DNA Copy Number Changes in Malignant Ovarian Germ Cell Tumors

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ABSTRACT

Malignant ovarian germ cell tumors (OGCTs) include immature teratomas (ITs), dysgerminomas (DGs), endodermal sinus tumors (ESTs), choriocarcinomas, and embryonal carcinomas. Knowledge about the genetic changes associated with malignant OGCT development is sparse. We therefore analyzed 25 OGCTs (12 DGs, 4 ESTs, and 9 ITs) for gains and losses by comparative genomic hybridization. In total, more gains than losses were observed, and the number of alterations ranged from 0–20 per tumor. The average number of changes among DGs, ESTs, and ITs was 10, 6, and 1.4, respectively. The most common changes in DGs were gains from chromosome arms 1p (33%), 6p (33%), 12p (67%), 12q (75%), 15q (42%), 20q (50%), 21q (67%), and 22q (58%); gains of the whole of chromosomes 7 (42%), 8 (42%), 17 (42%), and 19 (50%); and losses from 13q (58%). Two of three DGs with a gonadoblastoma component showed gains of 3p21 and loss of 5p, whereas none of the nine pure DGs had these changes, suggesting that they might be characteristic either of gonadoblastoma or of DG developing from a gonadoblastoma. Gain of 12p and gain from 1q were seen in three of four ESTs, whereas gains from 3p, 11q, and Xp and loss from 18q were each found in two tumors. Five of the ITs revealed changes (range, 1–4 changes/tumor), with gains from 1p, 16p, 19, and 22q each being found in two tumors. We conclude that ovarian DGs and ESTs seem to develop via the same genetic pathways that are already known for testicular germ cell tumors. On the other hand, ITs do not exhibit gain of 12p and also typically show fewer changes than other malignant OGCTs, indicating that they arise via different pathogenic mechanisms.

INTRODUCTION

OGCTs3 constitute 20% of all ovarian neoplasms (1). Most OGCTs are benign mature teratomas. Malignant OGCTs are classified into several subgroups, the most common of which, DG, is the female counterpart of testicular seminoma and comprises 1–2% of all malignant ovarian tumors (2). The other subgroups, all non-DGs, are EST or yolk sac tumor, IT, choriocarcinoma, and embryonal carcinoma, with EST and IT being the most frequent. Tumors composed of various tissue types are termed malignant mixed GCTs. OGCTs may also arise in phenotypic females with the genotype 46,XY and gonadal dysgenesis. These patients often have gonadoblastoma, from which a malignant GCT, most commonly DG, may develop (2, 3).

More than 300 benign mature teratomas of the ovary have been investigated cytogenetically, but only 4% have shown abnormal karyotypes, and only with numerical chromosome changes [reviewed in Hoffner et al. (4)]. Cytogenetic data on malignant OGCTs are sparse; only 50 cases [25 pediatric cases (patient ≤15 years), 24 adult cases (patient >15 years), and 1 case of unknown age] have been reported previously (Refs. 4–24; Table 1). Thirteen of these tumors were karyotypically normal, 17 had only numerical changes, and 20 had structural clonal chromosomal aberrations. The most frequently observed change was gain of material from chromosome 12, most often because of extra copies of chromosome 12 and/or the occurrence of an isochromosome of the short arm of chromosome 12, i(12p). Indeed, i(12p) has been the only recurrent structural chromosomal rearrangement detected by karyotype analysis of OGCTs (Table 1).

To our knowledge, no allelotype studies on this tumor type have been reported, and only one CGH study has been published previously (7).

Because so few OGCTs have been characterized genetically, we decided to examine a series of archival OGCTs using CGH, a molecular cytogenetic technique that allows screening of the entire genome for gains and losses, thereby providing a copy number karyotype for each tumor (25).

MATERIALS AND METHODS

Patients and Tumor Samples. The tumor material consisted of 25 formalin-fixed and paraffin-embedded samples of OGCT. The specimens were obtained from five pediatric patients (age, 11–15 years; mean age, 12.4 years) and 20 adult patients (age, 16–70 years; mean age, 29.5 years). The patients had been admitted to 13 different Norwegian hospitals for primary surgery and were subsequently referred to the Department of Gynecological Oncology at the Norwegian Radium Hospital for adjuvant treatment. The tumors were classified according to WHO recommendations (26) and included 12 DGs, 3 ESTs, 8 ITs, and 2 mixed GCTs (IT/EST; Table 2). To obtain specimens of tumor parenchyma with minimal admixture of stromal elements, H&E-stained sections from several blocks were reevaluated by a pathologist. From each case, the block with the purest tumor tissue and the least presence of normal cells (visually estimated to be <25%) was selected for sampling.

