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Glutamine-fructose-6-phosphate transaminase 2 (GFPT2) promotes the EMT of serous ovarian cancer by activating the hexosamine biosynthetic pathway to increase the nuclear location of β-catenin

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ABSTRACT

The hexosamine biosynthetic pathway (HBP), a branch of glucose metabolism, provides a substrate for glycosylation modification, which has a wide-ranging effect on cellular functions. Glutamine-fructose-6-phosphate transaminase 2 (GFPT2) has been reported to regulate the HBP as the first and rate-limiting enzyme. Given the inverse association between GFPT2 expression and survival of patients with serous ovarian cancer (SOC) observed in The Cancer Genome Atlas (TCGA) database, we attempted to investigate the role of GFPT2 and its related mechanisms in SOC. The results showed that GFPT2 was over-expressed in SOC tissues, and positive correlations with advanced stage (FIGO III/IV), suboptimal removal rate and poor survival were observed in 90 SOC patients. Cell migration and invasion were also inhibited in GFPT2 knockdown SKOV3 and HEY cells. The levels of O-linked β-N-acetylglucosamine (O-GlcNAc) and intranuclear β-catenin were evaluated and the observed increase in O-GlcNAcylation induced by GFPT2 may contribute to epithelial-mesenchymal transition (EMT). These data provide novel insights into the function of GFPT2 and O-GlcNAcylation in the EMT and thus the invasiveness SOC.

1. Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy, and the 5-year survival rate is currently less than 30% [1]. The incidence of EOC in China has increased over the past decade, with approximately 52,100 new cases reported in 2015, and approximately 22,500 deaths [2]. Serous ovarian cancer (SOC) accounts for 52% of all pathological types, and approximately 80% are diagnosed at an advanced stage (FIGO III/VI), with widespread metastasis primarily within the abdominal cavity. The mechanism of aggressive SOC is poorly understood.

Glutamine-fructose-6-phosphate amidotransferase (GFAT), also known as GFAT1, has been considered as the rate-limiting enzyme in the first step of HBP currently [3,4]. Interestingly, a new human GFAT subtype fragment was recently identified (GFAT2) due to its amino acid homology (75.6%) with GFAT1, and it has been reported in a few studies that the enzyme activity of GFAT2 in HBP, same as GFAT1 [3,4]. Therefore, more evidence would still be required to confirm its regulatory function on HBP. The genes encoding GFAT2 were designated as *GFPT2* [3,4]. *GFPT2* was previously found to be involved in insulin resistance in type 2 diabetes mellitus [5]. Few reports have described its role in cancer, although up-regulated mRNA level of *GFPT2* has been observed in glioblastoma, lung adenocarcinoma and breast cancer [6,7].

There is recent evidence of O-GlcNAcylation, mediated by an abnormal HBP, in the control of various types of cellular function. It is also implicated in tumorigenesis and resistance to treatment [8,9]. Uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) is the end product of the hexosamine biosynthetic pathway (HBP), and is catalyzed by O-GlcNAc transferase (OGT) to form O-GlcNAc which binds to serine/threonine residues of target proteins [10]. Conversely, O-GlcNAcase (OGA) can remove this modification from the glycosylated protein [11]. O-GlcNAcylation has been observed to act alone, or in concert with other post-translational modifications (PTMs) to alter the function, stability and localization of target proteins [12]. For example, O-GlcNAcylation of β-catenin at threonine 41 reduces modification by the phosphorylation, thus increases stabilization in colorectal cancer cells, indicating that glycosylation modification stabilizes β-catenin

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[13].

Actually, β-catenin plays a pivotal role in the canonical Wnt signal pathway and contributes to the tumorigenesis of EOC. Intranuclear β-catenin combined with T-cell factor and lymphoid enhancer binding factor transcriptionally activate downstream target genes, such as Zeb1 and Slug [14,15]. The serine/threonine residues of β-catenin are the basis for phosphorylation and provide the conditions for glycosylation [16–19]. However, few studies on the relationship of *O*-GlcNAcylation and β-catenin in EOC are available.

In this study, GFPT2 over-expression was detected in SOC and was found to be linked with poor survival. The aim of this study was to verify the role of GFPT2 in SOC and investigate the underlying mechanism of GFPT2 in the regulation of malignant behavior of SOC cells.

2. Materials and methods

2.1. Patients and tissue specimens

Paraffin sections of SOC (n = 90), normal ovarian epithelial tissues (n = 10) and oviducts (n = 30) were collected at the Department of Obstetrics and Gynecology from 2011 to 2018. Fresh tissue specimens of high grade serous ovarian cancer (HGSOC) (n = 7) and normal ovarian epithelial tissues (n = 6) were used to extract protein for further experiments. All patients underwent primary surgery without previous chemotherapy or radiotherapy. These patients had no other malignancies and the histological types and differentiation were independently evaluated by two pathologists. All patients signed an informed consent and the study was approved by the Ethics Committee of Chongqing Medical University (2,015,030,801).

2.2. Cell culture and reagents

HEY cells were obtained from GeneChem (Shanghai, China), and SKOV3 cells were acquired from the American Type Culture Collection (Manassas, VA, USA). ES-2, 3AO, and A2780 cells were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). All cell lines were cultured in RPMI 1640 (Sigma) culture medium containing 10% fetal bovine serum (Kangwei), in a humidified incubator (5% CO_2, 95% air, 37°C). Various concentrations of azaserine (ab145895, Abcam) and glucosamine (G1514, Sigma) were added as indicated.

