Malignant cells are not found in ovarian cortex from breast cancer patients undergoing ovarian cortex cryopreservation†

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Introduction

Breast cancer is the most common invasive tumour in western countries, accounting for 30% of all tumours and ~20% of all cancer-related deaths (Jemal et al., 2003). Approximately 25% occur before menopause and 15% before 45 years of age. Breast cancer incidence has increased by 0.5% every year during the last decade, but mortality has diminished by 1.4% per year during the same period, particularly in women under 50 years of age (Ghafoor et al., 2003). One of every 228 women will suffer from breast cancer before the age of 40. Delay in childbearing compounds the problem: many women who still have not fulfilled their reproductive desires have breast cancer and need treatment (Colleoni et al., 2002; Oktay et al., 2005; Sonmez and Oktay, 2006).

In 2005 our group started a fertility preservation programme consisting of extraction, cryopreservation and implantation of ovarian cortex in women at risk of developing premature ovarian failure (POF) after cancer treatment. To date, >200 women have participated in our Programme for Fertility Preservation, of whom 55% are breast cancer patients (Sanchez et al., 2008). Others have also recently reported breast cancer as the main indication for fertility preservation (Rosendahl et al., 2008).


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Ovarian cryopreservation is not free of limitations and concerns. In breast cancer patients one of the main concerns is the possibility of reintroducing tumour metastatic cells with the implant. In Hodgkin lymphoma and in T- and B-cell lymphoma, ovarian cryopreservation and implantation is considered a safe procedure regarding metastatic cells re-introduction (Radford et al., 2001; Donnez et al., 2004; Radford., 2004; Demeeestere et al., 2007; Andersen et al., 2008; Meirow et al., 2008). In the case of blood borne cancers, such as leukaemia, this procedure is considered unsafe because cancer cells are already in the bloodstream and it can be assumed that the blastic cells will always be present in the cryopreserved specimen (Oktyay and Buyuk, 2004). However, no studies have been published in this regard in breast cancer patients.

One of the problems found when such an important question is considered is the methodology employed to search for cancer cells in ovarian tissue. Since there is no established method, we decided to use the concept of the sentinel lymph node (SLN) study which consists of preparing serial sections per block, that are stained with morphological analysis

Seven serial sections of 4–6 mm were examined in an ovarian biopsy is 99% specific for breast origin, but a negative reaction does not exclude a breast origin (Lerwill, 2004). In order to increase the sensitivity and specificity of our study, MGB1 was added to our panel of proteins because it has been demonstrated that, in terms of diagnosis, MGB1 immunohistochemistry can serve as a differential marker of breast cancer origin (Sasaki et al., 2007; Moritani et al., 2008). GCDFP15 is a marker of apocrine differentiation that is expressed in 77% of breast carcinomas. A positive immunoreaction in an ovarian biopsy is 99% specific for breast origin, but a negative reaction does not exclude a breast origin (Lerwill, 2004). In order to increase the sensitivity and specificity of our study, MGB1 was added to our panel of proteins because it has been demonstrated that, in terms of diagnosis, MGB1 immunohistochemistry can serve as a differential marker of breast cancer origin (Sasaki et al., 2007). A positive reaction for GCDFP15 or/and MGB1 is consistent with a breast carcinoma metastasis, but a negative reaction is not informative. This is why we also selected a specific marker of ovarian tissue that is absent in breast cancer, namely Wilms’ tumour antigen-1 (WT1), that identifies the surface epithelium of the ovary and that is able to differentiate metastatic from primary ovarian tumour cells (Zhu and Michael, 2007). WT1 is a transcription factor that is strongly expressed in the nuclei of surface ovarian epithelium and in 100% of serous carcinomas (ovarian or extraovarian) and 80% of ovarian transitional carcinomas. In contrast, 100% of breast carcinomas are negative for WT1. Thus, a negative result for breast markers joined to a positive result for WT1 provides almost 100% specificity and sensitivity of standing with ovarian tissue, negative for breast tumour (Lerwill, 2004).

Finally, we also employed a universal marker of epithelia, cytokeratin CAM 5.2, with high sensitivity to detect ovarian surface epithelium, ovarian follicular cells and breast cancer cells (Klevesath et al., 2005).

