ORIGINAL PAPER

Enhanced chemoresistance and tumor sphere formation as a laboratory model for peritoneal micrometastasis in epithelial ovarian cancer

OLGA SORIŢĂU[1)], C. I. TOMULEASA[1)], EMÖKE PÁLL[1)], PIROSKA VIRÁG[1)], EVA FISCHER-FODOR[1)], V. FORIS[1)], OTILIA BARBOS[1)], CORINA TATOMIR[1)], G. KACSÓ[2)], A. IRIMIE[3)]

[1)]*Laboratory of Cell Cultures*
[2)]*Department of Brachytherapy*
[3)]*Department of Surgical Oncology*
"Ion Chiricuţă" Comprehensive Cancer Center, Cluj-Napoca

Abstract

Background and Purpose: Ovarian cancers are composed of heterogeneous cell populations, including highly proliferative immature precursors and differentiated cells that may belong to different lineages. The main reason why epithelial ovarian cancer is difficult to treat is the unusual mechanism of dissemination that involves local invasion of pelvic and abdominal organs. But, unlike many other carcinomas, initial dissemination rarely requires blood or lymph vessels. Because it has been proven that aggregates of malignant cells within the ascites of patients diagnosed with ovarian cancer represent an impediment to cure such cancers, in the present study we adopted suspension culture combined with anti-cancer regimens as a laboratory strategy for research of the initial process of peritoneal micrometastasis. *Experimental Design*: MLS human ovarian cancer cells were cultured in serum-free medium. Cells of passage eight were treated in combination with the anticancer agent doxorubicin at different peak plasma concentrations for 24 hours, and then maintained under suspension culture. The acquired increased aggressiveness properties was confirmed by multidrug resistance assays and by their ability to grow in an anchorage-independent manner *in vitro* as tumor spheroids. *Results*: Cells selected after chemotherapy had a increased proliferative potential, eliminated Rhodamine 123 in culture and also formed spheroids in suspension. *Conclusions*: Here we present direct evidence that the metastasis of human ovarian cancer may be a result of transformation and dysfunction of immature precursor cells in the ovary. Also, spheroid formation may represent a key component of chemotherapy recurrence and a better understanding of these 3D structures can contribute to the development of new treatments for metastatic carcinoma.

***Keywords*:** ovarian cancer, spheroid formation, resistance to chemotherapy, micrometastasis.

Introduction

Epithelial ovarian cancer is the eighth most common cancer among women and causes more deaths than any other female reproductive tract cancer, with approx. 25 000 new cases diagnosed and 16 000 deaths per year in the EU alone [1]. The average lifetime risk is about one in 70, with a strong family history of ovarian or breast cancer being the most important risk factor [2].

Patients may report abdominal fullness, dyspepsia, early satiety or bloating because of increased abdominal pressure due to ascites or involvement of the omentum [3]. Other physical examination findings include ascites, pleural affusions or the Sister's Mary Joseph nodule [4]. Paraneoplastic signs include hormonally mediated hypercalcemia, subacute cerebellar degeneration, the Leser–Trélat sign, migratory superficial thrombophlebitis, palmar fasciitis, dermatomyositis or even polyarthritis [5–9].

Ovarian epithelial neoplasia arises from the coelomic epithelium covering the ovarian surface or from inclusion cysts, but recent evidence has shown that ovarian cancer may also originate from cells other than the surface epithelia. The pathology subtypes are classified based on cell morphology into serous, mucinous, clear cell or endometrioid. These diverse histological types can be explained by the hypothesis that ovarian cancer develops from remains of the Müllerian duct structures [10–13], but another idea is that of ovarian surface epithelial cell metaplasia through dedifferentiation to adopt the various histology characteristics during transformation [14].

Ovarian surface epithelial cells are histologically organized as a single cell layer by a sheet of basement membrane and it is believed that the contact of ovarian surface cells with this membrane regulates cell growth and differentiation. In ovarian cancer, tumor cells often lose not only their ability to synthesize collagen IV and laminin, key components of the basement membrane, but also the apical-basolateral polarity [15]. As the cell-cell adhesion molecules are remodeled, the newly formed spheroids disaggregate on the mesothelium of the peritoneum. This is when the first step of metastasis occurs.

