Int J Clin Exp Pathol 2018;11(7):3426-3435
www.ijcep.com /ISSN:1936-2625/IJCEP0077189

Original Article

Over expression of GPR30, indicating poor prognosis and promoting proliferation, upregulates Beclin-1 expression via p38MAPK signaling in esophageal squamous cell carcinoma progression

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Received February 6, 2018; Accepted May 10, 2018; Epub July 1, 2018; Published July 15, 2018

Abstract: Background: Beclin-1 and GPR30, both very important proteins, have been associated with tumor development. In our pre-experiment, the co-expression of GPR30 and Beclin-1 was observed in esophageal squamous cell carcinoma (ESCC), an observation not reported in other studies. The aim of our research was to investigate the relationship of these two proteins in the further progression of ESCC. Methods: The over expression of GPR30 and Beclin-1 proteins was observed and confirmed by immunohistochemistry and immunofluorescence arrays. By interfering with GPR30 and p38 MAPK expression in EC-109, KYSE510, and KYSE3 cell lines, MTT and a scratch wound healing assay were used to investigate the impact of the GPR30 protein on the proliferative and migrative abilities of ESCC cells. A co-immunoprecipitation assay was used to observe the interaction between the p38 MAPK and Beclin-1 proteins; meanwhile, at a different time, in each group, the GPR30, MAPK, p ERK1/2, p38 MAPK, and Beclin-1 proteins were analyzed. The correlation between GPR30, Beclin-1 expression levels, and the clinical characteristics were evaluated by Mann-Whitney and chi-square tests. Using Kaplan-Meier plots and the Cox proportional hazard model analysis, we determined overall survival (OS) and progression free survival (PFS). Results: GPR30 and Beclin-1 were over expressed significantly in ESCC (both p=0.0000) and were distributed into cytoplasms the most (the former p=0.0223, latter p=0.0018). In contrast to the non-agonist group, the abilities of GPR30 in promoting cell proliferation and metastasis were observed in the agonist group, and the effects could be blocked by p38MAPK inhibitors. In further assays, GPR30 agonists, via binding to GPR30, up-regulated Beclin-1, MAPK, and p38 MAPK expression, and Beclin-1 expression was reversed in the p38MAPK inhibitor group. In the GPR30 agonist group, an interaction between p38MARK and Beclin-1 was observed, but no similar results were observed in the non-agonist group. The high expression of both GPR30 and Beclin-1 was observed with p-stage and pT advancing (both p<0.0001). In a Kaplan-Meier analysis, compared to GPR30's negative expression, high expression identified a group of patients with the shortest overall survival (OS, p=0.0072) and progression free survival (PFS, p=0.0074). The Cox proportional hazard models revealed that they predicted a short time to OS (p=0.0125). Conclusion: Over expression of GPR30 up-regulated Beclin-1 expression and indicated a poor prognosis and may promote ESCC development via p38 MAPK in ESCC progression.

Keywords: Esophageal squamous carcinoma (ESCC), prognosis, G protein-coupled receptor 30 (GPR30), G-1 (GPR agonist)

Introduction

Esophageal carcinoma is the 8th most common cancer and 6th in mortality worldwide, but it is one of the least studied [1, 2]. Esophageal squamous cell carcinoma (ESCC) is the main histological type that makes up more than 70 percent of the diagnoses of esophageal cancers [3, 4]. Recently, especially in China, there has been a dramatic rise in the incidence and mortality due to ESCC [5, 6]. ESCC carries a poor prognosis with an overall five-year survival rate, and more than 50 percent of patients are in an advanced stage of the disease at the time of diagnosis, having lost any chance of successful tumor resection [7]. Identification of

early, sensitive, and specific diagnostic and prognostic evaluation markers could provide important and rich information for diagnosis, prognosis, and possible treatment options of esophageal carcinoma.

The extensive functions of estrogen receptors in non-estrogen target tissue tumors (e.g., lung, colon, and liver cancer) have attracted more attention. Due to their complex structure and the many different types, the roles of estrogen receptors showing in these tissue tumors are very complex and very controversial [8, 9]. Recently, G protein-coupled receptor 30, a new receptor, has been recognized as important intermediary receptor for estrogen functions in many tissue tumors, e.g., breast and endometrial cancer [10-12]. Beclin-1, an essential mediator of autophagy, was originally discovered during the course of a yeast two-hybrid screen of a mouse brain c DNA library [13, 14]. Some researchers reported that the Beclin-1 protein may play a role in tumorigenesis due to the increased expression of the protein in the malignant colorectal and gastric epithelial cells [15, 16].

