B7-H4 Is a Novel Membrane-Bound Protein and a Candidate Serum and Tissue Biomarker for Ovarian Cancer

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Abstract
Using cDNA database mining strategies and real-time quantitative reverse transcription-PCR, we identified B7-H4 as a novel gene that is overexpressed in ovarian and breast cancer tissues when compared with normal tissues. The gene encodes a protein of 282 amino acids with a signal sequence, an immunoglobulin domain, and a COOH-terminal hydrophobic transmembrane domain. Immunohistochemistry experiments show plasma membrane staining in serous ovarian and breast cancer, confirming the tissue specificity and cell surface localization. We have developed a sensitive dual monoclonal antibody sandwich ELISA to analyze the level of B7-H4 protein in >2,500 serum samples, ascites fluids, and tissue lysates. High levels of B7-H4 protein were detected in ovarian cancer tissue lysates when compared with normal tissues. B7-H4 was present at low levels in all sera but showed an elevated level in serum samples from ovarian cancer patients when compared with healthy controls or women with benign gynecologic diseases. The median B7-H4 concentration in endometrioid and serous histotypes was higher than in mucinous histotypes, consistent with results of immunohistochemical staining. The multivariate logistic regression analysis of B7-H4 and CA125 measured in the same sample set resulted in an area under the curve (AUC) of 0.86 for all stages and 0.86 for stage I/II patients, which was significantly higher than the AUC for either marker alone. In early-stage patients, the sensitivity at 97% specificity increased from 52% for CA125 alone to 65% when used in combination with B7-H4. We conclude that B7-H4 is a promising new biomarker for ovarian carcinoma.

Introduction
More women die annually from ovarian cancer than from all other gynecologic malignancies combined. The incidence of ovarian cancer in the United States is estimated to be 14.2 per 100,000 women per year, and 9 women per 100,000 die every year from ovarian cancer. In 2004, ~25,400 women will be diagnosed with ovarian cancer: 70% to 75% of new diagnoses will be stage III and IV carcinoma with a predicted 5-year survival of 15% (1). However, when ovarian cancer is detected in stage I, before it has spread from the ovaries, 90% of women with ovarian cancer can be cured with currently available therapy. Because only 25% of ovarian cancers are diagnosed at stages I/II, detection of the disease at an earlier stage may significantly improve overall survival in women with ovarian cancer.

Currently, the only clinically approved serum marker for use in management of ovarian cancer is CA125, which is found elevated in the majority of serous cancers but is elevated in only half of those women with early-stage disease. The major clinical application of CA125 is in monitoring treatment success or detection of recurrence in women undergoing treatment for ovarian cancer (2). The use of CA125 as a screening marker is limited because it is frequently elevated in women with benign diseases, such as endometriosis. Hence, there is a critical need for novel serum markers that are more sensitive and specific for the detection of ovarian cancer when used alone, or in combination with CA125 (3).

Using cDNA database mining strategies that identify differentially expressed genes and verification of the differentially expressed mRNAs by microarray and real-time quantitative reverse transcription-PCR, we identified DD-O110 as a novel gene that is overexpressed in ovarian and breast cancer tissue, when compared with normal ovarian and breast tissues, or tissues from other organs (4). Initial data on this novel gene have been published using the name Ovr110 (5, 6). Recently, DD-O110 has been described as B7-H4, a new member of the B7 family that is expressed in activated T cells and may participate in the negative regulation of cell-mediated immunity in peripheral tissues (7–10). We will, henceforth, refer to human DD-O110 as B7-H4 for consistency with published literature.

The gene encodes a protein of 282 amino acids with a signal sequence, an immunoglobulin domain, a COOH-terminal hydrophobic transmembrane domain, and seven predicted N-glycosylation sites. Immunohistochemical studies showed membranous staining in serous ovarian and breast cancer, confirming the tissue specificity and cell surface localization predicted by bioinformatic analysis (11). The plasma membrane localization and its over-expression in ovarian cancer tissue make B7-H4 a promising target for antibody therapy and a potentially useful marker for detection of ovarian cancer (4).

