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EPITHELIAL PHENOTYPE OF CULTURED OVARIAN TUMOR CELLS

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ABSTRACT

Epithelial differentiation is an early or predisposing step in epithelial ovarian carcinogenesis which occurs in pre-neoplastic lesions, benign tumors and normal ovarian surface epithelium (OSE) of women with a familial history of ovarian cancer. During neoplastic progression, OSE acquires a more epithelial aspect, including the expression of CA125 protein and other epithelial markers, whereas mesenchymal characteristics diminish. In this study, we investigated 26 primary cell cultures, including benign and malignant OSE neoplasms, obtained from women who underwent surgical removal of the ovaries at the university hospital of the State University of Campinas (Campinas, SP, Brazil). Cell morphology was assessed from the time of cell adhesion to the substrate up to the third or fourth passage. CA125 was detected by immunohistochemistry at each passage. Serum CA125 levels were obtained from clinical records and heredograms were constructed using the information about the recurrence of familial cancer provided by the patients. Seventy-eight percent of the malignant OSE tumors analyzed showed an epithelial cell phenotype and 71% percent were positive for CA125. Benign and normal OSE cultures had a fibroblast-like cell phenotype, a negative CA125 expression and an inexpressive history of recurrent familial cancer, compared to malignant OSE tumors. We concluded that the expression of an epithelial phenotype *in vitro* may serve as an important tumor marker in malignant OSE neoplasms. In certain cases, this marker may be more reliable than the determination of serum CA125 levels. However, the relationship between the expression of the epithelial phenotype *in vitro* and a familial predisposition to tumor development remains to be determined.

Key words: CA125, hereditary ovarian cancer, immunohistochemistry, primary culture

INTRODUCTION

Despite the clinical importance of ovarian epithelial carcinomas, little is known about the early stages of their development [12,20]. Epithelial ovarian carcinomas are believed to arise in the ovarian surface epithelium (OSE) or in regions such as epithelial crypts and inclusion cysts. However, the ability of the OSE to give rise to ovarian neoplasms is still questionable [8].

The OSE is a simple mesothelium consisting of cube-shaped cells, derived from the embryonic coelomic epithelium. The OSE retains some mesenchymal features and can undergo epithelial-mesenchymal conversion [3]. *In vivo*, epithelial-mesenchymal conversion occurs immediately after ovulation, when OSE cells proliferate and migrate to heal the area wounded by oocyte expulsion. At this point, the cells change their phenotype to adquire a more elongated, fibroblast-like shape and express proteolytic enzymes which degrade the preexisting extracellular matrix and deposit new material to rebuild the damaged matrix [18].

In neoplastic progression, new epithelial features appear and become increasingly prominent and stable, whereas the mesenchymal characteristics of the OSE diminish [9]. During malignancy, OSE tumors acquire the morphological and functional properties of highly specialized epithelia of Müllerian duct origin, such as the Fallopian tube, endometrium and endocervix. Such properties include the gain of a columnar cell shape, the formation of papillae and glandular structures, and the expression of CA125 and other epithelial markers [3,20]. Many of these Müllerian characteristics are already present in metaplasias, thought to be weak pre-neoplastic lesions, and in benign tumors. Thus, epithelial ovarian carcinoma cells show a more complex differentiation than normal OSE cells, with

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aberrant epithelial differentiation being an early or predisposing step in epithelial ovarian carcinogenesis [3]. However, the mechanisms involved in this process are still unclear.