From three patients who had both DG and gonadoblastoma, (cases 4T, 9T, and 40T), only pure DG tumor components were analyzed, whereas from two patients with mixed OGCT (cases 36T and 45T), only the IT and EST tumor components were analyzed, respectively.

DNA Extraction and Measurement. DNA extraction of the 10 × 10- to 20 × 5-μm tumor sections was performed using a modification of the procedure described by Miller et al. (27). Briefly, deparaffinization of the tissue sections was performed four times by xylol treatment at 55°C for 10 min. The tissue sections were then repeatedly dehydrated (four times) in absolute ethanol, and 25% for 10 min. After removal of the ethanol, the pellet was dried at 55°C for 10 min. The control and reference DNA from a healthy female donor was used as reference DNA for the OGCT. DNA from peripheral blood lymphocytes from a healthy female donor was used as reference DNA for the OGCT. DNA from peripheral blood lymphocytes from a healthy female donor was used as reference DNA for the OGCT. DNA from peripheral blood lymphocytes from a healthy female donor was used as reference DNA for the OGCT. DNA from peripheral blood lymphocytes from a healthy female donor was used as reference DNA for the OGCT.
CGH study of malignant ovarian germ cell tumors

The DNA concentration of each sample was measured in a 1 × 10⁻⁴ mg/ml Hoechst solution (Hoechst 33258) with a fluorometer (TKO 100; Hoefer Scientific Instruments, San Francisco, CA).

CGH. The principal CGH procedure of Kallioniemi et al. (25) was used, with the modifications described by el-Rifai et al. (29). Test and reference DNAs were labeled with two different fluorochromes in a standard nick-translation reaction, and a mixture of two fluorochrome-conjugated nucleotides (New England Nuclear, Boston, MA; FITC-12-dUTP and FTC-12-dUTP for tumor DNA and Texas Red-6-dCTP and Texas Red-6-dUTP for normal DNA) was used to improve the labeling of GC-rich areas in the genome. The amount of DNase I (Roche Molecular Biochemicals, Mannheim, Germany) and DNA polymerase I (Life Technologies, Rockville, MD) and the amount of time used for nick translation were adjusted for each sample to achieve DNA fragment lengths of 500-2000 bp. This was accomplished by evaluating the degree of genomic DNA degradation in gel electrophoresis before nick translation, and the size of the fragments was then confirmed by a second gel electrophoresis after cutting. Equal amounts (1 μg) of labeled tumor and reference DNA were mixed with 20 μg of Cot-1 DNA (Life Technologies, Inc.), ethanol-precipitated, dried, and dissolved in hybridization buffer (Vysis, Downers Grove, IL). After denaturation, the DNA was hybridized to normal...
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Table 2 Summary of gains and losses detected by CGH in the 25 OGCTs

<table>
<thead>
<tr>
<th>Case no</th>
<th>Age (yrs)</th>
<th>Diagnosis</th>
<th>Component analyzed</th>
<th>Gains</th>
<th>Losses</th>
</tr>
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<tbody>
<tr>
<td>04T</td>
<td>18</td>
<td>DG/GB</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>09T</td>
<td>59</td>
<td>DG/GB</td>
<td>1p31-ter, 1q22-24, 3p14-q21, 3q21-23, 6, 7, 12, 16, 19, 20, 22, 24q</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14T</td>
<td>14</td>
<td>DG</td>
<td>1p33-ter, 6p21-21.3, 8p11-ter, 11q12-13, 12q24.1-ter, 14q31-ter, 16p12-ter, 16q23-ter, 17, 19, 20q, 21q22, 22q</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15T</td>
<td>17</td>
<td>DG</td>
<td>1q21-23, 7, 8, 9q33-ter, 1p2ter-q11, 17q24-ter, 20q12-ter, 21q22</td>
<td></td>
<td></td>
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<td>20</td>
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<td>32</td>
<td>DG</td>
<td>8, 12p</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27T</td>
<td>36</td>
<td>DG</td>
<td>1p33-ter, 6p21.3, 9q33-ter, 1p2ter-q11, 17q24-ter, 20q12-ter, 21q22</td>
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<td>35T</td>
<td>18</td>
<td>DG</td>
<td>1p2ter-q24, 1qter-q2, 7, 8, 12q23-ter, 15q, 17, 19q, 21q, 22q</td>
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<td>40T</td>
<td>21</td>
<td>DG/GB</td>
<td>3, 2p12-26.3, 6p11-21.3, 7, 8, 12, 15q, 19, 21q22</td>
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<td></td>
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<td>41</td>
<td>DG</td>
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<td>49T</td>
<td>28</td>
<td>DG</td>
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<td>52T</td>
<td>32</td>
<td>DG</td>
<td>2q12-13, 4p15.1-15.3, 8p12, 12q23-ter, 19q, 20, 21q22, 22q13</td>
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<td></td>
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<tr>
<td>33T</td>
<td>11</td>
<td>EST</td>
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<td>12</td>
<td>EST</td>
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<tr>
<td>45T</td>
<td>20</td>
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<td>3, 11, Xp</td>
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<td>55T</td>
<td>22</td>
<td>EST</td>
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<td></td>
</tr>
<tr>
<td>44T</td>
<td>11</td>
<td>IT</td>
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<td></td>
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<td>46T</td>
<td>15</td>
<td>IT</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>51T</td>
<td>25</td>
<td>IT</td>
<td>16p12-ter, 17q24-ter, 19</td>
<td></td>
<td></td>
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</table>

