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FERTILITY PRESERVATION

Is transplantation of cryopreserved ovarian tissue from patients with advanced-stage breast cancer safe? A pilot study

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Abstract
Purpose To assess the safety of reimplantation of cryopreserved ovarian tissue from advanced-stage breast cancer patients.
Methods Cryopreserved ovarian cortical fragments were obtained from 13 advanced-stage breast cancer patients aged 17–35 years. After thawing, part of the ovarian cortical tissue was grafted to severe combined immunodeficient mice for 6 months. The presence of malignant mammary cells in ovarian tissue was evaluated after thawing as well as after grafting by 1) histology and immunohistochemistry (epithelial membrane antigen, Her2/neu and gross cystic disease fluid protein 15 identification), and 2) detection of the MGB2 gene by qPCR.
Results No malignant cells were evidenced by histology and immunohistochemistry. None of the mice died during the 6-month grafting period, nor developed macroscopically visible masses. MGB2 gene expression was detected by qPCR and confirmed by sequencing in frozen-thawed ovarian tissue in 4 cases and in grafts in 1 case.
Conclusions This pilot study is the first to evaluate the risk of contamination of cryopreserved ovarian tissue from advanced-stage breast cancer patients by xenotransplantation for 6 months to immunodeficient mice, associated with more conventional screening methods. Our xenografting results are reassuring, but caution needs to be exercised, as MGB2 gene expression was detected in some cases. Larger numbers of ovarian tissue samples from patients with advanced-stage breast cancer are required to confirm our findings before ovarian tissue transplantation can be contemplated in these patients.

Keywords Fertility preservation · Breast cancer · Cryopreserved ovarian tissue · Ovarian tissue transplantation · Malignant contamination · Minimal residual disease · Xenografting · qPCR

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Capsule Cryopreserved ovarian tissue from advance-stage breast cancer patients is potentially unsafe for transplantation.

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Introduction

Breast cancer is the most common malignancy in women. Studies estimate that there were around 230,000 new invasive breast cancer cases in the US female population in 2011. Approximately 5 % of these cases were in women under the

age of 40 at the time of diagnosis [6]. For these young women, who have not had the chance to bear a child but require gonadotoxic treatment such as chemo- and/or radiotherapy, fertility preservation is often of paramount importance. To preserve fertility, three options can be proposed: in vitro fertilization (IVF) with embryo cryopreservation, oocyte cryopreservation, and ovarian tissue cryopreservation [17]. IVF with embryo cryopreservation or oocyte cryopreservation are potential options for breast cancer patients if stimulation can be done between surgery and the initiation of adjuvant chemotherapy [32]. When there is no time or ovarian stimulation is contraindicated, cryopreservation and transplantation of ovarian tissue has proved to be a promising approach to restore fertility, with 24 live births published to date [15, 16].

Breast cancer is the second most frequent indication (21.7 %) for ovarian tissue cryopreservation (OTC) in our department, after hematological diseases (39.9 %) [9, 10]. However, the risk of reintroducing malignant cells theoretically exists with this pathology [31]. Most occult metastases belong to the less common histological type, infiltrating lobular as opposed to infiltrating ductal carcinoma (IDC). Ovarian metastasis more commonly occurs in advanced-stage cancer [19, 27].

In the study by Azem et al. [3], histological examination of cryopreserved ovarian tissue from breast cancer patients revealed normal histology with no evidence of metastases. In women with breast cancer, neither Sánchez-Serrano et al. [38] nor Rosendahl et al. [36] detected the presence of ovarian metastases in cryopreserved ovarian tissue by morphological or immunohistochemical studies. Sanchez-Serrano et al. [38] analyzed 100 frozen-thawed ovarian cortical biopsies from 63 patients and 6 entire pieces of frozen-thawed cortex from patients diagnosed with IDC by histology or immunohistochemistry using cytokeratin CAM 5.2, gross cystic disease fluid protein-15 (GCDFP15), Wilms' tumor antigen-1 (WT1) and mammaglobin 1 as markers. Rosendahl et al. [36] examined cryopreserved ovarian cortical biopsies from 51 patients with breast cancer by histology and immunohistochemistry with another set of markers (cytokeratin 7, CK-AECAM, WT1 and cancer antigen 125). Neither of these studies found any sign of ovarian metastases. On the other hand, in a very large review of their national autopsy files, Kyono et al. [24] evidenced, by anatomopathological evaluation, ovarian metastases in 24.2 % of breast cancer patients.