CA125 glycoprotein antigen is derived from the coelomic epithelium, and is expressed on the surface of many epithelial ovarian cancers, but not in normal OSE. CA125 antigen is, however, expressed in normal tissues derived from coelomic epithelium, such as, the Fallopian tube epithelium, endometrium, endocervix, pleura and peritoneum [17]. The structure of this molecule has not yet been elucidated and its biological function is still unknown. It has been suggested that the locus for CA125 is closely linked to the 17p locus of the BRCA1 gene, mutations of which predispose to breast and ovarian cancers [25]. CA125 is considered a good marker for monitoring ovarian cancer, but its diagnostic value is still unclear [4,19], mainly because serum CA125 levels may also be increased in several other conditions, such as early pregnancy [13], endometriosis [11,23], pelvic inflammatory disease, uterine fibroids, tuberculosis, liver cirrhosis, congestive heart failure and various malignant disorders, including endometrial, colonic and pancreatic cancers [5,6]. Since Jacobs and Bast [15] reported that serum CA125 levels are elevated in 90% of advanced-stage epithelial ovarian cancers, while less than 50% of patients with stage I disease have abnormal levels, the measurement of CA125 has become a valuable parameter for prognosis of such a disease. Indeed, 75%-90% of patients with clinically demonstrable ovarian cancers have elevated (>35 U/ml) serum CA125 levels [7,16].

In the present study, we investigated the expression of CA125 and the maintenance of an epithelial phenotype in primary cultures of benign and malignant OSE neoplasms. Heredograms were also constructed to analyze a possible correlation between OSE cell behavior *in vitro* and the occurrence of familial ovarian cancer.

MATERIAL AND METHODS

Cell culture

Institutional approval for experimentation with human tissues was obtained prior to this study. Samples of OSE were obtained from 20 women (aged 15 to 77 years, mean 52.4 years) who underwent surgical resection of the ovaries at the university hospital of the State University of Campinas (Campinas, SP, Brazil). The samples were washed in Hank's solution to remove blood, fat and necrosed tissue and then cut into small fragments which were treated with collagenase (type II, Sigma) at 37°C, with stirring, for 30 min [14]. After enzymatic dissociation, the cells were harvested by centrifugation, washed in PBS and cultured in Ham-F10 medium (Nutricell), supplemented with 20% fetal calf serum (Nutricell) and 10% amniomax supplement (Gibco), in 4-well culture dishes (Nunclon MultiDishes, NUNC) with Thermanox coverslips (NUNC). Primary cell cultures were incubated at 37°C in a humidified incubator with 5% CO_2. On the third day of culture, the medium was changed to promote selective epithelial cell adhesion to the substrate. When the culture were semi-confluent, usually after 4-5 days, the coverslips were removed and replaced by new ones. The cell phenotype was observed up to the third or fourth passage of the cultures. Coverslips from each passage were fixed in PBS-buffered 10% formalin for 20 min for subsequent immunohistochemistry.

Immunohistochemistry

Formalin-fixed cells were washed in PBS. Endogenous peroxidase was blocked using 10% hydrogen peroxide. The coverslips were washed in PBS and non-specific binding sites were blocked with fetal calf serum (FCS) for 30 min. After washing with PBS, the coverslips were incubated with the primary antibody (mouse monoclonal anti-human CA125, clone OC125, Dako, 1:20 dilution) in a moist chamber overnight at 4°C. The coverslips were subsequently washed with PBS, incubated with a secondary antibody (Multi-link Sw α goat, mouse and rabbit biotinylated immunoglobulin, Dako) for 1 h at 37°C and then washed again with PBS before the addition of streptavidin biotinylated horseradish peroxidase (Dako) for 40 min at 37°C. Following washing with PBS, the reactive sites were visualized by incubation with diaminobenzidine (Sigma) and hydrogen peroxide for 5 min at room temperature. The reaction was stopped by washing in distilled water. The coverslips were counterstained with Harris' hematoxylin for 15 s and then mounted on slides using glycerol gelatin (Merck).

Serum CA125 quantification

As a routine procedure, before surgery for removal of the ovaries, serum CA125 levels were determined by immunoenzymatic assay using the mouse monoclonal anti-human antibodies M11 and OC125 (Dako) for antigen capture and detection, respectively. The cut-off value considered was 35 U/ml and the Ca125 levels were obtained from the patients' clinical records.

