

Expression and serum immunoreactivity of developmentally restricted differentiation antigens in epithelial ovarian cancer

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Cancer-embryo antigens or developmentally restricted differentiation antigens (DRDAGs), such as PLAC1 (CT92) and developmental pluripotency associated-2 (DPPA2/CT100), are expressed in pluripotent embryonic cells. They are also recognized as cancer-testis antigens (CT) which are proteins normally expressed only in the human germ line but that are also present in a significant subset of malignant tumors. These antigens may prove to be markers of 'repopulating' cells with stem cell-like characteristics and could be critical targets for immunotherapy in epithelial ovarian cancer (EOC). Our objective was to define the frequency of expression and immunogenicity of PLAC1 and DPPA2 in EOC and correlate expression with clinical outcome. One-step reverse transcriptase PCR was performed on 101 EOC samples and a panel of normal tissues. Expression of PLAC1 and DPPA2 in the EOC specimens was 21/101 (21%) and 31/101 (31%) respectively. In normal tissues, PLAC1 expression was restricted to the placenta while DPPA2 expression was restricted to the placenta and testis. Immunohistochemistry (IHC) and enzyme-linked immunosorbent assay (ELISA) were also performed on a subset of specimens. Humoral immunity was demonstrable in 2/12 serum samples from patients whose tumors expressed DPPA2. There was no demonstrable antibody response to PLAC1 in patients with PLAC1 positive tumors. The presence of PLAC1 and DPPA2 did not have a statistically significant effect on recurrence-free and overall survival. The tissue-restricted expression of PLAC1 and DPPA2, their expression in a significant proportion of EOC patients, and their potential to represent markers of stem cells make DRDAGs attractive targets for antigen-specific immunotherapy in EOC.

Keywords: human, ovarian cancer, PLAC1, DPPA2, RT-PCR, immunohistochemistry, humoral immunity

Introduction

There has been much speculation that cancers acquire characteristic traits by reactivating genes normally expressed in embryonic and fetal stages (1). The discovery of a growing number of proteins that appear to be expressed only in germ cells, trophoblasts and tumors – the cancer/testis (CT) antigens (2-4) – has led to the theory that aberrant expression of germline

genes in cancer reflects the activation of the silenced gametogenic program in somatic cells, and that this event is important for tumor progression (5). In previous studies, CT antigens such as NY-ESO-1 and LAGE-1 have been examined in a large panel of EOC tissues and cell lines using RT-PCR and IHC (6). Expression of NY-ESO-1 in EOC was demonstrated by RT-PCR and/or IHC in 82 of 190 (43%) specimens. LAGE-1 mRNA expression was present in 22 of 107 (21%) tumor tissues. These findings have led to the development of NY-ESO-1-based immune therapies for EOC (7, 8).

Due to the similarities between embryo implantation and the growth of cancer cells, there has been further discovery of new CT antigens. Two of these CT antigens are PLAC1 (CT92) (9), a human X-linked gene with placenta-specific expression, and developmental pluripotency associated-2 (DPPA2/CT100) (10), a non-X-linked gene which is expressed in pluripotent embryonic cells. These antigens may prove to be markers of 'repopulating' cells with stem cell-like characteristics that could be critical targets for immunotherapy in EOC. Our objective was to define the frequency of expression and immunogenicity of PLAC1 and DPPA2 in EOC patients and to examine the relationship between their expression and clinical outcome.

Results

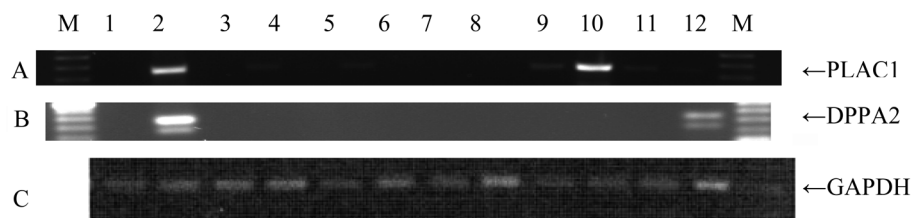
Study population

The characteristics of the study population are presented in Table 1. The median age of the study population was 65 years (range 32-89 years) and the median duration of follow-up was 34 months. The majority of patients presented at stage IIIC (70%) with grade 3 tumors (71%) of serous differentiation (58%). A complete response to therapy was achieved in 44 patients (44%) while the remaining patients achieved a partial response.

