

Importance of Anti-GRP78 Antibody in Pre-Eclampsia

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ABSTRACT

Background: Preeclampsia (PE) is a pregnancy specific syndrome that is associated with high maternal and fetal morbidity and mortality. Glucose regulated protein78 (GRP78) is an Endoplasmic Reticulum (ER) protein which is expressed on the cell surfaces of trophoblast cells under stress or hypoxic condition. GRP78 has a role in aggressive behavior of invasive cells and may play a role in normal placentation. **Objectives:** To investigate the autoantibody against GRP78 in the sera of patients with PE and to assess the correlation between antibody and severity of the disease. **Methods:** We evaluated the anti-GRP78 antibody within the sera of fifty pre-eclamptic (12 severe and 38 mild PE) and fifty healthy pregnant women using a home-made ELISA assay. Furthermore, western blot technique was used to assess the expression of GRP78 in placenta of healthy and pre-eclamptic women in their third trimester. The presence of anti-GRP78 antibody in the serum samples from pre-eclamptic and healthy women was also assessed. **Results:** GRP78 was expressed by placenta, and both healthy and pre-eclamptic women produced anti-GRP78 antibody. Although no significant difference was found between the pre-eclamptic and healthy women regarding the level of anti-GRP78 antibody, the difference between severe pre-eclamptic and healthy control women was statistically significant ($p < 0.003$). **Conclusion:** The findings of the present study indicated that measurement of anti-GRP78 antibody may provide a new marker for severe pre-eclampsia. Yet, future studies are required to confirm this notion.

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INTRODUCTION

Preeclampsia (PE) is a human specific pregnancy complication which is associated with high maternal and fetal morbidity and mortality (1). PE affects about 5-10 % of all pregnancies worldwide (2).

PE manifests after the 20th week of pregnancy with two diagnostic signs including maternal hypertension and proteinuria. PE is subdivided into severe and mild forms based on the level of blood pressure and proteinuria. The mild form is defined with at least 300 mg or 1+ urine dipstick testing proteinuria along with the new onset of blood pressure $\geq 140/90$ mmHg. On the other hand, the severe form manifests with at least greater than 160/110 mmHg blood pressure along with at least 5gr of protein in a 24 hour urine collection or 3+ urine dipstick testing (2,3).

Although the exact etiology of PE is not well known, a combination of several factors, such as environmental, racial, genetic, and immunological factors, could predispose the pregnant women to PE. The well known risk factors for PE include the history of infertility, multi pregnancies, obesity, vascular dysfunction such as lupus erythematosus and age more than 35 years in the first pregnancy (1). Moreover, the women with metabolic problems, renal disease or cardiovascular problems are more susceptible to PE (2).

Placenta is a transient pregnancy specific tissue and normal placentation is a key phenomenon for success of a pregnancy. Indeed, PE is a placentation disorder. It seems that in pre-eclamptics patients, impaired invasion of trophoblast cells to the maternal endothelial cells results to incomplete blood supply for the fetus and blood pressure along with proteinuria for the mother (4).

During the pregnancy period, trophoblast cells differentiate using two distinct pathways. In the extravillous pathway, cytotrophoblast cells are increased and diverge into an invasive phenotype. In the villous pathway, on the other hand, cytotrophoblast cells fuse to form the multinuclear syncytiotrophoblast (STB) cells (5,6).

PE is a placentation disease and it seems that a type of trophoblast invasion dysfunction or maternal endothelial dysfunction occurs in pre-eclamptic women. Abnormal placentation in pre-eclamptic patients, leads to an increased release of a group of placental factors, cytokines, and growth and angiogenic factors resulting in a systemic inflammation (7-10). Furthermore, oxidative stress and hypoxia, finally affect the maternal vascular inflammation leading to blood pressure and proteinuria (11,12)

Glucose-regulated protein 78 (GRP78) is an Endoplasmic Reticulum (ER) protein belonging to the heat shock protein 70 (HSP70) family (13). GRP78 is expressed on the cell surfaces of cancerous or other cells under stress or hypoxic conditions (14-17). Interestingly, GRP78 is over-expressed on the cell surfaces of the trophoblast cells (18). Moreover, a specific role has been proposed for GRP78 in embryonic development (19). Over-expression of GRP78 on the surfaces of both cancerous and trophoblast cells suggests that GRP78 plays a role in aggressive behavior of invasive cells (18). Mario et al. reported the presence of autoantibody against GRP78 protein within the sera of the patients with prostate cancer. Interestingly the level of autoantibody was reported to be correlated with cancer metastasis (20). The presence of antibody against GRP78 has also been reported within the sera of the pregnant women with PE (18).