2.3. Transfection with GFPT2-siRNA

To establish GFPT2 knockdown cells, three small interfering RNA (siRNA) sequences targeting GFPT2 were constructed by the GenePharma Company (SuZhou, China). GFPT2-homo-siRNA1, sense (5′-3′) GGAAUAAUCACGAAGUCAATT, antisense (5′-3′) UUGACUUCG UGAUUAUUCCTT

GFPT2-homo- siRNA2, sense (5′-3′) GCUUCUGAUGCAAGCGCU ATT, antisense (5′-3′) UAGCGCUUGCAUCAGAAGCTT

GFPT2-homo- siRNA3, sense (5′-3′) GGAAAUUUCUGGAAAGCAATT antisense (5′-3′) UUGCUUUCCAGAAAUUUCCTT

Parental cells were cultured with Lipofectamine 3000 (Invitrogen) and siRNA in RPMI 1640 medium.

2.4. Western blotting

Tissues and cells were lysed in radio immunoprecipitation assay (Beyotime) and 1% phenylmethanesulfonyl fluoride (Beyotime), and protease and phosphatase inhibitors (Beyotime) were added. The Nuclear Protein Extraction Kit (Beyotime) was used to extract the cytoplasm and nuclear proteins. Polyvinylidene difluoride membranes were infiltrated with primary antibodies overnight and then transferred and incubated with secondary antibody. Protein bands were detected by the chemiluminescence imaging system (Vilber Fusion FX5)

2.5. Immunohistochemistry

Paraffin sections were dewaxed with xylene and dehydrated by graded alcohol. Endogenous peroxidase in tissues was blocked by 3% H_2O_2, followed by sodium citrate buffer for antigen retrieval. The tissues were covered with normal goat serum and then incubated with primary antibodies overnight. The tissues were then incubated with secondary antibody at room temperature. Images were obtained using an Olympus optical microscope (Olympus, Japan). Staining intensity was evaluated using the histochemistry score (H-score).

2.6. Migration and invasion assay

During the cell migration experiment, SKOV3 cells (50,000 cells) suspended in serum-free medium were spread in the upper chamber (8-μm), and RPMI 1640 culture medium containing 10% fetal bovine serum was added to the lower chamber. The cells in the lower chamber were fixed with 2.5% paraformaldehyde and stained for imaging after 18–24 h. HEY cells (30,000 cells) were added to the upper chamber and cultured for 3–6 h. The procedure for the cell invasion experiment was the same as that for the migration assay, except that the upper chamber was pre-coated with 40 μl of 1 mg/ml Matrigel (BD Biosciences).

2.7. Wound-healing assay

For the wound-healing assay, cells covered the entire surface of the well after transfection with siRNA. A wound was regularly created in the fused cells. Serum-free medium was added to the cell culture for 24 h. The degree of wound healing was observed at 0 h and 24 h, respectively.

2.8. Immunofluorescence

Cells were fixed with 2.5% paraformaldehyde after transfection with siRNA, and goat serum was added. All cells were incubated with primary antibody overnight at 4°C and then with a fluorescent secondary antibody in the dark. Images were captured using a confocal laser scanning microscope.

2.9. Statistical analysis

Each experiment was performed at least three times independently. SPSS 21.0 and GraphPad Prism 5 were used for statistical analysis of the data. $P < 0.05$ was considered statistically significant. The Student's *t*-test was used to analyze the differences between two independent samples, and the chi-square test was used to analyze ovarian cancer patients' clinical data. Pearson's $\chi 2$ test was used to investigate the correlation between two independent groups.

3. Results

3.1. GFPT2 is over-expressed in serous ovarian cancer and is associated with poor prognosis

The GFPT2 expression data in 34 types of human tumors were analyzed by the cBioPortal for Cancer Genomics [20,21]. High GFPT2 expression was found in most malignancies (Fig. 1a). *GFPT2* amplification was more frequent in 1754 samples of SOC with *GFPT2* alteration, especially in HGSOC (Fig. 1b). As oviduct mucosa and the ovarian epithelium originate from the coelomic epithelium, we defined both as normal controls. Compared with normal controls, SOC tissues showed significantly stronger GFPT2 staining (Fig. 1c). The H-score was used to quantify the intensity of GFPT2 staining [22](Fig. 1d), and its relationship with clinical and pathological data is shown in Table 1. The expression level of GFPT2 was correlated with high human epididymis protein 4 (HE4) and cancer antigen 125 (CA125) levels, advanced FIGO