Expression of PLAC1 and DPPA2 mRNA in normal tissues and EOC specimens

Expression of PLAC1 and DPPA2 mRNA in a panel of human tissues and epithelial ovarian tumor specimens was investigated using RT-PCR (Figure 1, panels A and B). PLAC1 expression

Figure 1



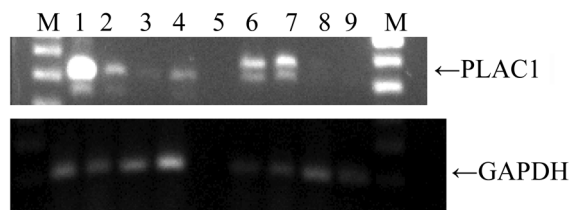
RT-PCR analysis for *PLAC1* and *DPPA2* expression in a normal tissue panel. (A) *PLAC1* mRNA expression yielded a 328 bp product. (B) *DPPA2* mRNA expression yielded a 430 bp product. (C) *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) served as an endogenous control and yielded a 204 bp product. Lanes: M, marker; 1, blank; 2, placenta; 3, adrenal gland; 4, bone marrow; 5, brain; 6, cerebellum; 7, fetal liver; 8, fetal brain; 9, heart; 10, placenta; 11, prostate; 12, testis.

Table 1

Patient characteristics.

Characteristic	No. of Patients
Evaluable Patients	101
Median Age (range)	65 yr (32-89 yr)
Cancer Type	
Ovarian	73
Primary peritoneal	28
FIGO Stage	
I/II	21
III	71
IV	9
Grade	
1	9
2	17
3	72
Not available	3
Histology	
Papillary serous	59
Clear cell	11
Endometrioid	4
Carcinosarcoma	5
Mixed	14
Other	8
Response to Frontline Therapy	
Complete response	44
Partial response	57
Recurrences	
No	27
Yes	22
Persistent disease	42
Not available	10
Current Status	
Alive with no evidence of disease	26
Alive with disease	32
Dead of disease	42
Dead from other causes	1
Antigen Status (RT-PCR)	
<i>PLAC1</i> positive	21
<i>PLAC1</i> negative	80
<i>DPPA2</i> positive	31
<i>DPPA2</i> negative	70
Both <i>PLAC1</i> and <i>DPPA2</i> positive	6
Either <i>PLAC1</i> or <i>DPPA2</i> positive	46

Figure 2



RT-PCR analysis for *PLAC1* expression in a subset of ovarian cancer specimens. *PLAC1* mRNA expression yielded a 328 bp product. *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) served as an endogenous control and yielded a 204 bp product. Shown is mRNA from placenta (lane 1). Lanes 2, 4, 6, and 7 are positive for *PLAC1* mRNA expression whereas lanes 3, 8, and 9 are negative. Lane 5 has a negative *GAPDH* control and hence *PLAC1* mRNA expression in this specimen is inconclusive.

was restricted to the placenta and *DPPA2* expression was restricted to the placenta and testis. The amplification products for *PLAC1* and *DPPA2* were confirmed by sequencing. Together, these results confirm the tissue-restricted expression of these two DRDAGs/CT antigens.

PLAC1 and *DPPA2* mRNA expression was tested in 101 epithelial ovarian tumor specimens. Twenty-one EOC samples were positive for *PLAC1* (Figure 2) and 31 were positive for *DPPA2*. Coordinate expression of *PLAC1* and *DPPA2* was found in 6 patients while 46 patient specimens were positive for either *PLAC1* or *DPPA2*.

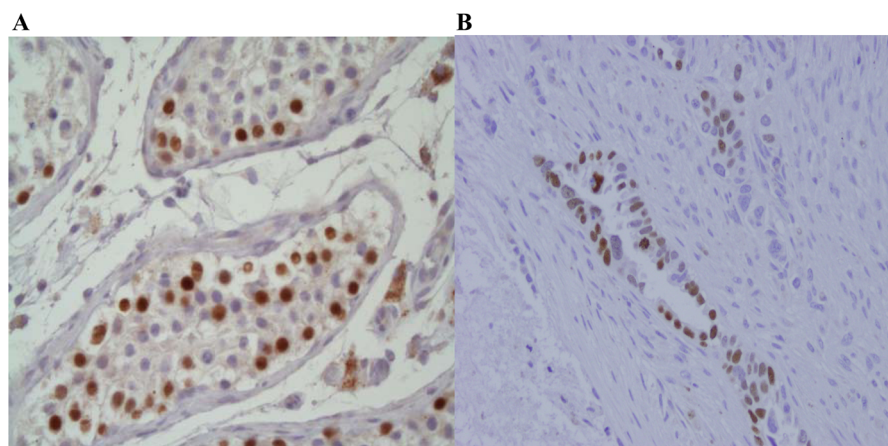
Expression of *DPPA2* examined by immunohistochemistry

