Maternal Blood Serum and Plasma Human Tumor-Associated Antigen RCAS1 During the Course of Uncomplicated Pregnancies: A Prospective Study

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Problem
We aimed to investigate the expression of the tumor-associated RCAS1 protein in maternal blood of uncomplicated pregnancies.

Method of study
Maternal blood was obtained from women with uncomplicated pregnancies ($N = 43$) at 11–13, 20–22, 32–34, 37–38 weeks of gestation, and immediately after delivery. Serum RCAS1 concentration was studied by ELISA, and plasma mRNA was subjected to real-time (RT)-PCR.

Results
Serum RCAS1 protein concentration was significantly up-regulated at 11–13 and 20–22 weeks than that at 32–34 weeks and after delivery. RCAS1 mRNA level was significantly increased at 11–13 weeks than that at 37–38 weeks. A significant positive correlation was defined between RCAS1 serum concentration at 11–13 weeks and gestational age at delivery and that between plasma RCAS1 mRNA levels at 37–38 weeks and umbilical cord blood base excess. A significant negative correlation was found between RCAS1 serum concentration at 37–38 weeks and umbilical cord blood pH at delivery.

Conclusions
RCAS1 protein might have importance in the development of uncomplicated pregnancies and for the prediction of pregnancy outcome.

Introduction

Normal pregnancy corresponds to a controlled systemic state of inflammation, and apoptotic or necrotic debris shed from the syncytial surface of the placenta constitute an inflammatory stimulus in all pregnancies.[1] During the adaptive process of endothelium to decidua, NK cells experience dramatic change: their numbers proliferate and more importantly, they undergo activation.[2] On the other hand, it was reported that human T and NK cells express the receptor for receptor-binding cancer antigen expressed on SiSo cells (RCAS1) at a high level after being activated and that RCAS1 inhibits the proliferation and induces the apoptotic cell death of the receptor-positive immune cells.[3] RCAS1 protein is involved in regulation of apoptosis of erythroid progenitor cells and may have a critical role in erythropoesis,[4] while other reported functions include participation in avoiding immune recognition and evading immune surveillance.[5] These findings led us to investigate RCAS1 protein in connection with normal course of human pregnancy.

RCAS1 is a homodimer with molecular weight of 25 kDa. It is a predominantly Golgi-localized membrane protein,[6] which consists of 213 amino acids,

with an N-terminal transmembrane segment and a coiled-coil structure in the C-terminal portion.[3] The tumor-associated antigen RCAS1 was defined by its immunoreaction with the 22-1-1 monoclonal antibody (mAb), which was raised by immunization of mice with the human uterine cervical adenocarcinoma cell line SiSo.[7] The gene product was termed 'receptor-binding cancer antigen expressed on SiSo cells' (RCAS1) and is identical with the estrogen-responsive protein EBAG9 (estrogen receptor-binding fragment-associated gene9), which is located on chromosome 8q23.[3,8,9] RCAS1 protein is widely studied in tumor malignancies as a biomarker that is associated with poor prognosis of cancers.[10–14] Moreover, knockdown of RCAS1 expression by RNA interference recovers T cell growth and proliferation *in vitro*,[15] while enhanced RCAS1 expression significantly promotes *in vivo* growth of tumors, derived from transfected COS-7 cells.[16] Furthermore, a comparative analysis of normal placenta and neoplasms has been reported.[17]

There have been several studies investigating the RCAS1 expression and immune tolerance during pregnancy,[18,19] and its connection with fetal maturity,[20] stillbirth,[21,22] the process of placental detachment.[5] Our recent studies revealed strong connection between RCAS1 antigen and gestational diabetes mellitus,[23] pre-eclampsia,[24] and its role in the maintenance of the normal pregnancy in mice has been studied.[25] The aim of this study was to assess the role of RCAS1 protein expression in maternal blood during the course of uncomplicated human pregnancies and to define its predictive value for pregnancy outcome.