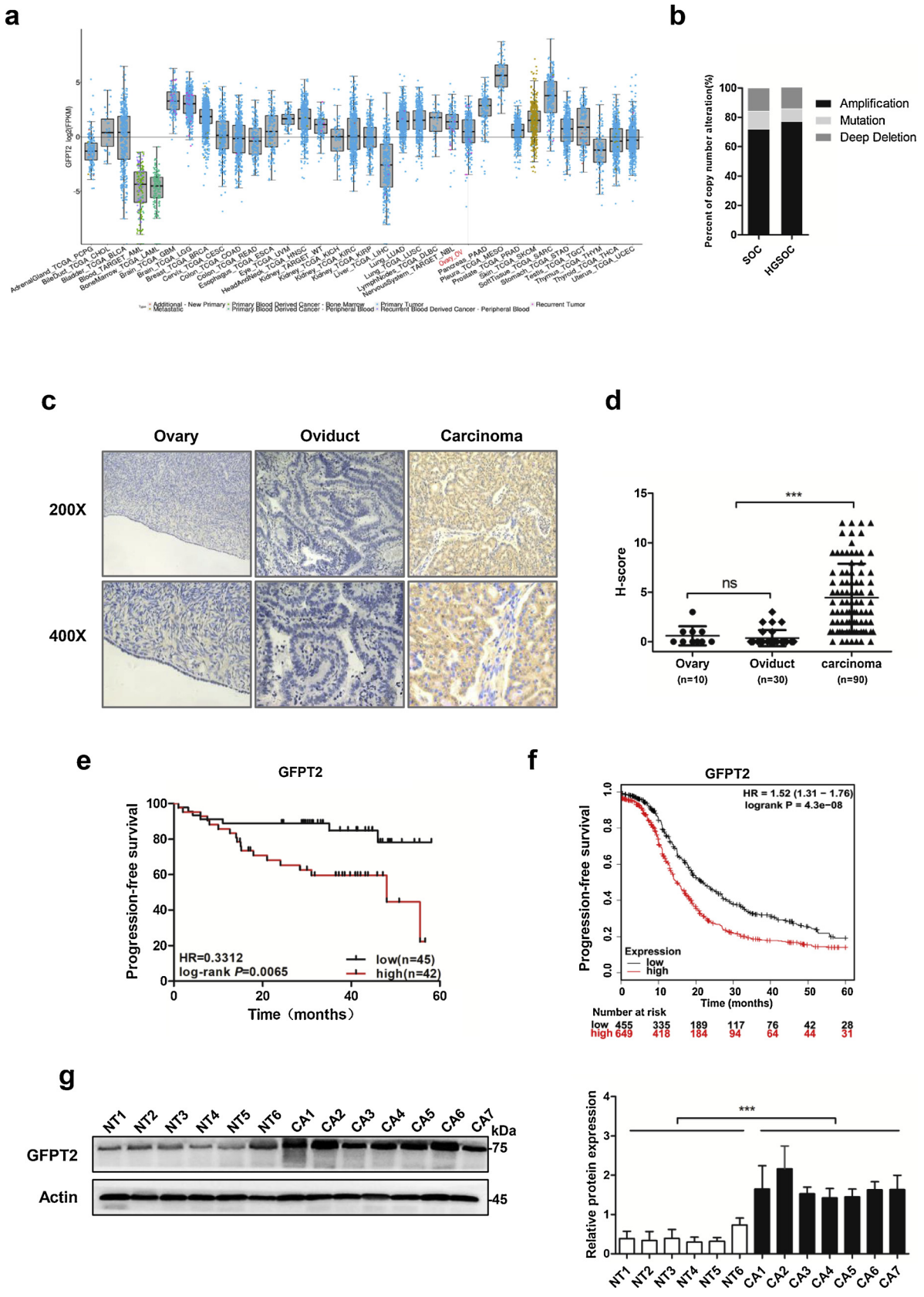

Fig. 1. Amplification of GFPT2 in SOC and its association with poor survival.
(a)The expression of GFPT2 in 34 types of human cancer. (b) The copy number of *GFPT2* in SOC. (c) GFPT2 expression was detected by immunohistochemistry in SOC tissues (n = 90), normal ovarian epithelial tissues (n = 10) and oviduct tissues (n = 30). Left: normal ovarian epithelium; Middle: Oviduct; Right: SOC (upper panel: 20 magnification; lower panel: 40 magnification). (d) H-score was determined to quantify the protein expression level of GFPT2 in SOC. (***p < 0.001, ns: not significant). (e) The PFS of 90 patients with SOC based on GFPT2 protein expression level. Patients lost to follow-up were excluded (n = 3). (f) The PFS of SOC patients with low (n = 455) and high GFPT2 expression (n = 649) from the TCGA database. (g) GFPT2 expression in normal ovarian epithelial tissues and HGSOC tissues was examined by western blot.

Table 1
Immunohistochemical analysis of GFPT2 expression on SOC.

Characteristics	n	Low expression	High expression	χ^2	*p*-value
All cases	90	47	43		
Age (years)					
< 55	56	29	27	0.011	0.915
≥55	34	18	16		
Stage					
I	16	12	4	4.046	0.044
II-VI	74	35	39		
Grade					
I-II	31	22	9	6.66	0.010
III	59	25	34		
CA125					
< 500	40	26	14	4.712	0.030
≥500	50	21	29		
HE4					
< 140	35	25	10	8.468	0.004
≥140	55	22	33		
Debulking surgery					
Optimal	52	32	20	4.284	0.038
Suboptimal	38	15	23		

stage and higher histological grade, which usually reflect the progression of SOC. As expected, high GFPT2 expression was linked to poor progression-free survival (PFS) in patients with SOC (Fig. 1e), which was consistent with the results obtained from the TCGA database [23] (Fig.1f). GFPT2 expression was also detected in seven fresh samples of HGSOC and six normal ovarian epithelial samples by immunoblotting, and the expression of GFPT2 in cancerous tissues was significantly higher than that in normal tissues (Fig. 1g). These data confirmed the over-expression of GFPT2 in most patients with SOC, and its association with aggressive features and poor prognosis.

3.2. High expression of GFPT2 promotes cell invasion and migration in SOC

346 co-expressed genes of *GFPT2* in SOC were also analyzed. Gene Ontology analysis classified these co-expressed genes into various terms using the online software Metascope [24]. The most significant terms were ranked by adjusted *p*-values, including vasculature development, cell-substrate adhesion, extracellular matrix organization and others (Fig. 2a). These data suggested that GFPT2 may affect the invasion of tumor cells.

To further investigate this issue, a panel of ovarian tumor cell lines was selected and GFPT2 expression was determined. Two SOC cell lines (SKOV3 and HEY cells) with high potential invasiveness and high GFPT2 expression were selected (Fig. 2b). Small interference RNA was employed to knock down the expression of GFPT2 (Fig. 2c, d). Two of the three target sequences displayed stronger knockdown efficiency in SKOV3 (siRNA1 and siRNA2) and HEY (siRNA1 and siRNA3) cells. Migration and invasion assays were performed and the results showed that both cell migration and invasion were significantly decreased when GFPT2 expression was silenced (Fig. 2e, f). Reduced wound healing (Fig. 2g, h) was consistent with these findings.