DPPA2 exhibited intense nuclear immunostaining in the spermatocytes of the testis (Figure 3A). Of the 31 tumors that tested positive for *DPPA2* by RT-PCR, 18 tumors were subjected to IHC and 3 (17%) tested positive. Of 25 tumors which were negative for *DPPA2* by RT-PCR, 4 (16%) tested positive by IHC (Figure 3B).

Antibody response to *PLAC1* and *DPPA2* in ovarian cancer patients

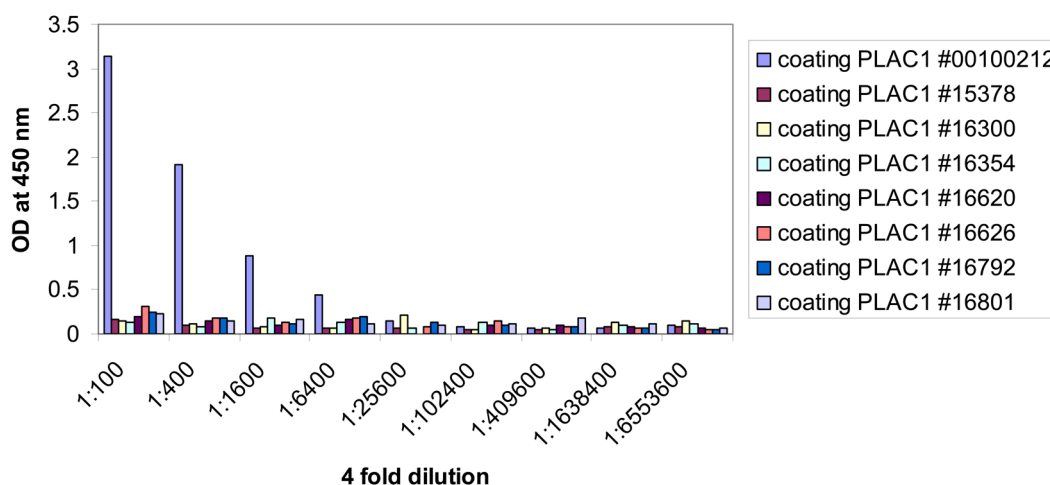
Archived sera were obtained from 20 patients with *PLAC1* positive tumors (determined by RT-PCR). A healthy donor serum specimen was included as a positive control. This healthy donor serum specimen was used as a positive control in a previous study by Silva *et al.* (11). There were no demonstrable antibody responses to *PLAC1* in these patients. Archived sera were also obtained from a subset of patients with *DPPA2* positive and negative tumors (determined by RT-PCR). A well-known cancer-testis antigen, NY-ESO-1, was used as a positive control. Two of the 12 patients who expressed *DPPA2* by RT-

Figure 3



DPPA2 expression in testis and an ovarian tumor specimen. Immunohistochemical staining was carried out with a polyclonal rabbit anti-DPPA2 antibody. (A) The spermatocytes of the normal testis show a nuclear pattern of staining of strong intensity. (B) Staining of an ovarian tumor specimen.

Figure 4



Serological reactivity of the recombinant PLAC1 protein in a subset of ovarian cancer patients. The presence of anti-PLAC1 antibodies in serum samples was assessed by ELISA. Serum obtained from a healthy donor (#00100212) was used as a known positive control.

PCR showed demonstrable antibody responses at a serum dilution of 1:400. In addition, 2 of the 3 patients with *DPPA2* negative tumors also showed this same seropositivity. Figure 4 and Figure 5 illustrate PLAC1 and DPPA2 ELISA titration curves with sera from selected ovarian cancer patients.

