Published in final edited form as:

Gynecol Oncol. 2011 July ; 122(1): 171–177. doi:10.1016/j.ygyno.2011.03.002.

High-grade, chemotherapy-resistant primary ovarian carcinoma cell lines overexpress human trophoblast cell-surface marker (Trop-2) and are highly sensitive to immunotherapy with hRS7, a humanized monoclonal anti-Trop-2 antibody

Joyce Varughese¹, Emiliano Cocco¹, Stefania Bellone¹, Marta Bellone¹, Paola Todeschini¹, Luisa Carrara¹, Peter E. Schwartz¹, Thomas J. Rutherford¹, Sergio Pecorelli², and Alessandro D. Santin^{1,*}

¹Division of Gynecologic Oncology, Department of Obstetrics, Gynecology, & Reproductive Sciences, Yale University School of Medicine, New Haven, CT, USA

²Division of Gynecologic Oncology, University of Brescia, Brescia, Italy

Abstract

Objective—We evaluated the expression of human trophoblast cell-surface marker (Trop-2) and the potential of hRS7, a humanized monoclonal anti-Trop-2 antibody, as a therapeutic agent against chemotherapy-resistant ovarian disease.

Methods—Trop-2 expression was evaluated by immunohistochemistry (IHC) in 50 ovarian serous papillary carcinoma specimens. Trop-2 expression was also evaluated by real-time PCR (qRT-PCR) and flow cytometry in a total of 6 primary ovarian cancer cell lines derived from patients with chemotherapy-resistant disease. Sensitivity to hRS7 antibody-dependent cellular cytotoxicity (ADCC) was tested in standard 5-hours ⁵¹Cr-release assays. The effect of serum and interleukin-2 (IL-2) on hRS7-mediated ADCC was also studied.

Results—Trop-2 expression was found in 41 of 50 (82%) tumor tissues tested by IHC. 83% (5 of 6) of the ovarian cancer cell lines tested by qRT-PCR and flow cytometry demonstrated high Trop-2 expression. All primary ovarian cancer cell lines expressing Trop-2 were highly sensitive to hRS7-mediated ADCC *in vitro* (range of killing: 19.3% to 40.8%) ($p < 0.001$). Negligible cytotoxicity against chemotherapy-resistant ovarian cancers was seen in the absence of hRS7 or in the presence of rituximab control antibody (range of killing: 1.1% to 8.9%). Human serum did not significantly inhibit hRS7-mediated-cytotoxicity while incubation with IL-2 in addition to hRS7 further increased the cytotoxic activity ($p = 0.04$).

Conclusions—Trop-2 is highly expressed in chemotherapy-resistant ovarian cancer cell lines at mRNA and protein levels. Primary ovarian carcinoma cell lines are highly sensitive to hRS7-

© 2011 Elsevier Inc. All rights reserved.

*corresponding author: Alessandro D. Santin, MD, Yale University School of Medicine, Department of Obstetrics, Gynecology, & Reproductive Sciences, Rm. 305 LSOG, 333 Cedar Street, PO Box 208063, New Haven, CT 06520-8063, Phone: 203-737-4450, Fax: 203-737-4339, alessandro.santin@yale.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

mediated cytotoxicity *in vitro*. hRS7 may represent a novel therapeutic agent for the treatment of high-grade, chemotherapy-resistant ovarian cancer.

Keywords

ovarian cancer; chemotherapy-resistant; Trop-2; trophoblast cell surface marker; hRS7; antibody-dependent cellular cytotoxicity

Introduction

Ovarian cancer continues to be a significant health problem, as it is the gynecologic malignancy with the highest mortality, with 21,880 new cases and 13,850 deaths estimated for 2010 in the United States [1]. Due to the asymptomatic nature of early stage ovarian cancer and lack of effective screening tests, most ovarian cancers are diagnosed in advanced stages. Despite aggressive surgical treatment and chemotherapy, the 5-year survival rate of patients with advanced stage disease is 30% [2]. While most patients initially respond to chemotherapy, they ultimately become resistant to the treatment [3]. Thus, there is great interest in developing targeted therapies against chemotherapy-resistant ovarian cancer.