3.3. GFPT2 knockdown inhibited O-GlcNAcylation in SKOV3 and HEY cells

To testify whether the change in GFPT2 expression reflected the level of O-GlcNAc in SOC, glucosamine was added to wild-type SKOV3 and HEY cells, as glucosamine can bypass GFAT to enter the HBP. As expected, the level of O-GlcNAc increased when the concentration of glucosamine increased (Fig. 3a, b). Conversely, the level of O-GlcNAc was suppressed when the concentration of azaserine (Fig. 3c, d), a nonspecific blocker of the HBP, was added. These findings demonstrated that the degree of glycosylation modification was reflected by the level of O-GlcNAc in SKOV3 and HEY cells. Meanwhile, the changes of HEY cells invasion/migration were changed accordingly (Fig. S1). Besides, GFPT2 knockdown by siRNA inhibited the production of O-GlcNAc in SKOV3 and HEY cells (Fig. 3e, f). However, the levels of OGT and OGA were unchanged, revealing no clear correlation with the change in O-GlcNAc level.

3.4. Down-regulated GFPT2 expression in SOC cells reduced the nuclear location of β-catenin and expression of Slug and Zeb1

Considering the association between increased glycosylated β-catenin level and its enhanced cellular stability [18,19], the impact of GFPT2 on SOC cells was evaluated. As shown in Fig. 4a and 4b, although the expression of β-catenin decreased slightly in GFPT2-siRNA cells, its target transcription factor proteins Slug and Zeb1 declined. Moreover, suppressed expression of β-catenin was mainly detected in the nucleus both in SKOV3 (Fig. 4c, d) and in HEY (Fig. 4e, f) cells, and reduced nuclear location of β-catenin was visualized in GFPT2 knockdown cells by immunofluorescence staining (Fig. 4g, h and Fig. S2a, b).

Slug and Zeb1 are known to negatively regulate E-cadherin and thus initiate EMT. To determine whether this occurred in GFPT2 knockdown SOC cells, the markers of EMT were assessed. In GFPT2 knockdown cells, increased expression of E-cadherin was observed, with a slight or moderate reduction in N-cadherin and Vimentin expression (Fig. S3a, b), although only the change in E-cadherin was observed by immunofluorescence staining (Fig. S3c, d).

3.5. Clinical correlation between O-GlcNAc and β-catenin in SOC

Given that studies have reported the combination of O-GlcNAc with β-catenin at the cellular level [13], we determined the levels of O-GlcNAcylation and β-catenin in SOC tissues and normal controls using immunohistochemistry to further elucidate the relationship between O-GlcNAc and β-catenin in clinical practice (Fig. 5a, b). The staining intensity of both O-GlcNAc and β-catenin in SOC tissues was significantly stronger than that in normal controls. Consistent staining of O-GlcNAc and β-catenin was confirmed in the same fields (Fig. 5c, d) in 52 patients. High O-GlcNAc level and β-catenin expression were confirmed by the H-score in SOC tissues compared with normal controls (Fig. 5e, f). The positive correlation between O-GlcNAc and β-catenin was observed, with correlation coefficient of 0.591 (Fig. 5g).

The impact of O-GlcNAc and β-catenin on the prognosis of patients with SOC was also assessed. Patients with a high level of O-GlcNAc or β-catenin expression had a worse PFS than those with low levels, although the difference was not statistically significant (Fig.5h, i). Given the close correlation between O-GlcNAc and β-catenin in tumor tissue and cells, patients with consistent distribution of both were analyzed, and the negative impact of O-GlcNAc and β-catenin on PFS was further validated (Fig.5j).

4. Discussion

Currently, the important role of GFPT2 in tumorigenesis has been gradually recognized. It has been reported recently that the expression of GFPT2 was up-regulated by NF-κB directly in mesenchymal lung adenocarcinomas cancer cells, to promote tumor migration [25]. And the increased GFPT2 expression has been associated with worse survival outcomes among patients with non-small cell lung cancer [25]. Additionally, the high GFPT2 expression induced by TGF-β in cancer-associated fibroblasts was thought to make lung adenocarcinoma more aggressive than squamous cell carcinoma [7]. However, the underlying mechanisms still remain unclear.

In this study, GFPT2 over-expression was found in patients with SOC and was associated with advanced FIGO stage, suboptimal surgery, and poor survival. Consistently, the decreased invasion and migration was observed in GFPT2 knockdown cells (Fig.2), implying that GFPT2 is an oncogene involved in the aggressive potential of SOC, and this

