IOR C2: A TUMOR ASSOCIATED ANTIGEN AS POTENTIAL TARGET FOR IMMUNOTHERAPY IN EPITHELIAL OVARIAN CANCER

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ABSTRACT
Ovarian cancer ranks the most lethal among gynecologic neoplasms in women. To assess the immunohistochemical expression of IOR C2 antigen in normal tissues as well as in ovarian cancer, the immunohistochemical expression of IOR C 2 in two ovarian cancer cell lines, frozen tissues from both fetus and adult healthy persons, tissue biopsies from normal ovary and ovarian tumors were investigated. IOR C2 immunoreactivity was also compared with the staining pattern of CEA and CA-125. IOR C2 was detected in both epithelial and glandular derivative cells of gastrointestinal tract and cell secretion substance. A limited number of normal tissues tested were positive. Normal human ovarian surface epithelium was no reactive. Intense and heterogeneous immunostaining was detected in 63, 6% of tumor samples studied. Nevertheless, a decreased or lack of IOR C2 over expression in benign cystadenomas was observed contrasting with strongest signal in ovarian carcinomas. IOR C2 was mainly found in mucinous tumors compared to serous and endometroid subtype. No reaction in clear cell carcinoma was observed. IOR C2 shows a differential expression during malignant transformation, as well as in ovarian tumors related to the histological subtype. This glycoprotein may be represents a potential cell surface target for the immunotherapy of cancer using monoclonal antibodies.

Keywords: Ovarian-Cancer, IORC2-Tumor-Associated-Antigen, ior c5 mAb, Immunohistochemistry.

INTRODUCTION

Ovarian cancer is a frequent cause of death among patients with gynecological malignancy in worldwide [0]. Unfortunately there is no screening tests used singly or in combination effective and applicable to the general population and the disease is not associated with any unique warning signs or symptoms [0]. The selection of specific surgery procedure and post-operation chemotherapy depends upon the stage and other clinicopathological prognostic factors; but it is often unsuccessful unless the disease is detected at an extremely early stage [0- 0].

The lack of preventive and early diagnostic methods, as well as effective therapies to treat recurrent ovarian tumors points toward a pressing need for novel treatment modalities in this localization. Immunotherapy with monoclonal antibodies or cancer vaccine-based approach should be an interesting modality to face this problem. Ongoing current clinical trials with these new drugs are promising, but immunotoxin, immunodirected radiotherapy or activation of immune mechanism requires the mediation of a highly selective targeting agent [5-6].

IOR C2 is a tumor-associated antigen overexpressed preferential and selectively on the surface of malignant colorectal cells [7-8]. This antigen is a 145-190 kDa *O*-Linked glycoprotein carbohydrate chain present essentially in the cytoplasm of normal colon and gastrointestinal tract and heterogeneously expressed in colon adenocarcinoma cells, also overexpressed in other epithelial derived tumors [7-9] preferentially localized in

the cell membrane. Previously we reported the expression of IOR C2 in mucinous and serous components in 6/8 ovarian tumors studied [9].

The ior c5 mAb is a previously described IgG1 murine antibody 0 that recognized IOR C2 antigen and has been used to detect colorectal and ovarian tumors in vivo [10-13].

The increasing clinical use of mAbs raised against cellular determinants requires their careful characterization. The immunohistochemical evaluation of these biological reagents is necessary to elucidate their tumor specificity as well as binding properties to normal tissues [14]. The aim of this study was to evaluate the expression of IOR C2 antigen in both fetal and adult human normal tissues as well as in ovarian epithelial derived tumor using ior c5 mAb.

MATERIALS AND METHODS

Tissue Specimens

Fetal tissue fragments were taken from two ribanol induced abortion (16 and 19 week's gestational age). Adult normal tissues were obtained from autopsy of three healthy persons within an hour after suffering clinical death. All fresh pieces of tissues were embedded in tissue-tek OCT compound, frozen in liquid nitrogen and stored at -70°C. Five-micrometer cryostat sections were cut, mounted on gelatin-chrome alum coated slides, air-dried and fixed by immersion in cold acetone for 10 min. Fixed sections were air-dried and stored (wrapped in foil) at -20°C. Prior to staining, slides were warmed up to room temperature before unwrapping and then air-dried.