Trop-2 is a surface glycoprotein originally identified in human placental trophoblast and subsequently reported to be highly expressed by various types of human carcinomas, but rarely in normal adult tissues [4–8]. Although the biological role of Trop-2 is still unclear, its overexpression has been found to correlate with invasive behavior and poor prognosis in various human carcinomas [9–12]. Consistent with this observation, our group reported Trop-2 as an independent marker for poor overall survival in ovarian cancer [13]. Overexpression of Trop-2 by epithelial tumor cells and its transmembrane location render Trop-2 an attractive target for cancer immunotherapy.

hRS7 is a humanized IgG1 monoclonal antibody (MAb) developed against Trop-2 using complementary-determining-region (CDR) and transfection techniques of the murine RS7-3G11 antibody (Immunomedics, Inc., Morris Plains, NJ) [14–16]. Since RS7-3G11 has been shown to rapidly internalize into target cells [14–16], hRS7 was initially tested labeled with ¹³¹I-IMP-R4 to evaluate its effectiveness in preclinical radioimmunotherapy studies on breast cancer xenograft models [16]. Results of these studies suggested that hRS7 may be promising as a carrier for radiometabolic therapy after labeling with suitable radionuclides. However, the ability of hRS7 to induce antibody-dependent cellular cytotoxicity (ADCC) against primary ovarian carcinoma cell lines has not previously been reported.

Therefore, in this study we investigated the potential of Trop-2 as a novel target against ovarian cancer by evaluating its expression at both mRNA and protein levels in multiple ovarian tumors and biologically aggressive primary ovarian cancer cell lines established from patients harboring chemotherapy-resistant disease. We also evaluated the *in vitro* cytotoxic activity of hRS7 against primary ovarian cancer cell lines resistant to multiple chemotherapeutic agents overexpressing Trop-2.

Methods

Establishment of ovarian cancer cell lines

Study approval was obtained from the Institutional Review Board, and all patients signed an informed consent form according to institutional guidelines. A total of six ovarian cancer cell lines were established after sterile processing of tumor samples from surgical biopsies as previously described [17]. Briefly, viable tumor tissue was mechanically minced in RPMI 1640 to portions no larger than 1–3 mm³ and washed twice with RPMI 1640. The portions

of minced tumor were then placed in 250 mL trypsinizing flasks containing 30 mL of enzyme solution [0.14% collagenase Type I (Sigma, St. Louis, MO) and 0.01% DNase (Sigma, 2000 KU/mg)] in RPMI 1640, and incubated on a magnetic stirring apparatus overnight at 4°C. Enzymatically dissociated tumor was filtered through a 150 µm nylon mesh to generate a single cell suspension. The resultant cell suspension was washed twice in RPMI 1640 plus 10% fetal bovine serum (FBS, Invitrogen, Grand Island, NY). The epithelial nature and purity of OSPC cultures were verified by immunohistochemical staining and flow-cytometric analysis with antibodies against cytokeratin, as previously described [17]. All cytotoxicity experiments were done with fresh tumor cultures that had at least 90% viability and contained more than 99% tumor cells. Four patients had ovarian serous papillary carcinomas (OSPC) and two patients had clear cell histology. Five of the six patients had Stage III or IV disease at the time of diagnosis. One patient with clear cell cancer was diagnosed at Stage IC. All patients had high-grade (G3) tumors. All patients received a combination of carboplatin and paclitaxel as their primary chemotherapy regimen. Five of the six patients whose cells were used for the establishment of cell lines demonstrated disease progression on chemotherapy. All six primary ovarian cancer cell lines were found highly resistant *in vitro* to multiple chemotherapy drugs including carboplatin, cisplatin, paclitaxel, doxorubicin, ifosfamide, gemcitabine and topotecan by Extreme Drug Resistant assays (Oncotech) [18].

Trop-2 immunostaining of formalin-fixed tumor tissues

A total of 50 OSPC specimens and 5 normal ovarian control tissues obtained from similar age women were evaluated by standard immunohistochemical (IHC) staining on formalin-fixed tumor tissue for Trop-2 surface expression. Ovarian carcinoma specimens were derived from primary, metastatic (i.e., omentum), and/or recurrent sites of disease from a total of 25 patients (means age $\pm$ SD = 60 ± 7 years) harboring advanced stage disease (i.e., stage III–IV). Nearly 80% of the patients presented between the age of 40–70 years. 8.6% of patients were below 40 years of age and 10% were above 70 years of age. All patients harbored tumors with serous papillary histology. Twenty-three OSPC were graded as poorly differentiated tumors (i.e., G3) with the remaining 2 samples graded as G2. IHC stains were performed on 4-µm-thick sections of formalin-fixed, paraffin-embedded tissue, as previously described [13]. The purified goat polyclonal antibody against the recombinant human Trop-2 extracellular domain (R&D Systems, Inc.; diluted 1:100) was applied for 1 hour. A secondary biotinylated anti-goat antibody (Vector Laboratories; diluted 1:250) and the streptavidin-biotin complex (StreptABCComplex/HRP) were applied, then 3'3'-diaminobenzidine (Dako) was used as chromogen and the sections were counterstained by hematoxylin (Dako). Cases with less than 10% membranous staining in tumor cells were considered negative for Trop-2 expression. The intensity of membranous immunoreactivity for Trop-2 in tumor cells was subjectively scored as follow: (a) 0, negative; (b) 1+, weak membrane staining; (c) 2+, medium staining; and (d) 3+, strong membrane staining (Figure 1). The percentage of positive tumor cell was also evaluated in all specimens. Appropriate negative and positive controls were performed with each case.

