**OBJECTIVE:** We studied ovarian cancers for the expression of membrane markers of hematopoietic origin.

**STUDY DESIGN:** We used flow cytometry to systematically characterize the expression of more than 30 hematopoietic antigens on ovarian carcinoma cell lines and to assess their stability under estrogen exposure. The expression of the antigens was validated by a bioinformatics survey and immunohistochemical staining of ovarian cancer specimens.

## RESULTS

Several antigens were expressed by the majority of the cells, such as CD15, CD71, and CD138, whereas others were found on small and distinct cellular subpopulations. The expression patterns of the different markers were unaffected by estrogen exposure, indicating their stability.

## CONCLUSION

The antigens described in our work may serve as potential targets for new and existing targeted drugs.

### Key words:

bioinformatics, flow cytometry, hematopoietic antigens, ovarian cancer

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**Systematic antigenic profiling of hematopoietic antigens on ovarian carcinoma cells identifies membrane proteins for targeted therapy development**

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**O**varian cancer is the fifth most common cancer in women, with the highest fatality to case ratio of all gynecological malignancies. Because symptoms tend to be nonspecific and develop late in the clinical course of the disease, patients present with advanced disease in more than two-thirds of the cases and prognosis is ultimately poor. The current approach to treatment of ovarian cancer consists of a combination of cytoreductive surgery and platinum-based chemotherapy. Like all chemotherapy, however, this approach is nonspecific.

Modern approaches in hematologic cancers have evolved over the past decade to include targeted therapy, which consists, in many cases, of 1 of 2 basic classes of drugs: monoclonal antibodies targeting tumor-specific antigens and highly specific chemical compounds directed against signal transduction pathways active in malignant cells. Examples for the first class include Myelotarg (gemtuzumab ozogamicin; Wyeth, Neopharm, Petach Tikva, Israel), a toxin-conjugated antibody directed against CD33 for acute myeloid leukemia, and Rituximab (MabThera; Roche Pharmaceuticals, Petach Tikva, Israel), which targets CD20, used predominantly in non-Hodgkin’s lymphomas and certain cases of chronic lymphocytic leukemias and autoimmune diseases. Examples for the second type of targeted agent include Gleevec (Imatinib; Novartis Pharmaceuticals, Hanover, NJ), a tyrosine kinase with significant activity against the BCR/ABL fusion protein in chronic myeloid leukemia and Velcade (bortezomib; Millennium Pharmaceuticals, Inc., Cambridge, MA), which targets the proteasome machinery in multiple myeloma.

Although the use of targeted therapy is rapidly expanding in hematologic cancers, drugs based on these concepts are sparse in the armamentarium against solid tumors. One of the few exceptions is Herceptin (Roche Pharmaceuticals), used in breast cancer. The insufficient targeted therapy in solid malignancies has prompted attempts to adopt drugs used for hematologic cancers in the treatment of solid tumors. A successful example for such an approach is Gleevec for the treatment of gastrointestinal stromal tumors, which apparently express mutated forms of the stem cell factor receptor c-kit (also designated CD117), containing a tyrosine kinase in its cytoplasmic tail, which is inhibited by Gleevec.

Targeted therapy requires the identification and localization of candidate cellular targets. Few potential targets have thus far been identified in ovarian carcinoma cells; however, the results of clinical trials using targeted drugs, such as anti-CA125 antibodies, anti-MUC1 antibodies, and...
Hence (anti-HER2/neu), have been disappointing.14,15

In the present study, we undertook a systematic approach for antigenic profiling of hematopoietic membrane proteins expressed by ovarian carcinomas to identify potential candidates for targeted therapy.

**Materials and Methods**

**Ovarian cancer cell lines**

BG1AS4 (a generous gift of Professor Lois A. Annab, National Institute of Environmental Health Sciences, Chapel Hill, NC), A2780PAR, and A2780ADR (a generous gift of Professor Jeremy Squire, University of Toronto, Toronto, Canada) and SKOV3 (American Type Culture Collection, Manassas, VA) were propagated in 75 mm² plastic flasks with RPMI 1640 growth medium (BG1AS4 and A2780) or McCoy’s growth medium (SKOV3) supplemented with 10% fetal calf serum, 1% L-glutamine, and 1% penicillin-streptomycin. The cells were maintained at 37°C in 5% CO₂ and 95% air. Cells were harvested upon reaching 70–80% confluence and replated or used for experiments.

