UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA

The role of adhesion molecule NCAM in ovarian cancer progression and its correlation with intrabdominal cancer dissemination.

Tesi di Dottorato

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INTRODUCTION

Epidemiology

Ovarian cancer is a highly metastatic disease and the leading cause of death from gynecologic malignancies. In 2009 in the United States, it was estimated that ovarian cancer will have been diagnosed in 21,550 women with an estimated 14,600 deaths per year (NCI program 2010). The majority of these deaths are from ovarian cancer of the serous histological type and around half of women who are diagnosed with ovarian cancer are 60 or older.

Genetic predisposition for familial early-onset breast cancer accounts for approximately 5–10% of all breast cancers and 7–10% of all ovarian cancers (1) Mutations in two autosomal dominant genes, *BRCA1* and *BRCA2*, have been linked to familial breast or breast and ovarian cancer (2,3). Women who carry *BRCA1* or *BRCA2* mutations have an estimated lifetime risk of developing breast cancer (of) between 60% and 85%, and a lifetime risk (of) of developing ovarian cancer between 26% and 54% for *BRCA1*, and between 10% and 23% for *BRCA2(4,5,6,7).*

Despite enormous progress in the understanding of ovarian cancer biology, this disease remains one of the leading cause of cancer death among women in most western countries due to the advanced stage of disease at diagnosis (stages III–IV) when the vast majority of women (are diagnosed) present with disseminated

intraperitoneal carcinomatosis. Epithelial tumors (carcinomas) account for approximately 60% of all ovarian (tumors) neoplasms.
They are also classified in five major histological subtypes designated as follows: serous, mucinous, endometrioid, clear cell, and transitional cell (or Brenner type)(8,9).
Although there have been a variety of epidemiologic variables correlated with ovarian cancer, such as talc use, galactose consumption, none has been so strongly correlated as low parity, infertility, and duration of reproductive career, infact early menarche and late menopause seem to increase the risk of ovarian cancer.

Clinical aspects

In early-stage disease, premenopausal patients may complain of irregular menses. If a pelvic mass is compressing the bladder or rectum, the patient may report urinary frequency and/or constipation. Occasionally, she may perceive lower abdominal distension, pressure, or pain, such as dyspareunia. Acute symptoms, such as pain secondary to rupture or torsion, are unusual. The presence of a pelvic mass at clinical evaluation in postmenopausal subjects can be an important sign of possible ovarian cancer. Abdominal discomfort or vague pain, abdominal fullness, bowel habit changes, early satiety, dyspepsia, and bloating are frequent presenting symptoms. Occasionally, patients may present with bowel obstruction due to intra-abdominal masses or shortness of breath due to pleural effusion.
Unfortunately due to the lack of specific symptoms most of the patients are diagnosed with a advanced-stage disease.
In such a setting, patients most often have symptoms related to the presence of ascites. The symptoms include abdominal distention, bloating, constipation, nausea, anorexia, or early

satiety. Occasionally, patients may present with bowel obstruction due to intra-abdominal masses or shortness of breath due to pleural effusion in stage IV disease. If nodal metastases are present, inguinal, supraclavicular, and axillary nodes may be enlarged at palpation.

Serum CA-125 level has been widely used as a marker for epithelial ovarian cancer both in the primary assessment of a suspect adnexal mass and in the follow-up.

Surgical staging for early stage disease requires a laparotomy by a midline incision for an adequate exposure and careful examination of the abdominal cavity according to the Federation of Gynecology and Obstetrics (FIGO) guidelines (Fig 1). If disease appears confined to the ovary staging procedure includes, beside total abdominal hysterectomy and BSO, biopsy of the diaphragmatic peritoneum, paracolic gutters, pelvic peritoneum, and complete lymphadenectomy of the pelvic and para-aortic lymph nodes, an infracolic omentectomy and 4 washings of the peritoneal cavity (diaphragm, right and left sides of the abdomen, and pelvis). An appendectomy is performed for mucinous tumors.

Stage I - limited to one or both ovaries

- IA - involves one ovary; capsule intact; no tumor on ovarian surface; no malignant cells in ascites or peritoneal washings
- IB - involves both ovaries; capsule intact; no tumor on ovarian surface; negative washings
- IC - tumor limited to ovaries with any of the following: capsule ruptured, tumor on ovarian surface, positive washings

Stage II - pelvic extension or implants

- IIA - extension or implants onto uterus or fallopian tube; negative washings
- IIB - extension or implants onto other pelvic structures; negative washings
- IIC - pelvic extension or implants with positive peritoneal washings

Stage III - microscopic peritoneal implants outside of the pelvis; or limited to the pelvis with extension to the small bowel or omentum*

- IIIA - microscopic peritoneal metastases beyond pelvis
- IIIB - macroscopic peritoneal metastases beyond pelvis less than 2 cm in size
- IIIC - peritoneal metastases beyond pelvis > 2 cm or lymph node metastases

Stage IV - distant metastases to the liver or outside the peritoneal cavity

*Para-aortic lymph node metastases are considered regional lymph nodes (Stage IIIC).

Figure 1. FIGO Staging for carcinoma of the ovary

Surgical treatment of advanced ovarian cancer

The standard approach to primary treatment of patients with advanced ovarian cancer (AOC) consists of up-front cytoreductive surgery followed by combination platinum-based chemotherapy. Tumor reduction prior to chemotherapy may synchronize cell division, improve drug availability to metastases, reduce the number of cycles of chemotherapy required to eradicate residual disease, and diminish development of subsequent drug resistance. Since the publication by Griffith in 1975 (10), the rationale for surgical cytoreduction has been evident, subsequently several publications confirmed the role for primary cytoreductive surgery in management of advanced-stage ovarian cancer. Though it is widely recognized that patients with stage IIIC disease and carcinomatosis, or widely disseminated peritoneal disease, carry a worse prognosis than patients without such features, however it has been shown that surgical cytoreduction to no macroscopic disease can improve survival to equal those with less initial disease volume (5,6,11).

Unfortunately many patients with ovarian cancer do not undergo optimal surgical treatment, the reasons explained by numerous studies that have shown that optimal cytoreduction rates grater than 50% often require the incorporation of a variety of extensive upper abdominal surgical procedures that requires the skill of gyn oncologists whereas most of those patient are operated on by general gynaecologists.

The extent of the disease before surgery could partly determines the ability to perform a complete cytoreduction and therefore to have a significant impact on prognosis. Cherau et al (12) compare 6 different score of peritoneal spread according to operative findings (The FIGO stages, the peritoneal cancer index (PCI),the Eisenkop score, the Fagotti laparoscopic based score, the Fagotti modiefied score, and the Aletti score)(13-17). The authors pointed

out that the most relevant scoring systems to predict a complete resection were the Fagotti modified score and the PCI score. Moreover they supported the Aletti score as the best predictor of postoperative complications(12).

Recently, in a retrospective study (18) aimed to identify a subgroups of patients who are unlikely to benefit from an aggressive surgical approach, thus avoiding unnecessary morbidity and short term mortality which dramatically impact and shorten remaining life of these women and dramatically raise cost of care. The high risk group was identified by combining three different factors: high tumor dissemination or stage IV, poor performance status (ASA >3) or nutritional status (preoperative albubim levels < 3.0 gr/dl), and age <75 years. The median overall survival of this group was only 17 months. Therefore the autors suggest neoadjuvant chemotherapy in this small high risk patient group as an alternative treatment instead of aggressive debulking as standard of care for the vast majority of patients with advanced ovarian cancer.

If initial maximal cytoreduction be cannot not carried out, interval debulking surgery (IDS) should be considered in patients responsive to chemotherapy or showing stable disease. IDS should ideally be carried out after three cycles of chemotherapy, followed by three further cycles of chemotherapy.

Beside the impact of cytoreductive surgery there are obviously other biological factors not yet fully understood that play a significant role in the prognosis of advanced ovarian cancer. Until such factors are not clearly identified to select those patients that would not take advantage of debulking surgery then all medically fit patients deserve an aggressive, primary surgical approach.

The 5-year survival of patients with stage III disease with microscopic residual disease only at the start of treatment is 63.5% compared with 32.9% for those with residual disease < 2

cm and 24.8 % for those with suboptimal residual disease (> 2m)(19).

Etiology

The relationship of parity and infertility to the risk of ovarian cancer has led to the hypothesis that suppression of ovulation may have an important role. Theoretically, the surface epithelium, during the ovarian cycle, undergoes repetitive disruption and repair. It is thought that this process might lead to higher probability of spontaneous mutations that can unmask germline mutations or otherwise lead to the oncogenic phenotype.

Ovarian carcinoma could originate from any of three potential sites: the surfaces of the ovary, the fallopian tube, or the mesothelium-lined peritoneal cavity. Ovarian cacinoma tumorigenesis then either progresses along a stepwise mutation process from a slow growing borderline tumor to a well-differentiated carcinoma (type I) or involves a genetically unstable high-grade serous carcinoma that metastasizes rapidly (type II). During initial tumorigenesis, ovarian carcinoma cells undergo an epithelial-to-mesenchymal transition, which involves a change in cadherin and integrin expression and up-regulation of proteolytic pathways(20). Carried by the peritoneal fluid, cancer cell spheroids overcome anoikis and attach preferentially on the abdominal peritoneum or omentum, where the cancer cells revert to their epithelial phenotype. The initial steps of metastasis are regulated by a controlled interaction of adhesion receptors and proteases, and late metastasis is characterized by the oncogene-driven fast growth of tumor nodules on mesothelium covered

surfaces, causing ascites, bowel obstruction, and tumor cachexia(20).