Quantitative real-time polymerase chain reaction

RNA isolation from a total of six primary ovarian carcinoma cell lines was performed using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Since Trop-2 is an intronless gene, all RNA samples were treated with TURBO DNase enzyme (TURBO DNA-free kit; Ambion, Inc., Applied Biosystems) to remove any contaminating DNA that was present. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in duplicate by using a primer set and probe specific for the Trop-2 (i.e., Trop2-EX56) with a 7500 Real Time PCR System using the manufacturer's recommended protocol (Applied Biosystems). The comparative threshold cycle (C_T) method (Applied Biosystems) was used

to determine gene expression in each sample relative to the value observed in the lowest nonmalignant ovarian epithelial cell sample, using glyceraldehyde-3-phosphate dehydrogenase (Assay ID Hs99999905_m1) RNA as internal controls.

Flow cytometry

The humanized anti-Trop-2 MAb hRS7 (Immunomedics, Inc.) was used for flow cytometry studies. Briefly, six primary ovarian cancer cell lines established from the above described patients were stained with 2 µg/ml of hRS7. The chimeric anti-CD20 MAb rituximab (Rituxan; Genentech) at the dose of 2.5 µg/ml was used as negative control. A goat anti-human F(ab')₂ immunoglobulin (BioSource International) was used as a secondary reagent. Analysis was conducted with a FACScan, using Cell Quest software (Beckton Dickinson).

Tests for ADCC

A standard five-hours chromium (⁵¹Cr) release assay was performed to measure the cytotoxic reactivity of Ficoll-Paque™ PLUS (GE Healthcare) separated peripheral blood lymphocytes (PBL) obtained from several healthy donors against all six ovarian cancer cell lines. The release of ⁵¹Cr from the target cells was measured as evidence of tumor cell lysis after exposure of tumor cells to 2 µg/ml of hRS7. Controls included the incubation of target cells alone or with PBL or MAb separately. The chimeric anti-CD20 MAb rituximab was used as a negative control for hRS7 in all bioassays. ADCC was calculated as the percentage of killing of target cells observed with hRS7 plus effector cells compared with ⁵¹Cr release from target cells incubated alone.

Interleukin-2 enhancement of ADCC

To investigate the effect of interleukin-2 (IL-2) on hRS7-mediated ADCC, effector PBL were incubated for 5 hours at 37°C at a final concentration of IL-2 (Aldesleukin; Chiron Therapeutics) ranging from 50–100 IU/ml in 96-well microtiter plates. Target cells were primary ovarian cancer cell lines exposed to 2 µg/ml of hRS7, whereas controls included the incubation of target cells alone or with PBL in the presence or absence of IL-2 or MAb, respectively. Rituximab was used as a control MAb. ADCC was calculated as the percentage of killing of target cells observed with MAb plus effector PBL, as compared with target cells incubated alone.

Tests for complement-mediated target cell lysis and γ-globulin inhibition

A standard 5-hours ⁵¹Cr-release assay identical to those performed for ADCC assays was used. However, human serum was added as a source of complement to test for complement-mediated target cell lysis. To test for the possible inhibition of ADCC against ovarian cancer cell lines by physiological human serum concentrations of γ-globulin, human serum diluted 1:2 was added in the presence or absence of effector PBL. The effect of heat-inactivated human serum (56°C for 60 minutes) was also tested in the presence of effector PBL. Controls included the incubation of target cells alone or with either lymphocytes or MAb separately. Rituximab was used as a control MAb.

Statistical analysis

qRT-PCR data were evaluated using unequal-variance *t*-test for ovarian carcinoma-versus-normal difference. Differences in Trop-2 expression by flow cytometry were analyzed by the unpaired *t*-test, and a *p*-value of <0.05 among samples was considered to be significant. The Wilcoxon rank-sum (WRS) test was used to compare ovarian cancer cell types to normal ovarian tissue for differences in IHC staining intensities. Kruskal-Wallis test and chi-square analysis were used to evaluate differences in hRS7-induced ADCC levels in primary

tumor cell lines. Statistical analysis was performed using PASW Statistics version 18 (SPSS, Chicago, IL).