In preparing for our research, we found that both GPR30 and Beclin-1 have a high expression, and there is a correlation between them in ESCC. Though there are some studies on GPR30 and Beclin-1 proteins in many kinds of tissue tumors, only very few of these discuss ESCC. Our initial research motivated us to analyze the relation between them, and to further explore their mechanisms in ESCC progression.

In this study, we identified that both GPR30 and Beclin-1 proteins have a high expression and may serve poorly in prognosis. These interesting results may imply a novel clinical treatment possibility for esophageal carcinoma.

Materials and methods

Human tissue samples, cell lines and reagents

159 cases with esophageal squamous carcinomas that underwent surgical procedures at the First Affiliated Hospital of Sun Yat-sen University were recruited to a tissue bank protocol approved by the Institutional Review Board (IRB), with the data ranging from 2003-2013. The relevant clinic pathological data collected included age; gender; p staging; pT and pN; follow-up of overall survival (OS) and progression free survival (PFS). EC-109, KYSE510 and KYSE30 cell lines were purchased from the American type culture collection (Rockville MD, USA) and were cultured in the RPMI1640 medium (Invitrogen, Carlsbad, CA, United States), supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2. cells were cultured in a phenol-red free medium supplemented with 5% charcoal stripped FBS at least 18 h before experimental treatment. Primary antibodies (GPR30, p38MAPK, Beclin-1, ERK1/2 and MAPK [17, 18]) shown to be highly specific in both Western blot and IHC in our laboratory.

Immunohistochemistry

Anti-GPR30 and Beclin-1 were used at 1:50 antibody dilution, with overnight incubation at 4°C. We set a negative control using PBS as the primary antibody. Similar steps were performed in normal esophagus tissue. Their expression was defined as positive if distinct staining of the cytoplasm, cytomembrane or nuclear was observed in at least 10% of the tumor cells. Cases were scored independently by the three authors. Discordant results were reevaluated jointly to reach a consensus.

Immunofluorescence assay

GPR30 and Beclin-1 antigen substrate were incubated with a dilution anti-GPR30 and Beclin-1 (1:200) at 4°C overnight. CY3-conjugated anti-IgG (Santa Cruz Biotechnology, Inc.) was used as the secondary antibody at a 1:100 dilution for 1 hour. Washed with PBS and counterstained cell nucleus with DAPI for 5 minutes. A fluorescence microscope (Leica DM1000, Germany) was used for examination.

Western-blot analysis

The cells were homogenized in protein lysis buffer (RIPA) containing 10% protease inhibitor (Sigma-Aldrich), and the protein concentrations were then quantified using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The following primary antibodies (anti-GPR30, p38 MAPK, MAPK, ERK1/2 and Beclin-1, Santa Cruz, CA, USA) were then