Considering the relationship between trophoblast invasion and PE in one hand and the relation between autoantibody against GRP78 and cell invasion on the other hand, the

present study aims to investigate autoantibody against GRP78 in the sera of the patients with PE. The correlation between antibody and severity of PE is assessed as well.

MATERIALS AND METHODS

Subjects. The present study was conducted on 100 (50 normal and 50 pre-eclamptic) pregnant women after obtaining written informed consents. The study was approved by the local Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran. Among the 50 pre-eclamptic women 12 were diagnosed with severe PE, while the remaining 38 were diagnosed with the mild form. All the participants were investigated clinically and diagnosed by the same gynecologist.

The normal subjects and the age-matched pre-eclamptic cases were selected among the primiparous women. The diagnosis of PE was based on two standard diagnostic markers, blood pressure and proteinuria. The women with above 160 mmHg systolic or 110 mmHg diastolic blood pressure plus at least more than 5 gr protein in a 24 hour urine collection or 3+ or greater on urine dipstick were considered as severe PE. On the other hand mild PE was diagnosed with at least 140 mmHg systolic or 90 mmHg diastolic blood pressure along with at least more than 1+ dipstick proteinuria. Finally, the normal women did not have any history of autoimmunity or malignancy and showed a normal pregnancy. Demographic characteristics of all the participants are summarized in Table 1.

Sampling and Measuring the Anti-GRP78 Antibody. In this study 2 ml peripheral blood was obtained from both cases and controls. Then the sera were separated from the blood samples and kept at -70°C until ELISA assay.

For evaluation of the antibody against GRP78 protein, a home-made ELISA assay was performed. In brief, 96 well MaxiSorp ELISA micro plates (Nunc, Denmark) were coated with 100 micro liter of human recombinant GRP78 protein containing 300 ng of protein (ab78432, Abcam, AL-Ain, UAE), using bicarbonate buffer in PH 8.8 and incubated at 4°C for 16 hours. After two washes with phosphate-buffered saline supplied with 0.05% Tween 20 (PBST), non specific binding was blocked by adding 3% Bovine Serum Albumin (BSA) in PBST at room temperature for 2.5 hours. Then 50 micro liter of 1/50 diluted of each serum was added and the plate were incubated at room temperature for 1 hour. After serum incubation, the wells were washed for four times using PBST. Anti-human IgG or total immunoglobulin peroxidase conjugated antibody (1/5000 in PBST, Abcam, AL-Ain, UAE) was used as the secondary antibody. After one hour incubation with the secondary antibody, the wells were washed with PBST for four times. Ready to use 3, 3', 5, and 5'-tetramethylbenzidine (TMB) solution was used as the developer of the reaction. After adding TMB, the plate was incubated in a dark place for 30 minutes. Finally, the optical densities of the wells were read at 450 nm. Human pure IgG solution (Sigma, USA) was used as the positive control, while omitting of the serum was considered as the negative control. All tests were performed duplicated and anti-GRP78 antibody was reported as mean \pm standard deviation based on the optical densities as arbitrary unit.

Intra and Inter-assay coefficients of variation for measurement of GRP78 were 7.1% and 8.3%, respectively.

Qualitative Evaluation of GRP78 and Anti-GRP78 Expression. Western blot technique was used for evaluation of the expression of GRP78 protein in the placentas

from four severe pre-eclamptic and four healthy pregnant women. Briefly, total protein was extracted from pre-eclamptic and normal placentas as described before (21). The extracted proteins were then separated using SDS-PAGE technique and transferred onto the PVDF membrane. Mouse anti-GRP78 monoclonal antibody (ab96483, Abcam, AL-Ain, UAE) was used as the primary antibody, while goat anti-mouse HRP conjugated antibody (ab97023, Abcam, AL-Ain, UAE) was used as the secondary antibody. Finally, sigma Fast 3–3' di-aminobenzidine (DAB) tablets (Sigma, Steinheim, Germany) were used for visualization of the blotted bands. For minimizing the variation, all the eight protein samples were separated, transferred and blotted simultaneously and anti-GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) antibody (ab8245) was used to normalize GRP78 expression between cases and controls. Moreover all tests were performed duplicated. Furthermore in order to evaluate the presence of anti-GRP78 antibody within the tested sera, the separated placental proteins were blotted with the pooled sera from the pre-eclamptic and healthy women. For each sample (Healthy controls, Mild and Severe pre-eclampsia), two pooled sera, each from five different sample, were checked.