Further studies are therefore required to investigate this controversial issue with more sensitive methods, such as real-time quantitative polymerase chain reaction (qPCR) approaches. Quantitative PCR was used to detect and characterize potential metastatic cells through identification of genetic features associated with tumor cells. A review of the literature by Lacroix [25] on the detection of metastatic cells by qPCR processing in lymph nodes and peripheral blood from patients with breast cancer defined some markers with low and high breast specificity. MGB1 and MGB2, lipophilin B, PIP (or GCDFP-15), and several others were classified as markers with high breast specificity. Breast cancers are composed of a heterogeneous collection of cells showing differing degrees of tumor marker expression [5]. Mammaglobin (MGB1) and mammaglobin B (MGB2) are 2 related genes of the uteroglobin gene family that are overexpressed in breast tissue and metastatic lymph nodes from patients with breast cancer [4, 47, 48]. After careful analysis of the literature on micrometastasis detection in sentinel lymph nodes, we selected the MGB2 gene as one of the most specific PCR markers to detect potential malignant breast cell invasion in our cryopreserved ovarian tissue [1, 4, 20, 30, 34].

The aim of our study was to assess, using real-time qPCR and long-term xenotransplantation for the first time, the potential risk of reintroducing malignant cells with cryopreserved ovarian cortical tissue from patients with advanced-stage breast cancer.

Materials and methods

Experimental design (figure in supplementary data)

Thirteen patients were included in the study. One or two cryovials of frozen ovarian cortical tissue (= 4 to 8 strips of different sizes from 1×2 mm to 4×10 mm) were thawed per patient. Frozen-thawed ovarian tissue from ten patients was divided into three groups: histology and immunohistochemistry processing, qPCR analysis, and 6 months' xenotransplantation. Recovered grafts were fixed in 4 % formaldehyde for histological evaluation and immunohistochemistry and frozen in Trizol for qPCR processing. For the last three patients, one cryovial containing a large strip of frozen ovarian tissue was thawed only for qPCR analysis.

Adequate positive and negative controls were included for qPCR analysis and for xenografting.

Patients

Use of human tissue for this study was approved by the Institutional Review Board of the Université Catholique de Louvain. All 13 patients (aged 17–35 years, mean 29 years) suffering from advanced-stage infiltrating ductal carcinoma agreed to donate tissue by signing the cryopreservation informed consent form. Cryopreserved ovarian cortical tissue from patients with advanced-stage disease (≥stage 2B) ($n=9$) or stage 2A with positive nodes or large tumors ($n=4$) (according the TNM classification) was thawed. Breast tumors were of intermediate grade (grade 2) in 6 patients and high grade (grade 3) in 7 patients. Two patients were positive for the BRCA 1 gene. The characteristics of the breast tumors are detailed in Table 1 [8, 22]. It should be noted that none of the patients had undergone chemotherapy before OTC.

Table 1 Clinical characteristics of patients with breast cancer included in the study and histological and immunohistochemical characteristics of tumors

Patient	Age	Obstetrical status	Type of breast cancer	Lymph nodes	TNM	Grade	Stage	ER	PR	Ki67	Her2/neu	FISH Her2/neu	Genetic features
1	29	G0P0	IDC with poorly differentiated intraductal carcinoma	3/9	pT2 pN1b M0	2	II B	8	4	20 %	2+	NA	NA
2	25	G0P0	IDC with intraductal carcinoma and lymphatic permeations	4/12	pT2 pN1 bii M0	2	II B	8	6	20 %	1+	NA	NA
3	33	G0P0	IDC with lymphatic emboli	8/23	pT2 pN2a	3	III A	0	7	60 %	1+	Positive	NA
4	27	G0P0	IDC	NA	cT2 N0 M1 (hepatic metastasis)	2	IV	Positive	Negative	66 %	3+	NA	NA
5	31	G0P0	IDC with a large range of poorly differentiated intraductal carcinoma	2/7	pT2 pN1b	3	II B	8	7	10 %	2+	Positive	Negative
6	32	G0P0	Poorly differentiated IDC with ductal carcinoma in situ	0/8	pT2 pN1 bi	3	IIB	6	0	15 %	0	NA	NA
7	35	G0P0	IDC	1/15	pT1c pN1 bi Mx	3	II A	0	0	60 %	1+	NA	NA
8	27	G1P1	IDC	2/12	pT1 pN1 bi	2	II A	Positive	Positive	NA	1+−2+	Negative	NA
9	28	G0P0	IDC	1	cT2 N1 Mx	3	IIB	0	0	80–90 %	1+	NA	BRCA-1 positive
10	17	G0P0	IDC	1/6	pT1c pN1b Mx	2	IIA	5	7	NA	0	NA	NA
11	35	G0P0	IDC	20/20	pT3 pN3 pMx	3	III C	0	0	50 %	0	NA	NA
12	29	G2P1	IDC with intraductal carcinoma and lymphatic permeations	0/8	pT2 N0 Mx	3	II A	0	7	80 %	1+	NA	BRCA-1 positive
13	29	G1P1	IDC	0/7	pT2 pN0(i-) pM1 (bone and pulmonary metastases)	2	IV	7	6	25 %	0	NA	NA

