Glucose-regulated protein 58 modulates β-catenin protein stability in cervical adenocarcinoma

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ABSTRACT

Background: Cervical cancer continues to pose a serious problem threatening women’s health worldwide. The incidence of cervical adenocarcinoma (AD) is rising in more developed countries. Previously, we showed that glucose-regulated protein 58 (Grp58) serves as an independent factor predictive of poor prognosis for cervical AD. However, the molecular mechanism underlying Grp58 involvement in cervical carcinogenesis is currently unknown. Methods: DNA microarray and enrichment analysis have been used to identify the pathways disrupted by knockdown of Grp58 expression. Results: Among these, the WNT signaling pathway is one of those that are significantly enriched. Our experiments showed that β-catenin, a critical effector of WNT signaling, is stably accumulated in stable Grp58 knockdown cells. Membrane localization of β-catenin was observed in Grp58 knockdown, but not control cells. Using the transwell assay, we demonstrated that accumulated β-catenin induced by Grp58 knockdown or lithium chloride treatment inhibits the migration ability of HeLa cells. Furthermore, an inverse expression pattern of Grp58 and β-catenin was observed in cervical tissues. Conclusions: In summary, our results demonstrate that β-catenin stability is negatively regulated by Grp58. Furthermore, overexpression of Grp58 may result in loss of or decrease in membranous β-catenin expression to promote tumor progression in cervical cancer.
Keywords:

Cervical adenocarcinoma; Grp58; β-catenin; migration
**Background**

Cervical cancer is the third leading cause of cancer-related mortality among women worldwide [1], although records show a marked decline in incidence over the past three decades. Despite the reducing occurrence of cervical squamous cell carcinoma (SCC), the frequency of cervical adenocarcinoma (AD) is increasing due to insufficient detection of cervical AD precursor lesions with the Papanicolaou smear test [2]. Therefore, identification of biomarkers specific for cervical AD is essential for early detection and prognosis. Persistent infection with high-risk human papillomavirus (HPV) is the major risk factor for both SCC and AD [3]. However, HPV alone is not sufficient to cause cervical cancer, and other molecular markers of cervical carcinogenesis are essential. Previously, we demonstrated that glucose-regulated protein 58 (Grp58) serves as an independent prognostic factor for cervical AD, but not SCC [4]. Cell-based studies revealed that Grp58 regulates the invasion and metastatic ability of HeLa cells. Grp58 is a multi-functional protein belonging to the disulfide isomerase family of proteins [5]. The functions of Grp58 in quality control of glycoprotein and major histocompatibility complex class I (MHC class I) maturation are well documented [6]. Recent evidence has suggested that Grp58 plays a role in cancers [7, 8], although the details are unclear. In the current investigation, we aimed to explore the role of Grp58 in cervical AD progression and
the precise molecular mechanism underlying Grp58 function.
Results

Altered WNT signaling pathway in Grp58-knockdown HeLa cells.

Previously, we performed an Affymetrix microarray to identify the genes disrupted upon knockdown of Grp58 expression. Differentially expressed genes were assessed with the MetaCore pathway analysis tool. A total of 1218 Affymetrix probe IDs (fold change > 1.2 and <0.8) were imported, among which 1208 were identified. Eighteen pathway maps were significantly enriched based on the differentially expressed genes (false discovery rate<0.01), which are listed in Table 1. Intriguingly, three WNT-related maps (listed in 2, 15 and 16 of Table 1) were enriched. Cohort genes participating in the WNT signaling pathway were disrupted in Grp58 knockdown cells, including frizzled drosophila homolog of 10, Myc, CD44, disheveled homolog 1, lymphoid enhancer-binding factor 1, vimentin, casein kinase I epsilon and casein kinase II alpha chain [4].

Grp58 regulates β-catenin protein stability.

β-Catenin is a crucial molecule of the WNT signaling pathway. Accordingly, the β-catenin mRNA and protein levels in Grp58 knockdown cells were examined. As shown in Figure 1A and B, β-catenin protein was more abundant in stable Grp58 knockdown cells (G#1 and G#2), compared to control cells (L#1 and L#2). However, no significant differences in β-catenin mRNA levels were observed between the cell
groups. We propose that Grp58 regulates β-catenin expression via modulating protein stability, but not gene transcription. β-Catenin protein expression was indistinguishable in control and knockdown cells treated with MG132, a proteasome inhibitor (Figure 1C). The half-life of β-catenin was subsequently analyzed. CHX was used to block new synthesis of protein. Notably, the β-catenin protein was stably accumulated in Grp58 knockdown cells after 4 h of CHX treatment (Fig. 1D, lanes 15 and 16). In contrast, traces of β-catenin were barely detectable in control cells at this time-point (Fig. 1D, lanes 13 and 14). The data suggest that Grp58 regulates β-catenin protein degradation, but not gene transcription, in HeLa cells.