Cancer is a genetic disease that results from a series of mutations in various cancer genes. Uncontrolled cancer growth occurs because of the accumulation of somatic mutations or the inheritance of one or more mutations through the germ-line followed by additional somatic mutations. The mutations in genes that are directly involved in normal cellular growth and proliferation can lead to the development of uncontrolled growth, invasion, and metastasis. Understanding the biology and molecular pathogenesis of ovarian epithelial tumors is a key factor to identify better prognostic indicators and possibly develop effective therapies. The main reason for the lack of success in effectively treating ovarian cancer is our limited understanding of its etiology and the very few molecular diagnostic markers and therapeutic targets known so far. Identification and characterization of ovarian cancer-associated genes are fundamental for unveiling the pathogenesis of its initiation and progression, especially the development of recurrent diseases. If there was a way to determine “key drivers” of carcinogenesis which could address this issue, those patients with tumors amenable to surgical cytoreduction could be offered surgery as the initial therapy and the others (suboptimal) could be offered neoadjuvant therapy, followed by surgery. These “key drivers” could represent potential markers for prognosis and therapy. It has been suggested that early genetic events may direct the differentiation of ovarian epithelial cells. Decades of research have investigated molecular events such as: oncogenic activities of KRAS, BRAF, and AKT, and silencing mutations of TP53, RB, and PTEN that lead to ovarian cancer development. However, this information has had surprisingly little clinical impact on the outcome of women diagnosed with ovarian cancer. Recent evidence suggests that metastasis is an earlier event than previously thought and that

only a very small number of shed malignant cells are capable of metastasizing (0.01%) (11,21).

The persistence of cancer cells in the vasculature does not necessarily result in seeding to distant sites and emerging evidence in breast cancer suggests that early tumors may already hold the genetic profile needed for metastasis. These early alterations in dominant genes may dictate the specific path that is followed with K-RAS leading to an LMP tumor and the early occurrence of a P53 or BRCA alteration leading to genetic instability and rapid progression to a high-grade phenotype. Characteristics common to both pathways include evasion of immune surveillance, invasion into the stroma, survival in the peritoneal cavity, attachment to intraperitoneal sites, and continued growth and angiogenesis (22). What is urgently needed is an effective approach to rapidly and maximally leverage available ovarian cancer patient data to create an understanding of the disease that is detailed enough and accessible enough to enable “what if” queries regarding how best to treat patients with specific tumor characteristics, in terms of both genetics (the potential for disease outcome), disease biology (how the potential has played out up to the point of measurement), and the connections between these and the clinical outcome, and can, in addition, incorporate the thousands of relevant variables.

The Epithelial- Mesenchymal Transition

Carcinogenesis involves the accretion of unprogrammed genetic and epigenetic changes, which lead to dysregulation of the normal control of cell number. But a key clinical turning point in carcinoma progression is the establishment by emigrant cells of secondary growth sites (i.e., metastasis). The metastatic ‘‘cascade’’ comprises numerous steps, including escape from the primary tumor site, penetration of local stroma, entry of local vascular or

lymphatic vessels (intravasation), aggregation with platelets, interaction with and adhesion to distant endothelia, extravasation, recolonization, and expansion (23) all the time avoiding effective immune clearance and being able to survive in these multiple settings. The large majority of ovarian malignancies are of epithelial origin; however, the human OSE consists of mesothelial cells. It has been reported that epithelial to mesenchymal transition (EMT) plays a role in carcinogenesis including ovarian cancers. The native ovarian surface mesothelium is of an 'uncommitted' phenotype and has the potential to change to the epithelial or mesenchymal phenotypes in response to signals such as those associated with ovulation. Due to the lack of an anatomical barrier, ovarian carcinoma can spread directly throughout the peritoneal cavity, mainly by intra-abdominal dissemination and by lymphatic dissemination, enabling in this way the attachment to peritoneum and omentum (24). .

Therefore, the main routes of dissemination are: intra-abdominal spreading by exfoliation of the primary tumor into the abdominal cavity and lymphatic spread through the lymphatic vessels mainly to the pelvic and para-aortic basins, on the other hand distant metastases through the bloodstream are rare(25).

Moreover, metastatic tumor cells undergo morphological and molecular changes during the transition from a benign to a malign phenotype to facilitate the interaction with the peritoneal stroma and mesothelium and the attachment to the distant peritoneum.

It is also important to understand what specific features or functions are associated with the terms epithelium and mesenchyme. An epithelium is a collection of cells forming a relatively thin sheet or layer due to the constituent cells being mutually and extensively adherent laterally by cell-to-cell junctions. The layer is polarized, the two sides showing nonidentical properties so that the sides can be defined as, say, inside or

outside, or more precisely, apical and basal. Cell-to-cell adhesion molecules typically involve (but are not restricted to) members of the cadherin axis, which are distributed widely but with a particular aggregation complex usually as a circumferential belt at the lateral border. In some circumstances, cells in an epithelial layer can alter shape, such as change from flat to columnar, or pinch in at one end and expand at the other.

Mesenchymal cells form a relatively diffuse tissue network: there is no complete cellular layer, and the cells typically have only points on their surface engaged in adhesion to their neighbors. These adhesions may also involve cadherin associations (i.e., withmolecular family similarity to those of epithelial cells). Mesenchyme gives the impression of much more relaxed organization, and this suggests flexibility, individualism, and motile propensities. In many cases, mesenchyme cells do participate in cell migrations.(26). The presence (epithelial) or absence (mesenchyme) of a basal lamina (although this need not be complete) is a typical correlate, but new mesenchyme generated by EMT may transiently retain basal lamina fragments (27) Capacity to move, individualistically or in groups, are not absolute defining characteristics for epithelia and mesenchymes, although, in general, the latter seem more dynamic and plastic. These same elements, histologic, molecular, and transcriptional, are commonly associated with carcinoma progression, leading to the obvious possibility of EMT as a part of the metastatic process. However, the execution of a development-like EMT by cancer cells is only one hurdle in achieving metastatic ‘‘success,’’ so one cannot expect sure and immediate metastasis even when the primary tumor shows signs of EMT. In addition, primary tumors are heterogeneous, and usually only a very small proportion, sometimes called the ‘‘invasive front,’’ shows the histologic and molecular EMT-like signature (26). A key feature of EMT is the switch from E-cadherin to Ncadherin, cells undergoing EMT

downregulate the expression E-cadherin accompanied by an increased expression of N-cadherin which promotes the interaction with endothelial and stromal components (Fig. 2). Cadherins are not the only cell adhesion molecules (CAMs) that play a critical role in tumor progression. Studies with human tumor biopsies and mouse tumor models have revealed that the neural cell adhesion molecule (NCAM) also plays an important role in the progression to tumor malignancy (28).

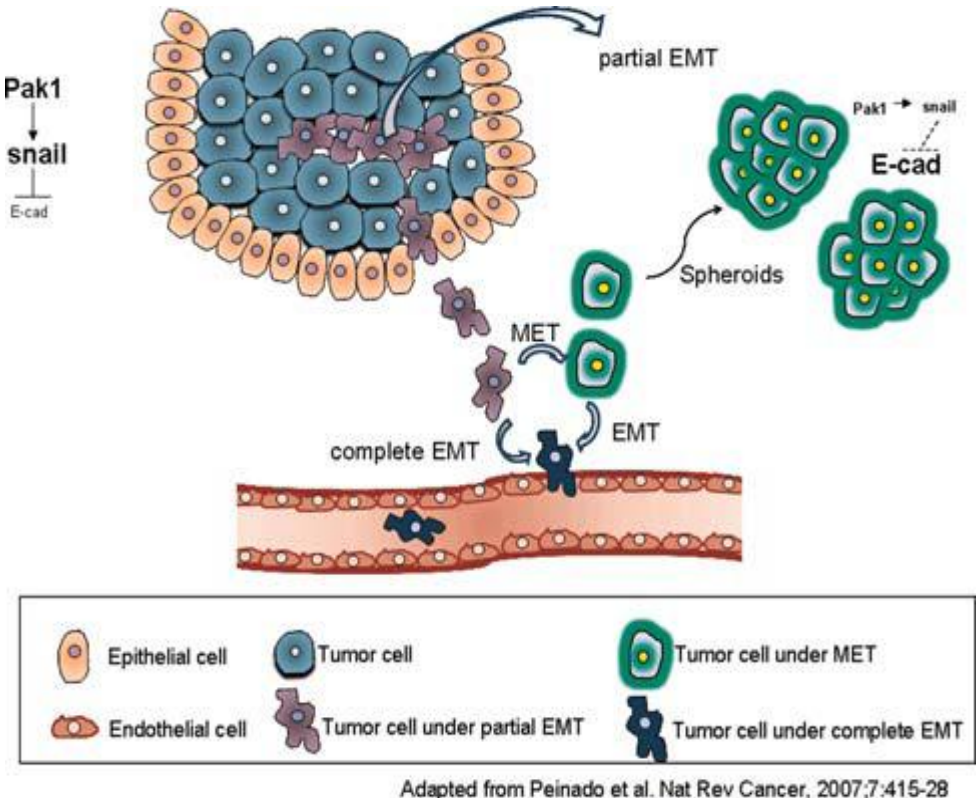

Fig.2 Cartoon showing the postulated EMT process in ovarian carcinoma. Tumor cells in the primary tumor undergo partial EMT causing them to become motile and invasive. These cells leave the microenvironment of the primary tumor and invade the peritoneal cavity. In this new microenvironment these cells can either undergo complete EMT and form solid metastases, or reverse to a less aggressive phenotype in a process of MET and grow in effusions. Letter size for Pak1, Snail and E-cadherin denotes expression levels at each anatomic site.