Material and Methods

Cell lines

MLS ovarian tumor cell line was kindly gifted by

Dr Yael Schiffenbauer, from the Drug Hypersensitivity and Tissue Typing Laboratory, Rabin Medical Center, Beilinson Campus, Tel-Aviv, Israel. As control groups for the Rhodamine 123 efflux assay, we used both the HFL human lung fibroblast cell line (European Collection of Cell Cultures, Budapest, Hungary) and human liver cancer stem cells, isolated from a hepatocellular carcinoma by our research team [16]. Cells were grown as monolayers in Dulbecco's modified Eagles Medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, non-essential amino acids, 100 U/mL penicillin and 100 µg/mL streptomycin (all from Sigma Aldrich, St. Louis, USA). For all lines, the medium was replaced every day with fresh one and the flasks were kept in a 37^0C incubator with 93% air and 7% CO_2.

In vitro propagation in serum-free culture medium

Every two passages, the FCS concentration was dropped by 2.5%. The cell morphology started to change by forming small tumor spheres, apparently suggesting a epithelial to mesenchymal transition. Cells were cultured in DMEM/F12 containing 20 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor (EGF), 10 ng/mL insulin growth factor (IGF) (all from Sigma Aldrich) and 2% B27 (Invitrogen, Carlsbad, USA) at a density of 1000 cells/mL. Cells and spheres were passages by enzymatic dissociation with trypsin to single cells through a gauge 21 needle of a 5 mL syringe every three days and then reseeded.

Anticancer regimens and Darwinian selection of tumor cells

Cells at passage 10 were plated in complete serum-free medium into 24-well plates for three days until formation of tumor spheres. Then doxorubicin was administered at 3 µg/mL and 5 µg/mL concentrations. Each concentration was repeated thrice. After incubation for 24 hours, cells were centrifuged, mechanically dissociated into single cells and recultured for seven days in fresh serum-free medium, in accordance to Li HZ *et al.* [17].

Tumor sphere formation as an *in vitro* model for *in vivo* peritoneal metastasis

Tumor cells that have survived the anticancer regimens screening assay were plated at a density of 10^5 cell/mL in serum-free culture media, as described by Casey RC *et al.* [18]. Because in ovarian carcinoma cells detach from the surface of the tumor into the peritoneal cavity, the unvascularized 3-D multicellular spheroids formed by culture in serum-deprivation culture represent an *in vitro* model of peritoneal micrometastasis, whose adhesive abilities have yet to be elucidated. Spheres were observed under an Olympus CKX 41 inverted light microscope, at 100× and 200× amplification.

Drug resistance assays

Highly proliferative ovarian cancer cells, obtained after chemotherapy selection, were compared with MLS ovarian tumor cells. Exponentially growing cells were cultured in 96-well plates before adding carboplatin and doxorubicin. After 24 and 48 hours, the relative cell number was determined by standard MTT assay.

Post-therapy ovarian cancer cells, hepatic cancer stem cells, MLS ovarian tumor cells and HFL human lung fibroblasts were seeded in specific culture media supplemented with FCS, non-essential amino acids, L-glutamine and antibiotics, at 20×10^4 cells/mL. Cells were stained with 10 µM Rhodamine 123 and then incubated for three hours at 37^0C and 7% CO_2. After culture, all cell types were washed three times with PBS before intracellular fluorescence studies, according to Donnenberg VS *et al.* protocols [19].

Statistical analysis

Statistical analysis was done using Prism 5.0 statistics program for Windows (GraphPad, San Diego, USA). Data were analyzed using one-way ANOVA with the Bonferroni multiple comparison test (Kruskal–Wallis as non-parametric). Statistical significance was set at $p<0.05$ and all experiments were performed in triplicate.

Results

MLS cell growth in serum-free culture media and enrichment of the subpopulation of putative ovarian proliferative cancer cells

The goal of this experiment was to test whether MLS ovarian tumor cells had the capability to divide in a serum-free culture system. After the FCS concentration was dropped systematically, cells had begun to show morphologic changes consistent with epithelial-to-mesenchymal transition (Figure 1).

Primary tumor spheres were initially observed after seven days of culture in complete serum-free media (Figure 2). Spheres were then disaggregated into a single cell suspension and serially passaged at clonal density of 1000 cells/mL. Secondary tumor spheres appeared on the second day of culture.