Heredograms

After giving their consent, the patients were asked about the occurrence of cancer in their families, including the parental and two subsequent generations. The ages of the affected family members when cancer was diagnosed and the deaths caused by cancer were recorded. Heredograms were then constructed based on this information and classified as follows:

- *Class 1*: expressive familial history (at least two first-degree relatives with ovarian/breast/colon cancer);
- *Class 2*: expressive familial history of types of cancer other than exclusively ovarian/breast/colon cancer;
- *Class 3*: inexpressive familial history of cancer in general, no occurrence or only isolated cases of cancer in the family (first- or second-degree relatives without a convincing familial or hereditary basis for these neoplasms).

RESULTS AND DISCUSSION

Twenty-six primary cell cultures of OSE (three of them from bilateral ovary samples) were obtained from 20 patients. Histopathological analysis showed that the cultures were derived from neoplasms (12 malignant, 1 borderline and 7 benign tumor samples) and non-neoplastic OSE (6 samples of normal OSE).

The cultured cells showed two phenotypes, a predominant epithelial cell phenotype, corresponding to malignant OSE tumor cells (Table 1), and a fibroblast-like cell phenotype, which included benign OSE tumors, normal OSE cells and also a borderline tumor (Table 2). The epithelial phenotype of malignant OSE tumor cell cultures persisted up to the third or fourth passage (Table 1). Thereafter, these cultures showed a gradual shift to a more fibroblast-like phenotype (Fig. 1A,B). This observation agreed with previous studies on the behavior of cultured malignant neoplastic OSE cells [3,14]. However, this phenotype was maintained up to the third or fourth passage only, in contrast to the fifth passage mentioned in previous reports [3,14].

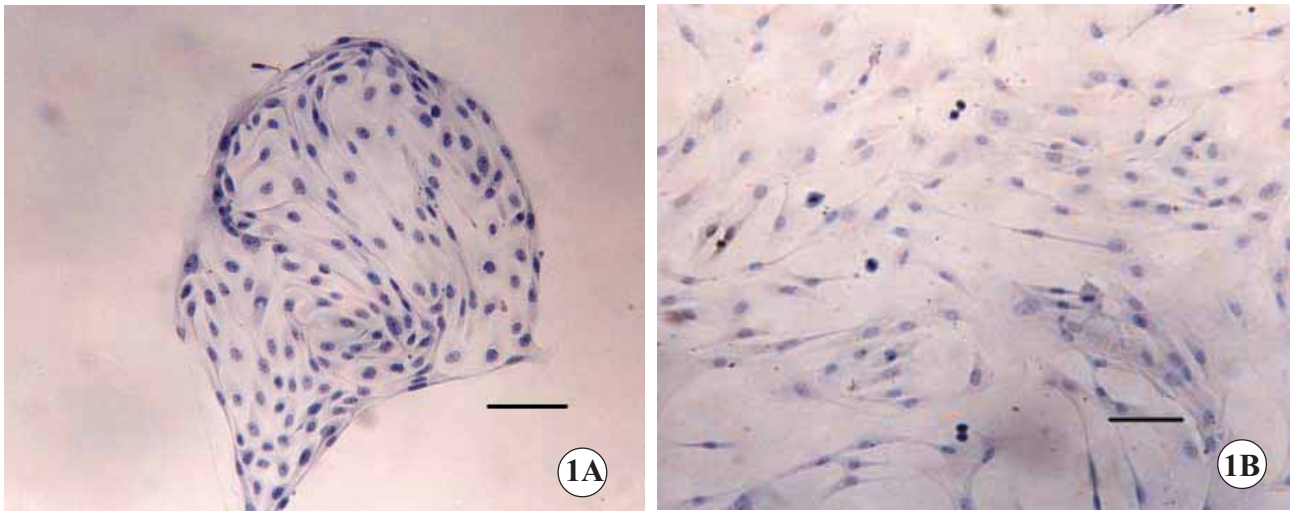

Figure 1A. Cystadenocarcinoma cells expressing the epithelial phenotype during the initial phase of adhesion to the substrate (CA125, avidin-biotin-peroxidase complex, negative control). **B.** Ovarian cystadenocarcinoma cells, after the third passage, showing loss of the epithelial phenotype and loss of CA125 positivity (avidin-biotin-peroxidase complex, Bars = 100 μm).

