Evaluation of quantitative polymerase chain reaction markers for the detection of breast cancer cells in ovarian tissue stored for fertility preservation

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Objective: To develop molecular tools increasing the sensitivity of breast cancer micrometastases detection within ovarian tissue cryopreserved for fertility preservation.
Design: Expression of breast markers was evaluated by quantitative polymerase chain reaction in ovarian tissue from patients with benign or cancerous diseases. Suspected tissues were long-term xenografted into mice.
Setting: Academic research institute.
Patient(s): Patients undergoing a fertility preservation procedure.
Intervention(s): Ovarian tissue was processed for RNA extraction and quantitative polymerase chain reaction analysis. Cryopreserved ovarian cortex from patients with breast cancer or benign disease was grafted for 6 months into severe combined immunodeficiency mice.
Main Outcomes Measure(s): Predictive values of mammaglobin 1 (MGB-1), gross cystic disease fluid protein-15 (GCDFP-15), small breast epithelial mucine (SBEM), and mammaglobin 2 (MGB-2) to detect breast cancer cells in ovarian tissue, and the potential development of cancerous disease after xenograft of ovarian cortex from breast cancer patients.
Result(s): MGB-1 and GCDFP-15 presented the highest predictive values to detect breast cancer micrometastases in the ovarian cortex, with an efficiency reaching 100% and 77%, respectively. The MGB-2 assay resulted in a high false-positive rate (47%) in the ovarian cortex but could be used to detect breast cancer cells in ovarian medulla. MGB-1 was detected in three of five ovarian cortex samples from early-stage breast cancer patients but not in the ovarian tissue from advanced breast cancer patients (none of 10). None of the mice grafted with ovarian tissue expressing these markers developed cancerous disease.
Conclusion(s): MGB-1, GCDFP-15, and MGB-2 can serve as molecular markers for the detection of breast cancer micrometastases within the ovarian tissue of breast cancer patients. However, the clinical relevance of such a highly sensitive assay must be further investigated. (Fertil Steril® 2015;104:410–7. ©2015 by American Society for Reproductive Medicine.)
Key Words: Ovarian cryopreservation, breast cancer, safety of autotransplantation, quantitative PCR, mammaglobin-1

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Over the past decade, cryopreservation of ovarian tissue before the administration of curative gonadotoxic treatment has become an important option to preserve the fertility of young patients with cancer. To date, almost 40 live births have been reported after autotransplantation of cryopreserved ovarian cortex (1, 2). However, one major limitation of this procedure is the risk of reintroducing malignant cells into the patient (3). This risk is especially high in cases of systemic disease, such as acute leukemia (4–7), but it is less obvious for solid tumors such as breast cancer. In many centers, breast cancer represents the major indication for fertility preservation (8–10). According to analyses of ovarian necropsies, the risk of ovarian involvement in young breast cancer patients reached 19.4% (11) but was considered as low to moderate at the time of diagnosis (12). However, only a few studies addressed this issue by investigating the presence of micrometastases in ovarian cortex of women with breast cancer undergoing the cryopreservation procedure (13–18). Histology and immunohistochemistry tests targeting cytokeratins and/or breast markers such as mammaglobin 1 (MGB-1) and gross cystic disease fluid protein-15 (GCDFP-15) did not reveal malignant cells in the ovarian cortex of breast cancer patients undergoing the cryopreservation procedure. However, the sensitivity of immunohistochemistry may be insufficient to detect micrometastases. Indeed, histologic examination of the ovarian cortex from leukemia patients may not reveal contamination, whereas recurrence of disease has been observed after graft of this tissue into mice (4). This finding highlights the need to develop highly sensitive methods to evaluate the safety of ovarian autotransplantation in breast cancer patients. Given its high level of sensitivity and specificity, the quantitative polymerase chain reaction (Q-PCR) method is an attractive approach. This strategy has been successfully used for the detection of leukemia or lymphoma cells in the ovarian cortex (4, 6, 7) and to monitor the number of circulating tumor cells (CTCs) in the peripheral blood of breast cancer patients (19, 20). Recently, two studies evaluated the presence of breast cancer micrometastases in cryopreserved ovarian tissue by molecular tools (17, 18). Hoekman et al. (18) did not observe any overexpression of *MGB-1* in the ovarian cortex stored for fertility preservation in breast cancer patients. In the second report, the authors showed that ovarian tissue from advanced breast cancer patients may carry cells expressing mammaglobin 2 (*MGB-2*) but did not confirm tumoral ovarian involvement after long-term xenotransplantation (17). Given the heterogeneity of primary breast cancer and micrometastases, the molecular analysis of ovarian cortex with a single marker may not be sufficient. Both *MGB-1* and *GCDFP-15* are widely used for CTC detection (21) and for immunohistologic evaluation of residual disease in the ovarian cortex (13), making them interesting for molecular detection of micrometastases within ovarian tissue. Moreover, the small breast epithelial mucine (*SBEM*) may also serve as an additional marker because it is expressed in mammary tissue but not in normal ovarian tissue (22).