Statistical Analysis. All the statistical analysis was carried out using the SPSS statistical software, version 16 for Windows (SPSS Inc., Chicago, IL, USA). Student's *t-test* or Mann-Whitney test was used to compare the mean levels of anti-GRP78 antibody between the groups. In addition, Fisher exact test was used to compare the smoking status between the pre-eclamptic and healthy women. Besides, p values less than 0.05 were considered as statistically significant.

RESULTS

As shown in Table 1, no significant difference was found between the cases and controls regarding the maternal age, gestational week at the sampling time, and smoking status, while as expected the systolic and diastolic blood pressure were significantly elevated in pre-eclamptic cases compare to the healthy controls ($p < 0.001$).

Table 1. Demographics characteristics of pre-eclamptic cases and healthy controls .

Characteristics	Pre-Eclamptics (n=50)	Controls (n=50)	P Value
Maternal age (years, mean \pm SD)	27.3 \pm 5.1	25.3 \pm 3.9	0.13
Gestation at sampling (weeks, mean \pm SD)	32.1 \pm 5.2	32.1 \pm 7.1	0.98
Smoking	3	5	0.71
Maximum systolic BP (mmHg, mean \pm SD)	147 \pm 9.3	128 \pm 3.1	<0.001
Maximum diastolic BP (mmHg, mean \pm SD)	96 \pm 7.1	73 \pm 1.9	<0.001

Table 2 presents the results of the comparison of anti-GRP78 antibody between the pre-eclamptic cases and healthy controls based on optical density.

Table 2. Anti-GRP78 antibody levels in pre-eclamptic and healthy women.

Subjects	Anti-GRP78 (mean \pm SD)	P Value
Healthy controls (n=50)	1.11 \pm 0.40	
Pre-eclamptics (n=50)	0.95 \pm 0.35	*
Mild Pre-eclamptics (n=38)	1.00 \pm 0.29	**
Severe Pre-eclamptics (n=12)	0.80 \pm 0.40	***

Antibody levels are presented based on the optical density as arbitrary unit.

* P=0.11 for pre-eclamptics versus healthy controls (Student's *t-test*)

** P<0.005 for severe pre-eclamptics versus mild pre-eclamptics (Mann Whitney test)

*** P<0.003 for severe pre-eclamptics versus healthy controls (Mann Whitney test)

As shown in this Table, although no significant differences between the pre-eclamptics and the healthy controls regarding the level of GRP78 antibody (0.95 v.s. 1.11, $p=0.11$) was found, a significant difference was observed between the severe pre-eclamptics and healthy controls in this regard (0.80 v.s. 1.11, $p<0.003$)

The results of the mean level of anti GRP78 antibody in all study groups are presented in Figure 1.

