Occasional involvement of the ovary in Ewing sarcoma

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BACKGROUND: Ewing sarcoma (EWS) is a highly metastatic malignancy in young patients. Ovarian cryopreservation is often an option for fertility preservation in cancer patients of reproductive age, specifically in minors. Thus, the possibility of ovarian involvement in EWS needs to be elucidated.

METHODS: Eight patients aged 13–20 years with EWS participated in the study. Ovarian samples were fixed and prepared for light microscopy, and frozen in liquid nitrogen for RNA extraction followed by RT–PCR. Histological studies, including immunostaining for the adhesion receptor CD99, were used to detect histopathological features. Sensitive molecular methods were used to detect translocations causing the formation of tumor-specific EWS–Friend leukemia virus integration site 1 fusion gene (EWS-FLI1).

RESULTS: In seven patients, there was no evidence of EWS in the ovaries from pathological/molecular studies. However, in one patient, the RT–PCR showed the EWS translocation, although there was no pathological evidence.

CONCLUSIONS: Ovarian involvement is possible in EWS. Therefore, in patients with EWS ovarian tissue should be examined for traces of malignancy at both the pathological and molecular levels prior to the grafting of cryopreserved tissue in order to minimize the risk of reseeding the cancer.

Key words: Ewing sarcoma / ovarian cryopreservation / ovarian metastasis / pathological markers / molecular markers

Introduction

Ewing sarcoma (EWS) is the second most common bone malignancy in children, adolescents and young adults and accounts for 8% of all pediatric malignancies (De Alava and Gerald, 2000; Athale et al., 2001; Carvajal and Meyers, 2005; Ludwig, 2008). The annual reported frequency in western countries is 1–3 per million for all age groups (Carvajal and Meyers, 2005; Riggi and Stamenkovic, 2007; Ludwig, 2008), and 5 per million at the onset of puberty (De Alava and Gerald, 2000; Carvajal and Meyers, 2005; Ludwig, 2008). In about 60% of cases, the primary tumor originates in bony central-axis sites, predominantly the diaphyseal regions of the long bones (Kovar, 1998; Carvajal and Meyers, 2005; Lazar et al., 2006; Riggi and Stamenkovic, 2007; Ludwig, 2008). The cytoplasm typically contains glycogen and diastase-degradable granules. Histological features include small round hyperchromic nuclei and inconspicuous nuclei with evenly distributed chromatin and little mitotic activity (De Alava and Gerald, 2000; Riggi and Stamenkovic, 2007). Because these findings are non-specific, immunohistochemistry is frequently required for the differential diagnosis (De Alava and Gerald, 2000; Lazar et al., 2006; Riggi and Stamenkovic, 2007). In more than 90% of cases, EWS cells express the adhesion receptor CD99 (p30/p32MIC2), a 32-kDa cell surface glycoprotein encoded by the MIC2 gene. However, CD99 is not a specific marker for EWS as it is often also expressed in lymphoid cells of many normal tissues.

Therefore, in some patients, an accurate diagnosis cannot be obtained from pathological studies and specific genetic alterations are sought (De Alava and Gerald, 2000). RNA-based RT–PCR or densely packed sheets usually with extensive necrosis and lacking intercellular material (De Alava and Gerald, 2000; Lazar et al., 2006; Ludwig, 2008). The cytoplasm typically contains glycogen and diastase-degradable granules. Histological features include small round hyperchromic nuclei and inconspicuous nuclei with evenly distributed chromatin and little mitotic activity (De Alava and Gerald, 2000; Riggi and Stamenkovic, 2007). Because these findings are non-specific, immunohistochemistry is frequently required for the differential diagnosis (De Alava and Gerald, 2000; Lazar et al., 2006; Riggi and Stamenkovic, 2007). In more than 90% of cases, EWS cells express the adhesion receptor CD99 (p30/p32MIC2), a 32-kDa cell surface glycoprotein encoded by the MIC2 gene. However, CD99 is not a specific marker for EWS as it is often also expressed in lymphoid cells of many normal tissues.