IDC infiltrating ductal carcinoma *TNM* TNM classification (tumor, node and metastasis) *ER* estrogen receptor, *PR* progesterone receptor: evaluated using the Allred score [22] Ki67-positive tumor cells are counted and expressed in %. A high percentage of proliferating cells is often correlated with a poor prognosis Her2/neu status is evaluated by immunohistochemistry and confirmed by FISH [8]

Control groups (Table 2)

For qPCR analysis, 21 normal ovarian cortical samples from 21 patients with benign gynecological disease (23–36 years of age) were used as negative controls. As positive controls, breast tumors ($n=4$) [4] and ovarian cancers ($n=3$) [40] were chosen.

To validate the xenografting model, one piece of tissue with infiltrating ductal carcinoma from an 85-year-old patient with breast cancer (stage IIA) was grafted for 6 months.

Freezing and thawing procedures

Freezing and thawing of ovarian tissue was undertaken according to the protocol described by Gosden et al. [21], as detailed in previous papers [14, 44]. Briefly, biopsy samples were cut into small strips (+/− 4 × 1–2 mm). The fragments were slow-frozen with 10 % dimethyl sulfoxide as cryoprotectant and placed in cryovials (7–16 per patient). For quality control and assessment of the absence of malignant cells, one or two cryovials were thawed.

Transplantation to SCID mice

Guidelines for animal welfare were approved by the Committee on Animal Research of the Université Catholique de Louvain.

Ten severe combined immunodeficient (SCID) 6- to 8-week-old female mice (Charles River Laboratories) were

Table 2 MGB2 PCR results of control groups

N°	Sample	Description	Conclusion (based on MGB2 PCR criteria)	Mean copy number	SD (copy number)
Negative controls					
1	11-169 N	Normal ovarian tissue	Negative		
2	07-395NA	Normal ovarian tissue	Negative		
3	214/1	Frozen- thawed ovarian tissue	Negative		
4	214/2	Frozen- thawed ovarian tissue	Negative		
5	214/3	Frozen- thawed ovarian tissue	Negative		
6	214/4	Frozen- thawed ovarian tissue	Negative		
7	214/6	Frozen- thawed ovarian tissue	Negative		
8	214/7	Frozen- thawed ovarian tissue	Negative		
9	214/8	Frozen- thawed ovarian tissue	Negative		
10	216/1	Frozen- thawed ovarian tissue	Negative		
11	216/2	Frozen- thawed ovarian tissue	Negative		
12	216/3	Frozen-thawed ovarian tissue	Positive	289	45
13	216/4	Frozen- thawed ovarian tissue	Negative		
14	216/5	Frozen-thawed ovarian tissue	Positive	2426	1007
15	216/6	Frozen-thawed ovarian tissue	Positive	233	7
16	216/7	Frozen- thawed ovarian tissue	Negative		
17	216/8	Frozen- thawed ovarian tissue	Negative		
18	216/9	Frozen- thawed ovarian tissue	Negative		
19	216/10	Frozen- thawed ovarian tissue	Negative		
20	216/11	Frozen- thawed ovarian tissue	Negative		
21	216/12	Frozen- thawed ovarian tissue	Negative		
Positive controls					
1	BMD 192/11	Infiltrating lobular carcinoma	Positive	67275	8279
2	07-749	Infiltrating ductal carcinoma	Negative		
3	08-366	Infiltrating ductal carcinoma	Positive	9	2
4	199/5	Infiltrating ductal carcinoma after xenografting to a SCID mouse for 6 months	Positive	1326	988
5	08-45 N	Ovarian endometrioid adenocarcinoma	Positive	3932	1843
6	08-99 T1	Ovarian serous papillary adenocarcinoma	Positive	7346326	269632
7	07-343 T	Ovarian endometrioid adenocarcinoma	Positive	9501	513

operated on. Breast tumor tissue (infiltrating ductal carcinoma) was grafted to an additional SCID mouse as a positive control for long-term xenografting.

The grafting protocol used in this study was the same as previously described [11]. Briefly, a small median slit was made in the abdomen and peritoneum of the animals, and human ovarian tissue strips were fixed to the inner side of the peritoneum with one or two stitches of 6–0 Prolene (Fig. 1a).