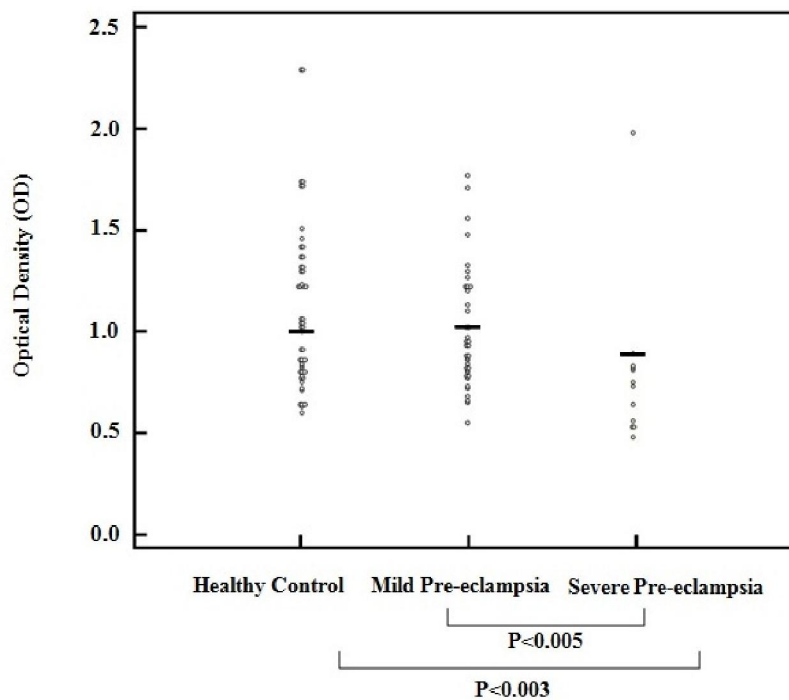


Figure 1. Dot blot graph of anti-GRP78 levels in severe pre-eclamptics, mild pre-eclamptics and healthy women. Antibody levels are presented and compared based on the optical density as arbitrary unit. All P values are calculated using Mann Whitney test.

As shown in Figure 1 and Table 2, the mean level of anti-GRP78 antibody significantly decreased in the women with severe form of the disease compared to those with the mild form and healthy pregnant women ($p < 0.005$ and $p < 0.003$ respectively).

The results of evaluation of GRP78 expression is presented in Figure 2. According to this Figure, western blot indicated that the expression of GRP78 protein was down-regulated in the severe pre-eclamptic placentas compared to the normal third trimester placentas. The density of the blotted bands in healthy controls was at least two times more intense compared to the bands from severe pre-eclamptic women when normalized with the control (GAPDH) bands (Figure 2). Furthermore, the results of western blotting with the pooled sera from cases and controls indicated that a band equal to GRP-78 protein was targeted by both case and control sera (data not shown).

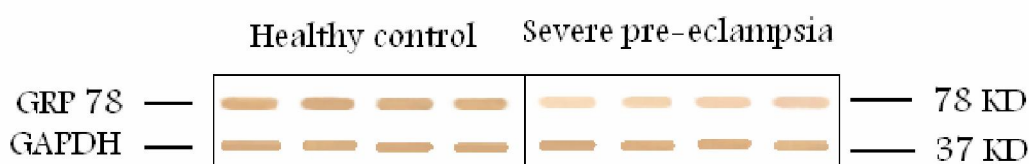


Figure 2. Different expression of GRP78 protein in severe pre-eclamptic placentas compared to the normal third trimester placentas evaluated using western blot technique. 40 microgram of the total placental proteins from four severe pre-eclamptic and four healthy controls was separated, transferred onto the PVDF membranes and blotted with anti-GRP78 monoclonal antibody. Anti-GAPDH antibody was used to detect GAPDH protein and normalized the density of the bands. Qualitative evaluation indicated that the density of the blotted bands in healthy controls was at least two times more intense compared to the bands from severe pre-eclamptic women.

DISCUSSION

The results of the present study indicated that anti-GRP78 was produced by both pre-eclamptic and healthy pregnant women in the pregnancy period. Moreover, our results indicated that the production of anti-GRP78 was significantly reduced in the severe pre-eclamptic women compared to the mild pre-eclampsics and healthy controls.

GRP78 is an ER chaperon protein that up regulates under hypoxic or stress conditions (22). GRP78 is also expressed on the cell surfaces of many tumor cells and there seems to be a relationship between the over-expression of GRP78 on the cell surfaces and metastasis of the cancerous cells (14,15,17,23). Over-expression of GRP78 protein has also been reported in metastatic cancer cell lines (24). Although the exact reason for the cell surface expression of GRP78 is not well known yet, it seems that the expression of GRP78 supports cancer cell mobility, helps the tumor cells to invade, and protects them from host stress conditions.

The expression of GRP78 on the cell surfaces of the trophoblast cells has also been reported previously (18,25). Interestingly, decreased expression of GRP78 protein on the cell surface of cytotrophoblast cells isolated from pre-eclamptic women was reported as well (26). PE is a placental disease and it seems that placentation is impaired in PE women. PE is characterized by an abnormal invasion of trophoblast cells to

maternal myometrium and arteries. Indeed, there seems to be a defect in the invasion of trophoblast cells and formation of syncytiotrophoblast (CTB) cells which are produced from fusion of cytotrophoblast (STB) cells in pre-eclamptic women (27).

Arnaudeau et al. indicated that GRP78 acted as a partner for P53 molecule and regulated the trophoblast cells invasion via inactivation and stabilization of P53 (25). In general, proper formation of the placenta needs invasion of trophoblast cells and formation of CTB cells and surface expression of GRP78 protein may control both processes. GRP78 protein is highly expressed on the cell surfaces of STB cells in the first trimester of pregnancy (28). We also indicated that GRP78 protein was over-expressed in the first compared to the third trimester of a normal pregnancy (Ghahresifard et al. submitted for publication).

