A new method for evaluating the risk of transferring leukemic cells with transplanted cryopreserved ovarian tissue

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Abstract

Purpose This study aimed to develop a method to detect ovarian residual disease by multicolor flow cytometry in acute leukemia patients.

Methods We designed an experimental model consisting in adding acute leukemia cells to a cell suspension obtained from healthy ovarian cortex. Leukemic cell detection within the ovarian cell suspension required the development of a specific myeloid antibody panel different from that commonly used for minimal residual disease (MRD) monitoring in bone marrow. The method was then used to detect ovarian residual disease in 11 acute leukemia patients.

Results Multicolor flow cytometry is able to evaluate the presence of viable leukemic cells in the ovarian cortex with good specificity and robust sensitivity of 10–4. We observed a good correlation between multicolor flow cytometry and quantitative polymerase chain reaction results. Ovarian residual disease detection by multicolor flow cytometry was positive in 3 out of 11 acute leukemia patients.

Capsule Ovarian residual disease detection by multicolor flow cytometry was positive for 3 of 11 acute leukemia patients. The safety of cryopreserved ovarian tissue autograft in case of acute leukemia remains of great concern.

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Conclusion Multicolor flow cytometry can potentially be applied to ovarian tissue from all acute leukemia patients and is essential to evaluate the risk of cancer re-seeding before autograft of ovarian tissue in case of acute leukemia.

Keywords Fertility preservation · Human ovarian tissue · Minimal residual disease · Multicolor flow cytometry · Acute leukemia

Nowadays, approximately 85 % of patients aged under the age of 45, who undergo chemotherapy and/or radiotherapy treatment, will survive for at least 5 years [1]. Premature ovarian failure is a major and common long-term side effect of these cytotoxic treatments [2–4]. The possibility for women to conceive a child after surviving cancer is a reality that can be envisaged thanks to advances in fertility preservation methods and assisted reproductive technology. Ovarian tissue cryopreservation is an option for preserving fertility in prepubertal girls or women for whom gonadotoxic treatment cannot be postponed [5]. Currently, frozen/thawed ovarian tissue can only be re-used by autograft and more than 40 live births have already been reported [6]. However, there is a risk of cancer re-seeding after ovarian tissue autograft especially in cases of acute leukemia [7]. Real-time quantitative polymerase chain reaction (RT-qPCR) [8] and multicolor flow cytometry (MFC) based on leukemia-associated immunophenotype (LAIP) [9] are used to identify persisting leukemic cells in blood or bone marrow. Three studies have investigated ovarian residual disease (ORD) in the setting of acute leukemia by RT-qPCR [10–12]. The objective of this paper was to describe a new method for ORD detection, by MFC, in cases of acute leukemia.

Human cortical fragments were cryopreserved and thawed before undergoing mechanical and enzymatic dissociation [13]. Healthy ovarian cortex was obtained from women

undergoing ovarian drilling for polycystic ovary syndrome. Blood or bone marrow (BM) cells originating from acute lymphoid leukemia (ALL) and acute myeloid leukemia (AML) patients were used for serial dilutions of leukemic cells in ovarian cell suspensions obtained from healthy ovarian cortex. Then, cryopreserved cortical strips from 11 leukemia patients were used for ORD assessment. The use of human ovarian tissue for this study was approved by the Clinical Ethics Committee of Besançon University Hospital (5 March 2013). All patients gave written informed consent. MFC was performed using a FACSCanto II™ flow cytometer and FACSDiva™ software version 6.1.3 (BD Biosciences, San Jose, CA, USA). The same combination of eight monoclonal antibodies was applied to leukemic cells alone, to different leukemic cell dilutions in healthy ovarian cell suspension, and to leukemic patient-ovarian cell suspension. MFC gating strategies have been described previously [14]. Briefly, a gating strategy is based on