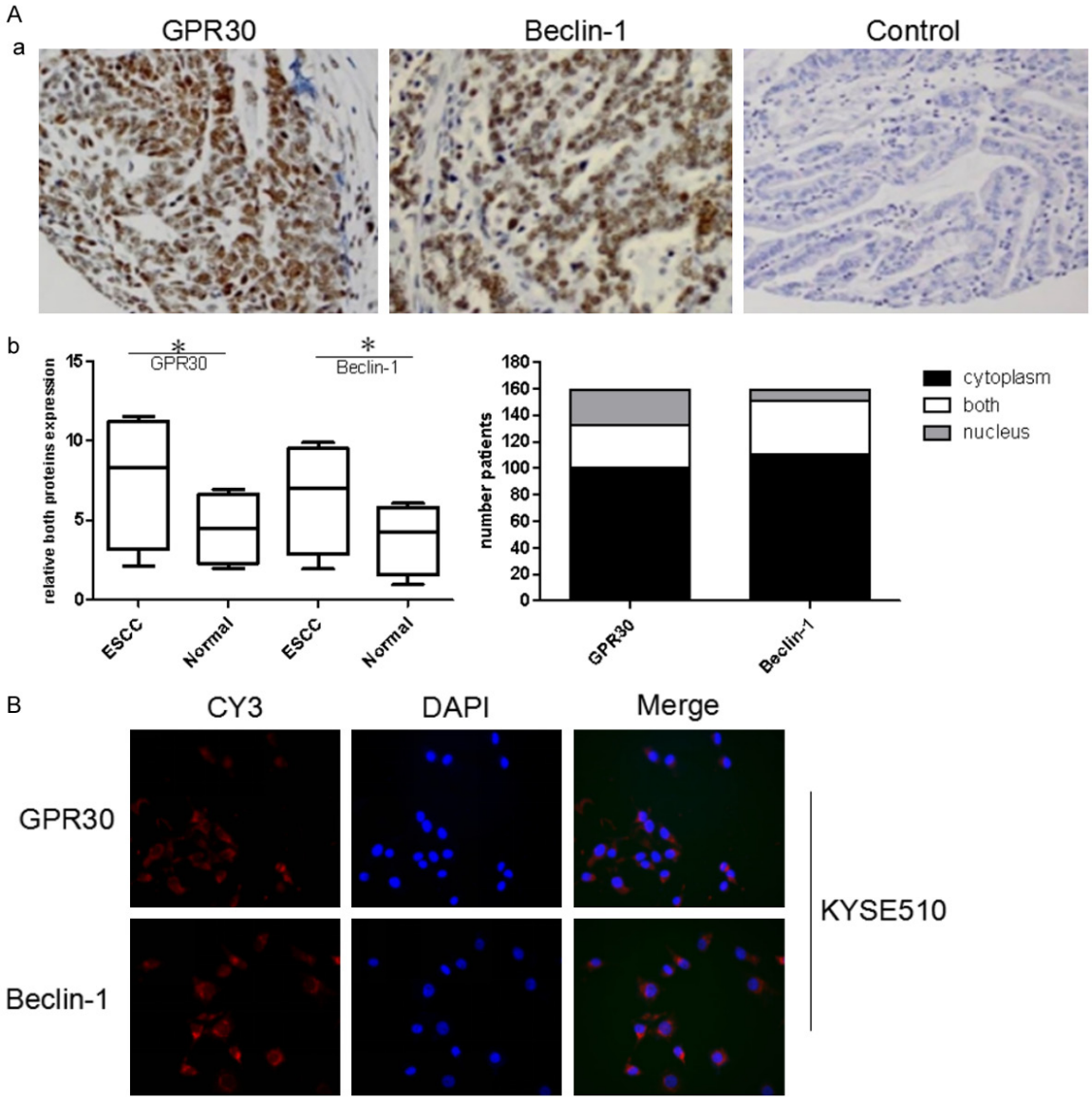

Figure 1. Expression of GPR30 and Beclin-1 in esophageal squamous cell carcinoma (ESCC) via IHC and IF assays. Expression of both proteins was defined as positive according to method. Aa: GPR30 and Beclin-1 expression (×200); Ab: Both proteins were located mainly in cytoplasms, and over expression in ESCC. B: To identify both proteins' expression and distribution, IF arrays were performed in ESCC cell lines (just KYSE510 was shown).

applied to the membranes according to the manufacturers' protocols. The membranes were washed and treated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology Inc.). A similar process was carried out for beta-actin. The results were visualized with chemiluminescence (ECL).

Plasmid constructs and transfection

The p38MAPK and Beclin-1 [40] over expression plasmids p3xFlag-p38MAPK and p XJ40-Myc- Beclin-1 were produced through the ligation of PCR-generated inserts into p3xFlag-CMV-7.1-2 and pXJ40-Myc-SOX4 respectively.

The purified plasmid p3xFlag-p38MAPK and p XJ40-Myc-Beclin-1 were transfected into 70% confluent EC-109, KYSE510 and KYSE30 cell lines respectively, using Lipofectamine 2000 reagents in a total volume of 1 ml of Opti-MEM (Invitrogen), as a study reported [19].