Results

Trop-2 transcript levels in ovarian carcinomas

A total of six primary ovarian cancer cell lines were tested for Trop-2 expression by qRT-PCR. Of the six tumors tested, five carcinomas (3 of 4 OSPCs and 2 of 2 clear cell carcinomas) showed a high mRNA copy number, ranging from 1774 to 9041 (Table 1). Trop-2 expression between these ovarian cancer cell lines and normal ovarian cells was statistically significant at $p < 0.05$. In contrast, lower Trop-2 expression by qRT-PCR was detected in one OSPC cell line (*i.e.*, 105 mRNA copy numbers, Table 1).

Trop-2 Expression by Immunohistochemistry on ovarian cancer cells versus normal ovarian tissue

To determine whether the high expression of Trop-2 gene detected by qRT-PCR assays in primary ovarian cancer cell lines also results in high expression of the molecule on the surface of ovarian tumor cells, we performed IHC analysis of Trop-2 protein expression on formalin-fixed, paraffin-embedded tumor tissues from a separate set of 50 OSPC specimens (including primary, metastatic and recurrent carcinoma from the same patient when available). As representatively shown in Table 2 and Figure 1, the intensity of staining for Trop-2 was significantly higher among the tumor specimens compared to normal ovarian surface epithelial controls ($p < 0.0001$). Trop-2 expression was detected in 41 out of the 50 (82%) OSPC tested and all tumors stained by IHC showed membranous positivity for Trop-2 (Figure 1, Table 2). In this regard, 18 (36%) of the specimens were found to have a low positivity (1+) for Trop-2 protein, while the remaining specimens available for IHC testing showed moderate (*i.e.*, 2+ : 16 samples, 32%) or strong (*i.e.*, 3+ : 7 samples, 14%) Trop-2 positivity (Table 2, Figure 1). No significant differences in Trop-2 expression were detected between primary versus metastatic or recurrent sites of disease (Table 2).

Trop-2 surface expression by flow cytometry in ovarian cancer cell lines

To determine correlation between qRT-PCR data and protein expression on the surface of tumor cells, we performed flow cytometry on all primary tumors. Once again, five cell lines showed high Trop-2 surface expression by flow cytometry (3 of 4 OSPCs, 2 of 2 clear cell carcinomas) (Table 1). These results were consistent with the mRNA copy numbers measured by qRT-PCR in the same tumors. The difference in Trop-2 surface expression between the cell lines with low and those with high Trop-2 expression was statistically significant ($p < 0.001$).

Ovarian carcinoma cell lines are highly resistant to natural killer (NK) cell activity but sensitive to hRS7-mediated ADCC

All six primary ovarian cancer cell lines were evaluated for their sensitivity to NK cells. These cell lines were exposed to PBL collected from multiple healthy donors and the cytotoxicity was measured using a standard 5-hours ^{51}Cr -release assay. Using dose titration experiments with different doses of hRS7, killing of the ovarian cancer cells was found to plateau at a hRS7 concentration of 2 $\mu\text{g}/\text{ml}$ (data not shown). Thus, this dose was used in all subsequent experiments. Ovarian cancer cell lines were found highly resistant to NK-mediated killing with exposure to PBL at an effector to target ratio (E/T) of 25:1 and 50:1 (mean killing: $2.8\% \pm 2.4 \text{ SD}$) (Table 3). In contrast, significant killing was demonstrated against the five high Trop-2 expressing cell lines after incubation with hRS7 to mediate ADCC (range of killing: 19.3–40.8%, mean $26.5\% \pm 11.8 \text{ SD}$; $p < 0.001$) (Figure 2, top &

middle panels; Table 3), while the one cell line demonstrating weak positivity to Trop-2 (OSPC-ARK-3) had a mean killing of $10.6\% \pm 2.3$ SD (Figure 2, bottom panel; Table 3). The results are consistent with the Trop-2 expression data by qRT-PCR and flow cytometry (Table 1). All cell lines were resistant to incubation with rituximab (2.5 μ g/ml) in the presence of PBL (Table 3).

IL-2 enhancement of ADCC against ovarian carcinomas

To investigate the effect of interleukin-2 (IL-2) in combination with hRS7 (2 μ g/ml) on ADCC against ovarian carcinoma cell lines overexpressing Trop-2, PBL from healthy donors were incubated with 50–100 IU/ml of IL-2 for 5 hours. As representatively shown in Figure 3 (top panel), hRS7-mediated ADCC was significantly increased in the presence of IL-2 in all primary cell lines tested ($p=0.04$). While the stimulation of PBL with IL-2 leads to a significantly higher ADCC in the presence of hRS7, it did not significantly increase tumor killing in the absence of hRS7 or in the presence of rituximab (Figure 3, top panel).

