets in KEGG. There is growing evidence that certain cancers are heavily dependent on oxidative phosphorylation (OXPHOS) and that OXPHOS inhibition is an effective means of targeting cancer [13, 58]. Our previous study also confirmed that  $\gamma$ -T3 treatment inhibited OXPHOS in gastric cancer cells [59]. The oxidative phosphorylation signaling pathway was inhibited in the treated group compared with the control group (Fig. 6a), which is consistent with our previous findings that  $\gamma$ -T3 can inhibit oxidative phosphorylation exerted to promote apoptosis in cancer cells. In a similar way, we found that the treated group inhibited the calcium signaling pathway compared to the control group (Fig. 6b), suggesting that  $\gamma$ -T3 acts on calcium signaling to inhibit cancer cells.

### $\gamma$ -T3 triggers apoptosis by notch signaling pathway

We performed KEGG analysis of mRNA down-regulated in RNA-seq assay of  $\gamma$ -T3-treated gastric cancer cells. The analysis showed that genes downregulated in  $\gamma$ -T3-treated gastric cancer cells play significant roles in HPV pathway, melanogenesis, inositol phosphate metabolism, notch signaling pathway, etc. (Figs. 7a and 5b). Next, we found that HPV pathway intersects with the notch signaling pathway. Additionally, the genes involved in the two pathways were screened and the results showed that there were 13 down-regulated genes in the HPV pathway and 4 down-regulated genes in the notch signaling pathway. In particular, the HPV pathway and the notch signaling pathway have the same down-regulated genes, as notch1 and notch2 (Fig. 7c). Studies have reported significantly elevated expression of notch1 and notch2 in gastric cancer tissues [23]. Our previous investigation



**Fig. 5**  $\gamma$ -T3 inhibits mitochondrial function and activates TNF signaling in gene function. **a, b** GSEA of  $\gamma$ -T3 in the BP of GO gene set. **c, d** GSEA of  $\gamma$ -T3 in the CC of GO gene set. **e, f** GSEA of  $\gamma$ -T3 in the MF of GO gene set.  $p < 0.05$ .  $n = 3$



**Fig. 6**  $\gamma$ -T3 inhibits oxidative phosphorylation in gastric cancer cells. **a, b** GSEA of  $\gamma$ -T3 in the molecular function of KEGG gene set.  $p < 0.05$ .  $n = 3$

identified that  $\gamma$ -T3 was able to induce apoptosis in gastric cancer cells [59]. Accordingly, the result proposed that  $\gamma$ -T3 may play a role in inducing apoptosis in gastric cancer cells by inhibiting the gene expression of notch1 and notch2.