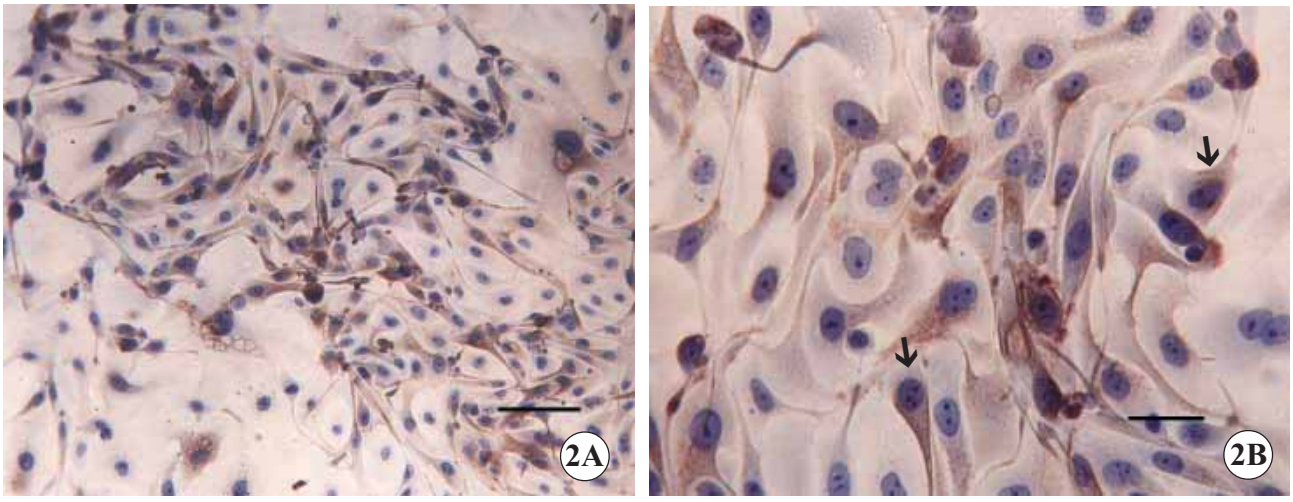

Figure 2A. Cystadenocarcinoma cells, after the first passage, showing the heterogeneity of the tumor in relation to the expression of CA125. Note the epithelial cells which are CA125 positive among cells with the same phenotype but which are negative for this marker (avidin-biotin-peroxidase complex). **B.** The same cystadenocarcinoma cell culture after the first passage, showing the polarity of CA125 expression (arrows) by cells with an epithelial phenotype (avidin-biotin-peroxidase complex, Bars = 100 μm and 40 μm).

Table 1. Phenotypes of cultures derived from malignant OSE neoplasms.

Case no. /Histological finding		Age at onset	Patient's history of neoplasms (age at onset)	Phenotype in culture passage (FB or EP)				Serum CA125 level (+ or -)	Heredogram class (1, 2 or 3)
				1st	2nd	3rd	4th		
1677	Small cell carcinoma	17	No	EP	FB			+	3
1690	Adenocarcinoma	38	No	EP				+	1
1744	Small cell carcinoma	51	No	EP				+	2
1740	Cystadenocarcinoma with borderline tumor areas	53	Breast (53)	FB	FB			–	2
1561-RO	Adenocarcinoma	56	No	EP	EP	EP	FB	–	1
1755	Serous cystadenocarcinoma	59	No	EP	FB			+	3
1637-RO	Serous cystadenocarcinoma	60	No	EP	EP	EP	FB	+	3
1637-LO	Serous cystadenocarcinoma			EP	EP	EP	FB	+	
1647	Serous adenocarcinoma	61	No	EP	FB	FB		+	2
1629	Mucinous cystadenocarcinoma	66	No	FB	FB			+	2
1564	Clear cell adenocarcinoma	67	No	EP	FB			+	1
1777	Clear cell carcinoma	72	Uterus (50)	EP	FB			+	2