We have cloned and expressed recombinant B7-H4, used it to generate a series of monoclonal antibodies (mAb), and developed a sensitive dual mAb sandwich ELISA. The ELISA was used to analyze the level of B7-H4 protein in tissue lysates, ascites fluids, and >2,500 serum samples. The results show that the B7-H4 protein was present at low levels in serum from normal healthy women but was elevated in serum samples from ovarian cancer patients when compared with healthy controls or women with benign diseases.

Materials and Methods

Generation of anti-B7-H4 antibodies. The generation of specific mammalian antibodies against recombinant DD-O110 protein (which is

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identical to B7-H4) is described elsewhere (4). Briefly, 10 μg of recombinant B7-H4 protein expressed in insect cells were used for footprint immunization of 7 to 8-week-old BALB/c mice twice weekly over a 5- to 6-week period. Lymphocytes were subsequently isolated and fused with P3X63Ag8.653 cells to form a hybridoma using standard techniques. Hybridoma supernatants were screened by ELISA for reactivity against B7-H4 and for the absence of cross-reactivity to an irrelevant protein. B7-H4-specific hybridomas were cloned by single-cell sorting using a Coulter EPICS Elite-ESP Flow Cytometer (Beckman-Coulter, Miami, FL). mAb A7.2 was used as the coating antibody in the first study, whereas mAb A57.1 was used in the confirmatory study. mAb A7.1 was used as the detecting antibody throughout.

Western blot. SDS-PAGE was done according to the method of Laemmli. All samples were reduced with 20 mM DTT in 1 x LDS Sample Buffer (Invitrogen, Carlsbad, CA) and heated at 70°C for 10 minutes. Fifteen micrograms of each cell lysate were loaded onto a 4% to 12% Bis Tris gel (Invitrogen) for 1 hour at room temperature, while shaking. After washing, 100 μL of sample for 90 minutes with shaking. The plate was washed, and 100 μL of 1 mol/L HCl, and the plates were read at 450 μm wavelengths.

Ascites fluids. Ascites fluids were obtained from patients with advanced-stage ovarian cancer presented at the Cleveland Clinic Foundation (Cleveland, OH) and were generously provided by Dr. Ram Ganapathi (Experimental Therapeutics Program, Cleveland Clinic Foundation). Ascites fluids were collected into a sterile bag by flushing the needle and bag with heparin and then were centrifuged at 10,000 × g for 30 minutes. The supernatant was filtered through a cheese cloth, aliquoted, and stored at −80°C until use.

mAb sandwich ELISA detection for B7-H4. High-binding polystyrene plates (Corning Life Sciences, Bedford, MA) were coated overnight at 4°C with 0.8 μg/well of anti-DD-O110/B7-H4 mAb. The coating solution was aspirated off, and free binding sites were blocked with 300 μL/well Superblock-TBS (Pierce Biotechnology, Rockford, IL) plus 10% calf serum with 0.8 A plates (Corning Life Sciences, Bedford, MA) were coated overnight at 4°C using commercially available reagents according to the manufacturer’s instructions. After blocking, the membrane was incubated with mAb A57.1 (1 μg/mL) followed by a 1-hour incubation with donkey anti-mouse horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) at 1:10,000 dilution. The blot was developed with Enhanced Chemiluminescence Plus developer (Amersham Biosciences, Piscataway, NJ) following the manufacturer’s instructions.

Tissue lysates. All tissues were lysed using Tris buffer (pH 8.0; 50 mM Tris, 150 mM NaCl, 5 mM EDTA) with 1% NP40 as described elsewhere (12).