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Therefore, in some patients, an accurate diagnosis cannot be obtained from pathological studies and specific genetic alterations are sought (De Alava and Gerald, 2000). RNA-based RT–PCR or
cytogenetics and fluorescence in situ hybridization may be used to identify tumor-specific chromosomal translocations that result in the formation of fusion genes and their protein products. In 85% of EWS cases, there is a translocation between chromosomes 11 and 22 (q24; q12), which results in the formation of the EWS–Friend leukemia virus integration site 1 (FLI1) fusion gene (Athale et al., 2001; Carvajal and Meyers, 2005; Riggi and Stamnenkovic, 2007; Ludwig, 2008). The most common functional EWS–FLI1 transcript is formed by fusion of EWS exon 7 with FLI1 exon 6 (type I fusion) (Carvajal and Meyers, 2005). Fusion transcripts serve as extremely sensitive and efficient markers of micrometastasis and minimal residual disease (Ludwig, 2008). Tumor cells can be detected at low levels even among normal cells, such as circulating cells in peripheral blood (PBL) and bone marrow (Kovar, 1998; De Alava and Gerald, 2000; Athale et al., 2001; Avigad et al., 2004; Ludwig, 2008).

With improvements in anticancer treatment, the survival rate of cancer patients is increasing (Feigin et al., 2008). However, therapy can cause premature ovarian failure in girls and women. The options for fertility preservation are currently limited; in many cases the only possibility is cryopreservation of ovarian tissue containing small immature follicles. So far, autologous transplantation of cryopreserved-thawed ovarian tissue in cancer survivors has resulted in 12 pregnancies, including seven live births (Meirow et al., 2008; Sanchez-Serrano et al., 2009; von Wolff et al., 2009; Ernst et al., 2010), two to an EWS survivor (Andersen et al., 2008; Ernst et al., 2010).

In some cancers, such as hematological malignancies and breast cancer, implantation of ovarian tissue following cryopreservation carries a risk of reseeding the malignancy (Meirow et al., 2005; Demmeestere et al., 2007; Andersen et al., 2008; Feigin et al., 2008; Sanchez-Serrano et al., 2009; von Wolff et al., 2009; Ernst et al., 2010), two to an EWS survivor (Andersen et al., 2008; Ernst et al., 2010).

The aim of the present study was to determine if ovarian grafting for fertility restoration in survivors of EWS poses a danger of reseeding the cancer. Specifically, we examined ovarian samples from patients with EWS for traces of malignancy at both the pathological and molecular levels (Ludwig, 2008).

**Materials and Methods**

**Patients and ovarian material**

From 2000 to 2009, we collected ovarian samples from eight female patients with EWS during cryopreservation for fertility preservation (Feigin et al., 2008) (Table I). All the patients were referred to the infertility and IVF Unit, Helen Schneider Hospital for Women, Rabin Medical Center, Beilinson Hospital from the Department of Pediatric Hematology Oncology, Schneider Children’s Medical Center of Israel. Patient details are given in Table I. In this and the following sections, patients are identified by their serial numbers in Table I. Patient age ranged from 13 to 20 years (mean ± SD 15.3 ± 2.5). Ovarian tissue was frozen after initiation of chemotherapy in three patients (patients 1, 4, 6), and before initiation of any form of anticancer therapy in five patients (patients 2, 3, 5, 7, 8). Hysterectomy and bilateral oophorectomy were performed in one patient with uterine EWS (patient 4), and partial unilateral oophorectomy was performed in the remainder (patients 1–3, 5–8). Two patients (patients 2, 8) died of the malignancy. The Ethics Committee of Rabin Medical Center approved the study protocol, and written consent was obtained from every adult patient or the parents of all minors.