After 6 months, ovarian grafts were recovered and fixed for histological analysis and immunohistochemistry (fixation in 4 % formol). If sufficient tissue was available, it was ground and resuspended in Tripure® (Invitrogen) and stored at −80 °C for further qPCR analysis.

Histological evaluation and immunohistochemistry

After thawing as well as after grafting, a piece of ovarian tissue was fixed in 4 % formaldehyde and embedded in paraffin. Serial 5 μm-thick sections were taken and every fifth slide was stained with hematoxylin and eosin (HE) (Merck) for histological analysis. The presence of ovarian follicles was analyzed in frozen-thawed ovarian tissue. All serial sections were evaluated by an experienced pathologist for malignant cell identification.

For each patient, one representative slide was stained by immunohistochemistry. Epithelial membrane antigen (EMA, also known as MUC-1) was the first-line choice of marker because its sensitivity is reported to be very high for detection of metastatic mammary cells in the ovary (95 %) [43]. However, this marker does not have good specificity, as ovarian carcinoma stains positive in 100 % of cases with EMA [43]. Other immunohistochemical stainings were therefore performed to confirm the mammary origin of EMA-positive cells: Her-2/neu and GCDFP15, depending on primary tumor characteristics. Anti-human EMA (monoclonal, mouse, M0613, DakoCytomation, 115 mg/L) used at a 1:100 dilution and Her-2/neu (monoclonal, rabbit, 800–2996, Ventana, 6