**β-Catenin stably accumulates in cell-cell adherens of Grp58-knockdown HeLa cells.**

In general, β-catenin mainly localizes to the cytoplasmic membrane as a component of adherens junctions. Subcellular localization of β-catenin in stable HeLa cells was determined using IF staining analysis, and the images visualized with confocal microscopy. As shown in Figure 2, Grp58 was mainly located in the endoplasmic reticulum (ER), consistent with previous reports [7, 9]. Notably, broad membranous staining of β-catenin was observed in Grp58 knockdown, but not control cells.

**β-Catenin inhibits HeLa cell migration.**

To explore the impact of stable accumulated β-catenin on cell migration, the GSK3
inhibitor, LiCl, was used to suppress β-catenin protein degradation. LiCl enhanced β-catenin protein levels in a dose-dependent manner (Fig. 3A). The migration abilities of cells treated with LiCl were further determined. As shown in Figure 3B, the migration abilities of L#1 and L#2 cells were significantly inhibited, along with β-catenin accumulation, in the presence of LiCl (Fig. 3B). The results indicate that β-catenin induced by Grp58 knockdown or LiCl treatment suppresses the migration ability of HeLa cells.

**Inverse expression patterns of Grp58 and β-catenin are observed in cervical cancer.**

Loss of membranous β-catenin is a common feature of various tumors, including cervical AD [10, 11]. Thus, expression patterns of β-catenin in our study population were determined. Intense β-catenin membranous staining was observed in adjacent normal epithelium, and most AD samples showed incomplete membranous staining (Fig. 4A). In paired samples, increased Grp58 histoscores were observed in all tumor tissues, compared with non-tumor regions, and decreased β-catenin histoscores recorded in 82.6% tumor tissues. An inverse expression trend of Grp58 and β-catenin was evident (Fig. 4B). However the correlation between Grp58 and β-catenin expression in tumor regions was not statistically significant.
Discussion

Grp58 regulation of cell invasiveness in cervical cancer was investigated in the present study. Our results demonstrated that β-catenin stably accumulates in the membrane in Grp58 knockdown cells to inhibit migration ability. To our knowledge, this is the first study to provide evidence that Grp58 regulates WNT signaling. In our microarray experiment, several genes downstream of the WNT canonical pathway were identified. However this phenomenon appears to reflect nuclear function and not a plasma membrane adherent function of β-catenin. Indeed, membrane-targeted β-catenin has been shown to increase the cytosolic β-catenin concentration, which is necessary for transduction signals to the nucleus [12]. Nuclear β-catenin is thought to play an oncogenic role in tumorigenesis. However, earlier studies suggest that some potent invasion-promoting genes, such as S100A4 and NEDD9, are inhibited by the WNT canonical pathway [13]. Shtutman et al. demonstrated that induction of progressive multifocal leukoencephalitis by β-catenin suppresses the tumorigenicity of renal carcinoma cells [14]. Microarray analysis led to the identification of S100A4 as a downregulated gene in the current study. The S100A4 protein level was verified using Western blot analysis (data not shown). Decreased S100A4 expression may result in attenuation of migration and invasion abilities. However, restoration of S100A4 expression was not sufficient to rescue the migration and invasion phenotype.
Identification of the key players involved in Grp58-β-catenin-mediated regulation of cell invasiveness is thus of considerable interest.

The prognostic value of β-catenin in cervical cancer is currently unclear. Abnormal β-catenin expression, observed as loss of or reduced membranous staining, is a common feature of cervical cancer [11, 15], and alterations in β-catenin-related cell adherence are thought to be involved in cervical carcinoma pathogenesis [10]. In our study population, membranous β-catenin was significantly decreased in a large proportion of AD, compared to adjacent normal epithelium, which served as the normal control since the adjacent normal columnar epithelium was rarely observed on the tissue slide. Conversely, Grp58 was overexpressed in AD. We observed inverse expression patterns of Grp58 and β-catenin in clinical specimens. In the cell-based study, knockdown of Grp58 expression resulted in accumulation of β-catenin around the plasma membrane of cells. Based on these results, we speculated that Grp58 acts as a regulator of β-catenin protein distribution and stability in cancer cells.