One portion of every ovarian sample (except from patient 6 whose tissue was not sent for pathological examination) was cut as uniform in size as possible (≏2 × 2 mm²) and fixed immediately in 4% formalin (Gadot, Binyamina, Israel) for light microscopy (LM). A second portion was placed in a cryogenic vial (Naïge Nunc International, Roskilde, Denmark) and immediately plunged into liquid nitrogen for subsequent RNA extraction.

**Table I** Details of the patients with EWS who were undergoing ovary cryopreservation for fertility preservation.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (yrs)</th>
<th>Treatment</th>
<th>Follicles/section</th>
<th>Ovarian pathology &amp; IMH (CD99)</th>
<th><strong>RT–PCR PBL</strong></th>
<th><strong>RT–PCR ovary</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13</td>
<td>After 6 cycles: ADR, VP16, I-FOS, ACTD, C, VCR + thoracic radiation</td>
<td>91</td>
<td>Normal</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>Before</td>
<td>101</td>
<td>Normal</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>Before</td>
<td>53</td>
<td>Normal</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
<td>After VCAIE</td>
<td>100</td>
<td>Normal</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td>Before</td>
<td>14</td>
<td>Normal</td>
<td>Positive</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>2 courses: VCR, D, C, E, I-Fos</td>
<td>unknown</td>
<td>Unknown</td>
<td>Negative</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>17</td>
<td>Before</td>
<td>1</td>
<td>Normal</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>Before</td>
<td>22</td>
<td>Normal</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

IMH, immunohistochemistry; ‘–’, molecular study not conducted; PBL, peripheral blood; ADR, adriamycin; C, cyclophosphamide; D, doxurobicin; E, etopside; I-Fos, I-fosphamide; VP16, etopside; I-FOS, I-fosphamide; ACTD, adriamycin, cyclophosphamide, actinomycin D; VCR, vincristine; VCAIE, vincristine, cyclophosphamide, arabinosidecytosine, idrarubucin.

*At time of ovary cryopreservation.

**for EWS–FLI1 fusion transcript.**
Histological preparation for LM
The histological preparation method has been described in detail previously (Abir et al., 2008; Farhi et al., 2009). These specimens were examined in the Department of Pathology for EWS-related pathological changes, and the number of follicles per section was counted (follicles were counted in one section per patient), as described by us previously (Abir et al., 2008; Farhi et al., 2009).

Immunohistochemistry for CD99
The immunohistochemical preparation method for unstained sections has been described in detail previously (Farhi et al., 2009). To validate the lack of pathological EWS traces in the ovaries, immunohistochemistry for CD99 was conducted in samples from all patients, except patient 6 whose ovarian tissue was not sent for pathological examination. Two sections per sample were utilized. The primary antibody was a concentrated mouse anti-CD99 antibody (Zymed Laboratories Inc., San Francisco, CA, USA; catalogue number: 18-023S). The samples were incubated with the primary antibody (diluted: 1: 100) for 45 min. The negative controls were incubated with phosphate-buffered saline at pH = 7 (Biological Industries, Beit Ha’emek, Israel). Thereafter, the samples were incubated with horseradish peroxidase (HRP) polymer conjugate against mouse, rabbit and guinea pig antibodies (SuperPicture HRP, Zymed Laboratories Inc., catalogue number: 878963). Finally, the sections were incubated with a diamino-benzidine urea H2O2 solution (Sigma Fast tablets, Sigma, St. Louis, MO, USA), and counterstained with Mayer’s hematoxylin (Pioneer Research Chemicals Ltd., Colchester, Essex, UK).

RT–PCR
Total RNA was extracted twice from every ovary from two different areas, and from PBL, with the TRI-reagent (MRC, Cincinnati, OH, USA) (Farhi et al., 2009). Unfortunately, we did not have access to the original biopsies (patients 1–5, 7, 8). In the patient with EWS in the uterus (patient 4) LM examination of the ovary sections stained with hematoxylin-and-eosin showed a few foci of cells with artifactual changes, but the CD99 immunohistochemical staining was negative. By contrast, immunohistochemical staining for CD99 was positive in all the primary tumors themselves (data not shown). The follicular counts in the ovarian sections are also given in Table I.