All tissue culture reagents were purchased from Biological Industries (Beit Ha’emek, Israel). The BG1AS4 cell line, known to express estrogen receptors,16 was propagated in the presence and absence of estrogen (17-β-estradiol, 10⁻⁸ mol/L, Sigma, St. Louis, MO).

**Ovarian cancer cell labeling**

Suspensions of cells harvested for analysis were incubated with fluorophore-conjugated monoclonal antibodies against each of the antigens investigated: CD19, CD79a, CD4, CD20, CD38, CD34, CD45, CD15, CD33 (Becton Dickinson Biosciences, San Jose, CA); CD8, CD14 (Caltag laboratories, Burlingame, CA); FMC7, CD7, CD56, CD3, CD5, CD11b, CD62p, CD41, HLADR (Immunotech, Beckman Coulter, Marseille, France); CD22, CD23, CD10, CD16, CD71, CD103, CD117, CD13 (Dako Cytomation, Glostrup, Denmark); CD138 (IQ Products, Groningen, The Netherlands); and CD133 (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). Background fluorescence was determined by suspending cell samples with nonspecific isotype murine antibodies conjugated to the same fluorophores (Immunotech or Becton Dickinson Biosciences) matched to the specified antibodies.

**Flow cytometry**

Six parameter flow cytometry was performed by the FACSCalibur analyzer, and data were processed using CellQuest software (Becton Dickinson Immunocytometry Systems). Analysis was performed on a minimum of 10,000 events, although 30,000 events were collected in most cases. Dot plots and histograms demonstrating the expression of the various antigens detailed in the study were generated, and subpopulations of ovarian carcinoma cells were identified based on their physical properties and antigenic profile.

**Bioinformatics analysis**

Mining Expression Data, a proprietary computational analysis developed by Compugen, is a platform for the collection of public gene expression data, normalization, annotation, and performance of various queries. Expression data from the most widely used Affymetrix microarrays are downloaded from the Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov/GEO). Data are multiplicatively normalized by setting the 95 percentile to a constant value, and noise is filtered by setting the lower 30% to 0.

Experiments are annotated, first automatically and then manually, to identify tissue and condition, and chips are grouped according to this annotation. A group may then be split if the expression pattern of the various experiments within it is not sufficiently similar. Each probe set in each group is assigned an expression value (the median of the expressions of that probe set in all chips included in the group), and the vector of expression of all probe sets within it is not sufficiently similar. Each group may then be split if the expression levels of a probe set representing the genes in the healthy ovary were compared with the malignant group.

**Immunohistochemistry**

After institutional review board approval, paraffin-embedded tissue blocks of 5 ovarian carcinoma samples were examined. Four millimeter unstained slides were prepared from each case. Detection in all tissue samples was performed using a rabbit polyclonal antibody (Biocare Medical, Walnut Creek, CA) in a 1:25 dilution. Immunohistochemistry was performed by deparaffinization of the slides in xylene and degraded alcohols 70% and 96%.

Immunoperoxidase staining was performed using the modified labeled streptavidine technique and run on an automated system (Ventana Autostainer Nexes, Tucson, AZ) using diaminobenzidine as a chromogen. All sections were counterstained with Mayer’s hematoxylin. Immunohistochemical cell membrane staining in all specimens was evaluated with microscopy by counting 10 high-power fields (×400), with a minimum of 1000 cells counted per slide.

Two pathologists assessed 2 parameters in each section:

a. The proportion of positively stained cells of the total number of cells counted in the fields examined, ranging from 0–100%.

b. The intensity of immunohistochemical staining graded subjectively on a scale of 0–3, with 0 reflecting no detectable staining and 3 representing very intense staining.
The scoring index was then calculated by multiplying the intensity grade of the stained cells by the percentage of the positively stained cells.