Statistical analysis. To determine the association between variables, Pearson and Spearman correlation coefficients and associated P values were calculated. Univariate receiver operating characteristic (ROC) analyses were done for CA125 and B7-H4. Multivariate ROC analysis combining CA125 and B7-H4 was facilitated by first performing multivariate logistic regression and using the regression coefficients for the individual terms to generate a composite marker based on the equation composite marker = (CA125 regression coefficient × CA125 value) + (B7-H4 regression coefficient × B7-H4 value). Areas under the ROC curve and sensitivity and specificity at defined points of the curve were determined (13). Wald test values were calculated from univariate and multivariate logistic regression models. The MedCalc Statistical Software (MedCalc Software, Mariakerke, Belgium) and JMP Statistical Discovery Software (SAS Institute, Cary, NC) were used for all statistical investigations.

Results

B7-H4 detection in tissue lysates and ascites fluids by Western blot and ELISA. mAb A57.1 described in Materials and Methods was used for detection of B7-H4 in Western blots. Recombinant B7-H4 protein was used as a positive control. Most mRNA-positive cell lines and tumor tissues showed a band at the predicted molecular weight of B7-H4 at around 28 kDa, which most likely represents intracellular, unprocessed B7-H4 (Fig. 1A). However, the most prominent band seen in these lysates was of much higher molecular weight between 50 to 100 kDa. This high molecular weight band could be reduced to a ~28-kDa band when the cell and tissue lysates were treated with N-glycosidase, an enzyme that specifically removes N-linked carbohydrates (data not shown; see ref. 4). All Western blot–positive ovarian cancer tissues and cell lines are also detected by the ELISA. The ELISA allowed more sensitive detection of B7-H4 and quantification in tissue lysates (Fig. 1B). The amounts of B7-H4 in normal ovary and in an ovarian cancer of mucinous histology type were below the MDC of the assay. The amount of B7-H4 expressed in positive ovarian cancer tissue lysates varied from 0.5 to 20 ng/mg of total protein (see Fig. 2). Analysis of tissue lysates from normal tissue showed that B7-H4 is expressed in normal breast tissue, kidney, seminal vesicles, liver, and trachea, in agreement with immunohistochemical staining results (11) and reported mRNA expression data (14).
The amount of B7-H4 in these tissues is around 1 ng/mg of total protein. B7-H4 can be detected at even lower levels in the adrenal and salivary gland, the esophagus, and the endometrium.

We quantified B7-H4 in 12 ascites fluids obtained from patients with late-stage ovarian cancer. All samples were positive for B7-H4, with levels varying from 50 pg B7-H4 per mg total protein to >2 ng/mg (concentration range, 1-74 ng/mL of ascites fluid). As expected, all ascites fluids were positive for CA125, ranging from 16 units/mg of total protein to 200 units/mg (concentration range, 360-13,000 units/mL of ascites fluid). As can be seen in Fig. 3, the levels of B7-H4 did not correlate strongly with levels of CA125 (Pearson correlation, $R = 0.52, P = 0.08$), suggesting that the expression of B7-H4 and CA125 are independent events in cancer progression.

**Detection of B7-H4 in serum of ovarian cancer patients.** The ELISA was used to analyze levels of B7-H4 in >2,500 serum samples. The serum samples included normal controls, samples from patients with colon, breast, lung, prostate, and ovarian cancers, and samples from patients with various benign diseases. B7-H4 was present in low levels in all sera (median, 0.55 ng/mL in women, 0.70 ng/mL in men) but showed an elevated level in serum samples from ovarian cancer patients when compared with healthy controls or women with benign gynecologic diseases. The B7-H4 concentration was not significantly elevated in serum samples from other cancer types and patients with any benign disease, confirming the tissue specificity of B7-H4. In contrast, the B7-H4 level was elevated by as much as 100-fold in patients with ovarian cancer. In study 1, the concentration of B7-H4 was determined in the sera of 147 women with serous or endometrioid ovarian cancer and 64 sera of women with mucinous cancer. All ovarian cancer histologic types had increased median B7-H4 concentration, but the median B7-H4 concentration in endometrioid and serous cancer patients was higher than in mucinous cancer patients (Fig. 4A), consistent with results of immunohistochemical staining.