Ovarian proliferative cancer cell sorting by suspension culture combined with Darwinian selection after chemotherapy

Cells at the eighth passage under serum-free suspension culture were incubated in combination with different concentrations of doxorubicin (3 µg/mL and 5 µg/mL) for 24 hours. The cells were afterwards washed thoroughly with PBS to remove all of the doxorubicin from the flask and maintained in suspension culture by gently agitating aggregates as single cells. By centrifugation to exclude all dead cells and debris, fresh culture media were replaced every two days. After six days of culture, cell viability was tested using the standard protocol of Trypan Blue staining. Almost all cells had underwent apoptosis and only 2–5% of cells were viable.

Acquired resistance to conventional chemotherapy

Our research team assessed the expression of

ABCG2, encoding a membrane efflux transporter expressed in hematopoietic stem cells and also associated with chemotherapy resistance. The cellular transport proteins of the ABC (ATP Binding Cassette) superfamily are active in the first step of the action of toxic substances, at the stage of drug penetration through the cell membrane and its intracellular accumulation. Because Rhodamine 123 uses the same pathways to pass through the membrane as conventional drugs used in oncologic treatments, by measuring the optical density (OD) of this fluorescent substance, we were able to determine indirectly whether the cell population isolated from the MSL cell line expressed the proteins responsible for multidrug resistance. Ovarian proliferative tumor cells were compared with hepatic cancer stem cells, HFL human fibroblasts and to MLS ovarian tumor cells (Figure 3).

To examine whether the isolated cells possessed a hypothesized chemoresistant phenotype, we also assessed the sensitivity of the cells to carboplatin and doxorubicin cultured under the same conditions. Compared with HFL fibroblasts and MLS tumor ovarian cells, both carboplatin and doxorubicin IC_{50} values were greater ($p<0.05$) (Figure 4).

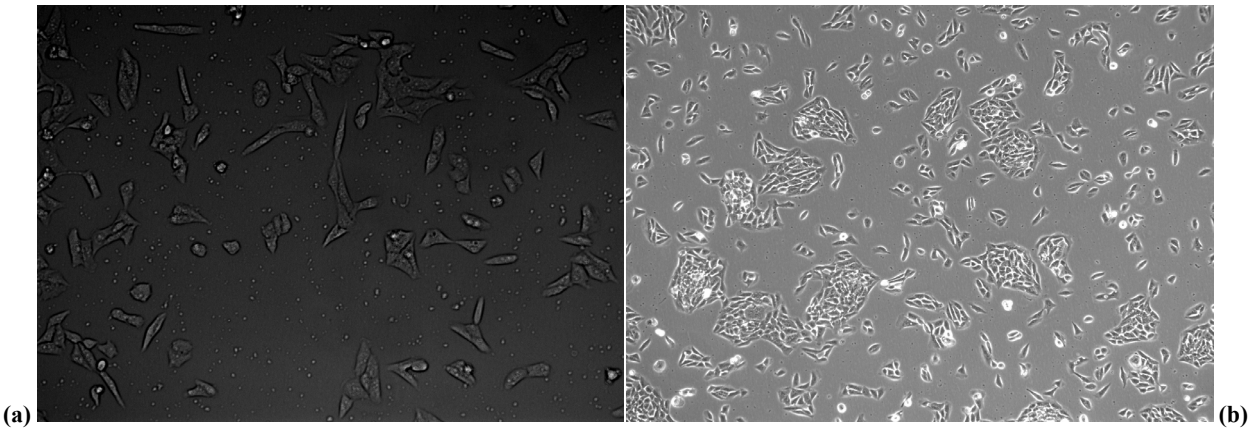

Figure 1 – *(a) MLS cells cultured in classic FCS medium. After the serum concentration was dropped, ovarian tumor cells changes, developing into spindle-shaped cells with loss of polarity and an increased intercellular separation (b).*