Statistical analysis

The expression of each antigen investigated on the sampled cells was determined by subtracting the background fluorescence of the isotype-matched control from the fluorescence of the sample examined in each analysis, using CellQuest software. Expression is represented as percentage of the total cell population. The results of 2-6 analyses for each cell line were averaged to determine the mean expression of each antigen on each cell line as well as the SD.

Results

Characterization of cell surface marker pattern of ovarian cancer cell lines

The cell surface antigen pattern was evaluated on the following ovarian cancer cell lines: the poorly aggressive A2780PAR cells, its drug-resistant variant A2780ADR, the platinum-resistant line SKOV3, and the estrogen-dependent BG1AS4. All cell lines were tested for the expression of a series of 30 known hematopoietic antigens, including specific markers of cells of the lymphoid, myeloid, monocytic, and erythroid lineages (Table 1).

The expression of immature cell markers was also tested (CD34, CD38, CD117, and CD133). As expected, the ovarian cell lines investigated did not express any of the pan-leukocyte markers, such as CD45 and CD11b (Table 1). Most of the CD markers were negative in our screen, but expression of some of the antigens was found on all the ovarian cancer cell lines, albeit at different levels (Table 1). Overall, the expression patterns of each of the antigens were very similar in the different cell lines (Table 1).

Among the antigens highly expressed by some of the cell lines was CD15 (Table 1 and Figure 1), a myeloid marker previously reported to be expressed in ovarian cancer cells. Between 70-80% of the cells in all cell lines were found to be positive for CD71 (a transferrin receptor) (Table 1). In addition, we found high levels of CD138 (Figure 1), a plasma cell marker known to be expressed by ovarian carcinoma cells, in all the cell lines. Significant numbers of ovarian cancer cells among the A2780PAR and BG1 lines also expressed CD4 (Table 1 and Figure 1). None of the other T cell lineage markers was expressed by ovarian carcinoma cells (Table 1).

Relatively small subpopulations positive for immature antigens were identified in the cell lines investigated. CD117, an antigen previously described in ovarian carcinomas, was found to be expressed in 3 of 4 lines (Table 1), but the levels of expression were relatively low and restricted to minor subpopulations (Figure 1). CD133 is another immature antigen that was found to be expressed by 3 of 4 ovarian carcinoma cell lines (Figure 1). Conversely, CD34 or CD38, both characteristic of immature hematopoietic cells, was not expressed by ovarian carcinoma cells (Table 1).

The expression pattern of some of the CD markers identified by flow cytometry was also confirmed by immunohistochemical staining of ovarian carcinoma tissue sections. As shown in Figure 2, CD138 stained powerfully in ovarian cancer tissue sections, with a clear pattern of membrane staining. In contrast, CD3, CD4, CD15, and CD20 expression was found to be low in ovarian carcinoma tissue sections (Figure 2).

An important prerequisite for a therapeutic target is the stability of its expression. We assessed the effects of estrogen exposure on the expression of CD markers on ovarian cancer cells. BG1AS4 cells, which express estrogen receptors, were propagated in the presence and absence of estrogen (17-β-estradiol, 10^-8 mol/L). Then flow cytometry was performed as described above. Exposure of the cells to estrogen had no apparent effect on these antigens’ surface expression (data not shown).

Candidate genes validated by gene expression analysis in ovarian carcinomas

The analysis of the gene microarray experiments is summarized in Table 2 and Figure 3. It is noteworthy that several CD genes are expressed at high levels in the various types of ovarian carcinomas. All types of ovarian carcinomas express higher levels of CD138 than normal ovarian tissue controls. Expression of CD15, CD16, CD133, and CD4 was also detected in several types of ovarian carcinomas. CD71, the major cell transferrin receptor, was prominent in both normal and malignant ovary samples (Table 2).
Ribonucleic acids (RNAs) encoding other CD markers (such as CD23) were low in most normal and malignant ovarian samples. Surprisingly, the expression of CD117 (c-kit), a stem cell factor receptor previously described in ovarian carcinoma, was found to be high in normal ovary samples and low in ovarian carcinomas. Whereas the expression pattern of most of the CD RNAs was similar among the different subtypes of ovarian cancer, relatively high levels of CD138 and CD15 were detected in mucinous ovarian carcinoma samples.