The neural cell adhesion molecule (NCAM)

Cell adhesion molecules mediating either cell-cell interactions or cell-matrix adhesion have emerged as key players throughout the natural history of EOC development, in that they have been implicated both in cancer cell survival upon detachment from the primary tumor and in the subsequent adhesion to and invasion of metastatic sites (25, 29). Neural cell adhesion molecule (NCAM) is a cell-surface glycoprotein with an extracellular portion composed by five Ig domains and two fibronectin type-III repeats. NCAM function has been extensively characterized in the nervous system, where it regulates intercellular adhesion, neurite outgrowth and neuronal migration. These activities are mediated both by homophilic interactions and by heterophilic binding of NCAM to a number of different membrane proteins or components of the extracellular matrix (30). Among the heterophilic partners of NCAM, the fibroblast growth factor receptor (FGFR) has attracted the attention of many investigators due to its functional implications. FGFR stimulation results in the recruitment and activation of specific effectors that, in turn, trigger a set of signalling pathways (31). The functional interaction between NCAM and FGFR was originally reported in neurons, where it was implicated in neurite outgrowth (32). Thereafter, some authors have provided extensive evidence of a physical association between the two proteins on different, non-neural cell types (33-36) All four members of the FGFR family as well as various FGFs have been found in EOC tissue (37-39) suggesting that dysregulated FGFR signaling contributes to ovarian carcinogenesis (39-41).and therefore it may represent a suitable therapeutic target (42). Based on the ability of NCAM to modulated FGFR function and on the proposed role of FGFR activity in ovarian cancer, we hypothesized that the NCAM/FGFR signaling axis is causally involved in EOC development.

OBJECTIVE

The primary objective of the present study was to investigate the expression and functional role of NCAM in EOC both in vitro and in vivo (mouse models).

The secondary objective was to investigate the correlation between NCAM expression in human OSE, benign ovarian lesion and primary tumors and metastasis. We further analize the correlation between NCAM and different tumor features.

The tertiary objective was to investigate the correlation between NCAM expression and ovarian cancer dissemination stratified in the major abdominal area in patients with advance ovarian cancer (stage III –IV).

PATIENTS AND METHODS

Lab Experiments

1. In vivo tumorigenesis

All experiments with mice were performed in accordance with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC) and approved by the Italian Ministry of Health. Mouse tumorigenesis assays and antibody treatment were performed as described (Arlt et al, 2006) with slight modifications. Briefly, pathogen-free, female C57/BL6 mice (7-9 weeks old; 20 g average body weight) from Charles River Laboratories (Wilmington, MA) were inoculated intraperitoneally with 2 x 106 ID8- GFP cells resuspended in 300 ml of PBS, leading to tumor formation within 1 months.

2. Immunohistochemistry in EOC mouse model.

Immunohistochemistry was performed using 5-µm serial sections from formalinfixed, paraffin-embedded tissue samples. Tissues were deparaffinized in Histolemon (Carlo Erba, Milano, Italy), hydrated through graded alcohol series, Epitope unmasking was performed in 0.25 mM EDTA (pH 8, at 98°C, for 50 min). Endogenous peroxidases were quenched in 3% H2O2 and slides were pre-incubated for 1 hour in blocking solution (PBS, 2% BSA, 2% goat serum, 0.01% Tween-20), followed by the incubation with 10 □g/ml mouse anti-NCAM (mAb 123C3) overnight at 4°C. Peroxidase-based Dako EnVision+ kit was used as a detection

system Slides were counterstained with hematoxylin for histological evaluation.

3. Immunofluorescence

Cells were cultured on glass coverslips and then fixed in 3% paraformaldehyde, 2% sucrose in PBS and blocked in 5% albumin in TBST (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20). After blocking, cells were stained with primary antibodies (anti-human NCAM (clone 123C3) 1 mg/ml, anti-mouse NCAM (clone NCAM13) 1:1000) in blocking solution for 1 hr at room temperature, followed by incubation with secondary antibodies conjugated with either FITC or Cy3 (Jackson Immuno Research Laboratories), diluted 1:400 in blocking solution. Cell nuclei were counterstained with 4'-6-Diamidino-2-phenylindole (DAPI). Images were acquired on a Olympus BX71 microscope equipped with analySIS software (Olympus Soft Imaging Solutions, Münster, Germany). Images were then processed using NIH ImageJ and Adobe Photoshop CS2 9.0.2 (Adobe Systems, Inc., San Jose, CA) softwares.

4. NCAM in human EOC

The expression of NCAM was investigated in two-hundred-fifty-two ovarian cancer patients who had undergone surgery at the European Institute of Oncology (Milano, Italy) from 1995 to 2004 and whose cancer specimen were available were evaluated in the study. Samples were collected in OR and stored at -80° degree. Representative areas were first selected on hematoxylin-eosin-stained tumor sections by a trained pathologist; two representative core biopsies for every tumor block were included in the tissue microarrays (TMAs). TMAs were assembled on a custom-built

tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA) as previously described (Zecchini et al, 2008).

Routine immunohistochemical staining was performed with anti-human NCAM mAb (clone 123C3) on paraffin-embedded TMA sections and on whole sections from 20 normal ovaries and 4 benign cystoadenomas. Whole section of randomly-chosen tumors included in the TMA were also stained to confirm immunoreactivity was preserved on the TMAs cores. Membrane staining for NCAM in tumor cells was scored as positive and the percentage of positive tumore cells was assessed by two independent pathologists. The average percentage of NCAM-positive cells of two cores was computed for analysis. For statistical purpose, a percentage of cell expressing NCAM of 10% or more was considered as positive, while less than 10% expressed NCAM were considered as negative.

5. Data collection

After obtaining Institutional Review Board approval, we reviewed all medical records of patients with epithelial ovarian cancer who were managed at the European Institute of Oncology between January 1995 and December 2004.

All patients included in the study underwent primary surgery at our institution and received no neoadjuvant treatment. De-identified data from patients charts collected in a database were: patients' demographics, surgical stage according to the International Federation of Gynecology and Obstetrics (FIGO) standards, specimens histology with grade. Extent of tumor burden at the beginning of the surgical procedure was also extracted from the surgical record. This was further stratified into four different groups based on dissemination:

(1) omentum: negative or with cancer nodules or forming an "omental cake"

(2) diaphragm surface (yes or no),

(3) mesentery (yes or no)
(4) presence of diffuse carcinomatosis defined as more than 100 nodules spread throughout the peritoneal surfaces (yes or no). Amount of residual disease as described in the surgical report at the end the debulking procedures was collected as well.
Histology was also stratified into two groups to verify any correlation with NCAM expression: group A (including serous, endometrioid, mixed hystotype) and group B (including clear cells, mucinous, and transitional hystotype).

6. Statistical analysis

Contingency tables, Pearson's chi-square and Exact Fisher test when necessary, were used to evaluate differences in the frequency of NCAM-positive (more than 10% of immunoreactive cells) tumors with respect to grade, stage, histotype and different intrabdominal areas of dissemination. T-Test was used to evaluate the differences in the numbers of migrating and invading tumor cells among the represented experimental conditions and to compare the number of metastases formed by tumor cell lines expressing wild-type, mutant or no NCAM. All statistical analyses were carried out with SAS statistical software (SAS Institute, Inc., Cary, NC) by a trained statistician. A p value of 0.05 or less was considered to be statistically significant.

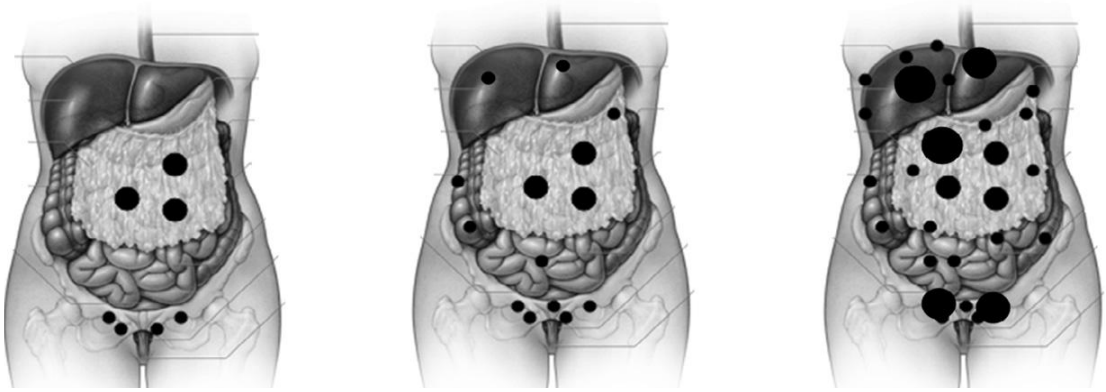

FIG.3 A model of intraabdominal dissemination in EOC. Figure from ref.18.

Results

Lab results:

NCAM stimulate EOC cell invasion via its interaction with FGFR

Tumor cell invasion is a key step during cancer progression and, therefore, we determined the role of the NCAM/FGFR interaction in the ability of EOC cells to invade Matrigel, a reconstituted basement membrane. The ectopic expression of fulllength NCAM resulted in a dramatic increase of the invasive potential of bothSKOV3 and OVCA-433 cells. We tested whether mAbs that prevent the binding of NCAM to FGFR had any impact on NCAM-dependent EOC cell invasion. By analogy to cell migration, the

stimulation of Matrigel invasion by ectopic NCAM expression was abolished by either 123C3 or Eric-1 mAbs (Fig. 4C). This supports the hypothesis that interfering with the NCAM/FGFR association has a dramatic impact on the promalignant function of NCAM in EOC cells.