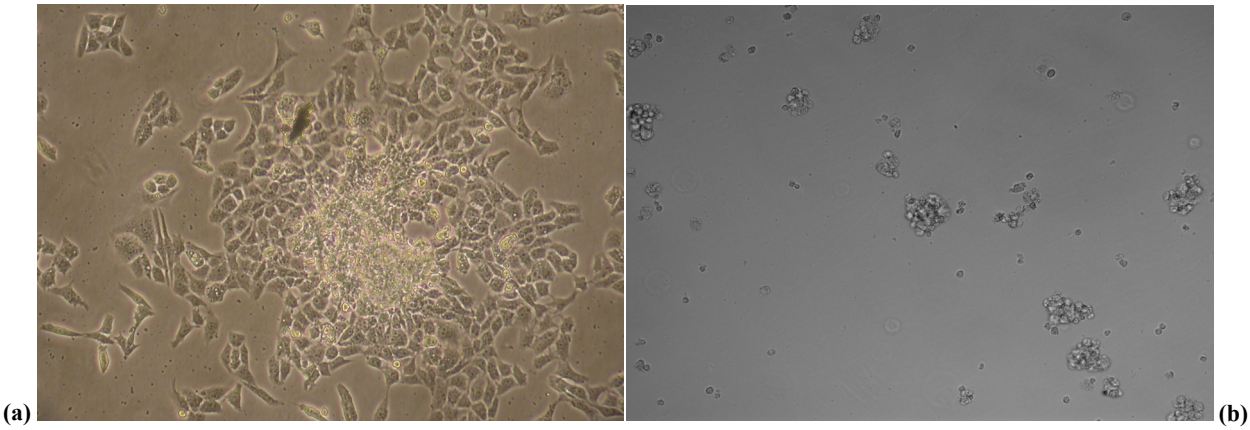

Figure 2 – *After gathering in small groups, cells had adopted an early 3D conformation (a). This structure later on started to form small tumor spheres (b).*