Co-immunoprecipitation

12 h after transfection, one group of cells was treated with mock (GRP30 against G-1, and no

Table 1. The relation between GPR30, Beclin-1 expression and esophageal carcinoma clinicopathological parameters

		GPR30		p	Beclin-1		*P*
		+	-		+	-	
Gender	Male	40	40	1.000	43	37	0.2055
	Female	39	40		34	45	
Age (years)	<60	40	42	0.8744	45	37	0.1129
	≥60	39	38		32	45	
p Staging	I	5	31	<0.0001	4	32	<0.0001
	II	11	26		12	25	
	III	32	13		29	16	
	IV	31	10		32	9	
pT	T1	8	31	<0.0001	10	29	0.0005
	T2	11	25		15	21	
	T3	30	15		27	18	
	T4	30	9		27	12	
pN	N0	42	38	0.5272	40	40	1.000
	N1-3	37	42		39	40	

G-1 in control group) for 24 h. 500 ug of protein from the cell lysates was incubated with 2 ug of anti-Myc antibody or normal rabbit IgG (Santa Cruz Biotechnology) for 16 h at 4°C. To each sample, we added 20 µl of protein A/G-agarose beads (Santa Cruz Biotechnology), incubated for 1 h and washed thrice with radio-immunoprecipitation assay buffer. Then, the complex was resolved on a 10% SDS-PAGE, transferred to the membrane and blotted with anti-Flag antibody (1 µg/mL, Sigma) or anti-Myc antibody. Membranes were incubated with ECL reagent (Super signal west Pico, Pierce) and exposed to autoradiographic film.

Cell proliferation assays

12 h after transfection, the cells were seeded in triplicate at a density of 5×10^3 cells per 96-well plates. Then, at 24 h, 48 h, 72 h, 96 h after G-1 treatment respectively, 10 µl of a modified 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide solution (Dojindo, Kumamoto, Japan) were added to the culture and reaction mixtures were incubated at 37°C for 2 h.

Scratch wound-healing assay

The cells were seeded onto 6-well tissue culture dishes and grown to confluence. Each confluent monolayer was wounded linearly using a 200 µl pipette tip and washed 3 times with PBS. Thereafter, the cell morphology and movement were observed and photographed at 0 h, 24 h, 48 h and 72 h.

Statistical analysis

The data were expressed as the means ± standard deviation. Mann-Whitney and chi-square tests were used for statistical analysis using Graphpad prism 5. Associations with overall survival (OS) and PFS were analyzed initially by Kaplan-Meier plots (Log-rank test). Cox multivariate proportional hazards regression models were used to assess the prognostic power of these parameters. The *P* Value of <0.05 was considered to be significant, and * indicates P<0.01.

Results

Expression of GPR30 and Beclin-1 protein in esophageal squamous cell carcinoma (ESCC)

IHC analyses revealed that GPR30 and Beclin-1 were over expressed in ESCC (former 84.21%, "80 out of 95"; latter 87.36%, "83 out of 95"). Compared to Normal (both <35%), both of their expressions were significantly higher (both p=0.0000) in ESCC and were mainly distributed in cytoplasms (**Figure 1A**). Moreover, similar results were observed in EC-109, KYSE510 and KYSE30 cell lines via an immunofluorescence assay (**Figure 1B**).

The relation between GPR30, Beclin-1 expression and clinical characteristics

The associations between GPR30 and Beclin-1 expression and a range of standard clinic pathologic parameters were tested. There was significant correlation between GPR30, Beclin-1 and p Staging (GPR30 vs p Staging, Beclin-1 vs p Staging, both p<0.0001), together with the relation between GPR30, Beclin-1 and pT (former p<0.0001, latter p=0.0005). Meantime, one interesting result, with p Staging advanced, the percentage of both proteins' positive expression cases increased. Compared to the expression in the lymph node metastasis negative group, there was no significant value in the positive group (**Table 1**).