Materials and methods

Patients

This study was designed as a prospective study during the course of pregnancy. For this investigation, we selected 43 healthy patients with singleton pregnancies. None of the patients had intrauterine fetal death, miscarriage, intrauterine growth restriction (IUGR), premature birth, or complicated previous obstetrical history. All the women visited the Osaka University Hospital's outpatient clinic for routine examinations and delivered between October of 2003 and October of 2005. Main characteristics and pregnancy outcome of the study group are given in Table I.

Table I Main Characteristics of Patients and Pregnancy Outcome

	Total number of patients studied (N = 43)
Nullipara (N, %)	19 (44)
Weeks of gestation at delivery	38.9 ± 1.4
Vaginal birth (N, %)	43 (100)
Pregnancy outcome	
Birth weight	2885.0 ± 54.0
Apgar score at 1st min	8.3 ± 0.1
Apgar score at 5th min	9.1 ± 0.1
Cord blood gases	
pH	7.3 ± 0.03
PO_2 mmHg	43.2 ± 3.2
PCO_2 mmHg	19.4 ± 1.1
Base excess mmol/L	−4.7 ± 1.1

Values are given as (Mean ± S.D.).

Sample Preparation

After approval by the local ethics committee of the Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, informed consent was obtained from each patient. For this study, we collected 43 maternal peripheral blood samples at each stage of pregnancy (11–13, 20–22, 32–34, 37–38 weeks of gestation) and at early puerperium after delivery from same patients. Samples were centrifuged at 4°C at 11,100 × g for 20 min. The sera and plasma were stored at −80°C.

ELISA to Determine RCAS1 Concentration

To determine the RCAS1 concentration in maternal blood serum, a commercial ELISA kit for RCAS1 detection (Cusabio Biotech Co., LTD, Wuhan, Hubei Province, P.R. China) was used. All the procedures were performed according to the manufacturer's protocols in duplicate using diluted samples. The RCAS1 concentration of samples is expressed by multiplying by the dilution factor (e.g., ×12). The intra- and inter-assay variabilities for RCAS1 were 3.4–5.9% and 2.3–7.0%, respectively.

RNA Extraction and Reverse-Transcription Reaction

Total RNA was extracted from 1.0 mL harvested maternal blood plasma (n = 15 from indicated stages

of pregnancy). The plasma was mixed with lysis buffer (8.99 g of NH_4Cl, 1.0 g of $KHCO_3$, 0.2 mL of 0.5 M EDTA per 80 mL of distilled water) at a proportion of 1:5 and centrifuged at $300 \times g$ for 10 min at 4°C. Pellet was resuspended into 1-mL lysis buffer followed by centrifugation at $700 \times g$ at 4°C, and resuspension in 800 µL of Sepazol-I (Nacalai Tesque Inc., Kyoto, Japan) and 200 µL Chloroform (Nacali Tesque Inc., Kyoto, Japan), followed by vortex. Samples were left on ice for 10–15 min and centrifuged at $15,100 \times g$ for 15 min at 4°C. The upper phase was removed and transferred to a microcentrifuge clean tube, and an equal volume of ice-cold isopropanol (Nacalai Tesque Inc., Kyoto, Japan) was added, followed by vortex,and left on ice for 15 min. After repeated centrifugation at $15,100 \times g$ for 15 min at 4°C, the pellet was rinsed with 400 µL of ice-cold 75% ethanol, followed by centrifugation at $4300 \times g$ for 8 min at 4°C. RNA was eluted in 40 µl of RNAse-free water and directly proceeded to the reverse-transcription reaction. For the reverse-transcription reaction, we used Oligo (dt) 12–18, 10 mM dNTPs, 5× First Strand Buffer, 0.1 M DTT, RNase inhibitor (Toyobo, Osaka, Japan), and Superscript ll reverse transcriptase (Invitrogen, Japan).