Effect of complement and physiologic concentrations of IgG on hRS7-mediated ADCC

In order to evaluate the effect of complement on hRS7-mediated ADCC as well as its potential inhibition by physiological IgG serum concentrations, human serum diluted 1:2 (with and without heat inactivation) was added to the ovarian cancer cell lines during standard 5-hours 51 Cr-release assays in the presence of PBL. As representatively demonstrated in Figure 3 (middle panel), in the majority of cell lines tested, no significant change in killing after incubation with serum compared to incubation without serum was noted. However, in one clear cell carcinoma cell line (CC-ARK-2), the addition of serum led to a significant increase in killing ($p=0.02$), while the addition of heat-inactivated serum with hRS7 and PBL to this cell line resulted in a decrease of killing compared to the killing in the presence of serum (Figure 3, bottom panel). These results suggest a peculiar high sensitivity of this ovarian clear cell carcinoma cell line to complement *in vitro*.

Discussion

In this study, we evaluated Trop-2 expression level in ovarian carcinoma tissues and in multiple biologically aggressive and chemotherapy-resistant ovarian cancer cell lines as well as their sensitivity to hRS7, a humanized MAb against Trop-2. Our findings demonstrate that (i) Trop-2 mRNA and/or protein are significantly upregulated in primary/metastatic/recurrent OSPC compared to normal surface epithelial ovarian tissues; (ii) normal ovarian cells express low Trop-2 transcript and showed a negative immunostaining for the protein; (iii) five out of six freshly established tumor cell lines derived from patients harboring biologically aggressive OSPC and clear cell tumors experiencing progression of disease on chemotherapy expressed high levels of Trop-2 on their cell surfaces as measured by flow cytometry, and (iv) primary ovarian cancer cell lines overexpressing Trop-2 are highly susceptible to ADCC mediated by hRS7, a humanized MAb developed for targeting Trop-2-expressing cancers. Our results may therefore have important implications for the treatment of chemotherapy-resistant ovarian cancer.

Given the high mortality of ovarian cancer patients with chemotherapy-resistant disease, the development of novel, target-specific, and more effective therapies is an urgent need for these patients. Trop-2, a surface glycoprotein overexpressed in a variety of human carcinomas, may represent a potentially effective target for the immunotherapy of chemotherapy-resistant disease. We have recently reported a significant association between increased expression of Trop-2 and poor overall survival in high grade ovarian serous carcinoma [13], as well as the potential benefit of anti-Trop-2 antibody therapy in uterine serous papillary carcinomas [19]. In the former study, Trop-2 protein expression was

significantly associated with tumor histology, along with the presence of ascites and lymph node involvement, suggesting a relationship between biological aggressiveness and Trop-2 overexpression in ovarian cancer. Although the exact function of Trop-2 is unclear and no physiological ligand has been identified, Trop-2 targeting with specific antibodies can cross-link cell surface receptors and induce signaling that may inhibit tumor cell migration and invasion [20]. Previous studies have also suggested that Trop-2 may function as a cell signal transducer and regulator of tumor cell growth and increase tumor cell resistance to apoptosis [21]. These observations support the possibility that aberrant Trop-2 expression may account for the enhanced invasive behavior and increased biologic aggressiveness of chemotherapy-resistant ovarian carcinoma.

Our present results suggest that targeting cancer cells with high surface expression of Trop-2 may be a novel, potentially effective option to treat chemotherapy-resistant high grade ovarian carcinomas. We found those primary ovarian tumors showing high Trop-2 expression, regardless of their serous or clear cell histology, to be highly susceptible to hRS7-mediated ADCC in the presence of effector cells. Although these tumor cells are resistant to multiple standard cytotoxic therapies in the clinic, they remain highly sensitive to lysis by NK cells when these are engaged by the Trop-2-specific antibody hRS7.

For effective cytotoxicity, the effector cells must be able to interact with the antibody at the target site *in vivo*, even in the presence of high concentrations of human IgG. In this study, we show that ADCC against ovarian carcinoma cells was not significantly decreased by high concentrations (up to 50%) of human serum. Indeed, in one cell line (CC-ARK-2), a consistent increase in cytotoxicity was detected in the presence of effector cells and human serum. These results indicate that the binding of hRS7 to the F_c receptor on mononuclear effector cells would likely succeed in the *in vivo* situation.

IL-2 treatment leads to the activation of NK cell cytotoxicity and expansion of the NK cell population within the PBL *in vivo* [22]. IL-2 has also previously been shown to work synergistically with monoclonal antibodies *in vivo* [23]. Low doses of recombinant IL-2 have been given by continuous infusion or subcutaneously resulting in high clinical and immunological activity and negligible toxicity [24]. These findings are important because suppressed ADCC responses have been reported in several cancer patients [22], but pre-treatment of PBL with IL-2 can increase the cytotoxicity levels in patients with suppressed ADCC to levels similar to those of normal donors [25]. Similarly, our *in vitro* experiments demonstrate a significant increase in ADCC after the pre-treatment of the PBLs with low doses of IL-2 compared to cytotoxicity in the absence of IL-2. The administration of low doses of IL-2 *in vivo* may therefore be a useful adjunct to increase the efficacy of hRS7 cytotoxicity in chemotherapy-resistant ovarian cancer patients.