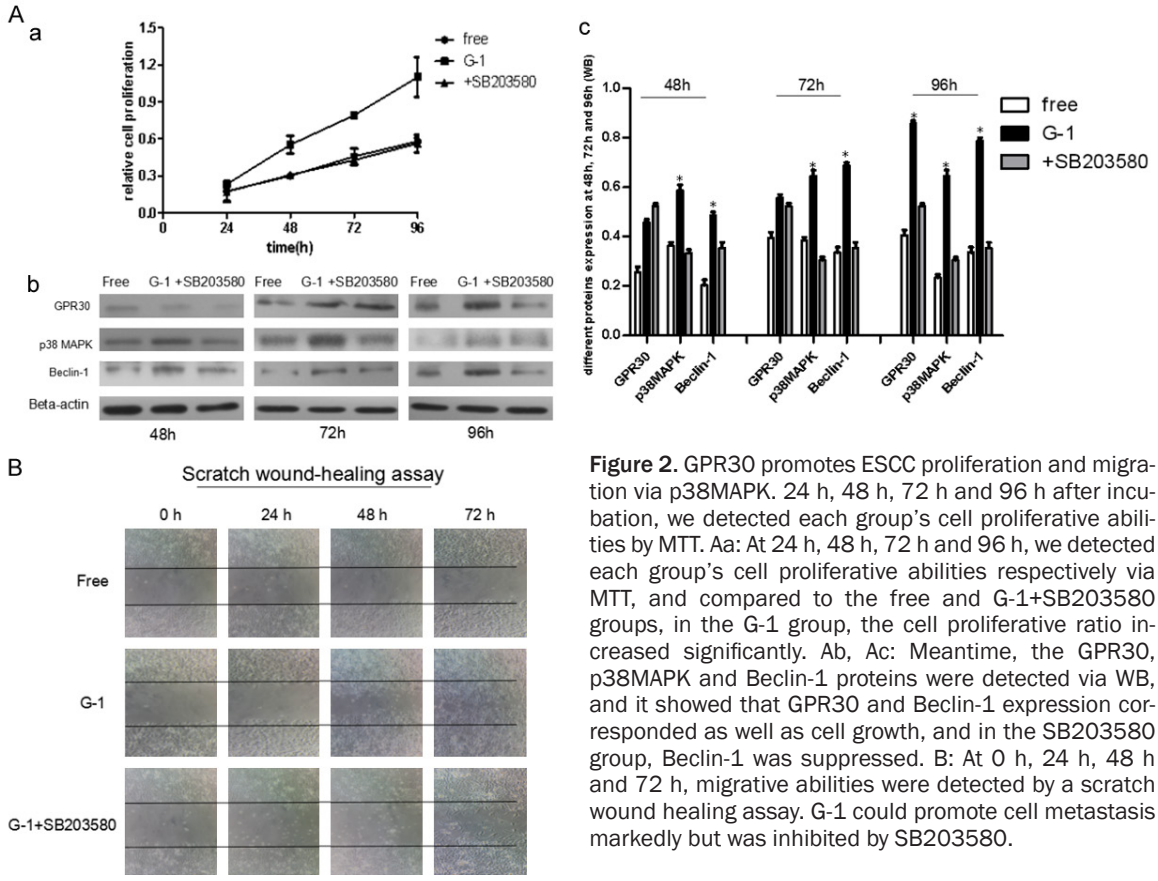

Figure 2. GPR30 promotes ESCC proliferation and migration via p38MAPK. 24 h, 48 h, 72 h and 96 h after incubation, we detected each group's cell proliferative abilities by MTT. Aa: At 24 h, 48 h, 72 h and 96 h, we detected each group's cell proliferative abilities respectively via MTT, and compared to the free and G-1+SB203580 groups, in the G-1 group, the cell proliferative ratio increased significantly. Ab, Ac: Meantime, the GPR30, p38MAPK and Beclin-1 proteins were detected via WB, and it showed that GPR30 and Beclin-1 expression corresponded as well as cell growth, and in the SB203580 group, Beclin-1 was suppressed. B: At 0 h, 24 h, 48 h and 72 h, migrative abilities were detected by a scratch wound healing assay. G-1 could promote cell metastasis markedly but was inhibited by SB203580.

GPR30 promotes ESCC cell proliferation and migration via p38 MAPK

In the study, we obtained some interesting results that agreed with our preliminary research. 24 h, 48 h, 72 h and 96 h after cells were incubated with G-1 or not, MTT and Scratch wound-healing assays were performed on all groups. The relative ratio of cell proliferation was detected. Compared to the free group, in the G-1 group, the cell proliferative ration increased significantly (**Figure 2Aa**). However, the promoting growth effect was suppressed by the p38MAPK inhibitor in the SB203580 group (**Figure 2Aa**). at 24, 48 and 72 h, via detecting GPR30, p38MAPK and Beclin-1 protein expression in every group, we found that Beclin-1 was highly expressed in G-1 group, and was consistent with GPR30 expression, but was blocked by the p38MAPK inhibitor (**Figure 2Ab**, **2Ac**).

In the scratch wound-healing assay, in the G-1 group, with a longer incubating time, the cell climbing effect was more apparent. Likewise, with the proliferation assay results, in the SB203580 group, the G-1 promoting effect was eliminated and showed similar results with the free group (**Figure 2B**).