Formalin-fixed and paraffin-embedded ovarian blocks of tissues from 6 normal ovarian biopsies and 33 patients bearing 11 benign cystoadenoma and 22 samples of malignant ovarian neoplasm were retrieved from the department of pathology at the San Cecilio University hospital (Granada, Spain) by searching the NovoPath database. Staging was made according to International Federation of Gynecology and Obstetrics (FIGO) classification. The study was carried out with the approval of the Institutional Ethical Committee.

Antibodies

ior c5 (IgG1 murine antibody) was obtained from a fusion experiment using the Sp20 Ag14 myeloma cells with spleen cells from balb/c mice immunized with SW1116 colon cancer cell line[9]. Mouse ascities fluid was purified by affinity chromatography in protein A sepharose in the Good Manufacturing Practice facilities of the Center of Molecular Immunology (CIM).

Monoclonal antibodies raised against carcinoembrionic antigen (CEA) (ior cea 1, an IgG1 murine antibody generated and produced at CIM facilities) and CA-125 (Ready to use antibody from Master diagnostic No. 001616QD1) were used as complementary characterization antibodies.

Cell lines

Two human ovarian cancer cell lines, A2780 and SKOV-3 were obtained from cell line service (Oviedo University, Spain), maintained under standard conditions at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Suspension of cultured cell lines were collected by trypsinization and 0,1 mL of a cell suspensions at a density of $1x10^6$ cells per mL of PBS containing 1% bovine serum albumin fraction v (Sigma A 9647) were cytocentrifuged onto clean chrome alum coated glass slides. The cytospin were air-dried overnight and stored (wrapped in aluminum foil) at -20°C. Prior to immunostaining, slides were warmed up to room temperature before unwrapping and then fixed in Acetone-Methanol for 90 seconds and transferred directly to TBS (Tris- buffered saline at pH 7,6) to be processed by Immunocytochemistry.

Flow-cytometric analysis

The reactivity of ior c5 mAb with the cell membrane of the two ovarian tumor cell lines was detected by indirect

immunofluorescence. Briefly, 0,25mL of a cell suspension of cultured cell lines at a density of $1x10^6$ cells per mL was incubated with ior c5 mAb, washed with PBS and sequentially incubated with FITC-conjugated rabbit anti-mouse IgG (dilution 1:200) as secondary antibodies (Dako F261). The percentage of florescent cells was calculated from 10, 000 events using a flow cytometer equipped with a 488 nm Argon ion laser (Beckman Coulter). Cells stained with secondary antibody only were used to account for the background fluorescence.

Immunohistochemistry

Staining was performed on 5 µm frozen or paraffin embedded tissue sections. Slides with paraffin embedded tissue were dewaxed and rehydrated through graded alcohols. After being heated in pressure cooker for 1 min in sodium citrate buffer (0.001M, pH 6.0). The slides were washed with TBS. Endogenous peroxidase was blocked with 1% solution of hydrogen peroxide during 10 min and rinsing in TBS. Primary antibody was incubated at optimal dilution (5 µg/mL) for 60 min. To enhance the primary signal the Streptavidin-Biotin peroxidase-complex-System (Master Diagnostica No. 001828QK) was used. All incubations were done in a moist chamber at room temperature and washings were repeated after each step. The final staining reaction was developed with 3-3 Diaminobenzidine (DAB). The slides were washed in tap water, lightly counterstained with Mayer´s hematoxylin solution and coverslipped. Sections of colon adenocarcinoma were used as positive control in each staining procedure. Negative controls were obtained by replacing the primary antibody with TBS.

Immunostaining reactions were evaluated using a simplified scale, providing merely a score for the intensities of the reactions (0, total absence of staining; 1, only faint staining; 2, moderate staining; and 3, strong intense staining).

Digital images were obtained from a single field of the preparation using an Olympus BX51 brightfield microscope with magnification of x 20 or x 40.