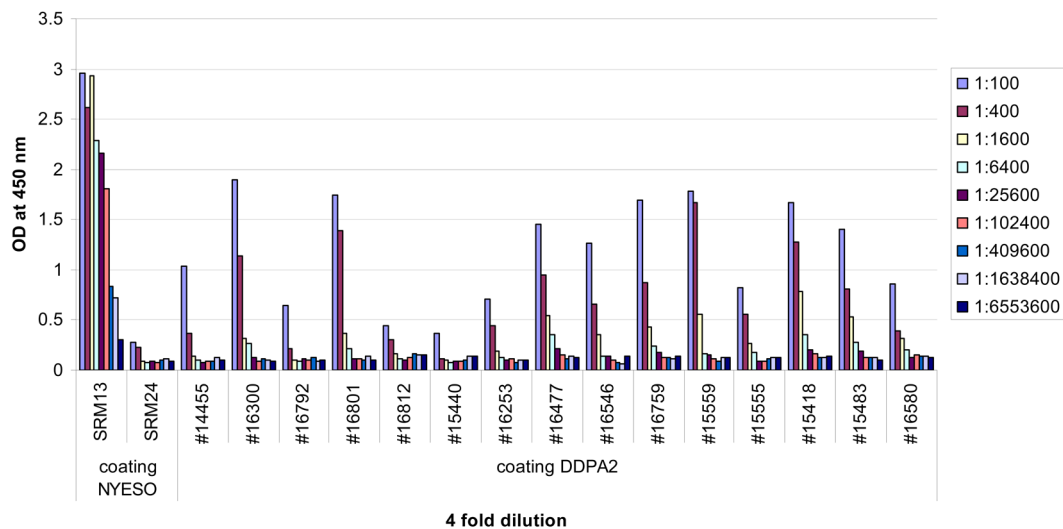
Relationship between *PLAC1*, *DPPA2*, *NY-ESO-1* and *BORIS* expression

It has been reported that *Brother of the Regulator of Imprinted Sites* (*BORIS*/*CTCF*), an autosomal CT antigen, functions as an oncogene in human cancer via dysregulation of the cancer epigenome (12). This raises the possibility that expression of *BORIS* may regulate the expression of developmentally restricted differentiation antigens/CT antigens. Therefore, we determined the expression of *BORIS* and *NY-ESO-1* by RT-PCR in the same panel of tissue specimens we previously described (6, 13). The pairwise relationships between *PLAC1* (CT92), *DPPA2* (CT100), *NY-ESO-1*, and *BORIS* expression were not

statistically significant (all *P* values were greater than 0.3 by the Pearson chi-square test), suggesting that *PLAC1* and *DPPA2* are not co-expressed with other classical cancer-testis genes.

Correlation of *PLAC1* and *DPPA2* expression with clinical outcome

The median estimated overall survival (OS) for all patients was 40.3 months [95% confidence interval (CI) 27.7-48.6] while the recurrence-free survival (RFS) excluding patients with persistent/progressive disease after initial therapy was 19.7 months (95% CI 16.1-Inf). *DPPA2* positivity was associated with older age at diagnosis (Wilcoxon-Mann-Whitney rank sum test, *P* = 0.0024) and larger residual tumor burden at initial surgery (Fisher's exact test, *P* = 0.030). The presence of *PLAC1* and *DPPA2* did not have a statistically significant effect on RFS or OS. Median RFS was 40.8 months in the *PLAC1* positive patients vs. 19.7 months in the *PLAC1* negative patients (log rank test, *P* = 0.37). Median OS was 49.9 months in the *PLAC1* positive patients vs. 40 months in the *PLAC1* negative patients

Figure 5

Serological reactivity of the recombinant DPPA2 protein in a subset of ovarian cancer patients. The presence of anti-DPPA2 antibodies in serum samples was assessed by ELISA. Serum obtained from a seropositive NY-ESO-1 patient (SRM13) was used as a positive control and that obtained from a seronegative NY-ESO-1 patient (SRM24) was used as a negative control. Sera #15559, #15555, and #15418 tested negative for *DPPA2* expression by RT-PCR.

(log rank test, $P = 0.52$). Median RFS was 36.1 months in the *DPPA2* positive patients vs. 19.1 months in the *DPPA2* negative patients (log rank test, $P = 0.46$). Median OS was 46.1 months in the *DPPA2* positive patients vs. 39.7 months in the *DPPA2* negative patients (log rank test, $P = 0.27$).