GPR30 up-regulate Beclin-1 expression via p38MAPK

To explore the relation between GPR30 and Beclin-1 protein further, some interfering, co-immunoprecipitation and Western-blot assays were performed. 24 h after cells incubated with G-1 or no interfering reagent, in the G-1 group, GPR30, ERK1/2, p38 MAPK and Beclin-1 were over expressed ($p<0.0001$, **Figure 3Aa**, **3Ab**). meanwhile, inhabiting p38MAPK expression, the Beclin-1 protein expression decreased significantly, but little interference was observed with the MAPK and ERK1/2 expressions (**Figure 3Aa**, **3Ab**).

In the co-immunoprecipitation assay, in G-1 group, Flag-p38 MAPK and Myc-Beclin-1 plasmid were constructed. Some interaction between p38 MAPK and Beclin-1 was found (**Figure 3B**), but similar results were not observed in any G-1 group.

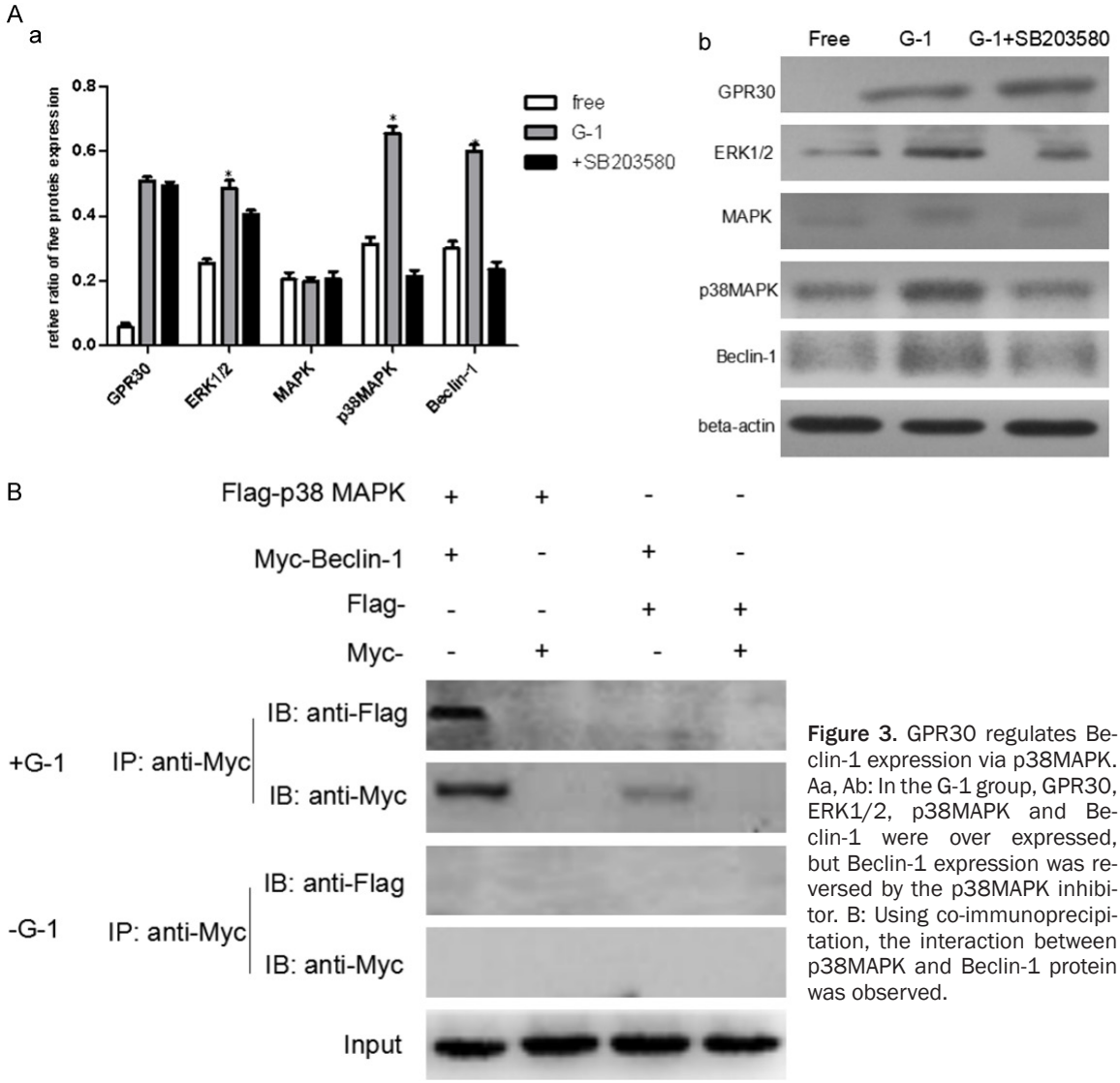

Figure 3. GPR30 regulates Beclin-1 expression via p38MAPK. Aa, Ab: In the G-1 group, GPR30, ERK1/2, p38MAPK and Beclin-1 were over expressed, but Beclin-1 expression was reversed by the p38MAPK inhibitor. B: Using co-immunoprecipitation, the interaction between p38MAPK and Beclin-1 protein was observed.

Correlation of GPR30 expression with overall survival (OS) and progression free survival (PFS)