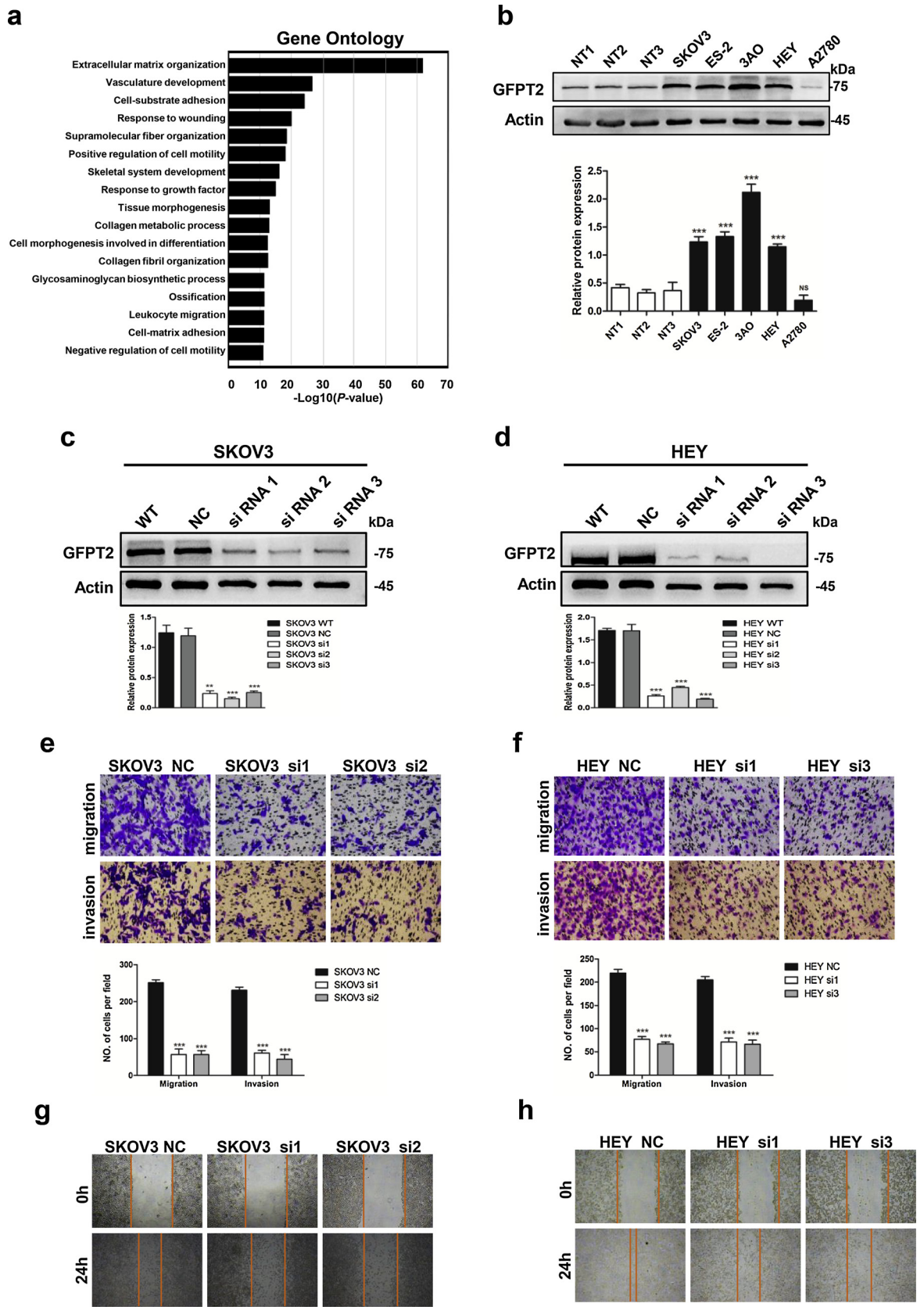

Fig. 2. GFPT2 knockdown decreased cell invasion and migration of SOC.
(a) Gene Ontology analysis identified genes co-expressed with *GFPT2*. (b) GFPT2 expression in ovarian cancer cell lines was examined by western blot. (c–d) SKOV3 (c) and HEY (d) cells were transfected with three siRNA sequences targeting *GFPT2* (siRNA1, siRNA2 and siRNA3). WT and NC were used as controls (WT, wild-type; NC, negative control). (e–f) Decreased migration and invasion due to down-regulated GFPT2 expression in SKOV3 (e) and (f) HEY cells (***p < 0.001). (g–h) Wound healing assay of SKOV3 (g) and HEY (h) cells.