Gonzalez-Gronow et al., in 2006 reported the presence of auto-antibody against GRP78 within the sera of the patients suffering from prostate cancer (20). They hypothesized that anti-GRP78 antibody facilitated prostate cancer cell invasion (20). Anti-GRP78 antibody within the sera of the patients with gastric cancer and hepatocellular carcinoma has been reported as well (29,30). Interestingly, Shao et al. indicated that the level of anti-GRP78 antibody was significantly increased in the patients with hepatocellular carcinoma compared to chronic hepatitis patients and healthy controls (30). The presence of anti-GRP78 antibody within the sera of pregnant women is also reported. Laverriere et al. showed that GRP78 was strongly associated with the aggressive behavior of trophoblast cells and the women with low levels of anti-GRP78 antibody in the first trimester of the pregnancy period were more susceptible to PE (18). The most important finding of the present study was detection of a low titer of anti-GRP78 antibody within the sera of severe pre-eclamptic women compared to those with the mild form and healthy controls. Considering the level of anti-GRP78 antibody, the findings of the current study are in line with those of the study by Laverriere et al. reporting a low titer of anti-GRP78 antibody within the sera of pre-eclamptic women in the first trimester of the pregnancy period compared to the healthy women (18).

In spite of the fact that the role of anti-GRP78 in trophoblast cells invasion and placentation is not well known, comparison of the formation of the placenta and invasion of tumor cells may shed light on the role of anti-GRP78 in a normal placentation. It seems that GRP78 functions are controlled by GRP78 protein level and anti-GRP78 antibody might affect several defined functions of GRP78 molecules, such as fusion of CTB cells, inactivation and stabilization of P53 molecule, and invasion of the trophoblast cells.

Our data regarding the expression of GRP78 and low levels of anti-GRP78 antibody in the severe pre-eclamptic women are well matched with this hypothesis. Interestingly, the results obtained by Laverriere et al. regarding the expression of GRP78 protein and anti-GRP78 in the pre-eclamptic women are also in agreement with our results (18). Finally, it can be concluded that the quantification of anti-GRP78 antibody may provide a new marker for severe PE. However, further studies are required to be conducted on the issue.