The Kaplan-Meier survival analysis was used to determine survival with respect to GPR30 expression as a method at the univariate model, and it was found that both protein's positive expression predicted significantly shorter time frames in OS and PFS [former p= 0.0072, latter p=0.0074]. At the same time, similar results were observed from other clinical characteristics, e.g., p staging, pT and pN (p=0.0252, p=0.0304 and p=0.0327, respectively, **Figure 4** and **Table 2**).

A Cox multivariate proportional hazards regression models was carried out to assess whether both protein expressions are prognostic of survival independently from other variables found to be significant in the multivariate analysis. The results show that GPR30 (p=0.0125), pStaging (P=0.0020), pT (0.0325) and Pn (P=0.0233) are independent prognostic factors (**Table 2**).

Discussion

As the 8th leading cancer in incidence and 6th in mortality worldwide, new treatments on esophageal carcinoma have attracted attention due to esophageal cancer's gradually increasing incidence worldwide [20, 21]. Many patients are found to be in advanced stages when are diagnosed, an indication of the value of early diagnosis. In this study, the clinical value of

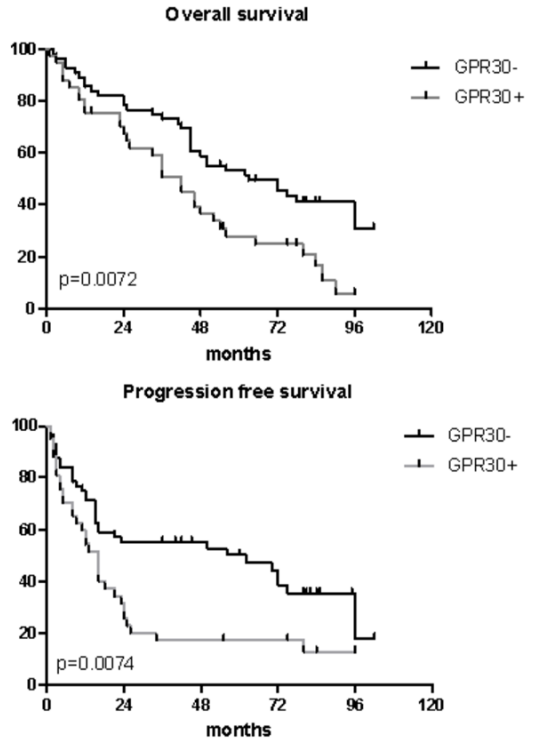

Figure 4. Evaluation of GPR30 as a predictor for OS and PFS by the Kaplan-Meier (KM) plot. There is significant correlation between high expression of GPR30 and OS or PFS. Moreover, GPR30 shows a potential role as a predictor for OS and PFS in ESCC.

GPR30 and Beclin-1 proteins was identified, and, the relation between them was evaluated further in ESCC.