## Discussion

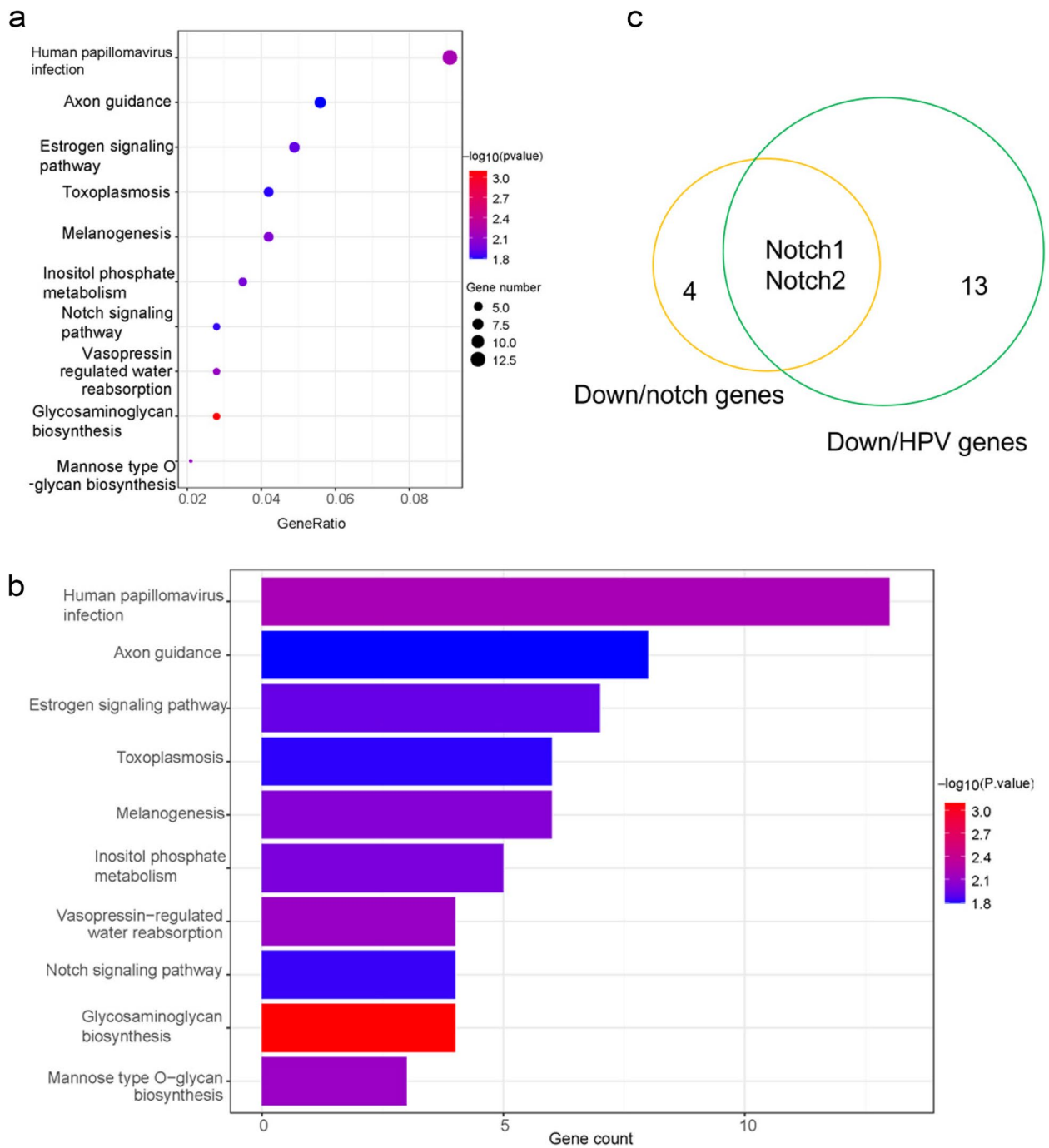
T3 is a natural vitamin E derivative, and the anticancer properties of T3 were identified and studied [5, 47].  $\gamma$ -T3 has been reported to inhibit the growth of a variety of human cancer cells in vivo and in vitro [1, 49, 56]. However, the mechanism of  $\gamma$ -T3 in gastric cancer research is not clear enough. Our previous study demonstrated that  $\gamma$ -T3 could induce apoptosis in gastric cancer cells [59]. Therefore, we performed RNA-seq assays on  $\gamma$ -T3-treated gastric cancer cells and untreated gastric cancer cells. There were 669 up-regulated genes and 320 down-regulated genes in mRNA in the  $\gamma$ -T3-treated group compared to the control group. In addition, there were 95 up-regulated genes and 42 down-regulated genes in ncRNA in the  $\gamma$ -T3-treated group compared to the control group. By KEGG analysis, we focused on the two pathways that ranked high in significant changes in  $\gamma$ -T3-treated gastric cancer cells, including HPV pathway and notch signaling pathway. We identified two common genes notch1 and notch2 in these two pathways. The expression levels of notch1 and notch2 were significantly downregulated in  $\gamma$ -T3-treated gastric cancer cells compared with the control group. The present results indicated that  $\gamma$ -T3 may play a role in inducing apoptosis in gastric cancer cells by down-regulating the expression

levels of notch1 and notch2 through the notch signaling pathway.

With a large number of new cases each year, gastric cancer is a diagnosed malignancy worldwide. Gastric cancer is frequently diagnosed at an advanced stage and has a high mortality rate, making it a common cause of cancer-related deaths [53]. The treatment of gastric cancer remains a major challenge at this time. A major challenge is to translate the latest discoveries in molecular biology into effective treatments for patients with gastric cancer. The exploration of drug mechanisms in gastric cancer remains a major obstacle in the development of targeted therapeutics. Despite the current progress in the treatment of gastric cancer, poor prognosis and death still exist [7, 27, 28, 64].