NCAM stimulates EOC cell migration via its interaction with FGFR

To investigate whether NCAM is involved in the malignant phenotype of EOC cells, we utilized the MOVCAR cell line, originally isolated from the cancer tissue of MISIIR-TAg transgenic mice, a genetic model of ovarian carcinoma (Connolly et al,2003). This cell line expresses high levels of NCAM, which is properly localized at the cell surface (Suppl. Fig. S1A). The migratory activity of MOVCAR-shNCAM was 2- fold lower as compared to cells transduced with a control shRNA. This effect was specifically due to NCAM gene silencing, since the expression of human NCAM restored the migratory potential of MOVCAR-shNCAM cells (Fig. 5A). Based on the notion that NCAM binds to FGFR and modulates FGFR activity we asked whether this interplay is involved in the NCAM-dependent migration of MOVCAR cells. We also employed the Matrigel invasion assay to determine whether NCAM and its functional interaction with FGFR were required for

the invasive potential of MOVCAR cells. The knockdown mices of NCAM resulted in the abrogation of MOVCAR cell invasion, which was restored upon reconstitution with human NCAM. Taken together, these results indicate that NCAM is required for both migration and invasion of MOVCAR cells and exerts its function via FGFR activity.

Following the observation that NCAM/FGFR interplay is necessary for EOC cell migration and invasion, we asked whether it is also sufficient. To address this question, we selected two human EOC cell lines, SKOV3 and OVCA-433, which express no endogenous NCAM. Both cell lines express various FGFR family members thus providing a suitable experimental system to investigate the impact of ectopically expressed NCAM on FGFR activity. In agreement with the data on NCAM silencing in MOVCAR cells, ectopic expression of NCAM in human EOC cells had no effect on cell proliferation (Suppl. Fig. S2C) Rather, NCAM-expressing SKOV3 and OVCA-433 cells exhibited a remarkable increase in their migratory activity as compared to mock-transfected cells.

To further confirm this notion, SKOV3 cells expressing NCAM or a control vector were either treated with a FGFR inhibitor or transfected with a dominant-negative version of FGFR1. FGFR inhibition resulted in the abrogation of NCAM-dependent migration supporting our results.

Peritoneal dissemination of EOC is regulated by the NCAM/FGFR interaction

We employed an assay based on the intraperitoneal injection of SKOV3 cells into immunodeficient mice, which is widely used as a model for peritoneal metastasis of human EOC (43-45). For this purpose, we used SKOV3 cells co-expressing GFP and either an empty vector, fulllength NCAM. After 5 weeks, peritoneal dissemination was assessed as the formation of GFP-positive

tumor masses attached to the bowel, liver and diaphragm, all typical sites of EOC metastasis in patients. As shown in Fig. 6A and B, NCAM expression resulted in increased number and size of bowel metastases as compared to mock-transfected cells (See Suppl Fig. S6 for additional images). NCAM also enhanced the dissemination of SKOV3 cells to the liver (Fig. 5C). Finally, NCAM-transfected cells also exhibited an increased ability to colonize the diaphragm, although the difference with control cells did not reach statistical significance (Fig. 5D). This implies that the association of NCAM with FGFR is a prerequisite for EOC spreading in vivo. We therefore asked if this interaction could be specifically targeted to interfere with tumor dissemination. To address this question, mice xenotransplanted with SKOV3- NCAM cells were subjected to intraperitoneal treatment with the mAb that, interferes with NCAM-mediated activation of FGFR. The dissemination of SKOV3-NCAM cells to bowel and diaphragm was dramatically reduced in mAb-treated mice, and a significant decrease was also observed in liver metastasis. In contrast, a control mAb showed no effect in SKOV3 cell dissemination (Fig. 7). These findings indicate that antibody-mediated targeting of the NCAM/FGFR can interplay suppresses the metastatic potential of EOC cells, an observation that could have relevant therapeutic implications.

Clinical results

The expression of NCAM was investigated in a panel of 276 surgically resected specimens, including 20 normal ovaries, 4 benign lesions (cystadenoma), and 252 primary EOC. Tumor feateres of the study specimens are shown in Table 1.

Table 2 summarizes the correlation between clinicopathological features of EOC specimens and NCAM expression.

Immunohistochemical staining with anti-NCAM antibody showed no signal in the surface epithelium of normal ovaries (Fig. 8A) or in preneoplastic lesions. In contrast, 60 (23.8%) primary EOC (Fig. 8B) were clearly positive for NCAM, thus indicating that NCAM expression occurs specifically in transformed ovarian epithelial cells (*P* value: 0.0003). Interestingly, increased levels of NCAM were frequently observed at the invasive front of the tumor lesions (Fig. 8B). Moreover we observed a statistically significant association with higher tumor grade, grade G2 and G3 compared to G1 (*P* value: 0.025) (Table 2). There was also a trend in the association of NCAM expression and advanced FIGO stages as compared to early ones, although this correlation did not reach statistical significance. We then investigated whether NCAM expression correlates with histology of the EOC specimens and we observed a stastically significant association (*P* value: 0.025) with group A (serous, endometrioid, mixed hystotype) compared to group B (clear cells, mucinous, and transitional-cell type). Based on the results obtained in the total number of cases we verified whether there was any correlation of NCAM expression with the same clinicopathological parameters in the group of advanced stages only (stage III: 165 patients and stage IV 38 patients). However, there were no statistically significant differences by hystotype, grade and distribution of intrabdominal dissemination (omentum, diaphragm surface, mesentery and presence of diffuse carcinomatosis) as shown in table 3.

Discussion

Over the past two decades, the 5-year survival for ovarian cancer patients has substantially improved owing to more effective surgery and treatment with empirically optimized combinations of cytotoxic drugs, but the overall cure rate remains approximately 30%. Many investigators think that further empirical trials using combinations of conventional agents are likely to produce only modest incremental improvements in outcome. Given the heterogeneity of this disease, increases in long-term survival might be achieved by translating recent insights at the molecular and cellular levels to personalize individual strategies for treatment and to optimize early detection (45,46).

Using targeted agents in ovarian cancer will discover not only how these novel therapies work but are also unveiling the complex 'wiring' of the disease itself, and the interconnections between what were previously believed to be distinct molecular pathways. The addition of targeted agents to our therapeutic armoury is likely to significantly and positively impact on patient survival.

Based on the ability of NCAM to modulated FGFR function and on the proposed role of FGFR activity in ovarian cancer, we hypothesized that the NCAM/FGFR signaling axis is causally involved in EOC development. Screening of tumor biopsies revealed that NCAM is expressed in a significant proportion of EOC samples, but not in normal tissue, where its levels are increased at the invasive front and correlate with tumor grade. Furthermore, we show that the NCAM/FGFR interplay induces EOC invasion and peritoneal dissemination, and that interfering with this interaction represents a promising strategy to inhibit EOC progression. Our findings, therefore, provide novel insights into molecular mechanisms involved in EOC aggressiveness and offer

the possibility to explore innovative therapeutic approaches for EOC treatment. This study reports for the first time that NCAM is not detectable in normal ovarian surface epithelium but is expressed de novo in EOC, an event that correlates with tumor aggressiveness. Our results are in line with the only screening published so far on NCAM expression in EOC, where NCAM was reported to be absent in healthy ovarian epithelium, poorly expressed in low-grade/early-stage tumors, but highly enriched in advanced EOC (46). The main novelty of our study lies in the functional contribution of this adhesion molecule to EOC progression. NCAM gene silencing and ectopic expression approaches supported the notion that NCAM is both necessary and sufficient to promote a migratory and invasive phenotype in EOC cells, with no major effect on cell proliferation. Interestingly, we have observed this specific function of NCAM in cell motility but not in cell proliferation also in other cell types, including fibroblasts (34) and different epithelial cell lines (48) suggesting that it is a rather general phenomenon. Along this line, NCAM has been recently implicated in cell migration in the context of epithelial-to-mesenchymal transition (49). In the same study, NCAM was found to be upregulated in tumor cells at the invasive front of neoplastic lesions, in agreement with our findings in EOC samples. Taken together, these observations point to NCAM as a novel pro-invasive factor with a causal role in cancer progression.

Our study demonstrates that the functional contribution of NCAM to EOC progression relies on its interaction with and activation of FGFR. The role of FGFR in mediating NCAM function has been originally proposed in neurons, where this interplay was implicated in neurite outgrowth (32). Subsequently, we have reported that NCAM is able to bind and activate multiple members of the FGFR family.

Our report provides the first evidence that NCAM-mediated stimulation of FGFR activity plays a causal role in cancer cell migration and invasion, two events strictly related to tumor malignancy. The observation that an antibody suppressing NCAM-induced activation of FGFR prevents peritoneal metastasis of EOC, on one hand, lends further support to the physiopathological relevance of this signaling axis in ovarian cancer malignancy. On the other hand, it has dramatic therapeutic implications, providing a strong rationale to explore the targeting of the NCAM/FGFR interplay in the context of novel strategies against EOC dissemination.

As for other tumor types, targeted therapy has progressively emerged in ovarian cancer as a suitable approach to complement the conventional chemoterapy protocols and, where possible, overcome some of their main limitations (e.g., chemoresistance and toxicity) (50,51). A significant percentage of primary EOC were clearly positive for NCAM where its levels are increased at the invasive front, as opposed to normal ovaries or benign lesions, thus indicating that NCAM expression occurs specifically in transformed ovarian epithelial cells. Similarly in a study published by Cho et al (47) , the possible roles of adhesion molecules (E-cadherin, α-, β-catenin, CD44s, CD44v6, CD56, CD99), was investigated in a series of benign, borderline, and malignant ovarian serous neoplasms, using immunohistochemistry. The study demonstrated that membranous expression of adhesion molecules is significantly higher in borderline tumors and adenocarcinomas compared with benign tumors, and reduced expression of E-cadherin is frequently seen in adenocarcinomas compared with benign and borderline tumors.