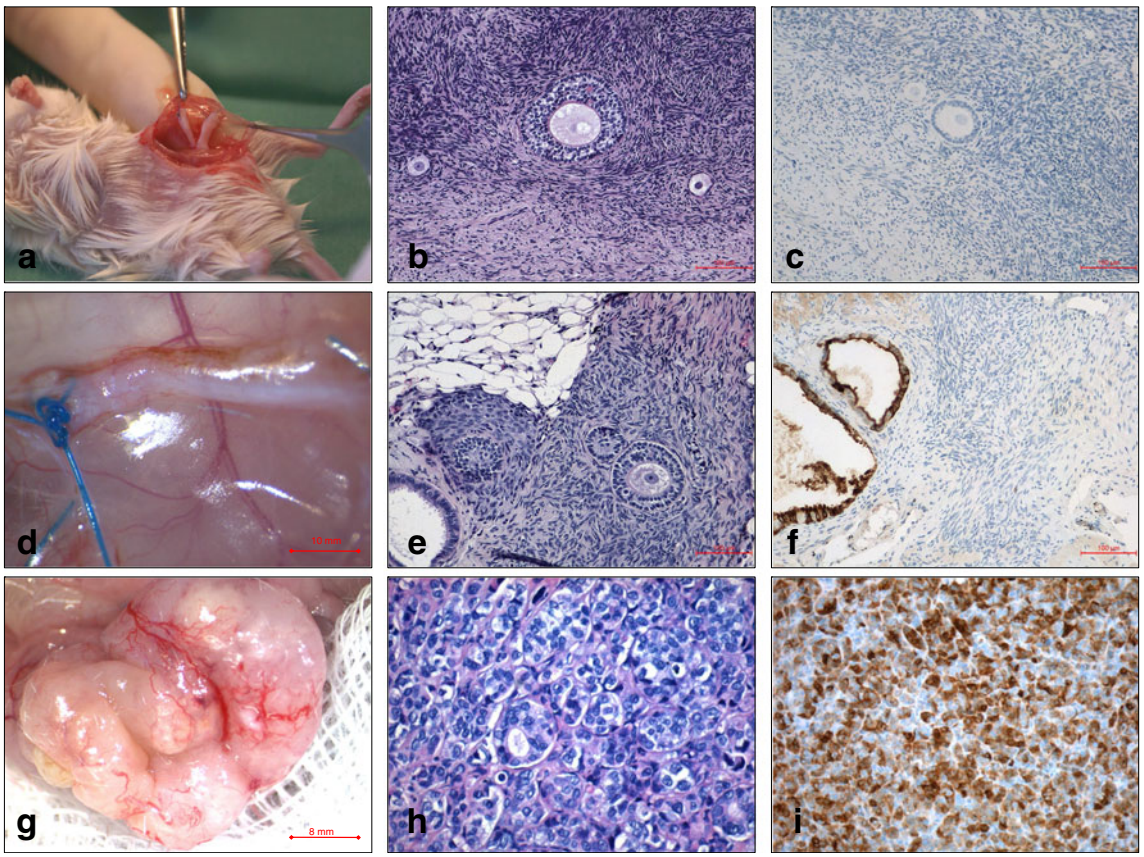

Fig. 1 Illustrations of macroscopic results, histology and EMA immunohistochemistry **a**–**c** Two frozen-thawed human ovarian tissue strips were grafted to the inner side of the peritoneum with one or two stitches of 6–0 Prolene. Macroscopic view (**a**). Histological aspect of a frozen-thawed ovarian fragment. Three ovarian follicles can be seen in the cellular stroma. No evidence of malignant mammary cells (**b**). Negative EMA immunohistochemistry of frozen-thawed ovarian tissue from a breast cancer patient. **d**–**f** Macroscopic view of a human ovarian graft after xenografting to a SCID mouse for 6 months (**d**). Note the stitches at both ends. Histological aspect of an ovarian xenografted fragment. Ovarian follicles can be seen in the cellular stroma near the murine omentum (**e**). EMA immunohistochemistry showed an ovarian epithelial inclusion cyst but no malignant cells (**f**). **g**–**i** Macroscopic view of an enlarged human breast tumor (infiltrating ductal carcinoma) after xenografting to a SCID mouse for 6 months (**g**) analyzed by histology (**h**) and EMA immunohistochemistry (**i**)

μg/ml, RTU) immunohistochemistries were automatically performed with the Ventana BenchMark XT® UltraView DAB detection kit (v3) with incubation with antibody for a period of 32 min. Finally, all slides were counterstained with hematoxylin II (Ventana, 790–2208) and bluing reagent (Ventana, 760–2037). Adequate positive and negative controls were included for each immunohistochemistry.

Molecular analyses

Total RNA isolation and cDNA synthesis

Total RNA was isolated using the Tripure® RNA reagent (Invitrogen) according to the manufacturer's instructions, quantified using a NanoDrop UV spectrophotometer and immediately frozen at −80 °C until use. For cDNA synthesis, 1.0 μg of total RNA was denatured at 70 °C for 10 min, chilled on ice, and subsequently reverse-transcribed for 1 h at 55 °C using 1 μL of random hexamers (0.7 μmol/L; Eurogentec; Belgium), 2U/μL of SuperScript III reverse transcriptase (Invitrogen, Merelbeke, Belgium), 0.35 mmol/L dNTPs, 50 mmol/L Tris-HCl, pH 8.3, 75 mmol/L KCl, 3 mmol/L MgCl2 and 100 mmol/L dithiothreitol, in a final volume of 50 μL.

Primers and standard curves

MGB2 cDNA (accession number NM_002407-) was amplified using MGB2-FOR (agcagtgtttcctcaaccagtca) and MGB2-REV (atagtctgtagccctctgagccaa) primers. These primers were designed using Primer Express™ software (version 3.0, Applied Biosystems) and by selecting intron-exon boundaries to exclude genomic DNA amplification. Amplification conditions involved initial pre-incubation at 95° for 10 min (polymerase activation), followed by amplification of the target cDNA for 50 cycles (95 °C for 10 s, 55 °C for 15 s and 10 s at 72 °C). Amplification reactions were performed in triplicate with the LightCycler 480 SYBR Green I Master kit in a final 20 μL volume with 0.5 μM of each primer and 2 μL cDNA. A negative control consisting of PCR-grade water instead of cDNA, as well as a positive control consisting of a TOPO®-XL recombinant plasmid carrying the MGB2 amplicon, engineered by incorporation of the 140-bp MGB2 DNA amplicon obtained through qPCR amplification of reverse-transcribed cDNA from a cell line expressing MGB2, was used. Plasmid copy numbers were determined by the equation: plasmid copy number (copies/μl) = (plasmid DNA concentration/plasmid molecular weight) × Avogadro's constant. Data were recorded as cycle quantification (Cq) on the Roche LightCycler® 480 System. Melting temperature (Tm) curves were automatically generated by the software and used to assess the specificity of the amplified product. Serial dilutions (with copy numbers ranging from 10^9 to 1) were submitted to qPCR analysis. Cq values obtained (each value considered was an average of triplicate fluorescence values for each plasmid dilution) were plotted against the logarithm of copy numbers to generate a calibration curve. The Cq value at intercept was calculated and set as the value for 1 plasmid copy. Absolute quantification of MGB2 mRNA was achieved using the comparative crossing point (Cq) method; the copy number of MGB2 mRNA in any unknown sample was calculated by comparing its Cq with the corresponding one on the standard curve. Sequencing of amplicons and comparison with public databases for identification purposes were carried out as previously described [18].