B7-H4 was not elevated in sera of women with benign gynecologic diseases (50 sera each of patients with endometriosis, enlarged ovaries/edema, and polycystic ovaries, respectively; Fig. 4A). The menopausal status for most of the healthy women was not known, but the B7-H4 level in women of menstrual age (18-45 years) was not different from women in menopausal age (45-60 years) or after menopause (over 60 years), whereas the CA125 level in premenopausal women was significantly different from the level in women during or after menopause (Table 1). B7-H4 levels did not change during pregnancy when sera of women in each trimester were tested ($n = 30$) and were also stable over the course of a menstrual cycle as measured by six to nine serial draws in 26 premenopausal women (data not shown). The median intraperson coefficient of variation was 12% between the multiple draws and ranged from 5% to 24%. This coefficient of variation was in the same range as the variation observed in interassay and interoperator comparisons (coefficient of variation between 10% and 15%).

![Figure 1. Detection of B7-H4 in lysates of cancer cell lines and ovarian cancer tissues.](image1)

**Figure 1.** Detection of B7-H4 in lysates of cancer cell lines and ovarian cancer tissues. A, Western blot: 15 μg total protein of each lysate was loaded. Ca, carcinoma. B, ELISA: 25 μL of each lysate (adjusted to 1.2 mg/mL total protein) was used under conditions described in Materials and Methods. Sample 4 had 13.8 ng B7-H4/mg total protein.

**Figure 2.** Detection of B7-H4 in lysates of normal tissue and six ovarian cancer (OC) tissues by ELISA. All tissue lysates were prepared as described in ref. (12) and diluted to 1 mg/mL total protein.
Serum B7-H4 levels also did not change in patients with inflammatory diseases, such as asthma, bronchitis, or Crohn's disease. These data suggest that increased levels of B7-H4 in serum are associated with the presence of ovarian cancer.

Diagnostic sensitivity and specificity of B7-H4 in combination with CA125. B7-H4 results from study 1 were analyzed by ROC curves, comparing women with ovarian cancer (all histologic types, \( n = 236 \)) with healthy women and women with benign gynecologic diseases (\( n = 410 \)). An area under the curve (AUC) of 0.76 was calculated for B7-H4 (Table 2). The AUC for CA125 in the same sample set was 0.81, which is in reasonable agreement with published results in other studies (15, 16). Correlation analysis between B7-H4 and CA125 in the cancer samples gave an \( R = 0.53 \) (Spearman’s Rho, \( P = 0.0001 \)).

To confirm these results, B7-H4 and CA125 were tested in another set of ovarian cancer serum samples (\( n = 57 \)) with pathology-confirmed stage information (Fig. 4B and C). We tested additional serum samples from healthy women above 50 years of age.

Figure 3. Analysis of B7-H4 and CA125 by ELISA in ascites fluid of 12 women with advanced ovarian cancer (OC-1 to OC-12). The concentrations of the markers were normalized to total protein.

Figure 4. A, study 1: distribution of serum B7-H4 in healthy women; women with serous, endometrioid, or mucinous ovarian cancer; and women with benign gynecologic diseases. The scatter plot displays the median as horizontal lines. B, study 2: distribution of B7-H4 serum levels in healthy women, women with ovarian cancer of all stages and histotypes, and women with benign gynecologic diseases. Median concentrations of B7-H4 in this study were 0.57 ng/mL (normal), 0.96 ng/mL (ovarian cancer), and 0.55 ng/mL (benign), respectively. C, study 2: distribution of CA125 serum levels in healthy women, women with ovarian cancer, and women with benign diseases. Median concentrations of CA125 are 11 units/mL (normal), 59 units/mL (ovarian cancer), and 14 units/mL (benign), respectively. D, study 2: ROC analysis of CA125, B7-H4, and B7-H4 + CA125 for differentiation of healthy women and women with benign diseases from women with ovarian cancer. The analysis resulted in an AUC of 0.73 for B7-H4, an AUC of 0.79 for CA125 and an AUC of 0.86 for CA125 + B7-H4 combination.
age and women with endometriosis. ROC analysis with these cancer samples and 522 normal control and benign samples yielded AUCs of 0.73 and 0.79 for B7-H4 and CA125, respectively (Fig. 4D), in good agreement with the results from the first study. The B7-H4 and CA125 values in this data set showed correlation with an R = 0.1 (Spearman’s Rho, P = 0.007) in an analysis of all samples and an R = 0.35 (Spearman’s Rho, P = 0.008) for cancer samples only.