Real-Time PCR

For real-time PCR, we used 1 µL of cDNA from each sample, Taq Man Gene expression assays (Hs99999905_m1 G3PDH, 250 µL, 20xMix and Hs00188444_m1 EBAG9, 250 µL, 20× Mix) (Applied Biosystems, Japan) and TaqMan Universal PCR Master Mix (Roche, Branchburg, New Jersey, USA). The final volume for the reaction was 20 µL. The absolute-quantification real-time PCR method was performed using computer program ABI PRISM 7000 (Applied Biosystems, Japan). 5(6) Carboxy Fluorescein Amidite was used as detector manager. Each reaction was performed in triplicate, and the results were normalized by the expression of G3PDH in each sample of cDNA but in separate tubes. The comparative Ct method was carried out on the data.

Statistical Analysis

Data, obtained from study groups on each designated period of gestation, were subjected to Kruskal–Wallis test with Dunn's post test. The relationship between the serum RCAS1protein concentration, the plasma RCAS1 mRNA levels, and pregnancy outcome was tested with correlation analysis. Furthermore, to determine whether the correlation coefficient was statistically different from 0, Fisher's r to Z transformation was performed on the correlation using the Statview statistics package (Abacus Concepts, Inc., Berkeley, CA, USA), with $P < 0.05$ considered to indicate significance. The data are presented as Mean ± S.E.M.

Results

To compare the concentrations of RCAS1 protein in maternal blood serum during the course of uncomplicated pregnancy, ELISA for RCAS1 protein detection was performed. As shown in Fig. 1, the concentration of RCAS1 protein in the maternal sera of patients at 11–13 weeks of gestation (213.0 ± 42.2 U/mL) was significantly higher than that at 32–34 weeks of gestation (116.0 ± 29.2 U/mL), and after delivery (55.0 ± 11.0 U/mL), whereas at 20–22 weeks of gestation, the RCAS1 protein concentration (191.2 ± 35.0 U/mL) was significantly up-regulated compared that to the early puerperium alone (55.0 ± 11.0 U/mL).

To compare levels of the RCAS1 mRNA transcripts in the maternal blood plasma, real-time (RT)-PCR,

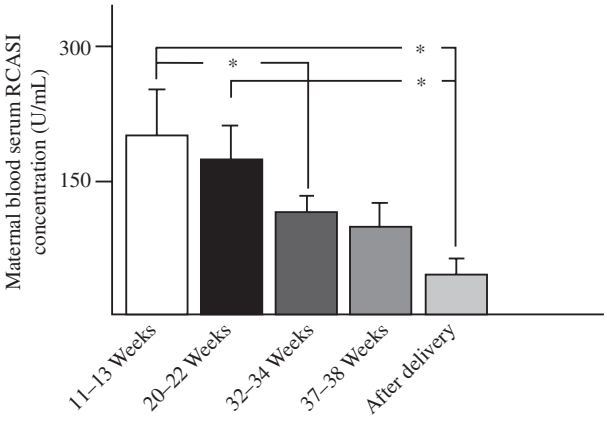

Fig. 1 The RCAS1 protein concentrations of maternal blood serum in patients at 11–13, 20–22, 32–34, and 37–38 weeks of gestation during the course of uncomplicated pregnancy and after delivery. The concentration of RCAS1 protein in the maternal blood sera of patients at 11–13 weeks of gestation was significantly higher than that at 32–34 weeks of gestation and after delivery. The concentration of RCAS1 protein in the maternal blood sera was significantly increased at 20–22 weeks of gestation than that after delivery. Samples evaluated at indicated stages of pregnancy $n = 43$ (*denotes $P < 0.05$).