the elimination of debris using forward (FSC) and side (SSC) light scatter characteristics. Nucleated viable cells are then selected (SYTO13⁺/7-AAD⁻) and used to quantify the ORD level. Within these cells, we identified CD45⁺ hematopoietic cells and CD3⁺ T lymphocytes. Four additional antibodies were used to identify leukemic cells by their specific LAIP. The sensitivity of the ORD detection method was obtained by 10-fold serial dilutions of leukemia cells in healthy ovarian cell suspensions (10^{-1} to 10^{-5}). Acquisition of at least 200,000 viable ovarian cells was performed by MFC for ORD assessment. A significant abnormal cell population was defined as a homogeneous cluster of at least 20 LAIP⁺ cells, referring to common cutoff level used for minimal residual disease assessment in hematology [9]. Molecular quantification of leukemia transcripts, *NPM1* mutation A (for AML) and *BCR-ABL1* (for B-ALL), was performed as previously described [13, 14]. The quantitative expression of mRNA was estimated thanks to standard *ABL1*.

Fig. 1 **a–c** Serial dilutions of leukemic cells among ovarian cell suspensions detected by MFC and RT-qPCR. **a** Modelization results for ALL (in green), AML with hematological panel (in red), and AML with adapted panel (in blue). The X-axis represents the theoretical values of MRD dilutions and the Y-axis the experimental MRD values of the same samples quantified by multicolor flow cytometry. For each dilution level, the standard deviation is represented by a vertical bar. Axes are in log scale. **b** Quantification of AML cells in an ovarian cell dilution experiment. Only living nucleated cells (SYTO13⁺/7-AAD⁻) which are

CD45^{low} are represented. A MRD-positive event must be contained in gates P3 (CD11b vs CD33), P4 (CD11b vs CD43), and P5 (CD15 vs CD33) (represented in black). Living nucleated ovarian cells are indicated in pink. **c** Correlation of MRD levels detected by MFC and RT-qPCR. The correlation between *BCR-ABL1* transcripts and MFC is represented in green and between *NPM1* variant A mutations and MFC in blue. Axes are in log scale. MFC multicolor flow cytometry, RT-qPCR real-time quantitative PCR, ALL acute lymphoblastic leukemia, AML acute myeloid leukemia, MRD minimal residual disease

Markers used routinely for acute leukemia minimal residual disease (MRD) detection by MFC in blood or BM were applied to abnormal cell detection on our experimental model of blood or BM leukemic cell dilutions (10^{-1} to 10^{-5}) within healthy ovarian cell suspensions. We observed a positive correlation between theoretical and experimental values for ALL ($r=0.9597$, $p<0.0001$, $n=45$) contrary to AML (Fig. 1a). Indeed, for AML, the markers used in hematology did not permit us to achieve a sensitivity of 10^{-4} , because of a background noise due to the expression of CD13, CD14, or HLA DR on ovarian cells. That is why we have used other potentially relevant markers (such as CD43 and CD361) for AML cell detection in serial dilutions and then for ORD detection in AML patients. For serial dilutions, a similar correlation ($r=0.97$, $p<0.0001$, $n=37$) was observed as for ALL (Fig. 1a). A MFC result is presented in Fig. 1b: for each dilution level, a number of MRD events were identified (represented in black) among healthy ovarian single cell suspension (represented in pink) which confers a MRD level. Molecular ORD assessment was performed in dilution experiments using leukemic cells originating from patients carrying *BCR-ABL* (B-ALL) or *NPM1 mutation A* (AML). On analysis of 24 dilution points by both MFC and RT-qPCR for each type of acute leukemia, a majority of these points are positive ($>1 \times 10^{-4}$) using both techniques with a correct correlation ($r=0.95$ for ALL, $r=0.96$ for AML, Fig. 1c). We investigated ORD assessment in cryopreserved ovarian tissue for 11 acute leukemia patients: 7 ALL and 4 AML (Fig. 2). ORD by MFC was performed for 11 patients (100 %), while only 2 patients had a molecular marker for ORD evaluation by RT-qPCR (18 %). Among these 11 patients, ORD results were positive by MFC for one T-ALL patient and two AML patients (27 %) but were negative for the

others ($n=8$, 73 %). For the two patients with molecular marker, ORD results obtained by RT-qPCR were negative, confirming the negative ORD results obtained by MFC.