Many studies have found that estrogen regulates a wide variety of biological processes, including differentiation, cell proliferation, and apoptosis, by binding estrogen receptors in many tissue tumors, e.g., colon and lung cancer [22, 23]. However, despite much research on the structure of estrogen receptors, their precise role, their intra-cellular location, and their role in mediating estrogen function remains controversial [24-26]. Recently, a new estrogen receptor, GPR3, also known as GPER, DRY12, FEG-1 or LERGU, was first identified as a GPCR involved receptor in membrane-mediated E2-signaling [27-29]. Moreover, it has been suggested that GPR30 can induce growth effects through the activation of GPER-mediated signaling in ER-negative breast tumors [30-32]. The existence of an alternative estrogen receptor like GPR30 may provide the basis for a better understanding of the remaining mechanisms on estrogen mediated function in many tissue tumors including lung and breast cancer. Though many studies on GPR30 in many tissues (e.g., lung cancer), have been completed and have demonstrated that the protein may play important roles in the progression of these tumors [17, 33] in esophageal carcinoma development, relatively little research has been done. Beclin-1, an essential mediator of autophagy, was originally discovered during the course of a yeast two-hybrid screen of a mouse brain c DNA library [5, 35], and the protein gene in humans has been located on chromosome 17q21 and has monoallelically deleted in up to 75% of ovarian cancers and 40% of prostate cancers [33, 34]. Recently, some studies have reported that Beclin-1 is overexpressed in esophageal cancer [14, 34]; however, no further mechanism of the protein was explored.

In our preparation for this study and in this study, the co-expression of GPR30 and Beclin-1 was identified, a finding which may provide a novel understanding for the Beclin-1 mechanism, especially the discovery of GPR30 expression up-regulating Beclin-1 expression and promoting ESCC progression; these results will expand research in estrogen.

In this study, via IHC, IF and other relative arrays, the over expression of GPR30 and Beclin-1 were identified, and after further analysis, we observed these proteins were located mainly in the cytoplasm, which provides some basis for their regulating mechanism via p38MAPK signaling. In other tissue tumors, on GPR30-mediated signaling, some parts were elucidated, e.g., in breast cancer cells, and the GPER-dependent ERK1/2 activation, which was an important molecule of the p38 MAPK signaling upstream, and was shown to be consequent to the Gβγ subunit-dependent transactivation of EGFR [17, 37-40]. In our interfering assays, the regulation of GPR30 and p38 MAPK proteins on Beclin-1 expression was seen and analyzed, and it confirmed that GPR30 up-regulates Beclin-1 expression via the p38 MAPK protein. Moreover, using a co-immunoprecipitation assay, we further found that there are interactions between p38 MAPK and Beclin-1. These similar results were observed further, in cell proliferation and migration assays, with GPR30 being activated, p38 MAPK expression

Table 2. Kaplan-Meier and Cox multivariate proportional hazard analysis

Factors	Univariate analysis		Multivariate analysis	
	Log-rank	P	Hazard ratio (95%)	P
Age				
<60	3.152	0.2371		
≥60				
GPR30				
Negative	3.552	0.0072	2.7 (1.32-6.41)	0.0125
Positive				
p Staging				
I-II	7.350	0.0252	2.56 (1.8-5.04)	0.0020
III-IV				
pT				
T1-T2	6.081	0.0304	2.89 (2.01-6.58)	0.0325
T3-T4				
pN				
N0	4.821	0.0327	4.86 (1.37-7.24)	0.0233
N1-3				

and Beclin-1 expression up-regulating, and the proliferation and migration effects of ESCC cells (EC-109, KYSE510 and KYSE30) were increased significantly. These results showed that, in ESCC intracellular, GPR30 may exert its effects resulting in tissue specific responses through estrogen nongenomic events, like other estrogen receptors (ERα and β) at least [17, 33].

Meanwhile, the correlation between these proteins' expression and clinicopathologic parameters was analyzed. With ESCC p-stage, and pT advancing, the percentage of GPR30 positive expression increased gradually and indicated a short time to OS and PFS. Using Kaplan-Meier plots and COX regression analysis, we showed that a positive GPR30 expression indicated poor OS and may be an independent prognostic factor.

Conclusions

Our results identified that GPR30 is over expressed in ESCC and may have important biological functions; meanwhile, we revealed the relation between GPR30 and Beclin-1 protein. We demonstrated that GPR30, a novel estrogen receptor, is mainly a cytoplasm receptor, and it takes part in regulating the cell proliferation effect via nongenomic pathways, e.g., ERK1/2 and p38 MAPK signaling. Although our study does not elucidate the mechanism clearly and is especially short of some assays on relative protein gene regulating, our future research will focus on these areas.

Acknowledgements

The study was supported by the National Nature Science Foundation of China (NSFC), Grant number: 81501964. We wish to thank dawei zou for retrieving the follow-up data and Dr. Yuanhui Lai and Prof. Rongbin Xiao for the research design.

All authors read the manuscript and agree to its publication.

Disclosure of conflict of interest

None.

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