New methods to improve the safety assessment of cryopreserved ovarian tissue for fertility preservation in breast cancer patients

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Objective: To develop a novel molecular panel of markers to detect breast cancer (BC) disseminated malignant cells in ovarian tissue, and to improve the safety of ovarian tissue transplantation.
Design: Experimental study.
Setting: University hospital.
Patient(s): Ten ovarian biopsies from healthy patients, 13 biopsies with diagnosed BC metastasis, and 4 biopsies from primary BC tumor for designing a diagnostic panel of BC cell contamination; 60 ovarian biopsies from BC patients undergoing fertility preservation for validating the panel.
Animal(s): Female nude mice.
Intervention(s): A novel panel for BC malignant cell detection by reverse-transcription polymerase chain reaction (RT-PCR), inmmunohistochemical analysis, in vitro invasion assay and xenotransplantation assayed in ovarian tissue from BC patients.
Main Outcome Measure(s): Expression of G*CDFP15*, *MGB1*, *SBEM*, *MUC1*, *WT-1*, and *NY-BR-01*, selected as markers, assessed by quantitative RT-PCR in samples with confirmed BC metastasis. The most sensitive markers were confirmed by immunohistochemistry, and tested in vitro and in vivo.
Result(s): *GCDFP15*, *MGB1*, and *SBEM* were the most sensitive and specific markers to detect BC metastatic cells when at least one was expressed by quantitative RT-PCR. The panel was validated in 60 patients and confirmed in an in vitro invasion assay, where no invasive cells were observed. Samples negative for BC cells cannot develop disease when xenografted.
Conclusion(s): *GCDFP15*, *MGB1*, and *SBEM* were the most sensitive molecules to create a diagnostic panel for BC malignant cell contamination, which may make ovarian tissue cryopreservation and transplantation a safe technique for fertility preservation in BC patients. (Fertil Steril® 2015;104:1493–502. ©2015 by American Society for Reproductive Medicine.)
Key Words: Breast cancer, GCDFP15, MGB1, ovarian cortex cryopreservation, SBEM

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Received May 28, 2015; revised and accepted August 6, 2015; published online October 1, 2015.
B.R.-I. has nothing to disclose. E.N.-M. has nothing to disclose. S.H. has nothing to disclose. C.D.-G. has nothing to disclose. N.P. has nothing to disclose. A.P. has nothing to disclose.
Supported by grants SAF 2011-30031-CO2-01 and FIS PI13/02353 from the Spanish Ministry of Economy and Competitiveness and by PROMETEOII/2014/045 of the Regional Valencian Ministry of Education; grant AP-2010–0675 from the Spanish Ministry of Education, Culture and Sport (to B.R.-I.) and grant CD11/00292 from the Spanish Ministry of Economy and Competitiveness (to S.H.).
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Fertility and Sterility® Vol. 104, No. 6, December 2015 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2015.08.009

Increased survival rates have been seen for the cancer forms that primarily affect children and young individuals, such as hematologic malignancies and certain solid tumor types (1). Breast cancer (BC), the most common malignancy in women of reproductive age (2), is also increasingly detected in women who wish to preserve their fertility (3). However, treatment with high-dose

chemotherapy and/or radiotherapy have deleterious effects on the ovaries, leading to premature ovarian failure (4, 5). The options available for fertility preservation in such patients include cryopreservation of the ovarian cortex (COC) followed by orthotransplantation (COC-OT) (6–8). Although these techniques are still considered experimental, they have been successfully applied in more than 60 patients (9), and the births of over 40 children have been reported (10).

In contrast to oocyte and embryo cryopreservation, COC does not require hormone exposure. The ovarian tissue can be obtained at the time of cancer diagnosis, and the procedure interferes only minimally with the patient's cancer treatment plan. Individual follicles can also be cryopreserved for future use in in vitro follicle maturation (11, 12), and they also allow the restoration of cyclical ovarian steroid secretion; the return of menstruation leads to increased quality of life as long as the graft remains functional.