A sample was considered positive for MGB2 if it met the following criteria: (1) generation of a fluorescence signal with a Cq value less than the intercept value of the standard curve; (2) an intra-assay coefficient of variation (CV) <0.05 between triplicates; (3) a Tm within the range of positive controls (CI 95 %); and (4) an amplicon sequence matching 100 % the *Homo sapiens* MGB2 cDNA sequence.

Results

Control breast cancer (infiltrating ductal carcinoma)

Macroscopic analysis (Fig. 1g) The mouse grafted with breast tumor tissue developed palpable abdominal tumor masses in the abdominal cavity after a period of 6 months.

Histology (Fig. 1h) Histological analysis of these abdominal masses confirmed the presence of infiltrating ductal carcinoma. Tumor cells were arranged in cords, nests and glandular structures. Neoplastic cells were moderately pleomorphic and showed some degrees of mitosis.

Immunohistochemistry (Fig. 1i) The majority of neoplastic cells exhibited EMA staining in cytoplasmic and membranous localizations.

qPCR As reported in Table 2, the positive control (breast cancer grafted for 6 months to a SCID mouse) revealed a high level of MGB2 gene expression.

Frozen-thawed ovarian cortical tissue from patients with breast cancer

Histology (Fig. 1b) Follicles were found in all patients by histological evaluation. The presence of malignant cells was suspected in patient 4. Indeed, a group of cells with a large nucleus and relatively abundant eosinophilic cytoplasm was detected in a frozen-thawed ovarian fragment. Although the nucleus of these cells showed an irregular profile, chromatin was regularly distributed and nucleoli were not visible.

Immunohistochemistry (Fig. 1c) Immunohistochemistry for EMA was carried out to investigate the potential mammary origin of these cells (patient 4), but the result was negative. Systematic EMA staining was also performed on one slide per patient. In two cases (patients 3 and 5), we observed a few EMA-positive cells. In one case (patient 3), the EMA-positive cells were seen lining a cystic structure, and in the other (patient 5), they were floating inside a cyst. At morphological analysis of the two cases, diagnosis of ovarian epithelial inclusion cysts was made, as the cysts were found to be lined with simple cubic-cylindrical epithelium, showing a few ciliated cells. Moreover, further analysis by immunohistochemistry with Her-2/neu was negative, so a tumor origin was excluded.

qPCR (Tables 2 and 3) A standard curve was established with serial dilutions of a plasmid carrying the MGB2 amplicon (see supplemental data). A Cq value corresponding to the intercept value (40.29; CI 95 % 40.08–40.50) was equivalent to 1 copy of MGB2 cDNA per qPCR assay (i.e. 10 copies per μg of total RNA). Accordingly, samples displaying a Cq value higher than 40.50 were considered negative. The Tm of amplified controls was 81.08 °C (CI 95 %°C: 80.46–81.70 °C).

On frozen-thawed ovarian tissue from breast cancer patients, in 4 out of 13 patients, the MGB2 gene was expressed at a low level (Table 3, patients 2, 4, 6 and 7). All other patients were negative. Table 2 shows qPCR results from positive and negative controls. All sequenced amplicons matched the human MGB2 cDNA sequence 100 %.

Sensitivity and specificity of the qPCR assay for detection of ovarian carcinoma were both 85.71 %. Positive predictive value (PPV) and negative predictive value (NPV) were 60.00 % and 95.45 % respectively.

Xenografting to SCID mice for 6 months

Macroscopic analysis (Fig. 1d) Ten SCID mice were grafted with frozen-thawed ovarian tissue from patients with breast cancer and one with breast tumor tissue (infiltrating ductal carcinoma). None of them died during the 6-month grafting period, nor developed macroscopically visible masses or cachexia.

Histology (Fig. 1e) Ovarian tissue grafts were retrieved after 6 months. By histology, follicles were found in all the grafts. No malignant invasion by breast cancer cells was observed on HE-stained sections.

Immunohistochemistry (Fig. 1f) EMA immunohistochemistry revealed an ovarian epithelial inclusion cyst (Fig. 1f) in two cases (patients 2 and 7). In three other cases (patients 5, 6 and 8), scattered EMA-positive stained cells were observed in murine omentum. By histology, these EMA-positive cells showed an abundant eosinophilic cytoplasm and an eccentric round nucleus with compacted chromatin. The morphology and localization of these EMA-stained cells did not correspond to mammary tumor cells, but to plasma cells. To characterize suspicious cells, other specific anti-human antigen immunohistochemical stainings were performed (Her-2/neu, GCDFP15) and proved to be negative.