One of the most widely studied functions of Grp58 is its role in the immune system. Grp58 participation in MHC class I antigen presentation is well documented [16]. Consistently, the “Antigen presentation by MHC class I” pathway was the third most significantly enriched in our microarray analysis. Alterations in adaptive immune
responses have been reported in cervical cancer [17]. Additionally, Cromme et al. demonstrated that MHC class I is downregulated in metastases, compared with primary cervical carcinomas [18]. Therefore, it is possible that Grp58 regulates the adaptive immune response to augment cancer invasion. Granzyme A (GZMA) signaling was the most significantly affected pathway in our enrichment analysis (Table 1). GZMA is a serine protease abundant in cytotoxic T lymphocyte (CTL) and natural killer (NK) cells, which triggers apoptosis in an oxidative stress-dependent manner by targeting the ER-associated SET complex [19]. Components of the SET complex, including SET, high mobility group box 2 (HMGB2), APEX nuclease 1 (APEX1) and acidic leucine-rich nuclear phosphoprotein 32 family member A (ANP32A), are affected by Grp58 silencing [4]. Downregulation of homeostatic ER stress responses via knockdown of Grp58 expression appears to enhance apoptosis induced by oxidative stress-inducing drugs [20]. Therefore, knockdown of Grp58 expression may disrupt ER homeostasis, resulting in accumulation of oxidative stress and influence on the status of the SET complex. The detailed molecular mechanism underlying Grp58-mediated apoptosis is unclear. It would be of interest to determine whether Grp58 regulates apoptosis through the SET complex and associated proteins that participate in cervical cancer progression and even drug resistance.
**Conclusions**

Patients with cervical AD are generally considered to have poorer prognosis than those with SCC [21]. However, our knowledge of the natural history and optimal management of cervical AD is limited. Early detection, prognosis and treatment strategies specific for AD should be explored in future studies. Previously we have identified Grp58 as an independent prognostic marker for cervical AD [4]. Here we have demonstrated that Grp58 appears to regulate WNT signaling by targeting β-catenin to augment cancer invasion. Regulation of the immune response and free radical homeostasis are possible mechanisms underlying cervical cancer progression. Further research is warranted to determine the detailed mechanism of Grp58 action in cervical cancer progression.
Methods

Pathway enrichment analysis.

Pathway enrichment analysis of a set of differentially expressed genes upon Grp58 knockdown was performed using the GeneGo MetaCore analysis tool (GeneGo, St. Joseph, MI). Genes displaying differential expression greater than 1.2 fold were uploaded. A pathway map with a false discovery rate of <0.01 was considered significant.

Cell lines and cultures.

The human cervical cancer cell line, HeLa, was obtained from American Type Culture Collection (ATCC, Number: CCL-2), and cultured as recommended. Stable Grp58 knockdown cells were established as described earlier [4]. For MG132 (Sigma-Aldrich, St. Louis, MO) and LiCl (Sigma-Aldrich) treatment, cells were seeded and incubated overnight. The cultured medium was refreshed, and MG132 (10 μM) or LiCl (20 or 40 mM) added to culture medium for 4 and 24 h, respectively, prior to harvest. For the Boyden chamber assay, LiCl was added to the upper and lower chambers during cell seeding. For analysis of β-catenin degradation, cells were pre-treated with MG132 for 4 h. The medium was refreshed and cycloheximide (CHX, 10 ng/ml; Sigma-Aldrich) used to block new protein synthesis. Cells were harvested at 0, 1, 2, and 4 h after treatment with CHX.
Real-time quantitative RT-PCR (qRT-PCR).

Total RNA was extracted from cells using TRIzol. The first cDNA strand was synthesized using the superscript III kit for RT-PCR (Life Technologies, USA). qRT-PCR was performed using SYBR Green, as described by shih et al. [22]. The primer sequences for β-catenin were as follows: forward, 5’-CCg CAA ATc ATg CAC CTT T-3’, and reverse, 5’-CTg ATg TgC ACg AAC AAg CA-3’.

Western blot analysis.

Western blot analysis was performed as described previously [23]. Grp58- specific primary antibody (1:10,000 dilution; Atlas, Sigma-Aldrich, St. Louis, MO), β-catenin specific antibody (1:2000 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and horseradish peroxidase-conjugated, affinity-purified secondary antibody to rabbit or mouse (Santa Cruz Biotechnology) were used. Immunocomplexes were visualized via chemiluminescence with an ECL detection kit (Amersham, Piscataway, NJ).