RESULTS AND DISCUSSION

Epithelial ovarian cancer, which compromises 90% of all ovarian malignancies, is the leading cause of death from gynecological cancer in the western world. These tumors are thought to arise from the mesothelial cell layer lining the ovarian surface (ovarian coelomic epithelium) and are classified into four main histological subtypes: serous, mucinous, endometrioid, and clear cell carcinomas [For a review see ref. 15]

The requirement of preventive methods, screening test for earlier detection of the disease, and effective therapies for recurrent ovarian tumors points toward a pressing need for identification of molecular targets, which could lead to a better strategies for diagnosis and therapy of patients with epithelial ovarian carcinoma. In this study, we investigated the possible use of IOR C2 tumor-associated antigen as target for immunorecognition and management of epithelial ovarian cancers.

The ior c5 antibody initially developed against colon cancer, showed heterogeneous reactivity with approximately 83% of human colon carcinomas, but only with a limited number of normal tissues, by immunohistochemistry [9].

Expression of IOR C2 antigen in fetal and adult normal human tissues

IOR C2 glycoprotein was present in both surface epithelium and glandular derivative cells of the gastrointestinal tract and also in cell secretion material in both embryonic as well as in its corresponding adult normal tissues. No signal was seen in muscle and mesenchymal components of connective tissue, blood vessels and lymphoid tissues. The material studied included a series of normal fetal tissues showed a slight to mild positive staining (Table 1).

Table 1. Immunohistochemical expression of IOR C2 antigen on normal fetal and adult human tissues.

Organ	Fetal	Adult
Trachea	2	3
Esophagus	2	3
Stomach	0	3
Small intestine	1	3
Colon	2	3
Fallopian Tube	NT	3
Lung	NT	3
Skin	0	0
Pancreas	0	0
Liver	0	0
Heart	0	0
Adrenal gland	0	0
Thymus	0	0
Brain	0	0
Cerebellum	0	0
Spinal cord	NT	0
Muscle	NT	0
Palatine Tonsil	NT	0
Spleen	NT	0
Kidney	NT	0
Thyroid	NT	0
Pituitary	NT	0
Prostate	NT	0
Uterus	NT	0
Ovary	NT	0

NT= Not tested

However, in trachea and digestive tract the reaction was higher in F1 than F2; in stomach, skin, pancreas, liver, heart, adrenal glands, thymus, brain and cerebellum no reaction was noticed.

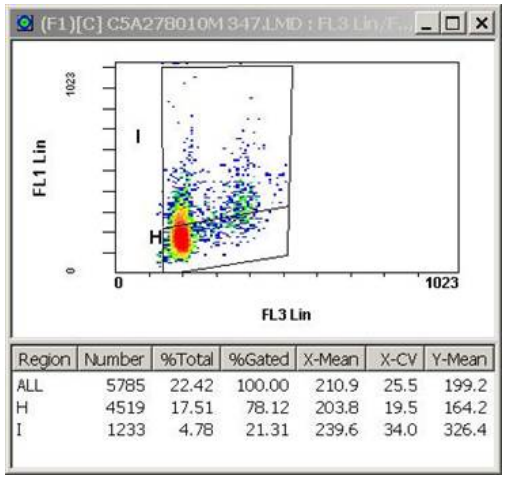
In adult as in fetal tissues, IOR C2 was significantly expressed on epithelial cells of digestive tube including stomach (Table 1). In the cytoplasm of normal colonic mucosa a homogeneous and intense staining was observed, also with reactivity in intraluminal secretion products. Bronchial epithelia showed similar intense and homogeneous inmunoreactivity with ior c5 mAb. In the fallopian tube a coarse and strong reaction of the apical membrane was observed. Other normal tissues tested such as ovary, uterus, prostate, palatine tonsil, spleen, spinal cord, kidney, thyroid and pituitary were non-reactive.

Expression of IOR C2 in ovarian- derived cell lines

For the detection of IOR C2 glycoprotein in ovarian carcinoma derived cells, both immunocytochemistry and FACS analysis were performed. Figure 1 shows the binding of ior c5 mAb with A2780 and SKOV-3 ovarian-tumor cell lines.