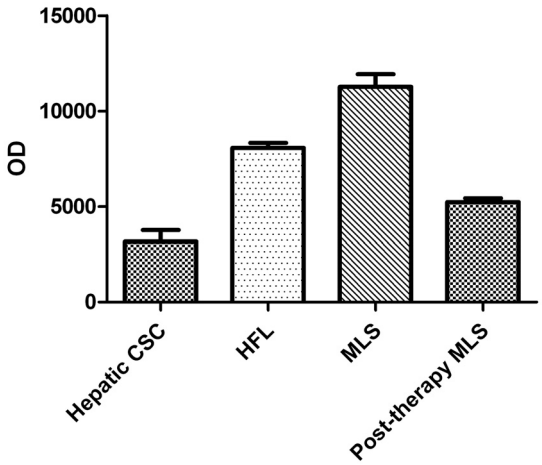

Figure 3 – *Direct dye efflux assay.*

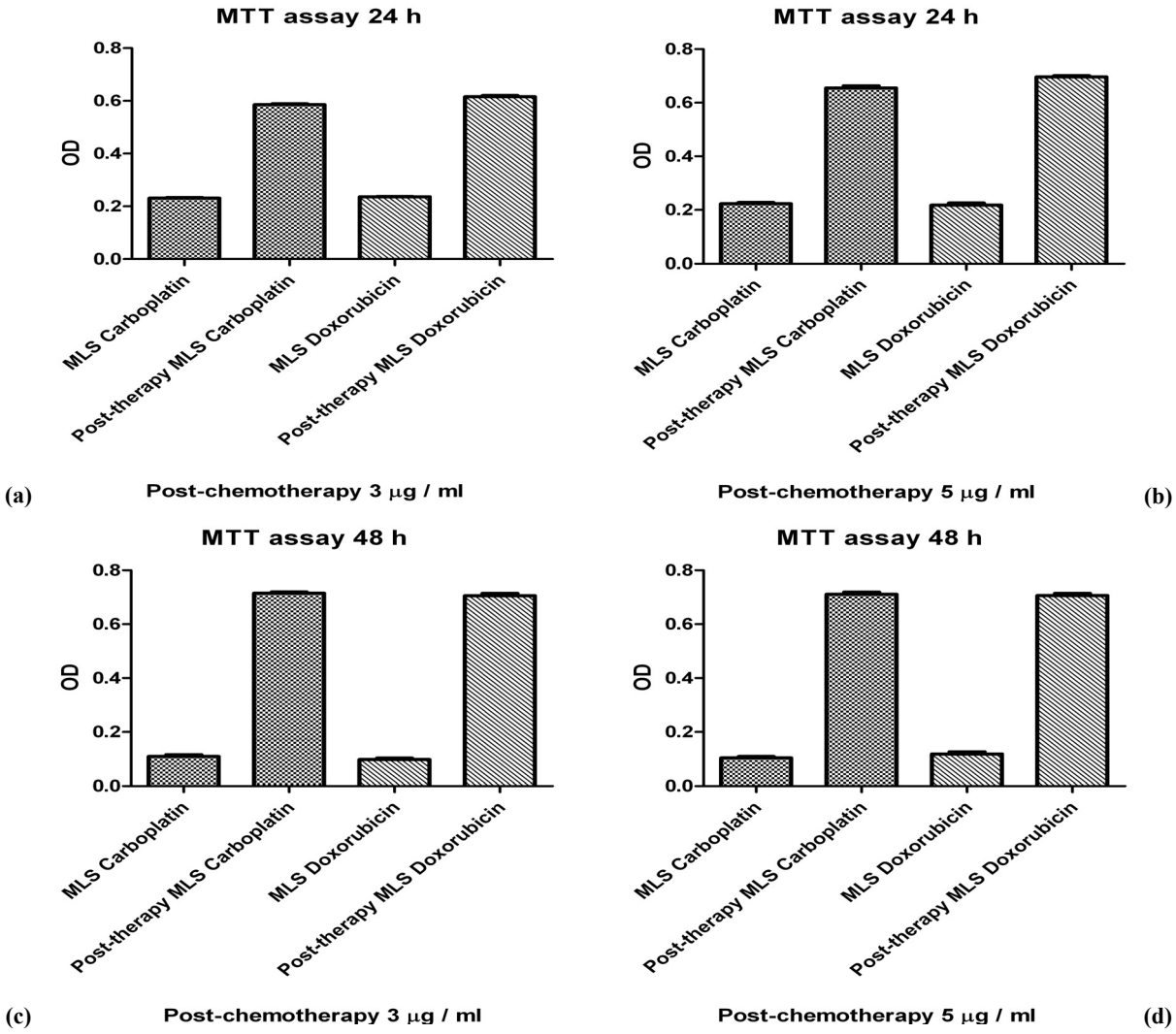

Figure 4 – ***Ovarian highly proliferative tumor cells were resistant to conventional chemotherapy. There was no significant difference between cells sorted by suspension culture with doxorubicin at 3 μg/mL (a, b) and cell sorted with chemotherapy drugs at 5 μg/mL (c, d).***

Discussion

The rate of mortality in ovarian cancer has changed little in the past three decades due to drug resistance. Early detection is critical and many genes specifically overexpressed in the context of ovarian cancer provide potential biomarkers for diagnosis, genes such as CA 125, osteopontin, MUC1 and HE4 [20]. Although chemotherapeutics target rapidly proliferating tumor cells and provide temporary remission, only the bulk of tumor cells is destroyed and drug-resistant cells remain.

According to the incessant ovulation hypothesis, the increased cancer risk is due to genetic mutations that might occur during proliferation of the surface epithelium in the process of postovulatory repair [21]. In ovarian carcinogenesis, the epithelial surface or inclusion cysts will become multicell layered and form adenomas. Subsequently, the epithelial cells can invade the surrounding stroma or the tumor cells may detach from the primary cancer bulk and spread in the peritoneal cavity as ascites. These cells have lost their basal-apical polarity because of genetic and epigenetic alterations, allowing them to survive and proliferate, resisting the positioning rules imposed on normal epithelial cells [22].

Characteristically, initial epithelial ovarian cancer dissemination is intra-abdominal and involves local invasion of the pelvic or abdominal organs, rarely involving blood or lymph vessels. Malignant cells are shed from the primary tumor into the peritoneal cavity where they are disseminated throughout the abdominal cavity by peritoneal fluid or ascites. Cells are shed as spheroids and later on settle onto the surface of the peritoneum, where disaggregation and metastatic outgrowth may occur. This process requires the remodeling of cadherins as the spheroids disaggregate on the mesothelium of the peritoneum while integrins anchor the spheroid body to the sub-mesothelial extracellular matrix. In serum-free culture, our cells confirm this hypothesis and may be key areas of deficiency in current treatment of cancer. Tumor spheres are present in malignant ascites and have a reduced response to chemotherapeutic drugs both *in vivo* and *in vitro*, representing a significant impediment to efficient treatment of FIGO stage II and III ovarian cancer.