Discussion

The identification of tumor-specific antigens is a prerequisite for the development of efficient immunotherapeutic approaches to cancer. In this study, we sought to explore genes that are highly expressed in placenta and ovarian cancer but show relatively restricted expression in normal tissues. These genes could be involved in processes that are similar between embryo implantation and growth of cancer cells and therefore represent new potential therapeutic targets (vaccines as well as monoclonal antibodies) for ovarian cancer. In the present study, we have demonstrated the expression of two developmentally restricted differentiation antigens/CT antigens, *PLAC1* (CT92) and *DPPA2* (CT100), in 46% of ovarian cancer specimens. Independent frequencies of expression were 21% and 31% respectively for *PLAC1* and *DPPA2*. Coordinate expression of *PLAC1* and *DPPA2* was found in only 6% of patients suggesting that some aspects of the regulation of the expression of both genes are different. However, the low frequencies of concordance between RT-PCR and IHC for *DPPA2* suggest shared features with regards to antigenic and tumor heterogeneity. It is unclear why there is a discrepancy in the detection of *DPPA2* expression by PCR and IHC. Since the antibody used in our study stained normal human testes (positive control) appropriately and had previously been shown to stain tumor tissues (10), our results are unlikely to be due to issues relating to sensitivity or specificity of the antibody. It is possible that, at least in ovarian cancer, there is a post-translational modification of the *DPPA2* protein such that the *DPPA2* antibody cannot recognize changes in the epitope. Similar conclusions were drawn by Tammela *et al.* (14) who investigated SCP-1, a cancer-testis antigen, in epithelial ovarian

cancer and found no correlation between SCP-1 expression at the mRNA and protein levels. Clearly, additional studies would be needed to clarify the mechanisms of *DPPA2* expression in epithelial ovarian cancer.

Although *PLAC1* (CT92) and *DPPA2* (CT100) are considered part of the cancer-testis antigen family, they appear to have a different expression pattern from that of other classic CT antigens, such as NY-ESO-1 and BORIS. These varied relationships suggest that *PLAC1* expression is probably regulated by independent mechanisms and questions whether *DDPA2*, a transcriptional regulator, is involved in the transcriptional regulation of the X chromosome CT antigens or is activated by the same mechanisms that activate the X chromosome CT antigens.

There are shared features between these cancer-placental antigens/DRDAGs and cancer-testis antigens with regards to tissue restriction and expression pattern. These features have led to the hypothesis that cancer is a somatic cell pregnancy and commandeers aspects of the gametogenic program for its own purposes (1). Based on this hypothesis, there may be signals and functions associated with the initiation and/or progress of deprogramming and/or maintenance of the undifferentiated proliferative stem cell state common to both human embryogenesis and cancer.

ECSA/DPPA2 has a shared function in embryos and cancers and is expressed in lung, liver, and colon cancers (10, 15). This indicates that some function in embryogenesis specifically confined to this stage of the emergence and/or maintenance of the embryonic stem cell is commonly involved in the cancer stem cell phenotype (15). Further evidence to support the shared regulatory mechanisms of human embryogenesis and cancer was presented by Monk *et al.* (15) who examined the real-time PCR expression profiles in human early development of the embryo cancer gene, *ECSA/DPPA2*, and the testis deprogramming gene *BORIS*, compared with the expression of the well-known pluripotency gene, *OCT4*. They concluded that, unlike *OCT4* which is expressed throughout preimplantation development, *BORIS* and *ECSA/DPPA2* are predominantly

expressed at early and late stages of preimplantation development, respectively, consistent with different roles in reprogramming during embryogenesis (15).

DPPA2 has been studied in non-small cell lung carcinoma (10). Expression of *DPPA2* was demonstrated in 30% of 110 non-small cell lung carcinomas by RT-PCR and/or IHC, as well as in a small percentage of melanomas, colorectal cancers, and lymphomas. On immunohistochemical analysis, the subpopulation of cells which stained positively for DPPA2 were large basally located cells adjacent to the stroma, an area reported to be a niche of cancer stem cells, thereby suggesting that these DPPA2 positive cells could be putative lung cancer stem cells.

Expression of *PLAC1* has also been found in many cancer cell lines (11), lung carcinoma (11), gastric carcinoma (16), and hepatocellular carcinoma specimens (10). Possible functions of *PLAC1* include involvement in early developmental events (11), cell motility, migration, and invasion (17, 18), all of which are normal physiological characteristics of the human trophoblast.