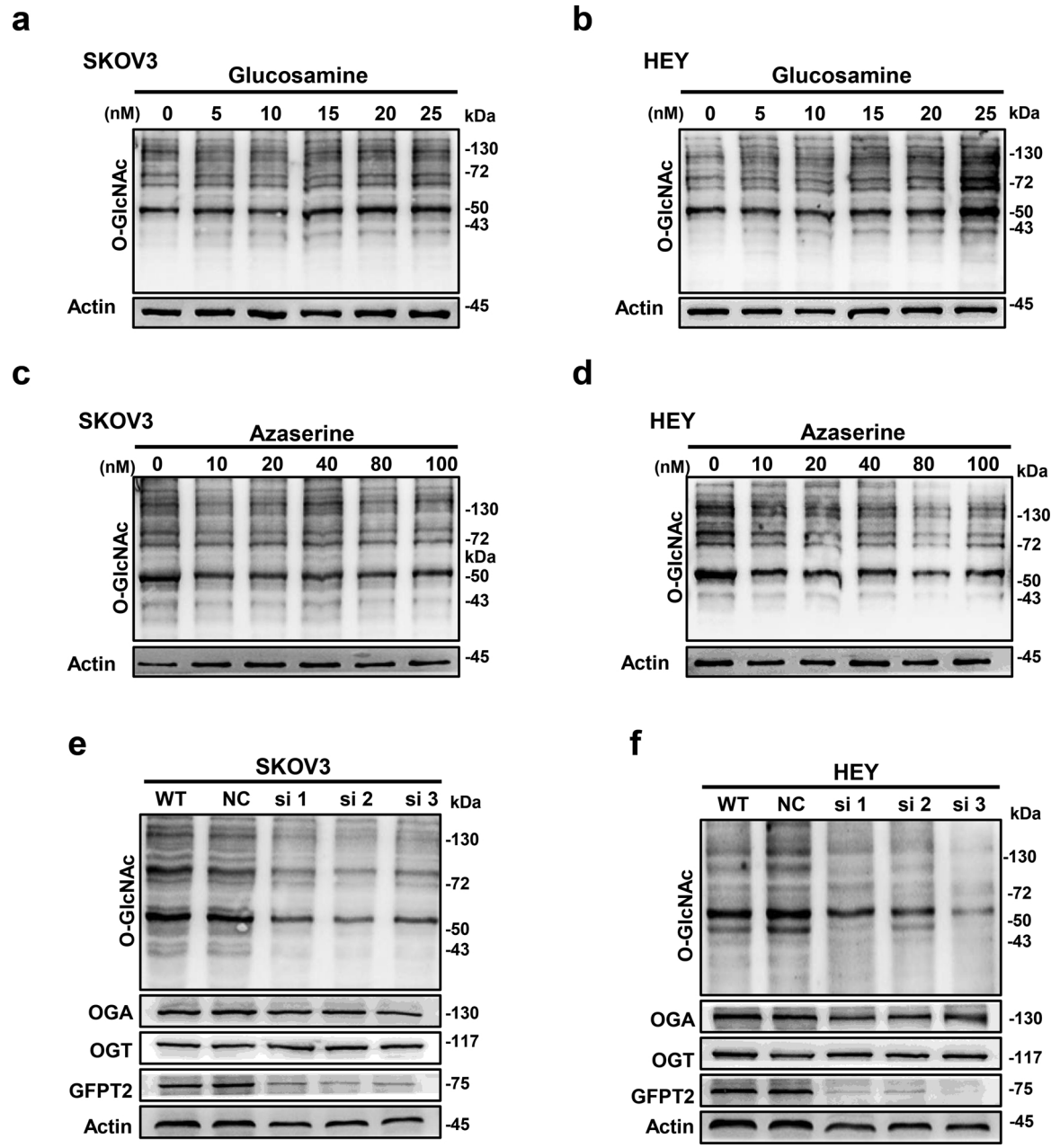

Fig. 3. Changes in O-GlcNAc level due to modulators or GFPT2 knockdown in SKOV3 and HEY cells.
(a–b) Following treatment with glucosamine for 24 h, western blot was performed to determine the change in O-GlcNAc in SKOV3 (a) and HEY (b) cells. (c–d), SKOV3(C) and HEY (D) cells were treated with azaserine, followed by evaluation of O-GlcNAcylation by western blot. (**e-f**) GFPT2 expression was knocked down by siRNA, and the level of O-GlcNAc in SKOV3 (e) and HEY (f) cells was detected by western blot.

provides a new sight into the function of GFPT2 in ovarian cancer. Referring to the association of the progression of lung cancer with GFPT2 and EMT [7], the elevated level of O-GlcNAcylation induced by GFPT2 in SOC might contribute to EMT and malignant invasion probably through increasing the expression of intranuclear β-catenin, which thus facilitates its translocation and signal activation (Fig.6). These results provided a good support for previous findings.

O-GlcNAcylation is a dynamic post-translational modification which is catalyzed by OGT and removed by OGA. Following high-glucose treatment, elevated levels of O-GlcNAc and OGT in cells were also reported to be linked with cancer development. But in SKOV3 and HEY cells with GFPT2 knocked down, a decreased level of O-GlcNAcylation was detected, while with no change in OGT or OGA expression, indicating that the impact of GFPT2 on O-GlcNAc formation and thus upon O-GlcNAcylation in SOC is independent of the effect of OGT/OGA. Obviously, this finding differs from previous studies.

Recently, glycosylation has become known for its role in PTM, which is involved in multifaceted cell events. Using glycomic analysis, O-GlcNAcylation was determined to have extensive cross-talk with phosphorylation, and acts, at least partially, via its interplay with phosphorylation [12,26,27]. On the basis of the consistency of high GFPT2 expression and O-GlcNAc level (Fig. 3), the allied nuclear location of β-catenin followed by the activation of its target genes Zeb1 and Slug (Fig. 4), we speculated that in SOC cells, GFPT2-mediated glycosylation might compete with phosphorylation in specific site(s) of β-catenin serine or threonine residues. This would facilitate the release of β-catenin from this complex and its translocation into the nucleus, with more intranuclear β-catenin driving transcription of Zeb1 and Slug, which control EMT. Given the multiple serine/threonine site(s) of β-catenin and properties of glycosylation, the specific modification site