The observed correlation of NCAM expression with EOC is in agreement with a statistically significant association of NCAM expression with higher tumor grade (G2 and G3 vs G1) and, though not statistically significant may be due to the unbalance in

the number of advanced cases (84.6%) as opposed to early ones, a trend in the association with advanced FIGO stages as compared to early stages. This raised the hypothesis that NCAM promotes EOC invasion, an issue that we have investigated at the cellular level as well as in mouse models. Moreover NCAM expression correlates with histology of the EOC specimens as we observed a statistically significant association with the group including serous, endometrioid, and mixed hystotype compared to the one including the other hystotypes. To this regards Shih and Kurman (52) have proposed a model that divides ovarian cancer into 2 groups designated type I and type II. Type I tumors are slow growing, generally confined to the ovary at diagnosis and develop from well-established precursor lesions so-called borderline tumors. Type I tumors include low-grade micropapillary serous carcinoma, mucinous, endometrioid, and clear cell carcinomas. They are genetically stable and are characterized by mutations in a number of different genes including *KRAS, BRAF, PTEN*, and *beta-catenin*. Type II tumors are rapidly growing, highly aggressive neoplasms that lack well-defined precursor lesions; most are advanced stage at, or soon after, their inception. These include high-grade serous carcinoma, malignant mixed mesodermal tumors (carcinosarcomas), and undifferentiated carcinomas. The type II tumors are characterized by mutation of *TP53* and a high level of genetic instability. The observation that in our study endometrioid subtype seems to behave as the more aggressive high-grade serous carcinoma could be due to the fact that most of the endometroid subtypes were poorly differentiated and advanced stage cases.

On the contrary when we look at the group of stage III and stage IV only there were no correlation of NCAM expression with the same clinicopathological parameters, most likely due to the fact that advanced stages specimens were mostly high grade and more aggressive histotypes.

Although our mice model suggested the association of NCAM with FGFR could be a prerequisite for EOC spreading in vivo as shown by the increased ability of NCAM-transfected cells to colonize the diaphragm. Regarding the tertiary objective of the present study in the stage III-IV group, we did not observe any statistically significant differences by hystotype, grade and distribution of intrabdominal dissemination (omentum, diaphragm surface, mesentery and presence of diffuse carcinomatosis). We therefore need more insights to verify whether the association of NCAM with FGFR could be specifically targeted to interfere with tumor dissemination. Taken together, these data point to NCAM as a novel biomarker of EOC associated with some of the clinicopathological features of cancer aggressiveness. Furthermore, our data show that the NCAM/FGFR interplay induces EOC invasion and peritoneal dissemination, and that interfering with this interaction represents a promising strategy to inhibit EOC progression. Our findings, therefore, provide novel insights into molecular mechanisms involved in EOC aggressiveness and offer the possibility to explore innovative therapeutic approaches for EOC treatment.

Conclusions

In summary, this study shows the aberrant expression of NCAM in human EOC tissue, and its association with cancer aggressiveness, and that the interplay of NCAM with FGFR enhances the migratory and invasive potential of EOC cells in vitro and their metastatic ability in vivo. Finally, we report that interfering with NCAM mediated activation of FGFR results in a dramatic reduction of EOC malignancy, providing the rationale for further assessing this approach as a novel therapeutic strategy.

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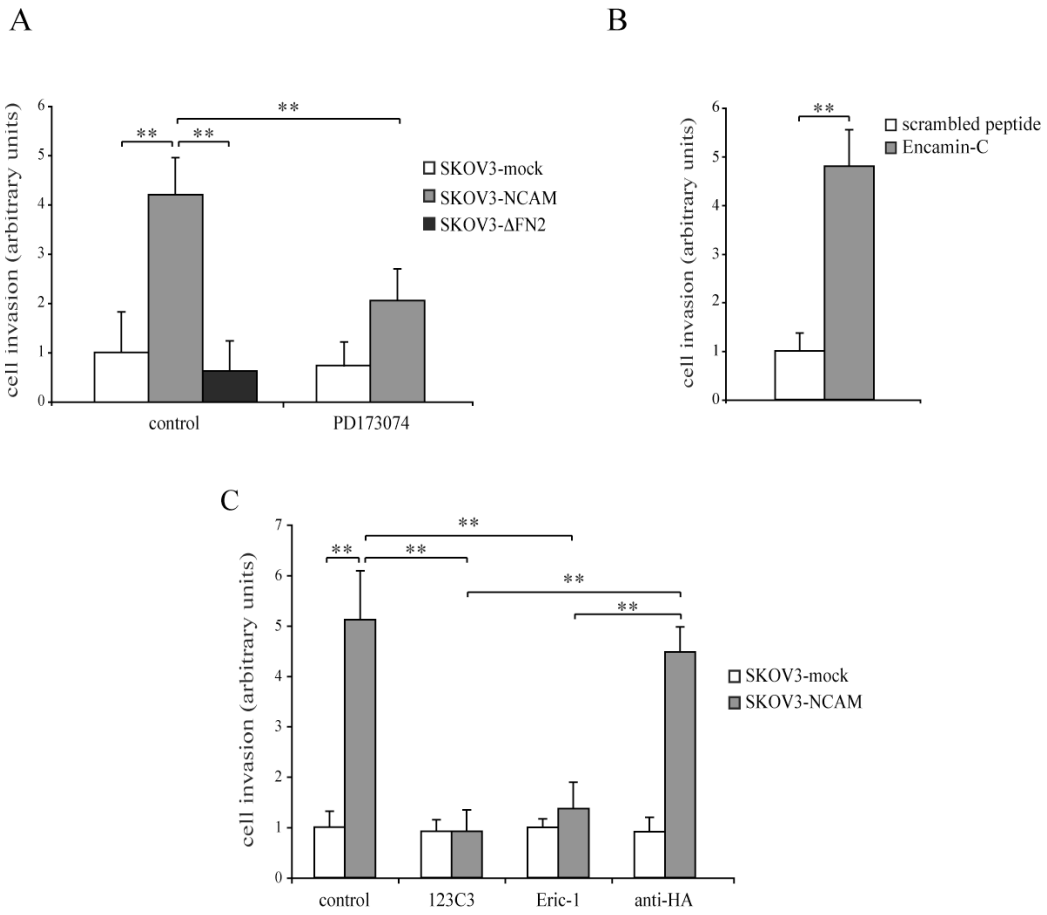

A
B
C
cell invasion (arbitrary units)
SKOV3-mock
SKOV3-NCAM
SKOV3-ΔFN2
control
PD173074
scrambled peptide
Encamin-C
123C3
Eric-1
anti-HA
**

Fig. 4 A-B-C.

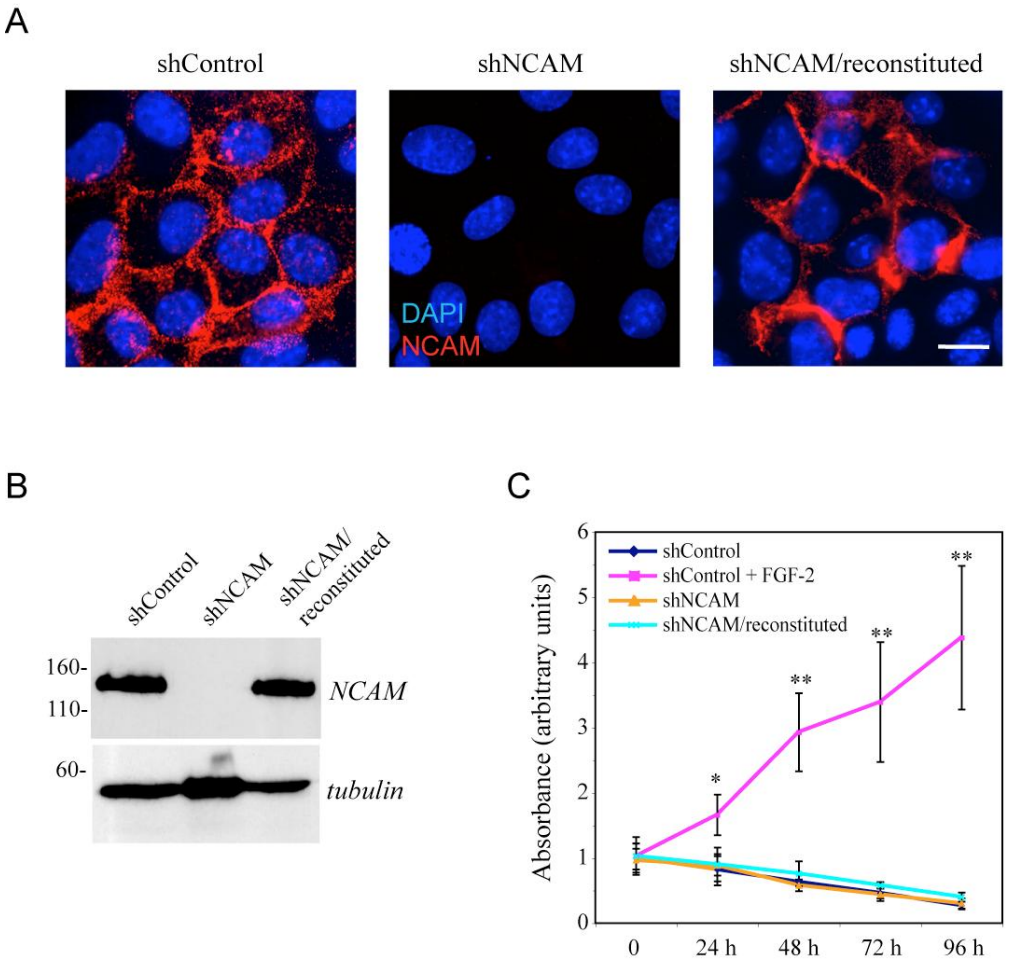
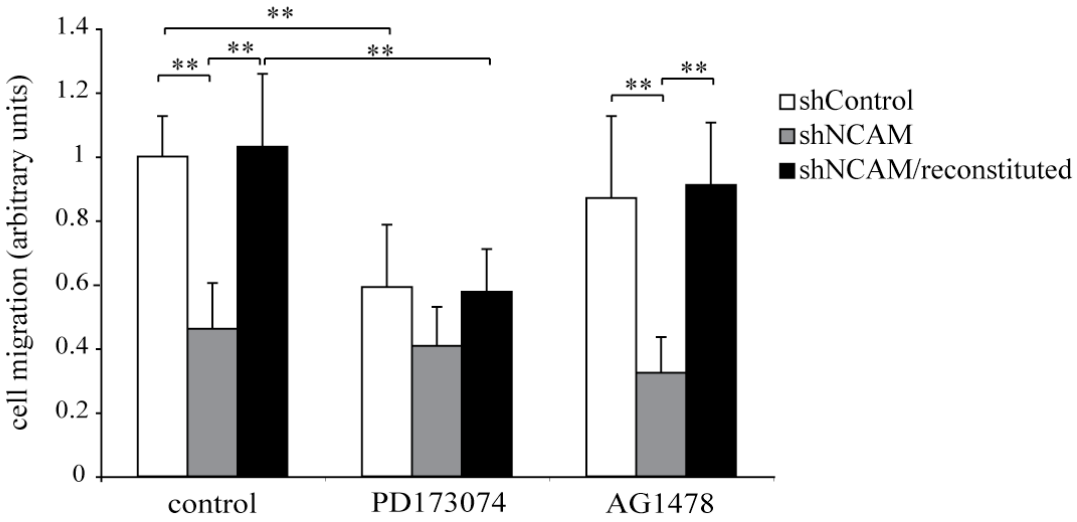