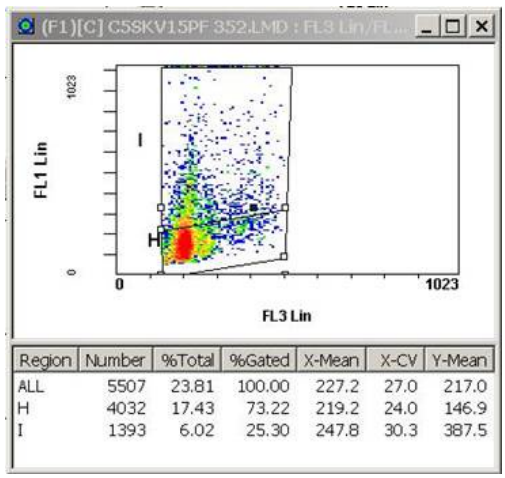
The mAb recognition was evidenced in membrane and cytoplasm of cells detecting as positive a 21, 3% of A2780 and 25, 30% of SKOV-3 cells. Particularly in cellular membrane.

A) **A2780**



Region	Number	%Total	%Gated	X-Mean	X-CV	Y-Mean
ALL	5785	22.42	100.00	210.9	25.5	199.2
H	4519	17.51	78.12	203.8	19.5	164.2
I	1233	4.78	21.31	239.6	34.0	326.4

B) **SKOV-3**



Region	Number	%Total	%Gated	X-Mean	X-CV	Y-Mean
ALL	5507	23.81	100.00	227.2	27.0	217.0
H	4032	17.43	73.22	219.2	24.0	146.9
I	1393	6.02	25.30	247.8	30.3	387.5

Fig. 1. Evaluation of IOR C2 expression in two ovarian cancer cell lines (A) A2780 and (B) SKOV-3 by FACS.

Expression of IOR C2 in ovarian tumors

A panel of 33 samples of ovarian tumors were carefully selected and subjected to histological evaluation. The sample included 11 benign cystoadenoma (serous and mucinous subtypes) and 22 malignant ovarian neoplasms. The immunohistochemical studies revealed a positive, intense and heterogeneous immunostaining in 21 out of 33 (63, 6%) cases (Table 2).

Table 2. Immunohistochemical expression of IOR C2 antigen in ovarian specimens.

Subtype	-	+	++	+++	Positive/Total
Benign Cystadenoma epithelium					
Serous	3	0	0	3	3/6
Mucinous	2	2	0	1	3/5
Invasive ovarian epithelial carcinoma					
Serous	2	1	4	2	7/9
Mucinous	0	2	1	3	6/6
Endometroid	4	0	0	2	2/6
Clear cell	1	0	0	0	0/1
Total					**21/ 33**

In some tumors, IOR C2 staining was heterogeneous with areas displaying a stronger reactivity. The intraluminal secreted material also reacted positively. The intensity of immunohistochemical staining decreased or was absent in benign mucinous and serous cystoadenomas showing a focal or heterogeneous positive staining limited to epithelial cells. IOR C2 was not detected in normal human ovarian surface epithelium (Fig 2).

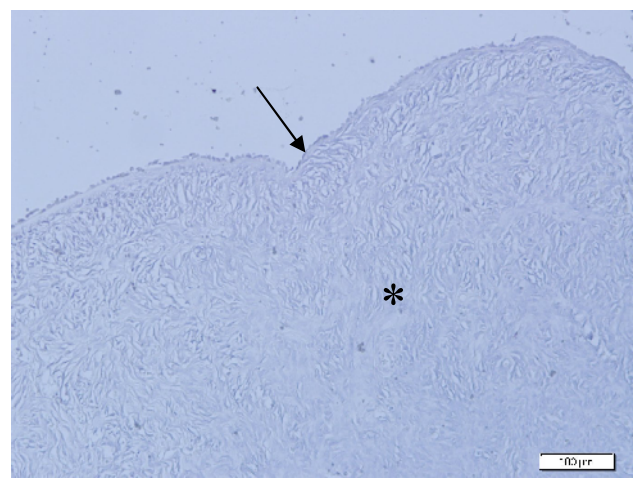


Fig. 2. Normal ovaries with surface epithelial cells (arrow) were no reactive with ior c5 Mab. No staining was also observed in the stromal cell (asterisk). Scale bar =100µm.