With this methodology, the present study was designed to detect occult disease in ovarian tissue from breast cancer patients undergoing ovarian cortex cryopreservation in order to preserve fertility so as to detect minimal disease in the ovaries and reduce the risk of storage and re-implantation of tissue with cancer.

Materials and Methods

Samples

The study includes 100 ovarian cortex biopsies from 63 out of 205 women entering our Programme for Fertility Preservation with the diagnosis of infiltrating ductal breast carcinoma, stages I–IIa in whom chemotherapy (ChT) was indicated. The stages were distributed among the studied population as follows: I: 16 women (25.4%); IIa: 23 (36.5%); IIb: 18 (28.6%); IIIa: 6 (9.5%). Concerning hormones receptors, 41 (65.1%) were positive for estrogen and progesterone, 7 (11.1%) were positive only for estrogen, 3 (4.7%) were positive only for progesterone and the remaining 12 (19.1%) were negative for both estrogen and progesterone receptors. Lymph node involvement was absent in 31 (49.2%) and present in 32 (50.8%) cases.

Mean age of the patients was 32.8 years (range 19–39) and 11 (17%) of them underwent ChT before ovarian cortex extraction. Neither BRCA-1, BRCA-2 nor HER2neu mutation carriers were present in the population studied. Before surgery, all patients underwent a pelvic examination including vaginal ultrasound to rule out ovarian disease. The definition of breast cancer stage as IIa follows the oncologist’s opinion, according mainly to the expected patient’s survival after treatment.

Our programme follows the principle of removing most of the right ovarian cortex for cryopreservation although the left ovary is left in place. If the patient has iatrogenic POF after cure, the left cortex is removed and the right thawed cortical pieces are implanted into the left ovarian medulla, in an attempt to reduce ischemia and to provide an anatomical site for the orthotransplantation that would potentially result in natural gestation (Sánchez et al., 2007). In all 63 patients, a random biopsy sized ~7 x 7 mm was taken from the surgically removed right ovary before cryopreservation. Additionally, a second biopsy was obtained from the left ovary in 37 cases in which a preoperative biopsy was taken before continuing the procedure. The results of the preoperative analysis were obviously negative in all cases and are not presented here because the subsequent analysis of each piece was performed in more detail. Thus, a total of 100 biopsies were available for analysis.

In addition, six entire ovarian cortex pieces (sized 4–6 x 2–3 cm) were donated for research after cryopreservation for a period of 24–40 months. These were thawed and were studied in the same fashion. The material came from four patients in whom a pregnancy was contraindicated: two developed pleural metastases (stages I and IIb at diagnosis) and two had bone metastases (both stage IIa at diagnosis) and from two patients (stages IIa and IIb at diagnosis) who donated their tissue because of socio-familiar reasons. The diagnosis of metastatic disease in these four donors was made around 2 years after cryopreservation. The entire ovarian cortex was processed in five to seven blocks per case, in the same fashion as the 100 biopsies.

Morphological analysis

The biopsies were fixed in 10% buffered formalin and embedded in paraffin wax. Serial sections were obtained following the SLN methodology and coded according to the official Tumour-Node-Metastasis Classification (TNM Classification of Malignant Tumours, Sixth Edition, 2002). A micro-metastasis was defined as a neoplastic focus of 0.2–2 mm, and Isolated Tumour Cells were defined as isolated neoplastic cells or metastatic foci <0.2 mm, detected by means of immunohistochemistry and morphology.

Seven serial sections of 4 μm were obtained per block: the first, third and seventh were stained with HE for morphological study. The serial sections were examined under an optical microscope by two trained pathologists in a double blind fashion searching for neoplastic cells according to the WHO system (World Health Organization, 1979).
Immunohistochemistry study

The second, fourth, fifth and sixth serial sections were incubated with different antibodies for immunohistochemistry. The antibodies were chosen in order to detect the presence of any metastatic cell originating from primary breast carcinoma. The panel included an anti-low molecular weight cytokeratin (CAM 5.2), two antibodies defined as specific for breast epithelium: GCDFP15 and MGB1; and an antibody present in the surface epithelium of ovary but absent in breast tumours (WT1). This panel of antibodies was chosen because of its sensitivity to detect epithelial cells (CAM 5.2) (Klevesath et al., 2005), the specificity to detect breast carcinoma cells (GCDFP15 and MGB1) (Monteagudo et al., 1991; Sasaki et al., 2007; Montani et al., 2008) and the ability to differentiate metastatic from primary ovarian tumour cells (WT1) (Zhu and Michael, 2007).