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REFERENCES

- 1 Eiland E, Nzerue C, Faulkner . Preeclampsia 2012. *J Pregnancy*. 2012; 2012:586578.
- 2 Grill S, Rusterholz C, Zanetti-Dällenbach R, Tercanli S, Holzgreve W, Hahn S, et al. Potential markers of preeclampsia--a review. *Reprod Biol Endocrinol*. 2009; 7:70.
- 3 Backes CH, Markham K, Moorehead P, Cordero L, Nankervis CA, Giannone PJ. Maternal preeclampsia and neonatal outcomes. *J Pregnancy*. 2011; 2011:214365.
- 4 Forbes k, Westwood M. Maternal growth factor regulation of human placental development and fetal growth. *J Endocrinol*. 2010; 207:1-16.
- 5 Al-Nasiry S, Spitz B, Hanssens M, Luyten C, Pijnenborg R. Differential effects of inducers of syncytialization and apoptosis on BeWo and JEG-3 choriocarcinoma cells. *Hum Reprod*. 2006; 21:193-201.
- 6 Ferretti C, Bruni L, Dangles-Marie V, Pecking AP, Bellet D. Molecular circuits shared by placental and cancer cells, and their implications in the proliferative, invasive and migratory capacities of trophoblasts. *Hum Reprod Update*. 2007; 13:121-41.
- 7 Redman CW, Sargent IL. Pre-eclampsia, the placenta and the maternal systemic inflammatory response--a review. *Placenta*. 2003; 24:S21-7.
- 8 Redman CW, Sargent IL. Latest advances in understanding preeclampsia. *Science*. 2005; 308:1592-4.
- 9 Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, et al. Excess placental soluble fms-like tyrosine kinase 1 (sflt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest*. 2003; 111:649-58.
- 10 Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA, McLaughlin MK. Preeclampsia: an endothelial cell disorder. *Am J Obstet Gynecol*. 1989; 161:1200-4.
- 11 Myatt L, Webster RP. Vascular biology of preeclampsia. *J Thromb Haemost*. 2009; 7: 375-84.
- 12 Redman CW, Sargent IL. Preeclampsia and the systemic inflammatory response. *Semin Nephrol*. 2004; 24:565-70.
- 13 Dudek J, Benedix J, Cappel S, Greiner M, Jalal C, Müller L, et al. Functions and pathologies of BiP and its interaction partners. *Cell Mol Life Sci*. 2009; 66:1556-69.
- 14 Fu Y, Lee AS. Glucose regulated proteins in cancer progression, drug resistance and immunotherapy. *Cancer Biol Ther*. 2006; 5:741-4.
- 15 Li J, Lee AS. Stress induction of GRP78/BiP and its role in cancer. *Curr Mol Med*. 2006; 6:45-54.
- 16 Quinones QJ, de Ridder GG, Pizzo SV. GRP78: A chaperone with diverse roles beyond the endoplasmic reticulum. *Histol Histopathol*. 2008; 23:1409-16.
- 17 Delie F, Petignat P, Cohen M. GRP78 protein expression in ovarian cancer patients and perspectives for a drug-targeting approach. *J Oncol*. 2012; 2012:468615.
- 18 Laverriere A, Landau R, Charvet I, Irion O, Bischof P, Morales M, et al. GRP78 as a marker of pre-eclampsia: an exploratory study. *Mol Hum Reprod*. 2009; 15:569-74.
- 19 Luo S, Mao C, Lee B, Lee AS. GRP78/BiP is required for cell proliferation and protecting the inner cell mass from apoptosis during early mouse embryonic development. *Mol Cell Biol*. 2006; 26:5688-97.
- 20 Gonzalez-Gronow M, Cuchacovich M., Llanos C, Urzua C, Gawdi G, Pizzo SV. Prostate cancer cell proliferation in vitro is modulated by antibodies against glucose-regulated protein 78 isolated from patient serum. *Cancer Res*. 2006; 66:11424-31.
- 21 Gharesi-Fard B, Zolghadri J, Kamali-Sarvestani E: Proteome differences of placenta between pre-eclampsia and normal pregnancy. *Placenta*. 2010; 31:121-5.
- 22 Lee AS. The glucose-regulated proteins: stress induction and clinical applications. *Trends Biochem Sci*. 2001; 26:504-10.
- 23 Shin BK, Wang H, Yim AM, Le Naour F, Brichory F, Jang JH, et al. Global profiling of the cell surface proteome of cancer cells uncovers an abundance of proteins with chaperone function. *J Biol Chem*. 2003; 278:7607-16.

- 24 Ramsay RG, Ciznadija D, Mantamadiotis T, Anderson R, Pearson R. Expression of stress response protein glucose regulated protein-78 mediated by c-Myb. *Int J Biochem Cell Biol.* 2005; 37:1254-68.
- 25 Arnaudeau S, Arboit P, Bischof P, Shin-ya K, Tomida A, Tsuruo T, et al. Glucose regulated protein 78: a new partner of p53 in trophoblast. *Proteomics.* 2009; 9:5316-27.
- 26 Fradet S, Pierredon S, Ribaux P, Epiney M, Shin Ya K, Irion O, et al. Involvement of membrane GRP78 in trophoblastic cell fusion. *PLoS One.* 2012; 7:e40596.
- 27 Ferretti C, Bruni L, Dangles-Marie V, Pecking AP, Bellet D. Molecular circuits shared by placental and cancer cells, and their implications in the proliferative, invasive and migratory capacities of trophoblasts. *Hum Reprod Update.* 2007; 13:121-41.
- 28 Kudo Y, Boyd CA, Sargent IL, Redman CW. Hypoxia alters expression and function of syncytin and its receptor during trophoblast cell fusion of human placental BeWo cells: implications for impaired trophoblast syncytialisation in pre-eclampsia. *Biochim Biophys Acta.* 2003; 1638:63-71.
- 29 Tsunemi S, Nakanishi T, Fujita Y, Bouras G, Miyamoto Y, Miyamoto I A, *et al*: Proteomics-based identification of a tumor-associated antigen and its corresponding autoantibody in gastric cancer. *Oncol Rep.* 2010; 23:949-56.
- 30 Shao Q, Ren P, Li Y, Peng B, Dai L, Lei N, Yao W *et al*. Autoantibodies against glucose-regulated protein 78 as serological diagnostic biomarkers in hepatocellular carcinoma. *Int J Oncol.* 2012; 41:1061-7.