Fig. S1. NCAM silencing in MOVCAR cells. MOVCAR cells were transduced with a control short-hairpin RNA (shControl) or with a short-hairpin RNA against mouse NCAM (shNCAM). A set of MOVCAR-shNCAM cells was also reconstituted by transfection with human NCAM (which is not targeted by shNCAM). (A) Immunofluorescence staining for NCAM (red), showing that shNCAM dramatically reduced the expression of endogenous NCAM. Ectopic expression of human NCAM in shNCAM-transfected cells resulted in reconstitution of NCAM expression and localization at the cell surface. Cells were counterstained with DAPI to visualize nuclei (blue). Scale bar, 10 μm. (B) Immunoblotting for NCAM on lysates from MOVCAR-shControl, MOVCAR-shNCAM and MOVCAR-shNCAM/reconstituted cells (upper panel), confirming the silencing of NCAM expression by shNCAM and its reconstitution upon transfection with human NCAM. Equal loading was verified by immunoblotting for tubulin (lower panel). (C) Cells were incubated in serum-free medium for the indicated time intervals. A set of MOVCAR-shControl cells were also stimulated with 10 ng/ml FGF-2 as indicated. At each time point, cells were fixed and stained with crystal violet. Stained cells were then solubilized with 10% acetic acid, and the absorbance at 595 nm was measured. Values are expressed as arbitrary units referred to the absorbance of MOVCAR-shControl cells at time 0. Error bars represent SEM. *$p<0.05$; **$p<0.005$.

A



B

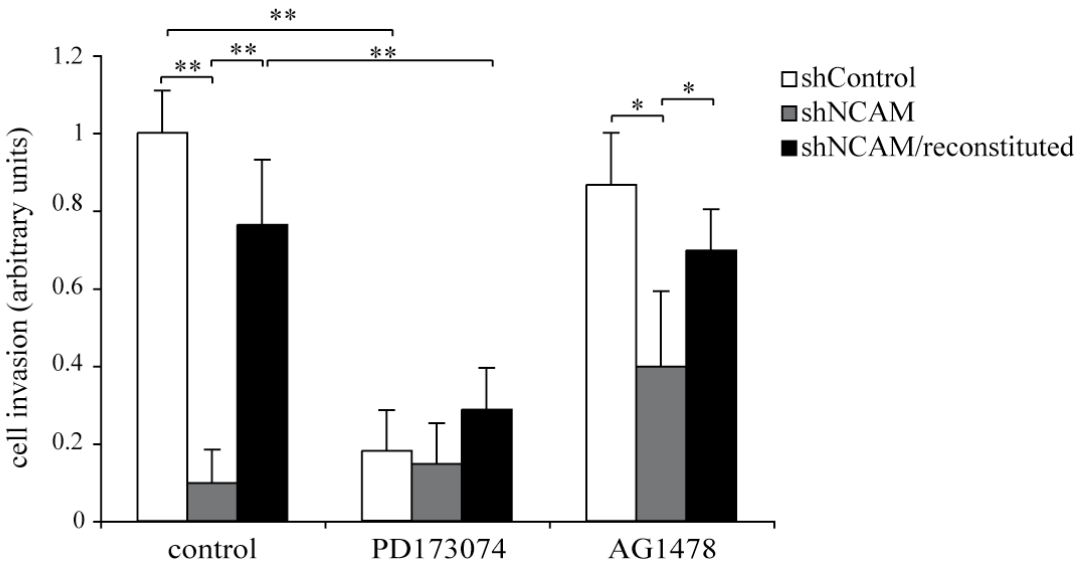


Fig. 5 A and B.