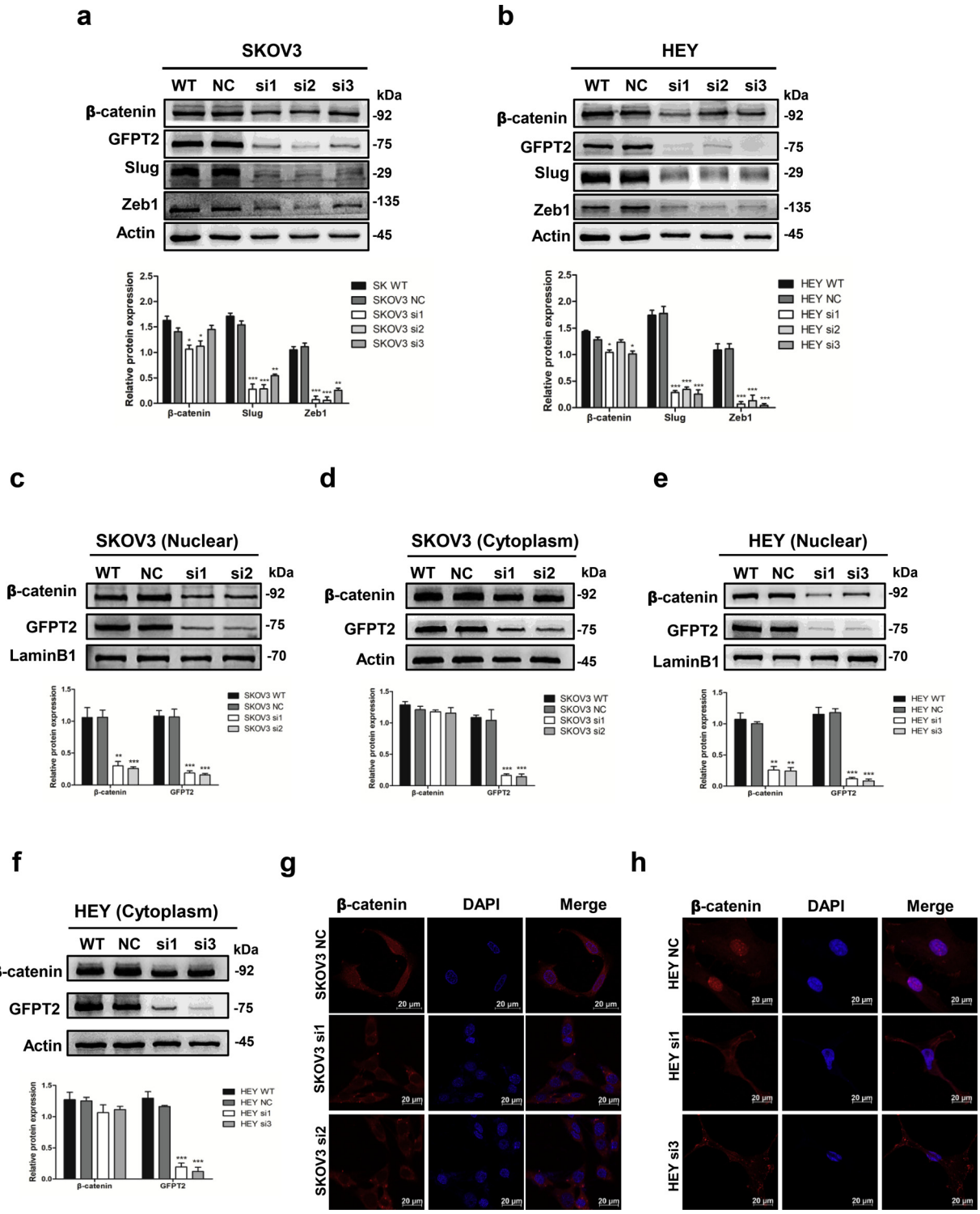

Fig. 4. Decreased nuclear translocation of β-catenin following GFPT2 knockdown.
(a–b) Western blot was carried out to examine the expression of ß-catenin, Slug and Zeb1 in SKOV3 (a) and HEY (b) cells with or without GFPT2 knockdown. (c–f) The level of ß-catenin expression in the nucleus and cytoplasm with or without GFPT2 knockdown was assessed by western blot in SKOV3 (c–d) and HEY (e–f) cells, respectively. (g–h) SKOV3 (g) and HEY (h) cells were transfected with siRNA against GFPT2 for 24 h, and nuclear location of ß-catenin was determined by immunofluorescence. Scale bar, 20 μm.

(s) and competition with phosphorylation should be identified in further studies.

Besides PTM, β-catenin per se was found to have a dual role in cells: a structural role and a signaling role. It was originally found to indirectly modulate the cytoskeleton by taking part in the formation of cadherin-based adherens junctions (structural role) [28]. O-GlcNAcylation of β-catenin was reported to regulate its nuclear localization and transcriptional activity, and to block its association with E-cadherin. The loss of E-cadherin transport to the surface of epithelial cells is critically important to the mechanisms underlying cancer cell metastasis [29]. The two downstream target genes of β-catenin, Zeb1 and Slug, inhibit the expression of E-cadherin [14,15]. Our data did not