**COMMENT**

The predominant focus of the study was to develop a novel, rapid screening approach to identify possible therapeutic targets in ovarian cancer and to validate this approach against other, common methodologies. Our results demonstrated that ovarian cancer cells, or their subpopulations, express a variety of hematopoietic markers. Some have been previously described, whereas several of the antigens found to be expressed by ovarian cancer cells in this study have never been investigated in this context.

Our study took a novel approach, beginning with a systematic screen for expression of a large number of hematopoietic antigens by ovarian cancer cell lines. This allowed us not only to look at the dichotomic result of positive/negative but also to detect small populations of cells that are positive for antigens that may disappear in more mature cells.

The work on cell lines may be biased because of the changes known to develop in immortalized cells and, therefore, needs to be validated in ovarian cancer samples. This may be done by immunohistochemical staining of tissue samples; however, this type of validation may be limited by the number of samples and antigens verified.

We utilized a novel validation technique based on a large cohort of samples present in public domain gene expression data libraries. This approach affords substantial advantages, including the large-scale screening of large, independent sample cohorts of primary ovarian cancer samples, and simultaneous com-

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**FIGURE 1**

Characterization of cell surface marker pattern of ovarian cancer cell lines

Representative flow cytometric histograms of surface marker expression (purple, isotype control; green, specific antibody). Among the antigens highly expressed by all the cell lines were CD15 and CD138. Relatively small subpopulations positive for immature antigens were identified, such as CD117 and CD133. It is noteworthy that a significant number of ovarian cancer cells expressed CD4.


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**FIGURE 2**

Immunohistochemistry staining

The flow cytometry findings were confirmed by immunohistochemical staining of ovarian carcinoma tissue sections. Significant CD138 expression was detected in ovarian cancer tissue sections, with a clear pattern of membrane staining. The CD3, CD4, CD15, and CD20 expression was low. Lower row describes the mean score index \( \pm SE \) (percent of cells that express the antigen).

Comparison with normal ovarian tissue samples. Although the control samples may contain only a small volume of cells compared with the malignant samples, a recent study indicated that bulk normal ovarian tissue can serve as a control for ovarian cancer in gene expression profiling.17

We found that several hematopoietic markers are prominently expressed by ovarian cancer cells. These include CD15, CD71, and CD138. Because CD71 is a transferrin receptor commonly expressed by cells with high proliferation rates, its expression by ovarian cancer cells is not surprising. CD15 is a cell surface carbohydrate (sialylated Lewis[x]), and its expression defines highly differentiated myeloid cells. It plays a significant role in selectin-mediated cell adhesion and has previously been reported to be expressed by ovarian cancer cells.18,19

CD138 (Syndecan-1) is a heparan sulfate proteoglycan that functions as a cellular receptor for components of the extracellular matrix (eg, collagen type I), and it is expressed on many epithelial tumors. It has a possible role in growth factor signaling in these cells. It has previously been described in ovarian cancer, and its expression has been correlated to tumor grade and stage, indicating a possible role for this antigen in tumor invasion and metastasis.

Therapeutically, it has been utilized as an attachment receptor for Capsid-modified AAV vectors to mediate enhanced gene transfer for gene therapy purposes.20 Although the levels of expression of the various CDs were similar in most types of ovarian carcinomas, both CD138 and CD15 were relatively high in the mucinous type (Figure 3).