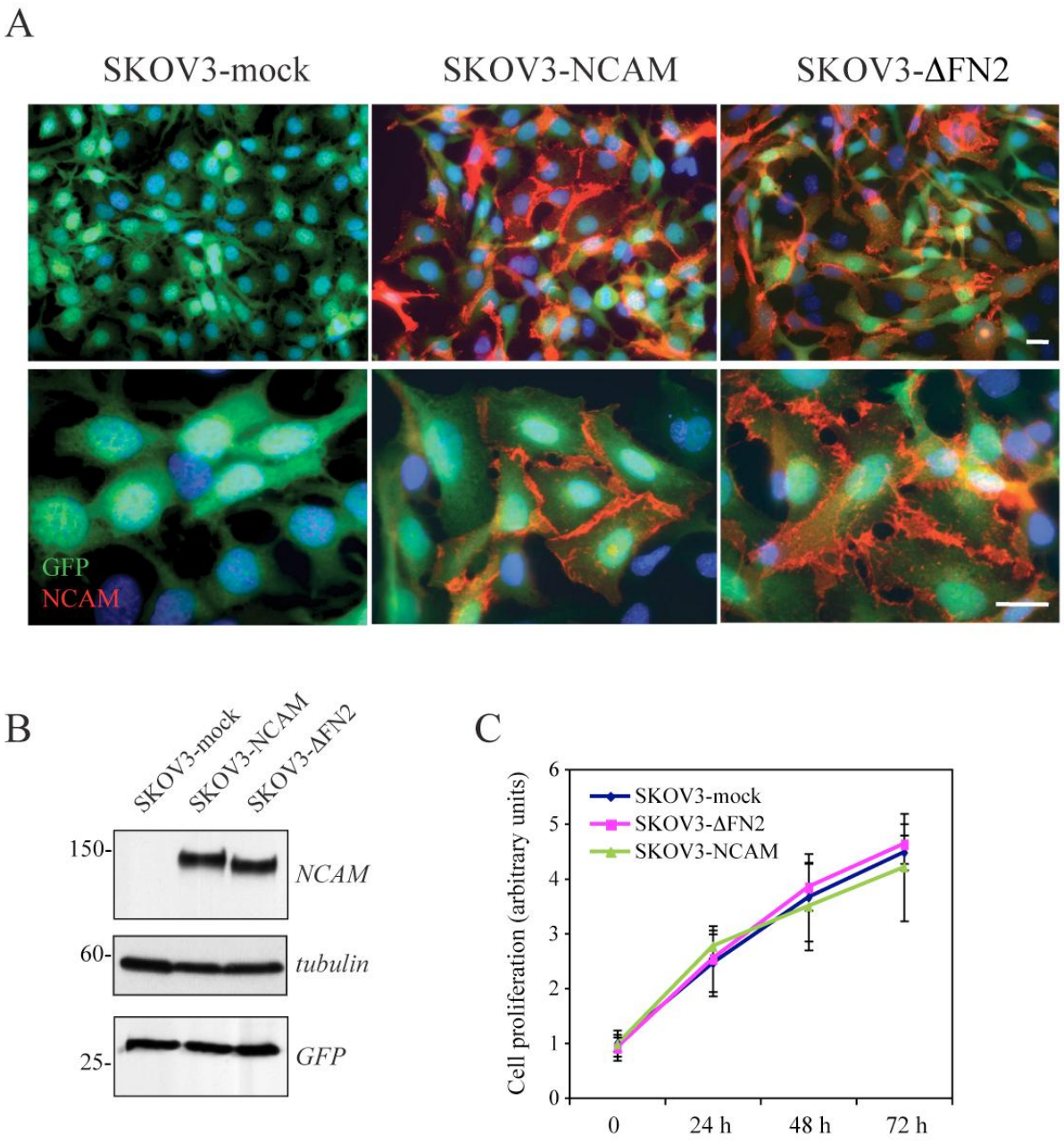


Fig. S2. Ectopic expression of NCAM and ΔFN2 in SKOV3 cells. SKOV3 cells were co-transfected with GFP (green) and with an empty vector, full-length NCAM or NCAM-ΔFN2. (A) Immunofluorescence staining for NCAM (red), showing that both full-length NCAM and ΔFN2 were correctly localized at the cell surface. Cells were counterstained with DAPI to visualize nuclei (blue). Scale bars, 10 µm. (B) Immunoblotting for NCAM on lysates from SKOV3-mock, SKOV3-NCAM and SKOV3-ΔFN2 cells (upper panel), showing that full-length NCAM and ΔFN2 were expressed at similar level. The amount of ectopically expressed GFP was also comparable in all transfectants (lower panel). Equal loading was verified by immunoblotting for tubulin (middle panel). (C) SKOV3-mock, SKOV3-NCAM and SKOV3-ΔFN2 cells were seeded at a density of 1 x 10^4 cells per well in 24-well plates, and and subjected to serum starvation. Cells were then stimulated with medium containing 10% FBS for the indicated time lengths, followed by fixation and staining with crystal violet. Stained cells were then solubilized with 10% acetic acid, and the absorbance at 595 nm was measured. Values are expressed as arbitrary units referred to the absorbance of SKOV3-mock cells at time 0. Error bars represent SEM.

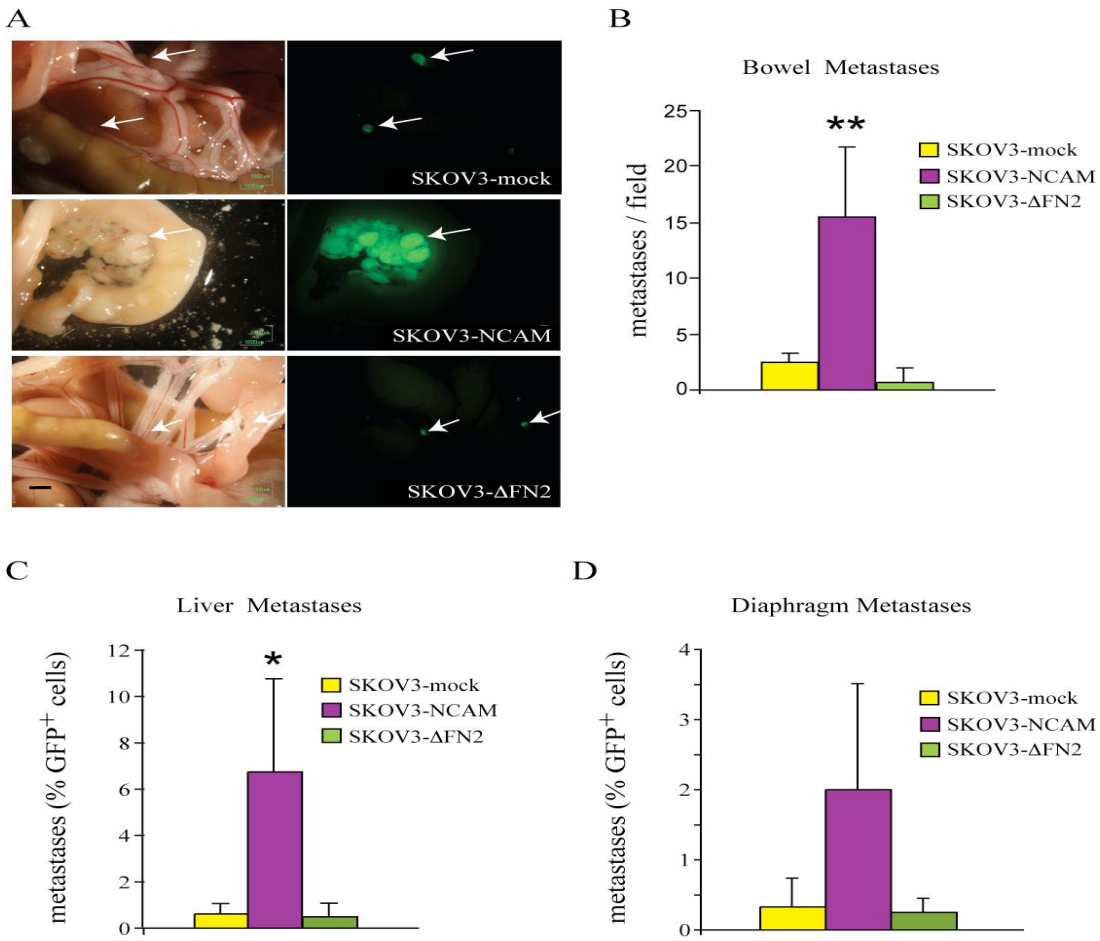

A
SKOV3-mock
SKOV3-NCAM
SKOV3-ΔFN2
B
Bowel Metastases
metastases / field
25
20
15
10
5
0
**
SKOV3-mock
SKOV3-NCAM
SKOV3-ΔFN2
C
Liver Metastases
metastases (% GFP+ cells)
12
10
8
6
4
2
0
*
SKOV3-mock
SKOV3-NCAM
SKOV3-ΔFN2
D
Diaphragm Metastases
metastases (% GFP+ cells)
4
3
2
1
0
SKOV3-mock
SKOV3-NCAM
SKOV3-ΔFN2

Fig 6 A-B-C-D.

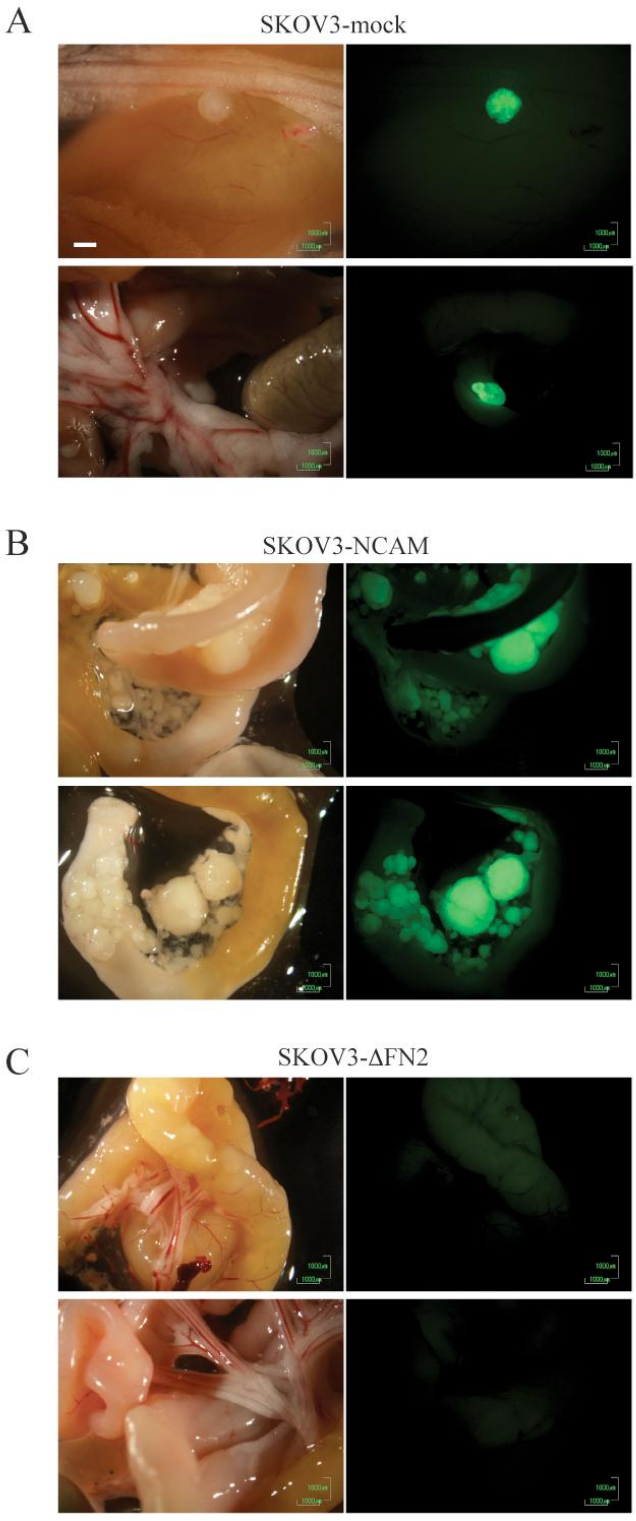


Fig. S6. NCAM promotes peritoneal dissemination of EOC through its FGFR-binding domain. SKOV3 cells co-transfected with GFP and with an empty vector (mock; panels A), full-length NCAM (B) or NCAM-ΔFN2 (C) were injected into the peritoneum of nude mice. Tumor dissemination to the bowel was then assessed as described in Material and Methods. Light (left panels) and fluorescence images (right panels) of the same fields are shown. Scale bar, 1 mm.