Nevertheless, COC-OT is associated with a risk of reintroducing cancer cells from the transplanted tissue. Patients with hematologic cancers such as leukemia are at increased risk for this adverse event (13–15). Various clinical approaches and laboratory-based techniques have been used to detect malignant cell contamination or the presence of metastatic disease in ovarian tissue before transplantation. These practices include preoperative imaging, histologic and immunohistochemical analysis, and polymerase chain-reaction (PCR). More refined screening techniques are being developed that provide support for the banking ovarian tissue at the time of cancer diagnosis with subsequent tissue evaluation before transplantation (15–25).

Breast cancer is the main indication for COC-OT (26). Although it was initially suggested that orthotransplantation after BC has been cured is safe (13), safety has not been properly addressed in women with non-advanced-stage BC. The reports that have been published have provided no evidence for malignant contamination by use of the classic methods of assessment, such as immunohistochemical analysis (16–18, 21). Although histology is still the gold standard in the clinical management of BC, new molecular methods are currently being introduced to provide more accurate techniques for the diagnosis and management of BC (27).

Well-characterized molecular tumor markers to detect occult BC cells currently are limited (28). In fact, employing genomic and proteomic analyses separately may be misleading because genes are up-regulated in some cases but protein is not detected (9) due to posttranscriptional regulation mechanisms (29). Therefore, to establish the accuracy and effectiveness of a definitive diagnostic tool, genomics should be complemented with new molecular proteomic techniques or with in vivo animal models. This is supported by several studies (15, 20) conducted with ovarian cortex from leukemia patients where the immunohistochemical analysis was negative for malignant cells but the PCR analysis demonstrated that malignant cells were already present in tissue. The same approach has been used for BC (24, 25); hence, the development of an improved diagnostic tool based on specific BC metastatic molecular markers and more sensitive molecular techniques cannot be delayed.

Therefore, based on a previous study into the sentinel lymph node concept (17), we are designing an improved BC diagnostic tool. Following preliminary results, highly specific molecular markers that were previously detected by classic techniques, such as mammaglobin-1 (*MGB1*) and gross cystic disease fluid protein-15 (*GCDFP15*), were retained in our new design. We have also included new BC metastatic-associated molecules such as small breast epithelial mucin-1 (*SBEM1*), whose gene and protein expression has correlated with metastases (30); mucin-1 (*MUC1*), a highly glycosylated protein aberrantly overexpressed in approximately 90% of human BC (31, 32); and the BC antigen *NY-BR-1* gene (*NY-BR-01*) (33), a differentiation antigen of the mammary gland that has been associated with 84% of BC (34).

MATERIALS AND METHODS

Study Design

In the first part of our study, the proposed molecular markers were tested by employing several types of positive and negative control samples by quantitative PCR to design the most sensitive and specific molecular panel for malignant contamination screening in clinical practice. The protein expression of the selected markers was then confirmed by immunohistochemical analysis.

The second part of the study was an internal validation of the panel in BC patients who had undergone fertility preservation to demonstrate the absence of malignant cells and the safety of the orthotransplantation procedures. These samples were submitted to analysis by quantitative PCR with the proposed metastatic panel, followed by immunohistochemical analysis. Absence of malignant cells was also confirmed by an in vitro invasion assay and by an in vivo animal model. A schema of the experimental design can be consulted as Supplemental Figure 1 (available online).

The use of human tissue and formalin-fixed, paraffin-embedded samples with diagnosed BC metastasis in this study was approved by the institutional review board of La Fe University Hospital (2011/0018). The in vivo animal model included in the study was approved by the institutional animal care committee at the Centro de Investigación Príncipe Felipe (13/0282).

Designing a Diagnostic Tool to Detect Breast Cancer Occult Micrometastases

Tissue samples and controls. To test and select the most specific molecular markers to detect BC metastasis in the ovarian cortex (OC), three experimental groups of samples and controls were included. Group 1, the reference control tissues, included 10 OC biopsies samples taken from healthy women who had undergone elective cesarean deliveries (mean patient age 30 years; range: 32–37 years). Group 2 comprised 13 formalin-fixed, paraffin-embedded samples with diagnosed BC metastasis from the Pathology Department of La Fe University Hospital (mean age 56 years; range: 49–69 years): eight samples from OC and five samples from other tissues (liver, lung, bone, eye, and uterine ligament). The latter group was used as the positive control for BC malignant contamination. Group 3 was composed of four fresh biopsies from primary