EP = Epithelial cell phenotype; FB = Fibroblast-like phenotype; LO = Left ovary; RO = Right ovary;
+ = elevated serum CA125 level (>35 U/ml); – = normal serum CA125 level (≤35 U/ml)

Immunohistochemistry and immunoenzymatic analysis of CA125 showed that the phenotypic changes (from flat epithelial cell to fibroblast-like cells) in malignant OSE tumor cell cultures were concomitant with the loss in the CA125 expression. Malignant OSE tumor cells expressed CA125 up to the third or fourth passage in culture (Table 1). Immunoenzymatic assays showed that the tumor cell population was heterogeneous, with CA125-positive cells having clear polarized localization of this marker, compared to other cells of similar morphology but negative for the marker (Fig. 2A,B). The loss of CA125 expression after the third or fourth passages may reflect changes in the cell membrane with a consequent loss of marker epitopes.

Cases 1629 and 1740 were exceptions to the expected behavior of malignant OSE cells in culture (Table 1), but the lack of CA125 expression seen in case 1740 agreed with the presence of a fibroblast-like phenotype. These cultures were probably derived from samples composed of benign tumors and/or borderline areas of tumor samples. Borderline OSE tumors have histopathological characteristics and a biological behavior intermediate to those of malignant and benign tumors, and are defined as "semi-malignant tumors" or "carcinomas of low malignant potential (LMP)" [22,24]. In this study, case 1624, a mucinous borderline tumor, behaved like the benign tumor samples analyzed (Table 2), and had a fibroblast-like phenotype and negative CA125 expression.

The elevated serum CA125 level (95.0 U/ml) in case 1629 did not agree with the fibroblast-like phenotype seen in culture. An explanation for this could be that this culture may have been derived from

tumors with areas that were apparently poorly representative of malignant neoplasm [21]. This case stresses the need for careful sample selection and collection, in order to be truly representative of the tumor population.

Case 1561-RO (Table 1) had a flat epithelial cell phenotype and normal serum CA125 level. The latter result may be a reflection of the cut-off value (35 U/ml) used for the serum CA125 levels. A more effective cut-off of 20 U/ml has been suggested for the follow-up of patients treated for gynecological adenocarcinomas [1]. Because of such discrepancies, we suggest that the epithelial phenotype expression in culture may be more reliable than serum CA125 levels for discriminating malignant tumors smaller than 2 cm^2. The existence of two populations of epithelial cells, one positive and the other negative for CA125, seen in primary cultures of malignant tumor cells (Fig. 2A) could explain the negative serum levels of some malignant tumors, especially of small tumors.

All of the samples from benign and non-neoplastic OSE tumors, had a fibroblast-like phenotype, except for cases 1565 and 1652 (Table 2), which had a flat epithelial cell phenotype up to the second passage. Events like this have been correlated with the precocious manifestation of OSE characteristics in women with a familial history of ovarian cancer [2]. However, considering the histological findings and the natural immunoreactivity of the epithelium of Fallopian tubes with the antibody OC125, we concluded that the findings for case 1652 resulted from adherence of the salpinx to ovarian tissue [17].

Table 2. Phenotypes of cultures derived from normal and benign OSE neoplasms.