Transwell assay.

Cells were trypsinized and re-suspended using serum-free medium. Equal amounts of cells (5x10^4 in 100 μl) were seeded in the upper chamber (Corning-Costar 3494 Transwell, Lowell, MA) in triplicate. Lower chambers were supplemented with 20% fetal bovine serum in medium. Traversed cells were stained with crystal violet after 24 h incubation.
Immunofluorescence staining (IF).

Cells were seeded on glass slides, fixed with 3.7% paraformaldehyde, permeabilized with 0.1% Triton X-100/PBS (PBST) for 10 min, blocked with 1% bovine serum albumin for 30 min, and stained with the indicated primary antibody for 3 h at RT. After washing three times with PBST, slides were incubated with secondary antibody for 2 h at RT. Fluorescence images were acquired using confocal microscopy (ZEISS LSM 510 META, Carl Zeiss Inc., Oberkochen, Germany). Grp58 and β-catenin primary antibodies were the same as those used for Western Blotting. The secondary antibodies employed were Alexa Fluor 488 goat-anti-mouse and 568 goat-anti-rabbit antibody (Invitrogen Co., Carlsbad CA).

Immunohistochemistry (IHC).

Formalin-fixed and paraffin-embedded tissues were examined using IHC, according to previously described procedures. The Grp58 antibody (Atlas) was used at 1:2000 dilution, along with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunocomplexes were visualized using the Envision kit (DAKO, Carpinteria, CA). Brown-colored cytoplasmic patches were considered Grp58-positive. Slides were scored separately by two independent pathologists (Y.L and S.M.J) blinded to all clinical data. Staining intensity was graded as absent (0), weak (1+), medium (2+) or strong (3+). The
histoscore \( (Q) \) was calculated by multiplying the percentage \( (P) \) of positive cells by intensity \( (I) \), according to the formula: \( Q = P \times I \). The mean \( Q \) of each cervical cancer type was selected as the cut-off value to divide the high/low expression groups, as described previously.

**Study population.**

Data from a total of 109 cervical carcinoma patients subjected to primary definitive surgery between 2000 and 2008 at Chang Gung Memorial Hospital (Taoyuan, Taiwan) were retrieved from the hospital database, and the histological types confirmed by pathologists. Thirty-four patients with cervical AD, classified as stage I to IIB according to the International Federation of Gynecology and Obstetrics (FIGO) staging system, were enrolled under the protocol approved by the Institutional Review Board (IRB: 95-1241B), with informed consent.
Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

CJL participated in design and acquisition of data and draft of the manuscript. TIW carried out the statistical analysis. YHH participated in discussion the data. TCC and CHL participated in clinical specimen and data collection. SMJ participated in scoring for immunohistochemistry slides. KHL participated in experimental design, coordination, and draft of the manuscript. All authors read and approved the final manuscript.

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**Figure legends**

**Figure 1. The β-catenin protein is stably accumulated in Grp58 knockdown cells.** Grp58 and β-catenin protein levels in stable Grp58 knockdown cells were determined with Western Blot (A, C and D) and qRT-PCR (B). (C) Cells were pretreated with vehicle control (DMSO) or MG132 for 4 h before harvest. (D) β-Catenin degradation assay. After pre-treatment with MG132 for 4 h, the medium was refreshed. Cells were subsequently treated with CHX and harvested at the indicated time-points.

**Figure 2. Distribution of Grp58 and β-catenin in cervical cancer cells.** Grp58 and β-catenin immunoreactive signals of control (upper panel, L#1) and Grp58 knockdown (lower panel, G#2) cells were captured with confocal microscopy.

**Figure 3. Cell migration ability is inhibited by β-catenin.** (A) Western Blot was used to determine β-catenin protein levels after treatment of cells with or without LiCl (20 and 40 mM) for 24 h. (B) The transwell assay was used to determine the migration abilities of cells treated with LiCl. Left panel, images of traversed cells; Right panel, Quantitative results of the transwell assay; **, *P*<0.01

**Figure 4. Grp58 and β-catenin expression patterns in cervical AD.** (A) IHC staining for Grp58 and β-catenin was performed in 34 cervical AD patients. (B) The
scatter plot shows Grp58 and β-catenin histoscores of patients. Paired samples are linked with lines.
## Tables

### Table 1 Pathway map enrichment analysis

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