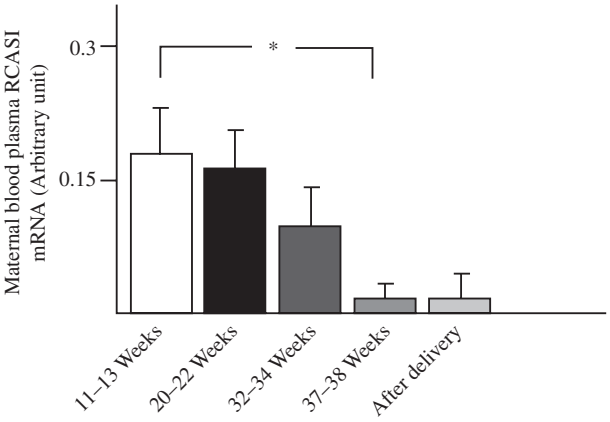

Fig. 2 The RCAS1 protein mRNA levels in maternal blood plasma at 11–13, 20–22, 32–34, and 37–38 weeks of gestation during the course of uncomplicated pregnancy and after delivery. The expression of RCAS1 protein mRNA/G3PDH was quantified by real-time PCR. The ratio (expressed in arbitrary units) in maternal blood plasma was significantly up-regulated at 11–13 weeks of gestation than that at 37–38 weeks of gestation. Samples evaluated at indicated stages of pregnancy $n = 15$ (*denotes $P < 0.05$).

using specific primers for RCAS1, was performed. Fig. 2 shows the expression levels of the RCAS1 transcripts in the plasma at different stages during the course of uncomplicated pregnancies. RCAS1 mRNA level was significantly higher at 11–13 weeks of gestation compared to 37–38 weeks of gestation.

We analyzed the relationship between the concentrations of RCAS1 in the maternal blood sera at different stages of gestation and the age at delivery, fetal weight, and umbilical cord blood pH/gases/BE. As shown in Fig. 3a, the maternal blood sera RCAS1 protein concentration at 11–13 weeks of gestation was positively correlated with gestational age at delivery ($r = +0.6$), whereas there was negative correlation between the maternal blood sera RCAS1 protein concentration at 37–38 weeks of gestation and cord blood pH after delivery ($r = -0.8$) (Fig. 3b). Correlation between other indices did not reach any significant difference.

After analyzing the relationship between the plasma RCAS1 mRNA expression and the sera RCAS1 concentrations at different stages of uncomplicated pregnancies, the age at delivery, fetal weight, umbilical cord blood pH/gases/BE, a significant positive correlation was defined between plasma RCAS1 mRNA at 11–13 weeks and serum RCAS1 concentration at 20–22 weeks of gestation ($r = +0.6$) (Fig. 4a). Furthermore, plasma RCAS1 mRNA levels

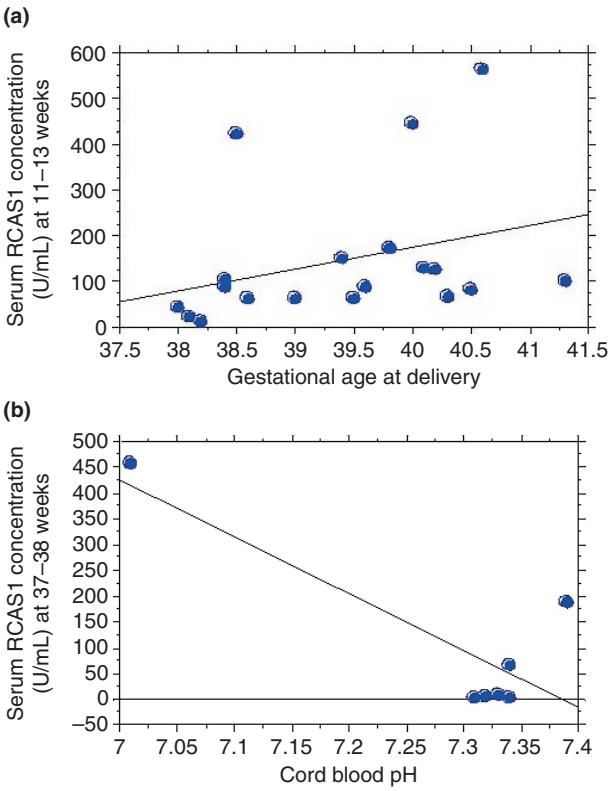

Fig. 3 Correlation of the maternal blood sera RCAS1 concentration at 11–13 and 37–38 weeks of gestation with pregnancy outcome in uncomplicated pregnancies. (a) The maternal blood sera RCAS1 protein concentration at 11–13 weeks of gestation positively correlated with gestational age at delivery; (b) Serum RCAS1 protein concentration at 37–38 weeks of gestation negatively correlated with umbilical cord blood pH after delivery. Samples evaluated at indicated stages of pregnancy $n = 43$ ($P < 0.05$).