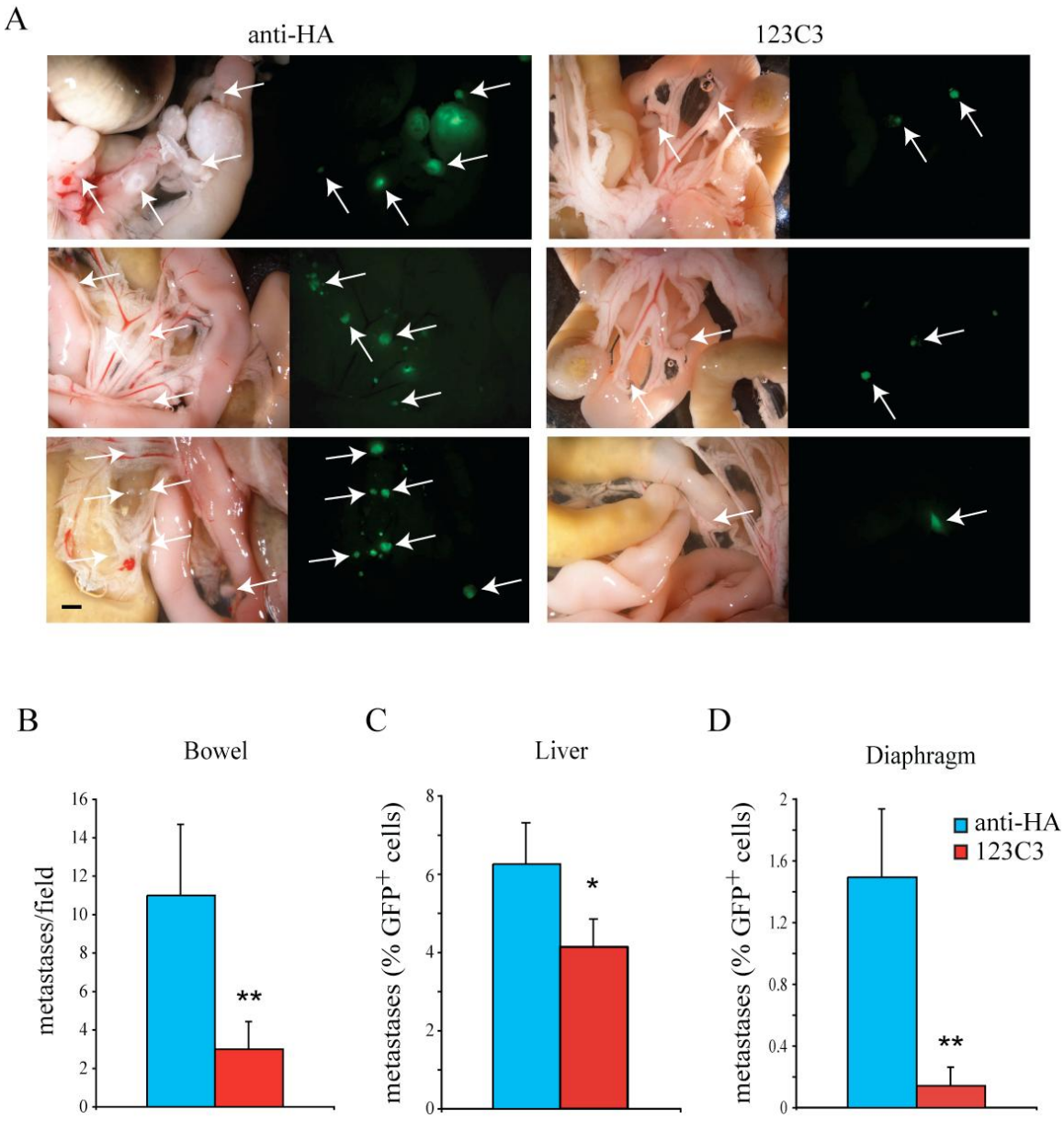

A
anti-HA
123C3
B
Bowel
metastases/field
16
14
12
10
8
6
4
2
0
**
C
Liver
metastases (% GFP+ cells)
8
6
4
2
0
*
D
Diaphragm
metastases (% GFP+ cells)
2
1.6
1.2
0.8
0.4
0
**
anti-HA
123C3

Fig. 7 A-B-C-D.

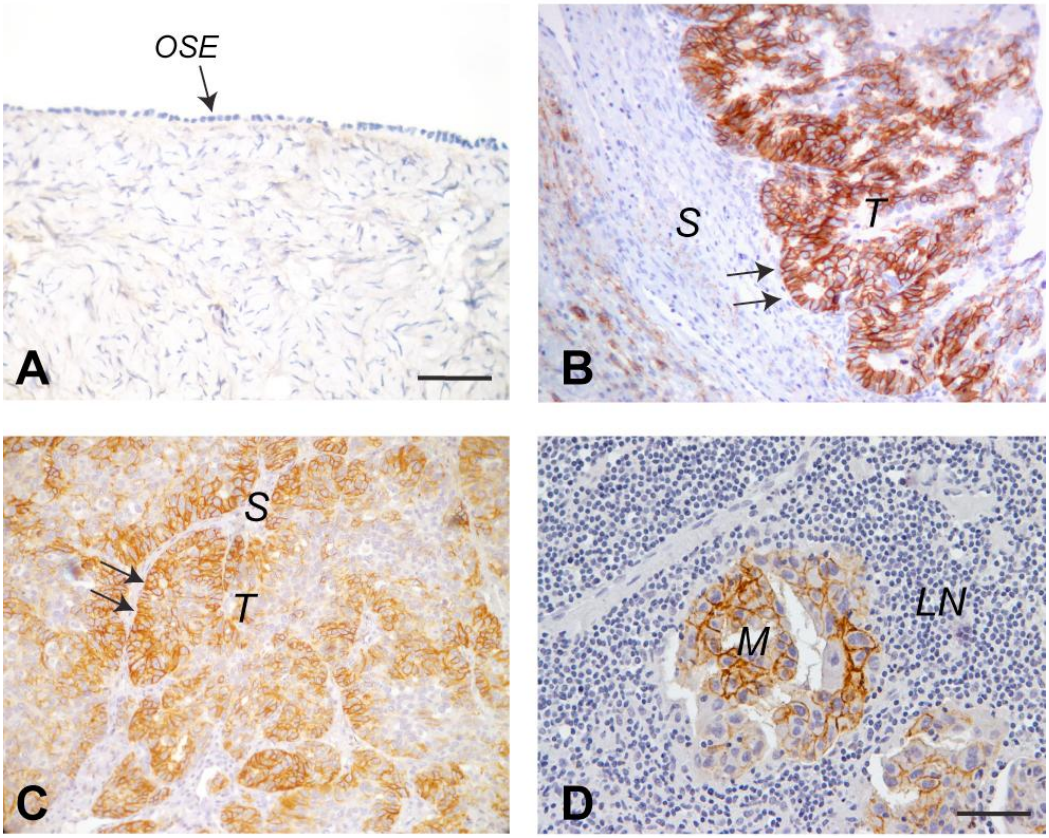


Fig. 8 A-B-C-D

Table 1.
Tumor features in 276 operative findings.

	N° patients	%
Normal Ovarian Epitelium	20	7.2
Cystadenoma	4	1.4
Epithelial Ovarian Carcinoma	252	91.3
Hystotype		
Serous	169	61.2
Endometrioid	43	15.5
Mixed	25	9.0
Clear cell	8	2.8
Mucinous	5	1.8
Transitional	2	0.7
Stage		
IA	9	3.5
IB	3	1.2
IC	9	3.5
IIA	4	1.5
IIB	7	2.7
IIC	6	2.3
IIIA	1	0.4
IIIB	8	3.1
IIIC	165	65.7
IV	39	15.4
Grade		
1	11	4.47
2	67	27.2
3	168	68.2

Table 2.
Cinicopathological features and NCAM expression in EOC patients.

	Ovarian Tissues		
	NCAM – positive	**NCAM – negative**	***P*-value**
Normal OSE	0 (0%)	20 (100%)	
Cysteadenoma	0 (0%)	4 (100%)	0.0003*
EOC	60 (23.8%)	192 (76.2%)	(OSEvs.EOC)
Metastases	71 (34.5%)	135 (65.5 %)	
Hystotype			
Serous	45 (26.6%)	124 (73.4%)	
Endometrioid	8 (18.6 %)	35 (81.4%)	0.025*
Mixed	7 (28%)	18 (72 %)	(Serous+Endometrioid+
Clear cell	0 (0%)	8 (100%)	Mixed vs Others)
Mucinous	0 (0%)	5 (100%)	
Transitional	0 (0%)	1 (100%)	
Grade			
G1	0 (0%)	12 (100%)	
G2	19 (27.9%)	49 (72.1 %)	0.003*
G3	41 (24.4 %)	127 (75.6%)	(G1 vs. G2/G3)
FIGO Stage			
Low (I-II)	6 (16.2%)	31 (31%)	N.S.[#]
High (III- IV)	54 (25.1%)	161 (74.9%)	

[#] Pearson's Chi Square test
*Fisher's exact test

Table 3.
Correlation between intrabdominal dissemination in stage IIIC–IV and NCAM expression.

	NCAM positive	NCAM negative
Stage		
III A/B/C	44 (26.6%)	121 (73.3%)
NS*		
IV	9 (23.6%)	29 (76.3%)
(0.70)		
Omentum		
Negative	4(26.6%)	11(73.3%)
Nodules	30(26.3%)	84(73.6%)
NS*		
Cake	19(26.0%)	54(73.9%)
(0.99)		
Mesentery		
Negative	20(22.7%)	68(77.2%)
NS*		
Positive	33(28.9%)	81(71.0%)
(0.31)		
Diaphragm		
Negative	19 (27.5%)	50 (72.4%)
NS*		
Positive	99 (74.4%)	34 (25.5%)
(0.76)		
Carcinomatosis		
Negative	16(22.2%)	56(77.7%)
NS*		
Positive	37(28.4%)	93(71.5%)
(0.33)		