B7-H4 and CA125 were concurrently elevated in most cancer patients, but each marker also detected unique samples. Therefore, B7-H4 and CA125 complemented each other in a multivariate logistic regression analysis. The multilogistic regression ROC curve of CA125 in combination with B7-H4 yielded an AUC of 0.86, indicating a significant improvement in the clinical performance over CA125 or B7-H4 alone (P = 0.01 and 0.0001, respectively; Table 2). When the 97th percentile of all normal and benign samples was used as a cutoff concentration for B7-H4 and 35 units/mL used as a cutoff for CA125, all women in the noncancer group with false-positive CA125 values (n = 31) were negative for B7-H4.

As reported in the literature, CA125 levels are below the critical cutoff concentration in ~50% of early-stage ovarian cancer patients (2). The sensitivity and specificity were, therefore, lower (AUC = 0.73) in patients with stage I/II disease (n = 29) than if all cancer stages were used for analysis. In this study, 17 patients (58%) had positive CA125 values. B7-H4 levels were also elevated in early stages of ovarian cancer with an AUC that was higher (AUC = 0.81) than for all stages. The multivariate logistic regression analysis of CA125 + B7-H4 for early-stage disease resulted in an AUC of 0.86. The sensitivity of ovarian cancer detection at 97% specificity increased from 52% to 65% when CA125 was used in combination with B7-H4 (Table 2). At a CA125 cutoff of 35 units/mL, a total of 21 of 29 patients with stage I or stage II disease were positive for either or both of the CA125 or B7-H4 markers. These results show that the combination of B7-H4 and CA125 increased the sensitivity and specificity of detection of early-stage ovarian cancer over the use of CA125 alone (P = 0.002).

### Table 1. B7-H4 and CA125 in healthy controls: the values for women in menstrual age (18-44 years) were compared with women during or after menopause

<table>
<thead>
<tr>
<th>Marker</th>
<th>Age 18-44 y (n = 54)</th>
<th>Age 45-60 y (n = 173)</th>
<th>Age 60-81 y (n = 105)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B7-H4 (ng/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>0.51</td>
<td>0.55</td>
<td>0.58</td>
</tr>
<tr>
<td>Mean</td>
<td>0.58</td>
<td>0.59</td>
<td>0.59</td>
</tr>
<tr>
<td>Central 90th percentile</td>
<td>0.19-1.1</td>
<td>0.15-1.1</td>
<td>0.2-1.1</td>
</tr>
<tr>
<td>CA125 (units/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>11.8</td>
<td>11.1</td>
<td>10.7</td>
</tr>
<tr>
<td>Mean</td>
<td>16.1</td>
<td>12.8</td>
<td>12.5</td>
</tr>
<tr>
<td>Central 90th percentile</td>
<td>5.1-38.7</td>
<td>5.5-23.9</td>
<td>5.2-25.2</td>
</tr>
<tr>
<td>P vs 18-44 age group</td>
<td></td>
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</tbody>
</table>

### Table 2. Results of logistic regression analysis: sensitivity at 97% specificity and AUC for CA125, B7-H4, and CA125 + B7-H4 for differentiation of women with ovarian cancer from healthy women and women with benign diseases