To be considered for antigen-specific immunotherapy of any tumor type, including EOC, an ideal candidate antigen should not only demonstrate high frequency expression in tumor tissues and restricted expression in normal tissues, but also evidence for inherent immunogenicity (6). In the study by Silva et al. (11), 14/226 plasma samples from NSCLC patients were found to have detectable titers to *PLAC1*, ranging from 1/100 to 1/1200. While our 20 ovarian cancer patients did not show *PLAC1* seropositivity, further testing of a larger panel of patients is warranted. In contrast, DPPA2 ELISA testing revealed seropositivity in both RT-PCR positive and negative patients. This could represent differences in assay sensitivity as well as tumor heterogeneity. In addition, reactivity to DPPA2 could suggest a possible gender-specific link with the immunogenicity of the antigen (11). The demonstration of DPPA2 seropositivity in healthy female donors and patients whose tumors did not express the antigen suggest previous exposure to DPPA2. Thus, spontaneous immunity to DPPA2 could be evoked as a consequence of pregnancy and may persist in the long term. Since the presence of antibody response to a tumor antigen could reflect evidence of a T cell response, it is also critical to determine *PLAC1*- and DPPA2-specific CD4+ and CD8+ T cell responses in future studies.

Although the lack of correlation between *PLAC1* and *DPPA2* expression and clinico-pathologic characteristics, such as recurrence and survival, may reflect the fact that the majority of the patients (Table 1) had advanced stage disease, it may also imply that the antigens are not solely responsible for tumor progression. The lack of correlation between *PLAC1* and *DPPA2* expression and clinical outcome is not unique. The expression of other CT antigens, such as NY-ESO-1 and LAGE-1, has also not been correlated with pathologic variables or survival outcomes (6). Despite this lack of correlation between CT antigen expression and clinical outcome, NY-ESO-1 has proven to be an attractive target for immunotherapy due to its tissue-restricted expression and immunogenicity (7, 8). Since *PLAC1* and *DPPA2* share these CT antigen characteristics, they also represent potential immunotherapeutic targets. Further discovery regarding the role of CT antigens in tumorigenesis, invasion, and metastasis in epithelial ovarian cancer is critically needed in order to further define the functional and therapeutic significance of CT antigens.

In summary, we have shown expression of two cancer-testis antigens in a significant proportion of ovarian cancer patients. We have also demonstrated that DPPA2 evokes a spontaneous

immune response in a subset of patients. Together with the potential of these antigens to be markers of 'repopulating' cells with stem cell-like characteristics, our findings indicate that *PLAC1* (CT92) and *DPPA2* (CT100) could be potential targets for immunotherapy in epithelial ovarian cancer. Further studies regarding the immunogenicity of *PLAC1* (CT92)- and *DPPA2* (CT100)-expressing tumors and characterization of DRDAG/CT antigen-specific T cells are warranted.

Abbreviations

CT, cancer-testis; EOC, epithelial ovarian cancer; IHC, immunohistochemistry

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Materials and methods

Patients and specimens

Formalin-fixed and paraffin-embedded tissue specimens were obtained from patients undergoing debulking surgery for epithelial ovarian cancer, primary peritoneal cancer, and fallopian tube cancers at the Roswell Park Cancer Institute, Buffalo, NY between 1998 and 2007. All tissue specimens were collected under an approved protocol from the Institutional Review Board (IRB). All pathology specimens were reviewed in our institution and tumors were classified according to WHO criteria (19). For patients whose tumors tested positive for PLAC1 and DPPA2 by RT-PCR and/or IHC, a subset of serum samples were also available for ELISA analysis. The medical records of patients were also retrospectively reviewed under an IRB approved protocol. The review comprised outpatient and inpatient treatment, including surgery and chemotherapy. Study outcomes included overall survival (OS) and recurrence-free survival (RFS). Both survival criteria were measured from time of definitive surgery. Recurrence was defined as objective evidence of progression as all therapy was given in an adjuvant setting. The duration of OS was the interval between diagnosis and death. Observation time was the interval between diagnosis and last contact (death or last follow-up). Data were censored at the last follow-up for patients with no evidence of recurrence or progression.

Total tissue RNA isolation

Total tissue RNA was isolated from frozen tumor tissues using the TRI Reagent (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer's protocol. After isopropanol, 75% ethanol treatment and drying, the RNA was dissolved in RNase-free water. The resulting RNA concentration was measured spectrophotometrically (DU Series 500 Spectrophotometer, Beckman Coulter, Fullerton, CA) and the quality of the RNA was checked by electrophoresis on a 1.5% agarose/ethidium bromide gel.