The origin of the antibodies and their dilutions were as follows: monoclonal mouse anti-human cytokeratin CAM 5.2 (prediluted; Becton-Dickinson, San Jose, CA, USA); monoclonal mouse anti-human WT1 (1:50; Novocastra, Newcastle, UK); monoclonal mouse anti-human GCDFP15 (1:100; Zymed Corp., South San Francisco, CA, USA); monoclonal mouse anti-human mammaglobin (1:50; Dako Corp., Glostrup, Denmark). As a positive control for MGB1, GCDFP15 and CAM 5.2, a section of breast infiltrating ductal carcinoma was employed, and a section of ovarian serous carcinoma was used for WT1 positive control. The immunostaining was performed in a DAKO autostainer, with a previous heat antigen retrieval (10 min in citrate buffer), and following the Labelled Streptavidin Biotin immunohistochemical method. To confirm the specificity of the immunoreaction, negative controls were assessed using normal serum as the primary antibody. All the immunohistochemical slides were evaluated as positive or negative, specifying their location: suspicious neoplastic cells; surface epithelium-epithelial inclusion glands; follicles (oocyte, granulosa cells and theca cells); luteinized granulosa cells, luteinized inner theca cells; stroma cells; rete ovarii and vessels. The slides were scored by a semi-quantitative method for intensity of staining (− = negative, +/− = slight, + = moderate, ++ = intense) by the two pathologists, but finally results were defined only as positive or negative result.

The results were considered as positive for metastasis when the samples showed histological findings consistent with metastatic disease, and the following panel of immunohistochemical results: positive for CAM 5.2, GCDFP15 and/or MGB1; negative for WT1. The results were considered as negative when the samples did not fit the morphological and immunohistochemical criteria. The samples were considered as suspicious when morphologic findings were seen, but not confirmed on the immunohistochemical panel (GCDFP15 and MGB1 negativity, WT1 positivity).

Results

Morphological analysis

Primordial and primary follicles (Fig. 1A) were found in 88 biopsies, secondary or antral follicles (Fig. 1B) and corpora lutea fragments were found in five biopsies. In 12 biopsies no follicles or luteinized cells were found, where the cortical tissue became only a fibrous stroma. Surface epithelium was detected in 95 biopsies, with inclusion glands in 20, and one biopsy had rete ovarii. In the six cases in which the entire ovarian cortex was analysed, surface epithelium, inclusion glands and primary follicles were found in all cases.

Ninety five biopsies were negative for neoplastic cells in the optical study, but five biopsies were considered suspicious. These five corresponded to different women from whom two biopsies, one from each ovary, were obtained and the suspicious features were found in only one of the two ovaries. Two biopsies, staged as IIa, showed elongated ducts with an atypical epithelial lining in a cauliflower pattern, which could be considered as metastases from ductal carcinoma or atypical inclusion glands (Fig. 1C). In three biopsies (stages I, IIa and IIIa), nests of small clear cells with atypical active nuclei were detected near atretic follicles. They were considered as suspicious neoplastic cells or irregular apoptotic granulosa cells (Fig. 1D). Concerning the six patients in whom the entire ovarian cortex was studied, no suspicious cells were found.

Immunohistochemical study

We further analysed the 95 biopsies in which no suspicious morphological pictures by staining for MGB1 and GCDFP15, which showed similar staining patterns although the results were more consistent for MGB1. Mammaglobin was positive in 24 biopsies and staining was detected in internal theca and luteal cells as intracytoplasmic granules (Fig. 2A, B). Slight positivity was also observed in three biopsies in the cytoplasm of the oocytes in primary follicles and in two biopsies on the surface epithelium. GCDFP15 was positive in 21 biopsies, in the same locations as MGB1 (Fig. 2C, D).

CAM 5.2 was positive in 90 biopsies, with a diffuse intracytoplasmic pattern (Fig. 2E). It was located in the surface epithelium (Fig. 2F) and the inclusion glands. CAM 5.2 was negative in the granulosa cells except for three biopsies, which presented follicular atretic cysts. In these biopsies, a soft cytoplasmic staining was detected in a few granulosa cells partially detached into the cyst.