In conclusion, this is the first report on the therapeutic activity of hRS7, an anti-Trop-2 antibody, in primary ovarian carcinoma. Our study has demonstrated that Trop-2 expression is highly and consistently expressed at mRNA and protein levels in primary cell lines established from patients with chemotherapy-resistant ovarian carcinomas. The high density and cell surface localization of Trop-2 on ovarian cancer cells suggests that this protein could represent an accessible tumor target for antibody-based therapies. hRS7 holds promise as one such therapy given its cytotoxic effects *in vitro* on Trop-2 positive cells.

References

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin.* 2010; 60:277–300. [PubMed: 20610543]
2. Cho KR, Shih I. Ovarian cancer. *Annu Rev Pathol.* 2009; 4:287–313. [PubMed: 18842102]

3. Martin LP, Schilder RJ. Management of recurrent ovarian carcinoma: current status and future directions. *Semin Oncol*. 2009; 36:112–125. [PubMed: 19332246]
4. Lipinski M, Parks DR, Rouse RV, Herzenberg LA. Human trophoblast cell-surface antigens defined by monoclonal antibodies. *Proc Natl Acad Sci USA*. 1981; 78:5147–5150. [PubMed: 7029529]
5. Miotti S, Canevari S, Menard S, Mezzanzanica D, Porro G, Pupa SM, et al. Characterization of human ovarian carcinoma-associated antigens defined by novel monoclonal antibodies with tumor-restricted specificity. *Int J Cancer*. 1987; 39:297–303. [PubMed: 2434438]
6. Alberti S, Miotti S, Stella M, Klein CE, Fornaro M, Menard S, et al. Biochemical characterization of Trop-2, a cell surface molecule expressed by human carcinomas: formal proof that the monoclonal antibodies T16 and MOv-16 recognize Trop-2. *Hybridoma*. 1992; 11:539–545. [PubMed: 1459581]
7. Nakashima K, Shimada H, Ochiai T, Kuboshima M, Kuroiwa N, Okazumi S, et al. Serological identification of TROP2 by recombinant cDNA expression cloning using sera of patients with esophageal squamous cell carcinoma. *Int J Cancer*. 2004; 112:1029–1035. [PubMed: 15386348]
8. Zhang L, Zhou W, Velculescu VE, Kern SE, Hruban RH, Hamilton SR, et al. Gene expression profiles in normal and cancer cells. *Science*. 1997; 276:1268–1272. [PubMed: 9157888]
9. Muhlmann G, Spizzo G, Gostner J, Zitt M, Maier H, Moser P, et al. TROP2 expression as prognostic marker for gastric carcinoma. *J Clin Pathol*. 2009; 62:152–158. [PubMed: 18930986]
10. Fong D, Moser P, Krammel C, Gostner JM, Margreiter R, Mitterer M, et al. High expression of TROP2 correlates with poor prognosis in pancreatic cancer. *Br J Cancer*. 2008; 99:1290–1295. [PubMed: 18813308]
11. Fong D, Spizzo G, Gostner JM, Gastl G, Moser P, Krammel C, et al. TROP2: a novel prognostic marker in squamous cell carcinoma of the oral cavity. *Mod Pathol*. 2008; 21:186–191. [PubMed: 18084248]
12. Fang YJ, Lu ZH, Wang GQ, Pan ZZ, Zhou ZW, Yun JP, et al. Elevated expressions of MMP7, TROP2, and survivin are associated with survival, disease recurrence, and liver metastasis of colon cancer. *Int J Colorectal Dis*. 2009; 24:875–884. [PubMed: 19421758]
13. Bignotti E, Todeschini P, Calza S, Falchetti M, Ravanini M, Tassi RA, et al. Trop-2 overexpression as an independent marker for poor overall survival in ovarian carcinoma patients. *Eur J Cancer*. 2010; 46:944–953. [PubMed: 20060709]
14. Stein R, Basu A, Chen S, Shih LB, Goldenberg DM. Specificity and properties of MAb RS7-3G11 and the antigen defined by this pancarcinoma monoclonal antibody. *Int J Cancer*. 1993; 55:938–946. [PubMed: 8253531]
15. Stein R, Govindan SV, Mattes MJ, Shih LB, Griffiths GL, Hansen HJ, et al. Targeting human cancer xenografts with monoclonal antibodies labeled using radioiodinated, diethylenetriaminepentaacetic acid-appended peptides. *Clin Cancer Res*. 1999; 5:3079s–3087s. [PubMed: 10541347]
16. Govindan SV, Stein R, Qu Z, Chen S, Andrews P, Ma H, et al. Preclinical therapy of breast cancer with a radioiodinated humanized anti-EGP-1 monoclonal antibody: advantage of a residualizing iodine radiolabel. *Breast Cancer Res Treat*. 2004; 84:173–182. [PubMed: 14999147]
17. Bellone S, Siegel ER, Cocco E, Cargnelutti M, Silasi DA, Azodi M, et al. Overexpression of epithelial cell adhesion molecule in primary, metastatic, and recurrent/chemotherapy-resistant epithelial ovarian cancer: implications for epithelial cell adhesion molecule-specific immunotherapy. *Int J Gynecol Cancer*. 2009; 19:860–866. [PubMed: 19574774]
18. Holloway RW, Mehta RS, Finkler NJ, Li KT, McLaren CE, Parker RJ, et al. Association between in vitro platinum resistance in the EDR assay and clinical outcomes for ovarian cancer patients. *Gynecol Oncol*. 2002; 87:8–16. [PubMed: 12468336]
19. Varughese J, Cocco E, Bellone S, de Leon M, Bellone M, Todeschini P, et al. Uterine Serous Papillary Carcinomas overexpress human trophoblast-cell-surface marker (Trop-2) and are highly sensitive to immunotherapy with hRS7, a humanized anti-Trop-2 monoclonal antibody. *Cancer*. in press.
20. Wang J, Day R, Dong Y, Weintraub SJ, Michel L. Identification of Trop-2 as an oncogene and an attractive therapeutic target in colon cancers. *Mol Cancer Ther*. 2008; 7:280–285. [PubMed: 18281513]