RT–PCR of EWS–FLI1 chimeric transcript
Ovaries from five patients were evaluated for contaminating EWS cells by identifying the EWS–FLI1 fusion transcript (Table I). RT–PCR and nested PCR were performed in all samples. One ovarian sample (patient 5) was found to be positive for the chimeric transcript EWS–FLI1 already in the RT–PCR reaction, before the nested PCR reaction (Fig. 1). The ovarian sample from the patient with EWS in the uterus (patient 4) was negative.

Three PBL samples that were obtained at the time of ovarian surgery (patients 2, 4, 7) were also subjected to RT–PCR analysis (Table I). The sample from patient 2 was positive for EWS–FLI1, but no evidence of malignancy was detected in the ovary.

Discussion
The present study provides evidence that in certain cases of EWS, the ovary is also involved in the malignancy. Therefore, there is a risk of reseeding the cancer after ovarian grafting in EWS survivors. It is noteworthy, however, that we identified the ovarian malignancy only by sensitive molecular methods (RT–PCR) and not by pathological studies, suggesting minimal involvement of the ovarian tissue in the disease.

Although the ovarian sample from the patient with minimal residual disease (patient 5) most likely contained only a few malignant cells that were below the threshold for pathological identification, because the sample that was examined pathologically was derived from a different ovarian portion than the sample tested by RT–PCR, positive pathological results might have been obtained had other specimens from the same patient been tested. Similarly, given that only minimal residual disease was identified by the sensitive molecular biology methods, it is possible that positive results would have been obtained also in different ovarian portions from some of the other patients.

The clinical relevance of contaminating EWS cells in the ovary is not completely clear. Earlier studies reported contaminating tumor cells in bone marrow/PBL and harvests (bone marrow cells or stem cells before autologous transplantation) from patients with EWS. Fagnou

Results
The results are summarized in Table I.

Pathological evaluation and follicular counts
The ovarian specimens of the seven patients whose tissue was sent for examination (patients 1–5, 7, 8) were negative for malignancy on pathological evaluation. Moreover, the immunohistochemical staining for CD99 did not yield evidence of malignancy in any of the ovarian biopsies (patients 1–5, 7, 8). In the patient with EWS in the uterus (patient 4) LM examination of the ovary sections stained with hematoxylin-and-eosin showed a few foci of cells with artifactual changes, but the CD99 immunohistochemical staining was negative. By contrast, immunohistochemical staining for CD99 was positive in all the primary tumors themselves (data not shown). The follicular counts in the ovarian sections are also given in Table I.

Figure 1 RT–PCR of EWS–FLI1 in patients with EWS. PCR products were analyzed on a 2% agarose gel. PCR product was 210 base pairs. M: marker 1: SKNMC cell line–positive control 2: non-EWS tumor–negative control 3: water–negative control 4: ovary of patient 5. Positive chimeric transcript is evident already after first round of PCR.
et al. (1998) found that patients harboring occult tumor cells in bone marrow at diagnosis had a significantly poorer outcome. Accordingly, in our previous study on the clinical relevance of circulating tumor cells during follow-up, the presence of the EWS–FLI1 chimeric transcript was significantly correlated with disease progression (Avigad et al., 2004). In addition, in 10 of the 11 patients in whom the disease progressed, bone marrow and/or PBL samples were positive for the chimeric transcript before overt clinical relapse became evident. However, four patients who were positive for the chimeric transcript throughout the follow-up had no evidence of disease in the very long term. In another study, all stem cell harvests obtained from 11 patients with EWS contained contaminating EWS cells, and nine of the patients relapsed following post-transplantation remission (Yaniv et al., 2004). Nevertheless, we should keep in mind that our methods today are very sensitive, so that we were able to identify even very small numbers of tumor cells which may have no clinical relevance. At the same time, it is noteworthy that in the sole case in which EWS cells were noted in the ovary (patient 5), the positive results were obtained after the first round of PCR, indicating the presence of a larger number of contaminating cells. Interestingly, patient 2 was positive for circulating EWS cells in the PBL but her ovary was negative for malignancy: this positive PBL result was obtained after nested PCR.