WT1 was expressed in all 100 biopsies, located in the surface epithelium and/or the inclusion glands as dark nuclear staining (Fig. 2G).
It was also expressed in the granulosa cells of primordial-primary follicles (Fig. 2H) and secondary follicles, although the expression was lost in mature or cystic follicles and corpora lutea. Cytoplasmic positivity was also detected in capillary vessels.

Five suspicious cases were found after histological evaluation. Two cases considered as possible inclusion cysts were MGB1 (--), GDCFP15 (--), CAM 5.2++ (Fig. 3A), WT1++ (Fig. 3B), a pattern consistent with surface epithelium, so these were re-evaluated and finally diagnosed as inclusion glands with hyperplastic surface epithelium. Three cases with clear cell nests were MGB1+ (Fig. 3C), GDCFP15++/-- (Fig. 3D), CAM 5.2-- and WT1--. The positive immunoreaction for breast cancer markers was localized in granulosa cells but not in the suspicious areas of small clear cells with atypical active nuclei by morphology. As a consequence, this pattern was consistent with the final diagnosis of internal theca cells or apoptotic granulosa cells, and not with metastatic ductal carcinoma.

In the six patients in whom the entire ovarian cortex pieces were studied, the pattern of immunohistochemical markers proved identical to the small biopsies, with positive results for CAM 5.2 in the primary follicles, as well as in the inclusion cysts and surface epithelium. Also, WT1 was positive in the surface epithelium, inclusion glands and follicular cells. MGB-1 and GDCFP15 were negative.

One patient underwent reimplantation of the ovarian cortex two and a half years after extraction. Most of the left ovarian cortex had been extracted via laparotomy by dissecting the cortex from the medulla with cold scissors. Employing biological glue and 3–0 stitches, the entire cryopreserved tissue, divided in two pieces, was implanted onto the medulla after thawing. This patient is menstruating regularly and free of disease after 18 months. The ethics Committee of the hospital allowed this surgery because of the age of the patient (41 years), the stage of disease (IIa) and the fact that she had received four cycles of ChT before extraction of the ovarian cortex.

**Discussion**

The present study employed the methodology currently used to screen the SLN to show the absence in ovarian cortex of metastatic disease in women undergoing ovarian cortical tissue extraction for fertility preservation, suffering from stages I to IIIa infiltrating ductal breast carcinoma.

Our hypothesis was that ovarian cortex preservation is a safe procedure. We know from the literature that even in stage IV breast cancer cases the most frequent sites of metastatic disease are bone, lung, liver and brain, although the ovary is not a common site of metastatic involvement. Indeed, in autopsies from women who died of
disseminated breast cancer (stage IV), the likelihood of finding ovarian metastases is <11% (Lee et al., 1987). Thus, investigators have suggested that in the absence of clinical and radiological evidence for distant metastasis (stage II or less) it is extremely unlikely to find ovarian involvement in breast cancer patients (Oktay and Buyuk, 2004).

Surprisingly no study has yet addressed this issue despite the fact that breast cancer is one of the most important indications for ovarian cortex cryopreservation in western countries (Sanchez et al., 2008; Rosendahl et al., 2008). Thus, we decided to search for the presence of micrometastases in cryopreserved ovarian tissue since the issue of safety is so critical in our fertility preservation programme. We employed the SLN methodology with the combination of different markers that have been demonstrated to be good indicators of the presence of cancer cells in breast and ovarian cancer. Certainly, SLN biopsy is not exempt from controversy and limitations, but it is considered a safe technique that allows correct staging (Blanco et al., 2007; Riccio et al., 2007). Moreover, for the development of a reproducible and useful method to detect micrometastases in ovarian cortex, the use of biopsies must be considered. Further, the method employed should also be applicable if the tissue is already cryopreserved and also be able to achieve high sensitivity in the detection of such micrometastases. Thus, a combination of morphology and immunohistochemistry in a similar fashion to that used for the SLN was considered to be the most appropriate method clinically available today.