21. Cubas R, Li M, Chen C, Yao Q. Trop2: a possible therapeutic target for late stage epithelial carcinomas. *Biochim Biophys Acta*. 2009; 1796:309–314. [PubMed: 19683559]
22. Caligiuri MA, Murray C, Robertson MJ, Wang E, Cochran K, Cameron C, et al. Selective modulation of human natural killer cells in vivo after prolonged infusion of low dose recombinant interleukin 2. *J Clin Invest*. 1993; 91:123–132. [PubMed: 7678599]
23. Hooijberg E, Sein JJ, van den Berk PC, Hart AA, van der Valk MA, Kast WM, et al. Eradication of large human B cell tumors in nude mice with unconjugated CD20 monoclonal antibodies and interleukin 2. *Cancer Res*. 1995; 55:2627–2634. [PubMed: 7540106]
24. Boyman O, Surh CD, Sprent J. Potential use of IL-2/anti-IL-2 antibody immune complexes for the treatment of cancer and autoimmune disease. *Expert Opin Biol Ther*. 2006; 6:1323–1331. [PubMed: 17223740]
25. Ortaldo JR, Woodhouse C, Morgan AC, Herberman RB, Cheresch DA, Reisfeld R. Analysis of effector cells in human antibody-dependent cellular cytotoxicity with murine monoclonal antibodies. *J Immunol*. 1987; 138:3566–3572. [PubMed: 2437198]

Figure 1. Representative immunohistochemical staining for Trop-2. A) normal ovarian tissue (0/1+ staining, magnification 20×), B) ovarian cancer (1+ staining, magnification 20×), C) ovarian cancer (2+ staining, magnification 20×), D) ovarian cancer (3+ staining, magnification 20×).

Figure 2.

Representative cytotoxicity experiments using hRS7 against CC-ARK-1 (top panel), OSPC-ARK-4 (middle panel), and OSPC-ARK-3 (bottom panel) cell lines. High levels of hRS7-induced cytotoxicity were evident against CC-ARK-1 and OSPC-ARK-4 (top and middle panels) with effectors to target ratios of both 25:1 and 50:1. In contrast, decreased cytotoxicity was noted in OSPC-ARK-3 (bottom panel) consistent with decreased levels of Trop-2 expression by qRT-PCR and flow cytometry. In all cell lines tested, no significant cytotoxicity was detected in the absence of hRS7 or in the presence of rituximab control MAb.

Figure 3.