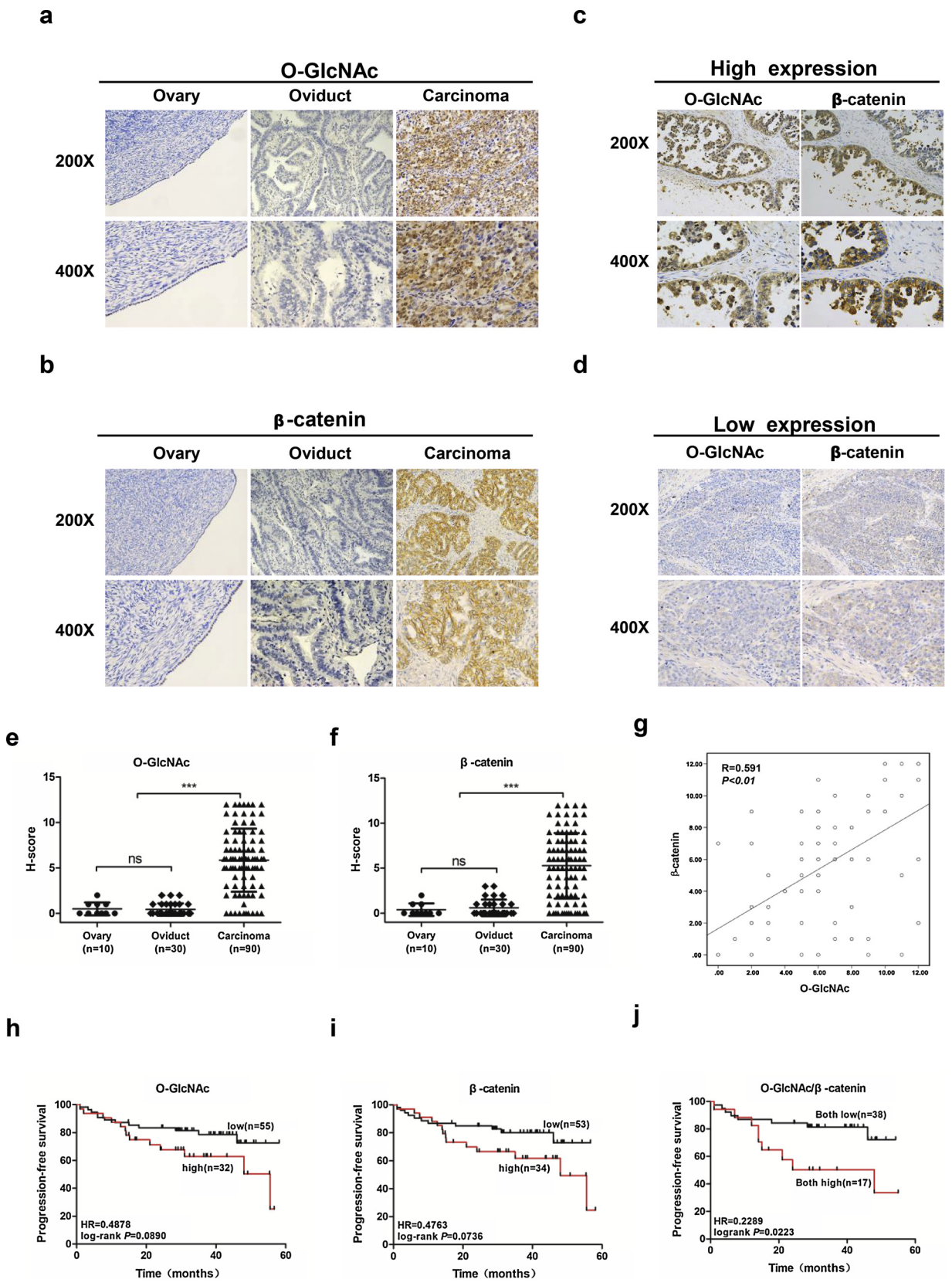

Fig. 5. High O-GlcNAcylation in SOC in vivo was positively correlated with ®-catenin.
(a–b) *O*-GlcNAcylation level (a) and ß-catenin expression (b) in SOC tissues (n = 90), ovarian epithelial tissues (n = 10), and oviduct tissues (n = 30) were determined by immunohistochemistry. (c-d) The positive relationship between O-GlcNAc and ß-catenin observed in the same field and images were acquired using tissues from patients with high (c) and low (d) expression. (e–f)H-score was calculated to assess the level of O-GlcNAc (e) and ß-catenin (f) in tissues (***p < 0.001, ns: not significant). (g) Correlation between O-GlcNAc and ß-catenin protein expression based on the H-score. (h–i) The PFS of SOC patients with high vs. low O-GlcNAc level (h) and with high vs. low ß-catenin expression (i) (n = 90). (j) The PFS of SOC patients with high vs. low O-GlcNAc / ß-catenin expression.

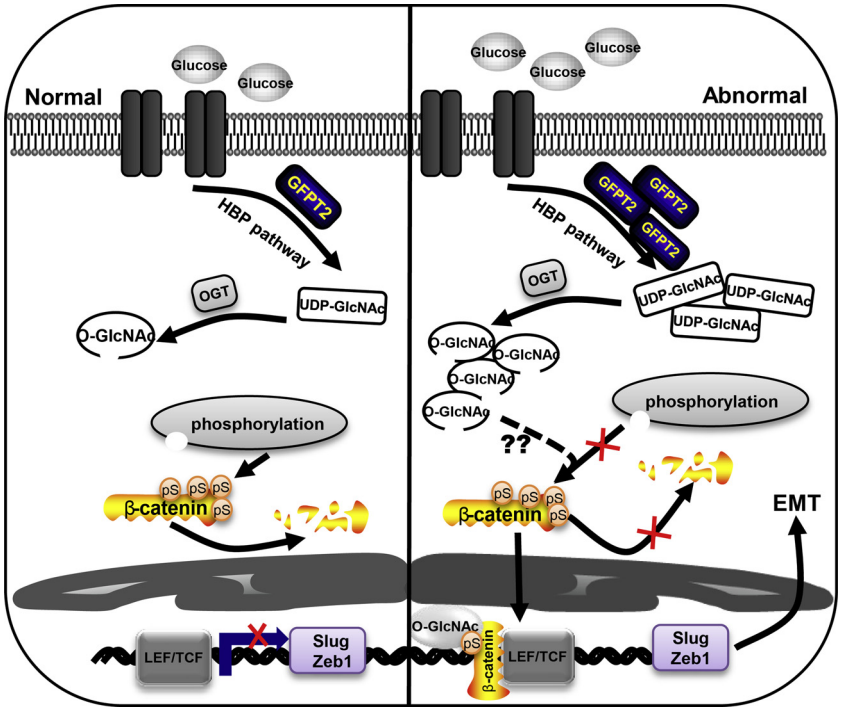

Fig. 6. Schema chart.

show a significant reduction of two other known EMT markers Vimentin and N-cadherin in GFPT2 knockdown cells, which was inconsistent with the expected results. In a recent report which analyzed tumor samples from 198 patients with HGSOC, it was concluded that the expression of EMT-related transition factors (such as Slug) other than the present EMT phenotypes was associated with advanced disease and poor overall survival [30]. It is still reasonable that GFPT2 is involved in the SOC EMT process.

On the other hand, E-cadherin was observed to combine with O-GIcNAc directly, indicating that E-cadherin is a substrate of O-GlcNAcylation [17]. The high level of glycosylation was reported to down-regulate the expression and activity of E-cadherin, thus enhances the invasion and metastasis of tumor cells [17,31,32]. Referring to these findings above, it would be reasonable to support the partial impact of GFPT2 inhibition upon the restoration of E-cadherin levels ovarian cancer cells.

In summary, these findings demonstrate that GFPT2 may act as a novel oncogene in SOC by activating the HBP independent of OGT or OGA. As a result, increased O-GlcNAcylation might interfere with the location of β-catenin and promote the progress of EMT.

5. Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of Competing Interest

The authors declare that they have no conflict of interest

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.prp.2019.152681.

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