<table>
<thead>
<tr>
<th>Study</th>
<th>CA125</th>
<th>B7-H4</th>
<th>CA125 + B7-H4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1: all stages</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC</td>
<td>0.81</td>
<td>0.76</td>
<td>0.83</td>
</tr>
<tr>
<td>Sensitivity at 97% specificity (%)</td>
<td>51</td>
<td>32</td>
<td>58</td>
</tr>
<tr>
<td>Study 2: all stages</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>AUC</td>
<td>0.79</td>
<td>0.73</td>
<td>0.86</td>
</tr>
<tr>
<td>Sensitivity at 97% specificity (%)</td>
<td>49</td>
<td>37</td>
<td>60</td>
</tr>
<tr>
<td>Study 2: stage I/II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC</td>
<td>0.73</td>
<td>0.81</td>
<td>0.86</td>
</tr>
<tr>
<td>Sensitivity at 97% specificity (%)</td>
<td>52</td>
<td>45</td>
<td>65</td>
</tr>
</tbody>
</table>

### Discussion

In this study, we report for the first time the identification and characterization of DD-O110 as a novel ovarian cancer serum marker. DD-O110 has recently been described in the literature as B7-H4, a molecule that negatively regulates T-cell immunity by the inhibition of T-cell proliferation, cytokine production, and cell cycle progression. The function of B7-H4 in ovarian cancer is not yet known, but the overexpression of B7-H4 on cancer cells may play a role in evading immune system surveillance (14).

We have generated a series of mAbs against the recombinant DD-O110/B7-H4 protein and developed a sensitive ELISA for measuring the native protein. We have shown overexpression of B7-H4 in ovarian cancer tissues when compared with normal tissues. B7-H4 was detectable in elevated levels in serum of ovarian cancer patients but is not typically elevated in patients with benign diseases. The sensitivity and specificity of this assay was comparable with the performance of CA125 in the same sample sets. In multivariate logistic regression analyses, B7-H4 was additive to CA125, especially in the detection of early-stage cancer, and improved the clinical performance of the individual marker. Our observation that B7-H4 is elevated in serum of patients with early-stage cancer is further supported by immunohistochemistry results showing that 60% of cells in stage I and in 90% of cells in stage II ovarian carcinomas overexpress B7-H4 (17).

Although CA125 is an important serum marker for ovarian cancer, its use is limited due to its frequent elevation in women with benign diseases and also because it is not elevated in 50% of women with early-stage ovarian cancer (16). The performance of CA125 as a means for early detection of ovarian cancer might be improved by using serial CA125 values (18), but it is likely that no single cancer biomarker will provide all of the necessary information for optimal cancer diagnosis and management. Therefore, current efforts in the research community are focusing on the identification of panels of biomarkers that can be used in combination (19). Simultaneous elevation of two or more markers may occur less frequently in healthy individuals than in women whose ovarian cancers coexpress multiple markers (3), thus potentially increasing the specificity of the test. Using CA125 and B7-H4 in our sample set, the multivariate analysis of these two markers increased sensitivity and specificity when compared with the analysis of each marker alone. Currently, other molecules, such as the tissue kallikreins (12, 20–22), prostasin (23), HE4 (24, 25), and soluble mesothelin related (26) are being characterized of DD-O110 as a novel ovarian cancer.
evaluated as potential ovarian cancer serum biomarkers and may serve as members of a multimarker panel to improve detection of ovarian cancer.

Our data indicate that B7-H4 is a promising new serum marker for ovarian cancer. B7-H4 should be evaluated in additional studies, using well-characterized samples, to confirm and expand our findings. In addition, the potential use of B7-H4 as a prognostic marker in tissue biopsies and for monitoring patients after surgery should be explored. Lastly, its cell surface localization and tissue specificity also qualify B7-H4 as a promising target for antibody-based therapy (4).

As more biomarkers are discovered and validated, efforts will focus on identifying an appropriate set of markers that can increase the rate and accuracy of detection of early stage ovarian cancers. The increase in sensitivity and specificity conferred by such a panel could potentially have a significant effect on the survival rate of patients with this devastating disease.

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References

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