Considering all 22 invasive samples analyzed in the present study, the IOR C2 protein was evidenced in the cytoplasm and membrane of tumor cells in 15 out of 22 (68, 18%) cases studied. A positive reaction was observed in 7 out of 9 serous adenocarcinomas, in all (6) mucinous adenocarcinoma, and in 2 out of 4 endometroid carcinomas (Fig 3) with a prevalent membranous staining with apical accentuation in polar cells. In a clear cell carcinoma no positive immunostaining was observed.

The origin of ovarian tumors is controversial and the theories about their cell derivation are reviewed [15]. Some morphological, embryological and molecular evidences support the idea of Mullerian tract (paramesonephric ducts) as potential site to ovarian epithelial tumors derivation. The increased membrane reactivity as well as the positive intraluminal material secreted by neoplastic cells could be related to antigen over expressions process associated to the malignant transformation. Several Mabs with similar pattern of expression have been used for in vivo imaging and immunotherapy [16, 17]

Tumor grading information was available for the 22 patients with invasive carcinoma (9 with grade 1, 12 with grade 3 and 1 with grade 4), but no relevant difference was detected in relation to IOR C2 expression. A positive correlation between the three antigens and the histological subtype (Table 3), IOR C2 was mainly present in mucinous tumors in comparison to serous and endometroid (100% vs 77, 5% vs 33, 3%).