Interestingly, both CD138 and CD15 contain high levels of carbohydrates, implying a negatively charged cell surface. Hence, either carbohydrate- or electrical charge-based adhesive mechanisms may be significant for the attachment, survival, or proliferation of ovarian cancer, possibly specific in the mucinous type, in addition to the well-established protein (eg, integrin)-based mechanisms.21,22 Such adhesion factors may potentially be used as therapeutic targets.23

CD4 is a novel hematopoietic antigen that was shown to be expressed by ovarian cancer cells in our current study by several distinct approaches. It is well known as a T-cell coreceptor, with an important role in major histocompatibility complex II-restricted antigen-induced T-cell activation. It has not been heretofore described on epithelial cells of any type, and its putative role in ovarian carcinogenesis is intriguing. Interestingly, the local production of an anti-CD4 antibody in vivo has been recently demonstrated. This methodology, based on viral-mediated gene transfer, may be effective against CD4-expressing cells.24

A nodal peripheral T-cell lymphoma was recently treated with a novel anti-CD4 therapeutic monoclonal antibody.25 These 2 latter studies indicate that this antigen may potentially be targeted in therapy against CD4-positive tumors.

Other hematopoietic antigens that are expressed to a limited extent by small subpopulations of ovarian carcinoma cells include CD133, CD103, CD16, and CD23. This pattern of expression is characteristic of immature, progenitor cell
populations, making these antigens potential candidates for tumor stem cell markers.

CD133 is known as a stem cell marker in at least 3 types of cells: hematopoietic cells, epithelium, and neural tissue. This recognized role, together with its pattern of expression in ovarian cell lines, makes it an especially strong candidate for an ovarian carcinoma stem cell marker.

It was disappointing to observe that possible targets for existing targeted drugs, such as CD20 and CD33, are not expressed to a significant extent by ovarian cancer cells, nor can their expression be induced by exposure to estrogen. An interesting example of possible bias in cell line analysis is CD117 expression in ovarian cancer. Although CD117 is expressed by ovarian cancer cells at significant levels (as reported in the literature and demonstrated in our current findings), the results of treatment with Gleevec, a targeted drug directed against CD117 and other tyrosine kinases, have been disappointing.

This may be explained by our current gene expression data analysis, indicating lower levels of CD117 in malignant ovarian tissue compared with their normal counterparts (Figure 3). The successful clinical experience with Gleevec in gastrointestinal stromal tumors apparently depends on mutations in c-kit in these malignancies. It is possible that ovarian cancer cells express the wild-type, non-mutated form of the molecule, explaining Gleevec’s poor efficacy in this disease.

Although hematopoietic antigens may serve as appealing therapeutic targets, this concept should be approached with caution. Treatment with antibodies against myeloid cell markers (eg, CD15) will most likely result in neutropenia and possibly also thrombocytopenia, corresponding to the well-documented side effects of Myelotarg.

In a similar manner, antilymphoid antibodies (eg, CD4 or CD138) may cause immunodeficiency, although previous experience points to relative minor side effects following treatment with anti-CD20 antibodies. However, such side effects are clinically manageable, and because these putative targets are not the common hematopoietic stem cell marker CD34, they should be temporary, allowing the full recovery of normal hematopoiesis.

In summary, we undertook a novel approach to identifying potential targets for therapy in ovarian cancer. Initial work with cell lines and a systematic screening process by flow cytometry allowed us to focus on several potential targets. These targets were validated by either immunohistochemistry or a new bioinformatics methodology, utilizing public microarray data. In the future, this methodology may also be used on proteomics data for further validation.

To the best of our knowledge, this is the first study that took a systematic approach to identifying potential targets for therapy in ovarian cancer. Initial work with cell lines and a systematic screening process by flow cytometry allowed us to focus on several potential targets. These targets were validated by either immunohistochemistry or a new bioinformatics methodology, utilizing public microarray data. In the future, this methodology may also be used on proteomics data for further validation.

To the best of our knowledge, this is the first study that took a systematic approach to the identification of potential targets for therapy in ovarian cancer. Moreover, the characterization of small cell populations with distinct antigen expression, potentially representing a stem cell subpopulation, may define an ideal target for such therapy.

**ACKNOWLEDGMENTS**

We thank Esther Eshkol for editorial assistance.

**REFERENCES**

196.e7 American Journal of Obstetrics & Gynecology AUGUST 2009