Table 3 MGB2 PCR results of ovarian tissue from study patients

PCR results	Frozen-thawed ovarian tissue			Frozen-thawed ovarian tissue after xenografting for 6 months to SCID mice		
Description	Conclusion (based on MGB2 PCR criteria)	Mean copy number	SD (copy number)	Conclusion (based on MGB2 PCR criteria)	Mean copy number	SD (copy number)
Patient 1	Negative			NA		
Patient 2	Positive	340	227	NA		
Patient 3	Negative			NA		
Patient 4	Positive	740	20	Negative		
Patient 5	Negative			Positive	1250	610
Patient 6	Positive	360	280	NA		
Patient 7	Positive	3400	470	Negative		
Patient 8	Negative			Negative		
Patient 9	Negative			Negative		
Patient 10	Negative			Negative		
Patient 11	Negative			NA		
Patient 12	Negative			NA		
Patient 13	Negative			NA		

NA not available

qPCR on graft Six of the 10 recovered grafts yielded enough tissue for qPCR analyses. MGB2 gene expression was revealed in one out of 6 analyzed cases (Table 3).

Discussion

Breast metastasis in ovarian tissue

Fertility preservation in breast cancer patients is an important topic of concern because of the frequency of this type of cancer. Staging in breast cancer is a fundamental parameter for prognosis. Breast cancer dissemination normally involves a succession of clinical and pathological stages, starting with breast carcinoma in situ, progressing to invasive lesions and culminating in metastatic disease [25]. The frequency of ovarian metastases in breast cancer patients can vary between 13.2 % and 37.8 %, depending on breast cancer type and on the published clinical series [24, 35]. It is therefore of major importance to detect the presence of metastatic invasion in cryopreserved ovarian tissue before transplantation.

In our study, EMA immunohistochemistry was selected because of its high sensitivity in breast carcinoma [26, 43]. Due to the lack of specificity, some other more specific immunostainings like Her2/neu were performed on EMA-positive immunohistochemical results. Neither histology nor immunohistochemistry evidenced the presence of any malignant cells, thereby confirming the results of the two published studies on patients suffering from more advanced breast cancer. Nevertheless, detecting micrometastases remains challenging. Morphological and immunohistochemical studies may not be sensitive or specific enough to identify malignant microinvasion of ovarian tissue, and more sensitive methods such as long-term xenografting and qPCR analyses are mandatory. These techniques were used to detect leukemic cells in cryopreserved ovarian tissue [11], with qPCR proving positive in 9 out of 16 ovarian tissue samples from leukemia patients. After xenografting, 4 out of 12 mice presented with malignant invasion.

Quantitative PCR analyses

In this study, frozen-thawed ovarian cortical tissue from 13 patients with advanced-stage breast cancer was examined. MGB2 gene expression was detected in 4 of the 13 frozen-thawed ovarian tissue samples and in one of 6 xenografts, while histological and immunohistochemical approaches were negative in all cases.

To validate the choice of PCR marker, negative and positive controls were performed. It should be pointed out that variability within positive controls does not suggest a direct link between MGB2 expression and cancer stage. Furthermore, sensitivity and specificity of the assay is based on the assumption that selection of both positive and negative controls is very tight. Indeed, 3 negative control cases with positive MGB2 expression were identified in this study. All 3 showed a gynecological pathology (benign serous ovarian cystadenoma, superficial ovarian endometriosis and rectovaginal endometriosis). MGB2 was also found to be expressed in endometrial carcinoma [28, 29] and it is known that endometriosis shares some common features with the oncogenic process. While no direct link between these conditions and MGB2 expression has been reported so far, it should be investigated. Tassi et al. [40] studied expression of the MGB2 gene in ovarian carcinoma and found 24 negative results out of 27 normal ovarian tissues (specificity: 88.9 %) and expression of the MGB2 gene in 88.8 % of epithelial ovarian carcinomas.

Although it is very important to evaluate the safety of ovarian tissue transplantation in cases where there could be malignant contamination, our study highlights the significant limitations we face when screening this tissue. Indeed, it is impossible to find a PCR marker which is 100 % sensitive and specific for breast cancer cells. Ideally, a disease-specific marker should be identified for each patient, but it is not always available [9–11]. In our study, we selected the MGB2 gene as one of the most specific markers to detect malignant breast cells, but because of the limited quantity of ovarian tissue available for this study, we were able to assess the presence of only one gene.

In the existing literature on axillary sentinel lymph nodes in breast cancer patients [20, 34, 46], the discrepancy between histological and immunohistochemical analyses and PCR approaches has already been described. It is most likely explained by the sampling procedure, because the parts of the lymph node analyzed using molecular techniques are different from those examined by histopathology and immunohistochemistry. We encountered the same discrepancy in the present study; as previously mentioned in the experimental design, ovarian fragments from each cryovial were divided into three separate groups.