at 32–34 weeks of gestation significantly positively correlate with serum RCAS1 concentrations at 32–34 weeks of gestation ($r = +0.7$) (Fig. 4b), and in early puerperium ($r = +0.8$) (Fig. 4c). A significantly positive correlation was defined between plasma RCAS1 mRNA levels at 37–38 weeks of gestation and umbilical cord blood base excess after delivery ($r = +0.8$) (Fig. 4d), whereas other indices did not reach any significant difference.

Discussion

To the best of our knowledge, this is the first investigation focused on the maternal peripheral blood human tumor-associated RCAS1 antigen in connection with the course of uncomplicated pregnancy. In

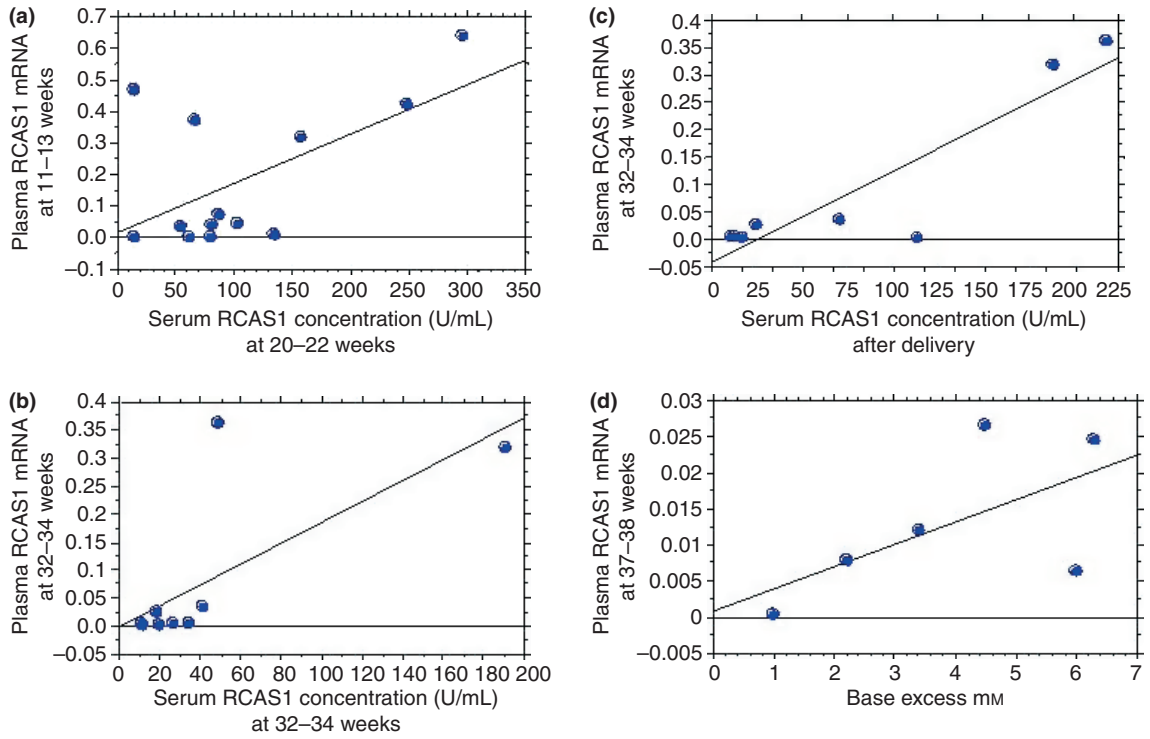

Fig. 4 Correlation of the maternal blood plasma RCAS1 mRNA levels at 11–13, 32–34, and 37–38 weeks of gestation with maternal blood sera RCAS1 protein concentration and pregnancy outcome in uncomplicated pregnancies. (a) The maternal blood plasma RCAS1 mRNA levels at 11–13 weeks positively correlated with maternal blood sera RCAS1 concentration at 20–22 weeks of gestation; (b) Plasma RCAS1 mRNA levels at 32–34 weeks of gestation positively correlated with serum RCAS1 protein concentration at the same stage of pregnancy; (c) Plasma RCAS1 mRNA levels at 32–34 weeks of gestation positively correlated with serum RCAS1 protein concentration after delivery; (d) Plasma RCAS1 mRNA levels at 37–38 weeks of gestation positively correlated with umbilical cord base excess after delivery. ($P < 0.05$).

this study, we confirmed a significant increase in RCAS1 protein concentration at 11–13 weeks of gestation in maternal blood serum. Moreover, we found that RCAS1 mRNA transcript levels were significantly increased at 11–13 weeks of gestation in maternal blood plasma. Previous studies showed that placenta is an important source of fetal RNA in maternal plasma.[26] Furthermore, the concentrations of such mRNA species in maternal plasma correlate with the known variations of the corresponding protein product with regard to gestational age.[27] Our results are consistent with these studies. Plasma RCAS1 mRNA levels significantly correlate with sera RCAS1 protein concentrations at different stages of gestation, and in early puerperium RCAS protein concentration falls down. Thus, the notion that the main source for RCAS1 protein in maternal blood is placenta is obvious. RCAS is expressed in the placenta during pregnancy in humans[5,18] as well as in mice with the higher expression levels on day 7.5 p.c, which corresponds to the period of placentation,[25] suggesting the possible role of RCAS1 in immune privilege during pregnancy.