The risk level for infertility after anticancer treatment for EWS is usually medium (Bath et al., 2002; Wallace et al., 2005; Feigin et al., 2008), and there are reports of spontaneous conception in EWS survivors (Sharon et al., 2001; Chihara et al., 2003; Bath et al., 2004). In one of these patients the malignancy was in the pelvis (Bath et al., 2004), similar to patient 4 in the present study. Thus, EWS of the pelvis does not necessarily pose an increased risk of involvement of the ovaries, despite their close anatomical proximity. Young age at diagnosis may have been an important factor in the spontaneous pregnancies reported in EWS survivors (Sharon et al., 2001; Chihara et al., 2003; Bath et al., 2004), as age is strongly correlated with high follicular density (Schmidt et al., 2003; Abir et al., 2008) even after anticancer therapy (Abir et al., 2008). Yet results regarding follicular density should be taken with caution as follicles are unevenly distributed throughout the human ovary (Schmidt et al., 2003).

One of our patients (patient 7) underwent ovarian cryopreservation at age 17 years. Five years later, she was amenorrheac but wanted to start a family. After both molecular and pathological studies for EWS traces in the ovarian specimens were found to be negative, her frozen-thawed ovarian tissue was transplanted into her streak ovaries, and this induced the recurrence of two menses. On two occasions transvaginal ultrasound examination identified antral follicles but attempts at egg collection were unsuccessful. An earlier study by another group described a patient in her early twenties with EWS who underwent ovarian cryopreservation (Andersen et al., 2008). Pathological studies for EWS traces in the ovarian specimens were negative. This patient became amenorrheac after treatment, and the ovarian tissue was transplanted into her streak ovaries: she became pregnant twice and gave birth to two healthy infants (Andersen et al., 2008; Ernst et al., 2010).

Hodgkin lymphoma is currently considered to be associated with an extremely low risk of ovarian metastasis (Meirow et al., 1998; Kim et al., 2001; Seshadri et al., 2006; Meirow et al., 2008; Kyono et al., 2009). In one of these studies of lymphoma patients (Kim et al., 2001) ovarian tissue was transplanted into immunodeficent mice, none of which developed malignancy. Although xenografting might possibly serve as a method for examining the malignant potential of human ovarian grafts, it is highly unlikely to be ethically accepted for clinical purposes.

Therefore, the development of sensitive molecular methods to detect minimal residual disease is essential. One study examined the ovaries of 19 of 58 patients with various hematological malignancies for the presence of minimal residual disease (Meirow et al., 2008). Findings were positive by real-time PCR in one patient with chronic myeloid leukemia (CML), despite negative pathological findings. A very recent study examined ovaries of 8 of 26 patients with various forms of leukemia for the presence of minimal residual disease (Rosenwald et al., 2010). Findings were positive by RT–PCR in six patients: four with CML, one with acute lymphoblastic leukemia and one with acute myeloid leukemia, despite negative pathological and immunohistochemical findings. Similarly, our study shows that certain patients with EWS may have traces of malignancy in the ovary. Since EWS is characterized highly metastatic (Kovar, 1998; De Alava and Gerald, 2000; Riggi and Stamenkovic, 2007; Ludwig, 2008), before considering ovarian transplantation of frozen-thawed tissue in survivors, ovarian samples from the patients should be examined by both pathological (Andersen et al., 2008) and molecular techniques. Only by combining these diagnostic methods can the potential danger of reseeding EWS be minimized.

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References