Collective data representing effect of low doses of interleukin-2 (IL-2) in combination with hRS7 on ADCC on primary ovarian cancer cell lines (top panel). hRS7-mediated ADCC was significantly increased in the presence of low doses of IL-2. No significant increase in cytotoxicity was detected after 5 hours of IL-2 treatment in the absence of hRS7 or in the presence of rituximab control MAb. Representative cytotoxicity experiments adding human serum to hRS7 against CC-ARK-1 (middle panel) and CC-ARK-2 (bottom panel). Addition of physiological concentrations of IgG (i.e., heat-inactivated serum diluted 1:2) to PBL in the presence of hRS7 did not significantly decrease the degree of ADCC. The addition of untreated serum to PBL in the presence of hRS7 significantly increased hRS7-mediated

ADCC in CC-ARK-2 (bottom panel) suggesting a peculiar sensitivity of this cell line to complement-dependent cytotoxicity.

Table 1

Trop-2 mRNA and protein expression in ovarian cancer cell lines

Cell line	Flow cytometry		qRT-PCR
	MFI* $\pm$ SD	Cells (%) $\pm$ SD	mRNA copy #
OSPC-ARK-1	151.78 $\pm$ 5.47	99.91 $\pm$ 0.06	9041.19
OSPC-ARK-2	127.93 $\pm$ 2.50	99.92 $\pm$ 0	1774.62
OSPC-ARK-3	26.34 $\pm$ 0.31	86.14 $\pm$ 1.07	105.882
OSPC-ARK-4	107.79 $\pm$ 2.68	99.95 $\pm$ 0.08	1867.90
CC-ARK-1	253.65 $\pm$ 29.96	99.56 $\pm$ 0.05	2341.57
CC-ARK-2	347.57 $\pm$ 21.16	99.95 $\pm$ 0.02	5368.28

* MFI: Mean Fluorescence Intensity

Table 2

IHC data showing Trop-2 protein expression in ovarian tumors and control tissues

Patient	Normal Ovary	Primary		Metastatic		Recurrent	
		staining	% positive cells	staining	% positive cells	staining	% positive cells
PT. 1	-	0		2+	25	0	
PT. 2	-	0		2+	20	1+	10
PT. 3	-	1+	10	2+	10	2+	30
PT. 4	-	0		0		0	
PT. 5	-	2+	70			1+	20
PT. 6	-	1+	10			0	
PT. 7	-	1+	10	2+	10	1+	10
PT. 8	-	2+	50	1+	30	1+	10
PT. 9	-		-		-	1+	10
PT. 10	-		-		-	2+	25
PT. 11	-	1+	50	3+	80		-
PT. 12	-		-	2+	70		-
PT. 13	-	0		1+	30		-
PT. 14	-	1+	10	2+	10		-
PT. 15	-	2+	60	2+	70		-
PT. 16	-	1+	20	1+	10		-
PT. 17	-	2+	50	2+	40		-
PT. 18	-	1+	80	2+	70		-
PT. 19	-	3+	80	2+	90		-
PT. 20	-	3+	50	1+	10		-
PT. 21	-	3+	30	1+	10		-
PT. 22	-		-	1+	10		-
PT. 23	-	3+	80	3+	90		-
PT. 24	-	0					-
PT. 25	-	3+	80		-		-

Patient	Normal Ovary	Primary		Metastatic		Recurrent	
		staining	% positive cells	staining	% positive cells	staining	% positive cells
PT. 26	0/1+	-	-	-	-	-	-
PT. 27	0	-	-	-	-	-	-
PT. 28	0	-	-	-	-	-	-
PT. 29	0	-	-	-	-	-	-
PT. 30	0	-	-	-	-	-	-

Table 3

hRS7-dependent cytotoxicity results in ovarian cancer cell lines

Cell line	Control $\pm$ SD	Rituximab $\pm$ SD	hRS7 $\pm$ SD	<i>p</i> -value *
OSPC-ARK-1	1.6% $\pm$ 1.1	2.3% $\pm$ 0.6	19.5% $\pm$ 3.9	
OSPC-ARK-2	1.9% $\pm$ 0.1	2.9% $\pm$ 1.7	19.3% $\pm$ 9.8	
OSPC-ARK-3	1.9% $\pm$ 0.2	3.4% $\pm$ 0.3	10.6% $\pm$ 2.3	
OSPC-ARK-4	1.1% $\pm$ 1.7	1.7% $\pm$ 2.3	22.5% $\pm$ 5.6	
CC-ARK-1	5.4% $\pm$ 2.1	8.9% $\pm$ 1.8	35.0% $\pm$ 7.5	
CC-ARK-2	5.6% $\pm$ 3.3	5.3% $\pm$ 3.0	40.8% $\pm$ 18.8	
Average **	2.8% $\pm$ 2.4	4.0% $\pm$ 3.1	24.4% $\pm$ 12.3	<0.001

* Cytotoxicity results in all cell lines versus controls by Kruskal-Wallis test.

** Average of 23 cytotoxicity experiments.