[18] Our present results show significant up-regulation of RCAS1 protein and RCAS1 mRNA expression at 11–13 weeks of gestation. Taken together, these findings suggest that RCAS1 protein produced by placenta and expressed in maternal blood at a high level might be of importance in process of decidualization, placentation, possibly through negative correlation with receptor-expressing NK cells. Thus, RCAS1 may play a role in the down-regulation of the maternal immune response, thereby maintaining pregnancy.[18]

According to recent investigations, a reduction in RCAS1 and FasL expression seems to be closely associated with activation and infiltration of maternal NK cells and destruction of uterine glands, resulting in rejection of the fetus.[18] At the same time, the level of the immune tolerance at the moment of the delivery drops independently of the fetal maturity.[20] RCAS1 drop may lead to the activation of cytotoxic immune response. The differences in the relative amount of

RCAS1 depend on the onset of labor, with the highest level in induced labor and the lowest in spontaneous labor.[19] Moreover, the alteration of RCAS1 expression in the human placenta may be involved in the changes of the maternal immune system that take place during stillbirth.[22] Taken together, we can speculate that the importance of RCAS1 protein in maintenance of pregnancy somehow might be extended to fetal well-being during pregnancy and labor, though no appropriate studies have been performed to clarify this issue.

Our present data reveal the positive correlation between the maternal blood sera RCAS1 protein concentration at 11–13 weeks of gestation and the age at delivery and that between the maternal blood plasma RCAS1 mRNA level at 37–38 weeks of gestation and umbilical cord blood base excess as well as negative correlation between the maternal blood sera RCAS1 protein concentration at 37–38 weeks of gestation and umbilical cord blood pH after delivery. Thus, the importance of the relationship between the placental RCAS1 protein and the fetal well-being during pregnancy and postpartum might be plausible.

The limitation of our study might be connected to the small number of patients studied. Moreover, we focused our studies only to uncomplicated course of pregnancy excluding patients with complicated pregnancies. Further studies evaluating a possible correlation between maternal blood RCAS1 concentration and pregnancy outcome should be performed with a larger number of specimens from uncomplicated as well as complicated pregnancies.