The limitations of this study could also be explained by the different sensitivity and specificity of the various techniques used. To improve the sensitivity of the biomolecular approach, one option is using a multi-marker gene panel [20]. Since circulating tumor cells in a given patient with breast cancer may not express the particular tumor marker being assayed, a multiple marker assay, taking into account tumor heterogeneity and mRNA expression variability, may improve detection [20, 39]. This might be a good option when limited tissue is available, as is the case with cryobanked tissue.

Sensitive PCR analysis is able to evidence trace contamination of ovarian tissue with cancer cells, but the biological potential of these scarce cells is unknown. Therefore, to

evaluate the biological potential of trace contamination, transplantation studies were performed.

Xenografting

We believe that xenotransplantation experiments are probably the best way to accurately evaluate the risk of recurrence after transplantation of ovarian tissue. Indeed, from a clinical point of view, it may be very difficult for physicians to decide if it is safe or not to transplant ovarian tissue with trace contamination detected via PCR using markers that are not 100 % sensitive or specific. This is the first study to evaluate breast cancer cell contamination in cryopreserved ovarian tissue by xenografting experiments. These transplantation results are quite reassuring, although some PCR tests were positive. On the other hand, it is also true that xenografted ovarian fragments may not necessarily reflect the content (or malignant potential) of the remaining cryobanked fragments.

Xenotransplantation was applied and validated by the occurrence of tumor masses after grafting of breast cancer tissue (positive control) to SCID mice. In our study, no malignant masses were macroscopically evidenced after grafting of frozen-thawed ovarian tissue from breast cancer patients. Surprisingly, in patient 5, we detected MGB2 gene expression in the xenografted ovarian fragment, but did not evidence malignant cell contamination in the frozen-thawed fragment by PCR. Conversely, in 4 cases (patients 2, 4, 6 and 7), the MGB2 gene was detected in frozen-thawed ovarian tissue by PCR analysis and confirmed by sequencing. However, none of the mice grafted with ovarian fragments from these patients developed tumor masses. In 2 cases (patients 4 and 7), enough ovarian tissue could be retrieved after grafting for PCR analysis, but the MGB2 gene was not identified. These contradictory results should be interpreted with caution, as they may be explained by the fact that ovarian fragments from each cryovial were divided into three groups: (i) histology and immunohistochemistry, (ii) PCR analysis and (iii) long-term xenotransplantation (described in the experimental design). Hence, the fragment used for PCR was not the same as that used for xenografting. Our xenotransplantation results are currently 100 % reassuring because none of the mice developed tumor masses, despite the presence of MGB2 gene-positive cells (patient 5). However, xenografting for 6 months may not be a sufficient period of time to observe the development of breast tumor masses, but if more than 2 years is required, the murine model may not be appropriate.

Clinical implications

After careful analysis of our qPCR results and the clinical characteristics of these patients, no relation could be found between the histological grade of the tumor and qPCR positivity of ovarian tissue. Among the five positive cases, 2 were of grade 2 and 3 of grade 3. Among the negative cases, 4 were of grade 2 and 4 of grade 3. Similarly, analysis of tumors, TNM classification and tumor characteristics (receptors and proliferation index) did not suggest any correlation between an aggressive tumor and positive qPCR results in the ovary. Nevertheless, some teams allow transplantation of frozen-thawed ovarian tissue from patients with early-stage breast cancer [2, 23, 33, 37]. However, while most breast cancer recurrences manifest within 10 years of the initial diagnosis, the disease can recur much later, emphasizing the need for long-term surveillance in clinical follow-up of these patients [7].

Conclusion

This pilot study evaluates the risk of contamination of cryopreserved ovarian cortical fragments by breast metastases with conventional screening methods (histology and immunohistochemistry) combined with a single-marker PCR assay, confirmed by gene sequencing and xenotransplantation. It demonstrates that cryopreserved ovarian tissue from patients with advanced-stage breast cancer may contain cells expressing the MGB2 gene. The real malignant potential of these cells is not yet known. Nevertheless, as some limitations were observed in our study, more investigations are required to confirm our results by including more patients, especially with advanced-stage breast cancer, and by increasing the sensitivity and specificity of screening methods using a multimarker PCR approach [20] associated with xenografting experiments. Only then can ovarian tissue transplantation be contemplated in patients with advanced-stage disease.

For these patients, who run the potential risk of having malignant cells in their cryopreserved ovarian tissue, other options could be of interest, such as follicle culture with in vitro maturation [41, 42] or grafting of isolated follicles enzymatically purified from frozen-thawed ovarian tissue [12, 13, 45].

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