Vitamin E has classic antioxidant properties. Natural vitamin E is a mixture of two compounds, tocopherols and T3 [41, 60]. Studies have reported that T3 have more significant neuroprotective and anticancer effects than tocopherols [31, 38, 45]. Several studies have found that T3 can induce apoptosis in a variety of cancer cells [3, 48, 54]. Furthermore, studies have reported a role for  $\gamma$ -T3 in inhibiting cancer cell proliferation [20, 50]. Our previous study showed that  $\gamma$ -T3 could induce apoptosis in gastric cancer cells and thus exert anti-cancer effects [59]. To further explore the specific mechanism by which  $\gamma$ -T3 exerts its anti-cancer effects, we treated gastric cancer cells with  $\gamma$ -T3 and performed RNA-seq assays on  $\gamma$ -T3-treated gastric cancer cells and untreated. The analysis revealed that consistent with our previous study,  $\gamma$ -T3 inhibited the mitochondrial complex and oxidative





**Fig. 7**  $\gamma$ -T3 down-regulates notch1 and notch2 expression levels. **a, b** The KEGG pathway involved in the mRNA of the genes downregulated in the  $\gamma$ -T3 treatment group compared to the control group. **c** Venn diagram of genes involved in the HPV pathway and the notch signaling pathway in the  $\gamma$ -T3-treated group down-regulated pathway compared to the control group.  $p < 0.05$ .  $n = 3$

phosphorylation pathway and activated the TNF signaling pathway. This further confirmed the role of  $\gamma$ -T3 in treating gastric cancer cells.

Notch signaling is evolutionarily conserved [18]. Notch signaling is a cascade response that plays a key

role in developmental processes, homeostasis and cell differentiation [8]. In mammals, notch signaling includes four receptors (notch1-4), and five ligands (Delta-like ligand-1, -3, -4 and jagged -1,2) [22]. Both receptors and ligands are transmembrane proteins and

intercellular interactions induce signal transduction [66]. The notch signaling pathway regulates cell proliferation, cell cycle progression, differentiation and apoptosis [11, 19]. Notch-induced signaling has been reported to be associated with cancer progression, such as epithelial-to-mesenchymal transition and tumor development [6]. It has been demonstrated that the notch pathway plays a role in tumorigenesis [46]. It was found that activation of the notch pathway can promote the progression of gastric cancer [25, 43]. Moreover, notch signaling was found to be upregulated in gastric cancer, and gene expression of notch1, notch2, and notch3 was elevated in gastric cancer tissues [23, 24]. The mRNA expression levels of notch1 and notch2 were down-regulated in the  $\gamma$ -T3-treated group compared with the control group by analysis of RNA-seq assay. It is suggested that  $\gamma$ -T3 may exert anti-cancer effects through inhibiting notch signaling pathway.

To sum up, our previous study found that  $\gamma$ -T3 induced apoptosis in gastric cancer cells. The  $\gamma$ -T3-treated gastric cancer cells and the untreated group were subjected to RNA-seq assay. The results revealed that  $\gamma$ -T3 may play an anti-cancer role by inhibiting the notch signaling pathway through down-regulating the expression levels of notch1 and notch2. It is indicated that  $\gamma$ -T3 may become a new drug and a new therapeutic target for the treatment of gastric cancer by acting in the notch signaling pathway. To provide a basis for the clinical treatment of gastric cancer.

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#### Authors' contributions

L.X. and J.Y. conceived and designed the experiments. L.X. performed the experiments. L.X. and J.Y. analyzed the data. L.X. and J.Y. wrote the article. All authors read and approved the final manuscript.

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#### Availability of data and materials

Our data is not subject to ethical issues or other conflicts of interest.

#### Declarations

#### Ethics approval and consent to participate

Not applicable.

#### Consent for publication

All authors consent to the publication of this study.

#### Competing interests

The authors declare no conflict of interest.

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