We can speculate that the maternal blood RCAS1 protein concentration changes at early stages of pregnancy might be indicative of placentation problems. Future investigations would be necessary to determine the exact function of RCAS1 protein in uncomplicated pregnancies and its possible role in the development of pregnancy pathologies. A possible role of RCAS1 protein in fetal well-being during pregnancy, in labor, and in postpartum should be evaluated.

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References

1 Sargent IL, Borzychowski AM, Redman C: NK cells and human pregnancy-an inflammatory view. *Trends Immunol* 2006; 27:399–404.
2 Yagel S: The developmental role of natural killer cells at the fetal-maternal interface. *Am J Obstet Gynecol* 2009; 201:344–350.
3 Nakashima M, Sonoda K, Watanabe T: Inhibition of cell growth and induction of apoptotic cell death by the human tumor-associated antigen RCAS1. *Nat Med* 1999; 5:938–942.
4 Matsushima M, Nakashima M, Ohshima K, Abe Y, Nishimura J, Nawata H, Watanabe T, Muta K: Receptor binding cancer antigen expressed on SiSo cells, a novel regulator of apoptosis of erythroid progenitor cells. *Blood* 2001; 98:313–321.
5 Wicherek L, Klimek M, Dutsch-Wicherek M, Kolodziejski L, Skotniczny K: The molecular changes during placental detachment. *Eur J Obstet Gynecol Reprod Biol* 2006; 125:171–175.
6 Reimer TA, Anagnostopoulos I, Erdmann B, Lehmann I, Stein H, Daniel P, Dorken B, Rehm A: Reevaluation of the 22-1-1 antibody and its putative antigen, EBAG9/RCAS1, as a tumor marker. *BMC Cancer* 2005; 5:47.
7 Sonoda K, Nakashima M, Kaku T, Kamura T, Nakano H, Watanabe T: A novel tumor-associated antigen expressed in human uterine and ovarian carcinomas. *Cancer* 1996; 77:1501–1509.
8 Ikeda K, Sato M, Tsutsumi O, Tsuchiya F, Tsuneizumi M, Emi M, Imoto I, Inazawa J, Muramatsu M, Inoue S: Promoter analysis and chromosomal mapping of human EBAG9 gene. *Biochem Biophys Res Commun* 2000; 273:654–660.
9 Tsuchiya F, Ikeda K, Tsutsumi O, Hiroi H, Momoeda M, Taketani Y, Muratasu M, Inoue S: Molecular cloning and characterization of mouse EBAG9, homolog of a human cancer associated surface antigen: expression and regulation by estrogen. *Biochem Biophys Res Commun* 2001; 284:2–10.
10 Akahira JI, Aoki M, Suzuki T, Moriya T, Niikura H, Ito K, Inoue S, Okamura K, Sasano H, Yaegashi N: Expression of EBAG9/RCAS1 is associated with advanced disease in human epithelial ovarian cancer. *Br J Cancer* 2004; 90:2197–2202.
11 Chatterjee M, Mohapatra S, Ionan A, Bawa G, Ali-Fehmi R, Wang X, Nowak J, Ye B, Nahhas FA, Lu K, Witkin SS, Fishman D, Munkarah A, Morris R, Levin NK, Shirley NN, Tromp G, Abrams J, Draghici S, Tainsky MA: Diagnostic markers of ovarian cancer by high-throughput antigen cloning and detection on arrays. *Cancer Res* 2006; 66:1181–1190.