In clinical practice, chemotherapy alone may lead to massive cytoreduction but seldom cures the disease. The majority of patients who respond to primary chemotherapy will develop recurrent, usually drug-resistant disease. By acquiring a more aggressive phenotype, malignant ovarian tumor cells will re-express high levels of ABC drug transporters. The two ABC transporter-encoding genes that have been studied most extensively are ABCB1, which encodes P-glycoproteins, and ABCG2, a promiscuous transporter of both hydrophobic and hydrophilic compounds [23]. These proteins have important role in normal physiology in the transport of drugs across the placenta and in the retention of drugs in the intestinal lumen, but also are important components of the blood–brain and blood–testis barriers. By using the energy of ATP hydrolysis, these proteic components of the cell membrane actively efflux drugs from cells, serving to protect them from cytotoxic agents as also proven by our results. The ovarian tumor cells derived from the MLS line eliminate Rhodamine 123 from in the surrounding medium, results confirmed by international data, where scientists have proven that highly proliferative cells (both normal and malignant) actively eliminate Rhodamine 123 and Hoechst 33342 whereas most mature, differentiated cells accumulate these fluorescent dyes [24–28].

Conclusions

Despite advances in surgery and chemotherapy, most patients diagnosed with ovarian cancer relapse and become drug-resistant. Tumor spheroids are found in ascites, have migratory abilities and can establish metastatic lesions after intraperitoneal injection in NOD/SCID mice. Spheroids are a very likely source of recurrent disease as the vast majority of therapies are ineffective in preventing their growth and dissemination. Further characterization of these tumorigenic populations will allow oncologists to identify different markers and molecules and use them as targeted therapy. With these targets known, the small fraction of cells responsible for relapse and tumor progression will be eliminated. Consequently, defining the unique properties of ovarian tumor spheres remains one main priority for future development of early diagnosis and effective therapies.

Acknowledgements

We are grateful to the excellent assistance of Dr Ion Dan Postescu regarding statistical analysis and interpretation of the data.

References

[1] ***, *National Institute of Health Consensus Conference. Ovarian cancer. Screening, treatment and follow-up. NIH Consensus Development Panel on Ovarian Cancer*, JAMA, 1995, 273(6):491–497.

[2] VELCULESCU VE, *Defining the blueprint of the cancer genome*, Carcinogenesis, 2008, 29(6):1087–1091.

[3] MARKMAN M, *Antineoplastic agents in the management of ovarian cancer: current status and emerging therapeutic strategies*, Trends Pharmacol Sci, 2008, 29(10):515–519.

[4] ALBANO EA, KANTER J, *Images in clinical medicine. Sister Mary Joseph's nodule*, N Engl J Med, 2005, 352(18):1913.

[5] TSUNEMATSU R, SAITO T, IGUCHI H, TSUKAMOTO N, *Hypercalcemia due to parathyroid hormone-related protein produced by primary ovarian clear cell adenocarcinoma: case report*, Gynecol Oncol, 2000, 76(2):218–222.

[6] FURNEAUX HM, ROSENBLUM MK, DALMAU J, WONG E, WOODRUFF P, GRAUS F, POSNER JB, *Selective expression of Purkinje-cell antigens in tumour tissue from patients with paraneoplastic cerebellar degeneration*, N Engl J Med, 1990, 322(26):1844–1851.

[7] RAFFAEL GD, GRAVALLESE EM, SCHWAB P, JOSEPH JT, CANNISTRA SA, *Diagnostic dilemmas in oncology: Case 2. Dermatomyositis and ovarian cancer*, J Clin Oncol, 2001, 19(23):4341–4343.

[8] MEDSGER TA, DIXON JA, GARWOOD VF, *Palmar fasciitis and polyarthritis associated with ovarian carcinoma*, Ann Intern Med, 1982, 96(4):424–431.

[9] CANNISTRA SA, *Cancer of the ovary*, N Engl J Med, 2004, 351(24):2519–2529.

[10] AOKI Y, KAWADA N, TANAKA K, *Early form of ovarian cancer originating in inclusion cysts. A case report*, J Reprod Med, 2000, 45(2):159–161.

[11] FEELEY KM, WELLS M, *Precursor lesions of ovarian epithelial malignancy*, Histopathology, 2001, 38(2):87–95.

[12] BOYD J, *Whence epithelial malignant carcinoma?*, Gynecol Oncol, 2008, 109(2):161–163.

[13] DUBEAU L, *The cell of origin of epithelial tumors and the ovarian surface epithelium dogma: does the emperor have no clothes?*, Gynecol Oncol, 1999, 72(3):437–442.