Several approaches have been explored to improve the detection of breast cancer occult metastatic disease at different sites (Schröder et al., 2004; Koga et al., 2004; Kreunin et al., 2007; Sasaki et al., 2007). In fact others have suggested that using RT–PCR for the MGB1 genes might identify patients at higher risk for micrometastases in SLN compared with patients with negative RT–PCR results (Ouellette et al., 2004). Another interesting approach to search for metastatic cells would be epithelial tumour cell lysis obtained using cytotoxic T cell retargeting through the bispecific antibody BIS-1, which has a combined affinity for the T-cell receptor and epithelial glycoprotein-2 (Schröder et al., 2004). The authors demonstrated in vitro that purging of added epithelial tumour cells from ovarian tissue with BIS-1 may contribute to safer replacement of ovarian tissue in female cancer survivors. This is an encouraging approach, but since it involves tissue desegregation it is not useful for ovarian cortex transplantation.

A major issue during the analysis of tissue for the presence of cancer is the size of the sample. In this case this is particularly relevant because we had two main objectives to fulfill: it was our purpose to establish a safe procedure that could be applied to all women affected with breast cancer and this required the analysis of as much tissue as possible; simultaneously, we tried to preserve fertility and therefore in the interest of the patient most of the tissue had to be frozen. However, over time we acquired six cases where tissue was cryopreserved and subsequently donated for research. Unfortunately four cases had distant metastases while the remaining two decided to donate their ovarian tissue because of socio-familiar circumstances. Thus, the entire tissue of these six patients was completely assessed in serial sections by regular pathology and immunohistochemistry. In addition to the 100 biopsies studied in our initial study, we found no neoplastic cells in any of our six additional complete ovarian cortical tissue cases, confirming our initial results. Thus, we currently believe that ovarian tissue preservation is a safe procedure in women with early stages of breast cancer, although it has to be clearly stated that the future may bring new methods of cancer screening that may change our perception of this procedure.

Concerning haematological diseases, Seshadri et al. (2006) examined retrospectively harvested ovarian tissue for subclinical involvement by Hodgkin lymphoma using morphology/immunohistochemistry and no subclinical involvement was identified in ovarian tissue. Recently Meirow et al. (2008) analysed ovarian tissue stored from haematological cancer patients for minimal residual disease using real-time RT–PCR and found no positivity in Hodgkin lymphoma. The authors also highlighted the importance of using preoperative imaging of the ovaries before ovarian tissue extraction. Our running protocol includes transvaginal ultrasound and/or other imaging techniques to ensure that the ovaries are in good condition. Thus, it seems that ovarian cortex autotransplantation can be considered today as a safe method to preserve fertility and may be employable in as many as 80% of the cases in western countries (Meirow et al., 2008; Sanchez et al., 2008). However, future studies may provide more accurate methods of cancer detection that may either confirm or change our perception of ovarian cortex cryopreservation as a safe procedure for our patients. It must be also mentioned that distant metastases at other sites may appear at a later stage, contraindicating pregnancy and ruling out re-transplantation.

The inclusion criteria in a programme of fertility preservation must still be properly established. The relevance of having received ChT prior to cryopreservation is still debatable. In the present study follicular histology did not reveal significant differences concerning morphology and follicular count. These findings, together with the results displayed by the re-transplanted patient in terms of restoration of ovarian function, suggest that fertility preservation procedures might be considered even after four cycles of ChT. However, an individualized and complete study of every single case should be performed before making any decision.

Another finding from our study which deserves consideration is the fact that primordial and primary follicles were considered free of disease and there was only slight cytoplasmic staining of oocytes with MGB1 and GCDFP15 in a few antral follicles. Therefore, if metastatic cells were to be found, primordial and primary follicles could be isolated and matured in an attempt to avoid any neoplastic contamination (Gook et al., 2004; Dolmans et al., 2006).

Finally it should be also stressed that, even though we obtained random sections of the ovarian tissue sent to Pathology, part of the tissue was never examined because it was kept for fertility restoration purposes, although we had the opportunity to study the entire cortex of six patients. Although this study deals with breast cancers in early stages, we cannot entirely rule out the possibility that advanced breast cancer and/or types of breast cancer different to infiltrating ductal cancer may present metastases in the ovary. Thus, caution recommends that in every individual case in which fertility preservation is attempted employing cryopreservation and reimplantation, multiple biopsies should be performed and a balance between the analysed and the cryopreserved tissues made on an individualized basis. Also, careful screening of the patient is recommended before reimplantation is planned.

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