12 Giaginis C, Giagini A, Theocharis S: Receptor-binding cancer antigen expressed on SiSo cells (RCAS1): a novel biomarker in the diagnosis and prognosis of human neoplasia. *Histol Histopahol* 2009; 24:761–776.
13 Kaku T, Sonoda K, Kamura T, Hirakawa T, Sakai K, Amada S, Ogawa S, Kobayashi H, Nakashima M, Watanabe T, Nakano H: The prognostic significance of tumor-associated antigen 22-1-1 expression in adenocarcinoma of the uterine cervix. *Clin Cancer Res* 1999; 5:1449–1453.
14 Sonoda k, Miyamato S, Yamazaki A, Kobayashi H, Nakashima M, Mekada E, Wake N: Biologic significance of receptor-binding cancer antigen expressed on SiSo cells (RCAS1) as a pivotal regulator of tumor growth through angiogenesis in human tumor uterine cancer. *Cancer* 2007; 110:1979–1990.
15 Han Y, Qin W, Huang G: Knockdown of RCAS1 expression by RNA interference recovers T cell growth and proliferation. *Caner Lett* 2007; 257:182–190.
16 Sonoda K, Miyamoto S, Yotsumoto F, Yagi H, Nakashima M, Watanabe K, Nakano H: Clinical significance of RCAS1 as a biomarker of ovarian cancer. *Oncol Rep* 2007; 17:623–628.
17 Wicherek L, Dutsch M, Mak P, Klimek M, Skladzien J, Dubin A: Comparative analysis of RCAS1 level in neoplasms and placenta. *Acta Biochim Pol* 2003; 50:1187–1194.
18 Ohshima K, Nakashima M, Sonoda K, Kikuchi M, Watanabe T: Expression of RCAS1 and FasL in human trophoblasts and uterine glands during pregnancy: the possible role in immune privilege. *Clin Exp Immunol* 2001; 123:481–486.
19 Wicherek L, Dutsch-Wicherek M, Mak P, Klimek M: The Role of RCAS1 and oxytocinase in immune tolerance during pregnancy. *Fetal Diagn Ther* 2005; 20:420–425.
20 Wicherek L, Klimek M, Dutsch-Wicherek M: The level of maternal immune tolerance and fetal maturity. *Neuro Endocrinol Lett* 2005; 26:561–566.
21 Galazka K, Wicherek L, Sikora J, Czekierdowski A, Banas T, Bednarek W, Obrzut B, Blecharz P, Reron A, Kalinka J: RCAs1 decidual immunoreactivity during stillbirth: immune cell presence and activity. *Am J Reprod Immunol* 2008; 60:513–522.
22 Wicherek L, Klimek M, Czekierdowski A, Popiela TJ, Galazka K, Tetlak T, Gilowski A, Dutsch-Wicherek M: The placental RCAS1 expression during stillbirth. *Reprod Biol Endocrinol* 2005; 3:24.
23 Tskitishvili E, Komoto Y, Kinugasa Y, Kanagawa T, Song M, Mimura K, Tomimatsu T, Kimura T, Shimoya K: Relationship between human tumor-associated antigen RCAS1 and gestational diabetes mellitus. *Am J Reprod Immunol* 2007; 58:440–446.
24 Tskitishvili E, Komoto Y, Kinugasa Y, Kanagawa T, Song M, Mimura K, Tomimatsu T, Kimura T, Shimoya K: The human tumor-associated antigen RCAS1 in pregnancies complicated by preeclampsia. *J Reprod Immunol* 2008; 77:100–108.
25 Tskitishvili E, Nakamura H, Kinugasa-Taniguchi Y, Kanagawa T, Kimura T, Tomimatsu T, Shimoya K: Temporal and spatial expression of tumor-associated antigen RCAS1 in pregnant muse uterus. *Am J Reprod Immunol* 2010; 63:137–143.
26 Ng EK, Tsui NB, Lau TK, Leung TN, Chiu RW, Panesar NS, Lit LC, Chan KW, Lo YM: mRNA of placental origin is readily detectable in maternal plasma. *Proc Natl Acad Sci USA* 2003; 100: 4748–4753.
27 Lo DYM, Chiu RWK: The biology and diagnostic applications of plasma RNA. *Ann NY Acad Sci* 2004; 1022:135–139.