[14] HANAHAN D, WEINBERG RA, *The hallmarks of cancer*, Cell, 2000, 100(1):57–70.

[15] SEIDMAN JD, RUSSEL P, KURMAN RJ, Surface epithelial tumors of the ovary. In: KURMAN RJ (ed), *Blaustein's pathology of the female genital tract*, 5th edition, Springer Verlag, New York, 2002, 791–904.

[16] TOMULEASA C, SORITAU O, RUS CIUCA D, POP T, TODEA D, PINTEA B, MOSNEANU O, FORIS V, SUSMAN S, KACSO G, IRIMIE A, *Isolation and characterization of hepatic cancer cells with stem cell properties from hepatocellular carcinoma*, J Gastrointest Liver Dis, 2009, *in press*.

[17] LI HZ, YI TB, WU ZY, *Suspension culture combined with chemotherapeutic agents for sorting of breast cancer stem cells*, BMC Cancer, 2008, 8:135–142.

[18] CASEY RC, BURLESON KM, SKUBITZ KM, PAMBUCCIAN SE, OEGEMA TR JR, RUFF LE, SKUBITZ APN, *β 1-integrins regulate the formation and adhesion of ovarian carcinoma multicellular spheroids*, Am J Pathol, 2001, 159(6):2071–2080.

[19] DONNENBERG VS, MEYER EM, DONNENBERG AD, Measurement of multiple drug resistance transporter activity in putative cancer stem/progenitor cells. In: YU JS (ed), *Cancer stem cells. Methods and protocols*, Springer, Los Angeles, 2009, 261–281.

[20] BAST RC JR, BADGWELL D, LU Z, MARQUEZ R, ROSEN D, LIU J, BAGGERLY KA, ATKINSON EN, SKATES S, ZHANG Z, LOKSHIN A, MENON U, JACOBS I, LU K, *New tumor markers: CA 125 and beyond*, Int J Gynecol Cancer, 2005, 15(Suppl 3):274–281.

[21] CAPO-CHICHI CD, SMITH ER, YANG DH, ROLAND IH, VANDERVEER L, COHEN C, HAMILTON TC, GODWIN AK, XU XX, *Dynamic alterations of the extracellular environment of ovarian surface epithelial cells in premalignant transformation, tumorigenicity, and metastasis*, Cancer, 2002, 95(8):1802–1815.

[22] CVETKOVIĆ D, *Early events in ovarian oncogenesis*, Reprod Biol Endocrinol, 2003, 1:68.

[23] DEAN M, RZHETSKY A, ALLIKMETS R, *The human ATP-binding cassette (ABC) transporter superfamily*, Genome Res, 2001, 11(7):1156–1166.

[24] SUMMER R, KOTTON DN, SUN X, MA B, FITZSIMMONS K, FINE A, *Side population cells and Bcrp1 expression in lung*, Am J Physiol Lung Cell Mol Physiol, 2003, 285(1):L97–194.

[25] ZHOU S, SCHUETZ JD, BUNTING KD, COLAPIETRO AM, SAMPATH J, MORRIS JJ, LAGUTINA I, GROSVELD GC, OSAWA M, NAKAUCHI H, SORRENTINO BP, *The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype*, Nat Med, 2001, 7(9):1028–1034.

[26] STAVROVSKAYA AA, STROMSKAYA TP, *Transport proteins of the ABC family and multidrug resistance of tumor cells*, Biochemistry (Moscow), 2008, 73(5):592–604.

[27] HIRSCHMANN-JAX C, FOSTER AE, WULF GG, NUCHTERN JG, JAX TW, GOBEL U, GOODELL MA, BRENNER MK, *A distinct "side population" of cells with high drug efflux capacity in human tumor cells*, Proc Natl Acad Sci U S A, 2004, 101(39):14228–14233.

[28] KONDO T, SETOGUCHI T, TAGA T, *Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line*, Proc Natl Acad Sci U S A, 2004, 101(3):781–786.

Corresponding author

Ciprian I. Tomuleasa, Laboratory of Cell Cultures, "Ion Chiricuţă" Comprehensive Cancer Center, 34–36 Republicii Street, 400015 Cluj-Napoca, Romania; Phone: +40741–337 480, Fax +40233–235 249, e-mail: ciprian.tomuleasa@gmail.com

Received: October 12th, 2009

Accepted: March 10th, 2010