This report clearly shows that ORD assessment by MFC in the case of acute leukemia is a promising technique to evaluate the presence or absence of residual leukemic cells in ovarian tissue. For acute leukemia patients (mostly children), ovarian tissue cryopreservation is the only fertility preservation option possible because some of these patients are of prepubescent age and/or patients require no delayed cytotoxic treatment. However, there is a legitimate risk of ovarian infiltration by leukemic cells which could lead to the recurrence of the leukemia after ovarian tissue autograft. Some studies have investigated ORD detection by RT-qPCR in acute leukemia patients [11, 12, 10]. Indeed, immunochemistry results have showed that this technique is unable to detect a few leukemic cells in the ovarian cortex [11, 12, 10]. Rosendahl et al. practiced ORD detection on ovarian tissue from 13 ALL patients [11]. Three of them had a molecular marker for ORD assessment, and one patient was positive by RT-qPCR. This study was complemented in 2012 by Greve et al. with three ALL supplementary patients [10]. One patient had molecular marker for ORD assessment, and ovarian tissue analysis was negative. Finally, 16 ALL patients were included in these two complementary studies, 4 of them had a molecular marker suitable for ORD assessment (25 %) and one of them was positive by RT-qPCR (25 %). For AML patients, 10 patients were included in these studies and only 1 patient had a molecular marker (10 %) and they were positive by RT-qPCR (100 %). Dolmans et al. also evaluated the presence of acute leukemic cells in cryopreserved ovarian tissue by RT-qPCR [12]. PCR analysis was positive for 7 of the 10 ALL patients with available molecular

Fig. 2 Schematic representation of ORD results obtained by multicolor flow cytometry and quantitative PCR for 11 acute leukemia patients

marker (70 %) among the 12 ALL patients included in the study. The PCR technique was finally only applied in 15 of the 38 patients (39 %) with acute leukemia in these different studies. At the same time as PCR analysis, the Danish and Belgian teams performed subcutaneous xenotransplantations of ovarian cortex into SCID [12] or nude [10] mice. For the Belgian's study, four mice showed macroscopic proliferation and another mouse presented a microscopic invasion. On the contrary, none of the mice xenotransplanted with ovarian cortex from acute leukemia patients showed signs of disease in the Danish study, although the ovarian cortex showed a positive RT-qPCR result in two of the four acute leukemia patients tested (three ALL and one AML). So, maybe ovarian tissue xenograft alone is not the best model for ORD detection but is the only available method to evaluate the potential development of leukemic cells in vivo.

We describe in this short report a new MFC method to quantify live ALL and AML cells in the ovarian cortex. Experimental models of leukemic cell dilutions in an ovarian single cell suspension enabled a robust sensitivity of 10^{-4} for ALL and AML to be validated. It is very important to determine the best antibody combination in order to reach a sensitivity of 10^{-4} (as seen for AML results), in order to obtain a similar sensitivity observed for MRD in blood or bone marrow. A positive correlation was observed between MFC and RT-qPCR results for ALL and AML. This MFC method was then applied to 11 acute leukemia patients (7 ALL and 4 AML), and ORD assessment was positive for 3 cases (27 %). However, we do not know if the ORD level detected can induce a relapse after ovarian tissue autograft. Two other methods are in development to avoid reintroduction of malignant cells with ovarian tissue autograft: in vitro folliculogenesis [15] and isolated ovarian follicle graft [16, 17].

In conclusion, this MFC technique can be applied to all ALL and AML patients and is currently the only available technique for ORD when no molecular markers are available [13, 14]. This technique is essential to be used alone or associated with PCR and/or xenograft to evaluate the risk of cancer re-seeding which will encourage cryopreserved ovarian cortical strips to be re-use by autograft.

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