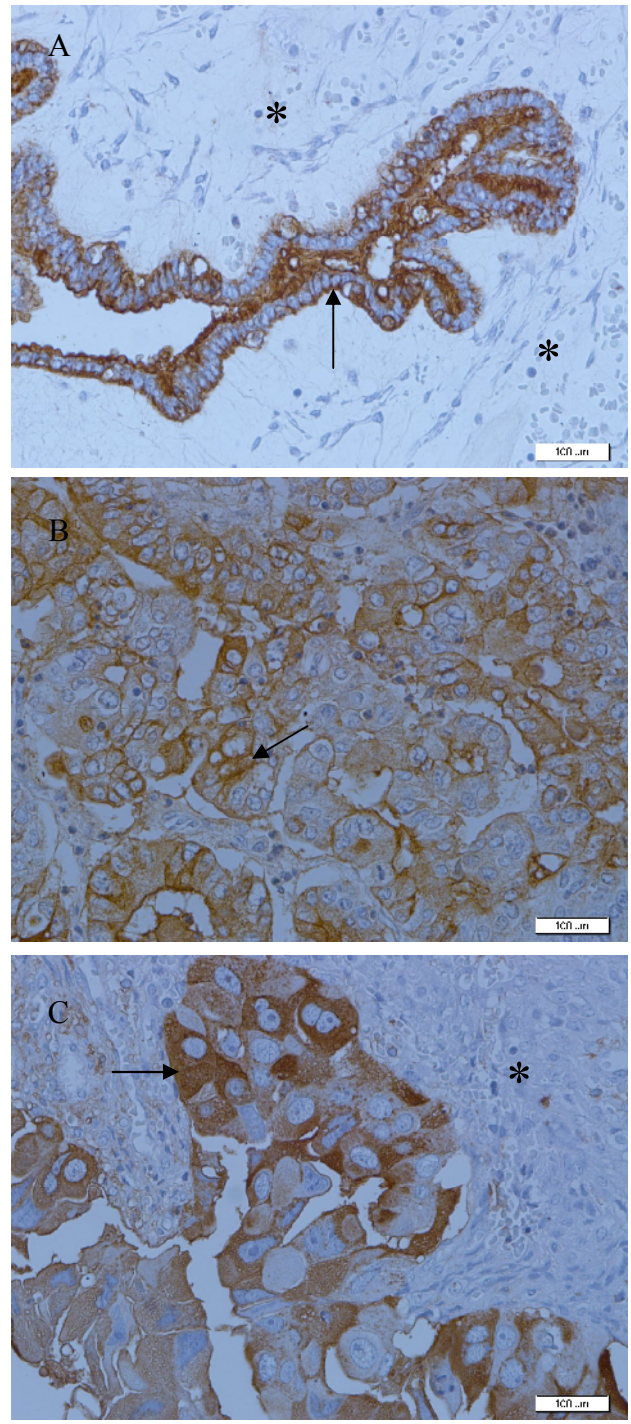


Fig. 3. Expression of IOR C2 tumor-associated antigen on representative examples of ovarian tissues. Strong immune staining (arrow) was detected in serous cystadenocarcinoma (A), mucinous tumor (B), and in endometrioid carcinoma (C). No staining was also observed in the stroma cells (asterisk).
Scale bar =100µm.

Table 3. Immunohistochemical positivity for the three tested antigens (IORC2, CEA and CA-125) in invasive ovarian epithelial carcinoma.

Subtype	Cases	IORC2+	CEA+	CA 125+
Serous	9	7 (77,5)	5 (55,5)	9 (100)
Mucinous	6	6 (100)	5 (83,3)	1 (16,6)
Endometroid	6	2 (33,3)	4 (66,6)	4 (66,6)
Clear cell	1	0 (0)	1 (100)	1 (100)
Total	22	15	15	15

This is the first report comparing IOR C2 with other antigens (CEA and CA-125) in paraffin embedded tissues from patients bearing invasive ovarian carcinoma. CA-125 antigen was also positive in 15 out of 22 cases (68, 18%) mainly in serous and in the clear cell carcinomas, nevertheless only one mucinous subtype (16, 6%) showed positive staining. CEA was detected in 15 out of 22 specimens tested. Interestingly, CEA was present only in five out of six mucinous (83, 3%) and in five out of nine serous (55, 5%) ovarian cancer. CA-125 and CEA immunostaining were observed in four out of six endometroid and in a clear cell carcinoma studied. The pattern of expression of each of these antigens on ovarian carcinomas was mainly at the luminal surface of positive cells.

In normal tissue, IOR C2 glycoprotein was located in the cell cytoplasm showing a homogenous pattern (data no shown), but in colon and ovarian malignant neoplasm a change to an apical membrane and secretion expression occurred (see Fig 3). Probably, during malignant transformation this antigen could be secreted through endoplasmic reticulum membrane toward to an external secretion.

Numerous candidate ovarian cancer biomarkers have been identified up to now, the multitude of antigens and several biological factors tested do not seem to be useful in predict chemoresponse or to serve as biomarkers of clinical characteristics including diagnosis and prognosis [3,18-20].

IOR C2 over expression in colorectal [11-13] and ovarian cancer bearing patients was demonstrated by Inmunoscintigraphy with 99mTc-ior c5 [10] with high specificity and accuracy. In the initial biodistribution/pharmacokinetics analysis of mAb ior c5 in human, it was evidenced good tumor localization with very limited accumulation in normal tissues [11, 12]. And it was also possible to evaluate a distance metastatic lesion not previously identify [10, 13].

Overexpression of mucins in ovarian tumors have been reported accompany or not of detectable level in normal ovarian tissues [21, 22] and with pathological functions in ovarian cancer development [For a review see ref. 23, 24]. Also IORC2 antigen non expressed in normal surface epithelium is abnormally present in cancer cells showing a differential expression during malignant transformation. In this way, might be considered a potential target for immunotherapy of ovarian cancer with antibodies and vaccines.

The pattern of expression of the IOR C2 has been studied in various tumors such as lung, breast tumors, endometrial and endocervical carcinomas [9], as yet these findings have not been incorporated into the clinical assays up to now. With the identification of IOR C2 as the molecular target of mAb ior c5, not only expressed in colorectal and ovarian cancers but also in a series of other epithelial neoplasms potentially expanding the possible use of the ior c5 mAb.

CONCLUSIONS

IOR C2 shows a differential expression during malignant transformation, as well as in ovarian tumors related to the histological subtype. This glycoprotein represents a potential cell surface target for the immunotherapy of cancer using monoclonal antibodies.

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