Case no. /Histological finding		Age at onset	Patient's history of neoplasms (age at onset)	Phenotype in culture passage (FB or EP)				Serum CA125 level (+ or -)	Heredogram class (1, 2 or 3)
				1st	2nd	3rd	4th		
1638	Cyst	15	No	FB	FB	FB		–	3
1573-RO	Cyst	40	---	FB	FB	FB		–	3
1573-LO	Cyst			FB	FB	FB			
1624	Mucinous borderline tumor	42	No	FB	FB			–	2
1646	Mucinous cystadenoma	43	No	FB	FB	FB		–	---
1652	Salpingitis adherence to ovarian tissue (non-neoplastic)	43	Vulva (42)	EP	FB			+	3
1565	Serous cystadenofibroma	50	No	EP	EP	FB		–	---
1561-LO	Cyst	56	No	FB	FB			–	1
1572	Mucinous cystadenoma	56	No	FB	FB	FB	FB	–	3
1648	Atrophic ovary (non-neoplastic)	61	No	FB	FB	FB		–	3
1497	Serous cystoadenoma	62	---	FB	FB	FB	FB	–	---
1613	Mucinous cystadenoma	68	No	FB	FB	FB	FB	–	---
1713	Mucinous cystadenoma	69	Uterus (51)	FB	FB			–	---
1664	Mucinous cystadenoma	77	No	FB	FB			–	---

EP = Epithelial cell phenotype; FB = Fibroblast-like phenotype; LO = Left ovary; RO = Right ovary;
+ = elevated serum CA125 level (>35 U/ml); - = normal serum CA125 level (≤35 U/ml); – = Non-available data

Table 3. Results for OSE cultures obtained from bilateral ovaries.

Case no. /Histological finding		Age at onset	Patient's history of neoplasms (age at onset)	Epithelial phenotype in culture (Yes or No)	Serum CA125 level (+ or -)	Heredogram class (1, 2 or 3)
1637-RO	Serous cystadenocarcinoma	60	No	Yes	+	3
1637-LO	Serous cystadenocarcinoma			Yes	+	
1561-RO	Adenocarcinoma	56	No	Yes	–	1
1561-LO	Cyst			No	–	
1573-RO	Cyst	40	No	No	–	3
1573-LO	Cyst			No	–	

LO = Left ovary; RO = Right ovary; + = elevated serum CA125 level (>35 U/ml); – = normal serum CA125 level (≤35 U/ml)

The different responses of malignant and non-malignant OSE were confirmed by the cell cultures from bilateral ovaries (Table 3) in which the epithelial phenotype was maintained solely in malignant tumors. These distinct morphologies may be used in the differential diagnosis of malignant and non-malignant tumors, independent of the minimum area of 2 cm^2 required for the determination of the serum CA125 level [10].

Müllerian differentiation is maintained in normal OSE of women with hereditary ovarian cancer. Studies *in vitro* have shown a high percentage of cells with a flat epithelial phenotype and the expression of CA125 after five passages in cultures. In contrast, OSE cells from women with no familial history of ovarian cancer were modulated in what is lost at the first passages of the culture [2,10]. In the present study, 13 heredograms were constructed for the OSE malignant tumor group, 10 of which showed a familial recurrence of cancer (Class 1 or 2). In the normal and benign OSE groups, ten heredograms were constructed and six of them showed no familial recurrence of cancer (Class 3).

Our results clearly showed that the phenotypic expression *in vitro* can be an important tumor marker in malignant OSE neoplasm, and may sometimes be more reliable than the assessment of CA125 expression. However, considering the possibility of false negatives with samples which are not representative of risk areas of the tumor, it may be more advisable to consider these two variables as being complementary to each other.

The epithelial phenotype seen *in vitro* appeared to be associated with a familial predisposition to neoplasms although more studies are required to confirm this hypothesis. In the present study, there was a tendency for epithelial phenotype to be expressed in cases of familial recurrence of ovarian and/or breast cancer, and in cases where other types of malignant neoplasms presented as part of the familial history of cancer. Possible deficiencies in the DNA repair process, leading to a high expression of the epithelial phenotype, and a reduction in the cellular response to growth *in vitro*, may represent some of the earliest changes in the process of ovarian carcinogenesis.

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