This study sought to evaluate the feasibility of adapting mammary CTC molecular detection methodologies to ovarian cortex stored for fertility preservation. The expression of *GCDFP-15*, *MGB-1*, *MGB-2*, and *SBEM* was evaluated in breast and in ovarian tissue. Ovarian cortex from patients with advanced-stage breast cancer or benign disease were long-term xenografted into severe combined immunodeficiency (SCID) mice, to evaluate their potential involvement by breast micrometastases.

MATERIALS AND METHODS

Tissue Specimens

Ovarian cortex was obtained from patients who underwent ovarian tissue cryopreservation for fertility preservation before the treatment of benign or cancerous disease (Supplemental Table 1) or who underwent oophorectomy for gynecologic pathology. In this study, breast cancers were classified according to tumor-node-metastasis staging: advanced and early breast cancer stages were defined as T $\geq$3 and/or N1 and as T1 or T2 and N0, respectively. Ovarian cortex from non–breast cancer patients (n = 17) and from breast cancer patients (n = 15, 5 early and 10 advanced stage) were thawed as previously described (23). Additionally, ovarian medulla removed from the ovarian tissue during processing for cryopreservation was stored at -80°C in RNAlater (Applied Biosystems). Ovarian medulla was obtained from patients with benign disease (n = 8), early-stage breast cancer (n = 4), and advanced-stage breast cancer (n = 9). Formaldehyde-fixed, paraffin-embedded (FFPE) specimens including tumoral breast (n = 3) and ovarian tissue with metastases from breast adenocarcinoma origin (n = 9) were used as positive controls.

RNA Isolation

Ovarian tissue was thawed and homogenized using MagNA Lyser tubes (Roche) in the presence of TRIzol (Life Technologies). Ribonucleic acid was isolated after chloroform treatment (Sigma-Aldrich) and purified from aqueous phase with the RNAqueous kit (Life Technologies) according to the manufacturer's instructions. Genomic DNA was digested by incubating samples with recombinant DNAse I (Ambion, Applied Biosystems). Ribonucleic acid from FFPE tissues was purified with the ReliaPrep FFPE total RNA miniprep system (Promega) according to the manufacturer's instructions.

Reverse Transcription and Q-PCR

Ribonucleic acid yield and purity were assessed by spectrophotometry (NanoDrop). Ribonucleic acid was reverse transcribed using the High Capacity Reverse Transcription Kit (Life Technologies). For each condition, a negative control was performed by replacing the reverse transcriptase with water.

Five nanograms (in the case of positive controls) or 25 ng (in all other cases) of complementary DNA (cDNA) were used as template in Q-PCR experiments in the presence of 10 μL of master mix (PowerSYBR Green PCR master mix; Applied Biosystems) and 200 nM of each primer (Supplemental Table 2) in a final volume of 20 μL. Each sample was analyzed in triplicate using a 7500 cycler (Applied Biosystems). Cycling was

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