RT-PCR analysis of PLAC1 and DPPA2 expression

Two micrograms of each RNA sample was subjected to cDNA synthesis using the Ready-To-Go™ RT-PCR beads (GE Healthcare, Buckinghamshire, UK). PCR was subsequently performed to analyze expression of PLAC1 (CT92) and DPPA2 (CT100) in a panel of human tissues (Human Total RNA Master Panel II; Clontech, Mountain View, CA) and 101 epithelial ovarian tumor specimens. A 328 bp PCR product was amplified using PLAC1 (CT92)-specific sense 5'-CCC CTC TTC AGT TCC AGT GA-3' and antisense 5'-CCA GTC TAT GGA GCA CAG CA-3' primers. A 430 bp PCR product was amplified using DPPA2 (CT100)-specific sense 5'-GCC CTT TGT TTA TGG

CCT GA-3' and antisense 5'-ACG CTT GGT TCC ATT TGT TC-3' primers. Glyceraldehyde-3-phosphodehydrogenase (*GAPDH*)-specific sense 5'- TCT TCA CCA CCA TGG AGA AG-3' and antisense 5'-CAA AGT TGT CAT GGA TGA CCT TGG-3' primers were used to obtain a 204 bp PCR product as a control. PCR was performed using 35 amplification cycles at an annealing temperature of 60°C. PCR products were then visualized on a 1.5% agarose/ethidium bromide gel.

Immunohistochemistry (IHC)

For detection of DPPA2 proteins, five micron thick sections were deparaffinized and pretreated in EDTA buffer for 20 minutes using an ordinary vegetable steamer. Sections were cooled for 20 minutes and were then incubated for 10 minutes with 3% H₂O₂ to quench endogenous peroxidase activity. Blocking was performed using serum-free protein block (Dakocytomation, Carpenteria, CA) for 30 minutes. Anti-DPPA2 antibody was added at a 1:150 dilution to each section and incubated for 60 minutes at room temperature. Rabbit IgG from Sigma (St. Louis, MO) was used as our negative isotype matched control. Labeled streptavidin biotin (LSAB+) reagents (Dakocytomation, Carpenteria, CA) were used according to the manufacturer's instructions followed by a 5 minute incubation with 3,3'-diaminobenzidine (DAB) (Dakocytomation, Carpenteria, CA). Sections were counterstained with hematoxylin and coverslipped. DPPA2 IHC was scored based on staining intensity as negative, weakly positive, moderately positive and strongly positive. For statistical analysis, cases were classified into two groups: Group 1 (negative) included the cases with negative staining and group 2 (positive) included cases staining with weak, moderate, and strong intensity.

ELISA

Recombinant PLAC1 and DPPA2 (1 µg/ml) in 100 µl PBS/well were absorbed to 96-well plates at 4°C overnight. Plates were washed three times with PBS and blocked with 5% NF milk/PBS and again incubated at 4°C overnight. Plates were washed three times with PBS. Four-fold serum dilutions in blocking buffer were prepared in 96-well dilution trays. After blot drying, 100 µl/well of serum dilution was added and incubated at 4°C overnight. Plates were washed three times with PBS/0.1% Tween and three times with PBS. After blot drying, plates were incubated with secondary antibody (HRP goat anti-human, Jackson ImmunoResearch Lab, Inc., West Grove, PA) at 1:5000 dilution for 1 hour at room temperature. Plates were washed and incubated in the dark with 100 µl tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, MD) for 10 minutes. After addition of 0.2 N H₂SO₄ (50 µl), the absorbance was determined with a microplate reader (Dynex Technologies, Inc., Chantilly, VA).

Statistical analysis

All statistical analyses were performed with R software (20). Estimated survival distributions were calculated by the method of Kaplan and Meier and tests of significance with respect to survival distributions were based on the log rank test. The Pearson chi-square is the most common statistic used to test the significance of the relationship between categorical variables. In this study, the Pearson chi-square test assessed the significance of the relationship between *PLAC1* and *DPPA2* expression with the expression of two other well-known CT antigens, *NY-ESO-1* and *BORIS*, which we previously reported to be frequently expressed in ovarian cancer (6, 13).

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