a DG/GB, the patient had DG and GB; Mixed IT/EST, the patient had IT and EST.
b DG, dysgerminoma; EST, endodermal sinus tumor; GB, gonadoblastoma; IT, immature teratoma.

denatured metaphase spreads and incubated for 2–3 days at 37°C. Finally, the slides were washed and mounted in Vectashield (Vector Laboratories, Burlingame, CA), an antifade counterstain solution with DAPI.

Microscopy and Analysis. Good metaphase spreads were selected and evaluated microscopically for each fluorescent dye, and single-color images (FITC, Texas Red, and DAPI) were sequentially acquired with a Cohu 4900 microscope with an automated filter wheel (Zeiss Axioplan, Oberkochen, Germany) using Cytovision software and hardware (Applied Imaging, Newcastle, United Kingdom). Chromosomes were identified based on their inverted DAPI banding pattern. The background fluorescence was subtracted, and the green and red signals of each metaphase were normalized to 1.0. The tumor and reference fluorescence profiles for each chromosome were obtained by integrating the corrected pixel values along slices orthogonal to the chromosome axis. Thereafter, ratio profiles (green:red fluorescence) were calculated for each chromosome, and data from at least 13 representative copies of each chromosome (range, 13–23 representative copies) were combined to generate average ratio profiles with 95% confidence intervals. CGH results were controlled by performing two independent analyses for each fluorescent dye, and single-color images were generally rare, loss from chromosome arm 13q was seen in 58% of the cases.

Thresholds. Upper and lower threshold values of 1.25 and 0.75, respectively, were used to interpret the gains and losses of DNA sequences. The theoretical reasoning for the use of these cutoff values is that they correspond to the gain or loss of one chromosome homologue in 50% of the cells analyzed, given a diploid tumor. To detect the gain of one homologue present in 50% of the cells, a one-dimensional plot of the ratio profile was used. The two-dimensional ratio profile was used to identify areas with gains or losses along a given chromosome arm, which were not accepted as informative with regard to defining SRO borders.

SRO. At least two tumors were required to identify each of the two borders of a SRO of chromosomal gain or loss, i.e., only two tumors were required if the region of gain or loss was completely the same in those two tumors. If the region of gain or loss did not exactly overlap in the two tumors, one or two additional tumors were necessary to define the SRO, ensuring that at least two tumors define each border. Tumors showing a harlequin pattern, i.e., two or more areas with gains or losses along a given chromosome arm, were not accepted as informative with regard to defining SRO borders.

RESULTS

The regions of gains and losses detected by CGH in the 25 OGCTs are summarized in Table 2 and Fig. 1. The most common chromosomal imbalances are illustrated in Fig. 2.

DGs. All 12 DGs showed chromosome alterations by CGH, and the average number of changes was 10 changes/case (range, 1–20 changes/case). More gains than losses were observed. The most common changes were gains from 12p (8 of 12 tumors; 67%), 14q (9 of 12 tumors; 75%), and 21q (8 of 12 tumors; 67%). Other chromosome arms frequently showing partial or complete gain were 1p (4 of 12 tumors), 6p (4 of 12 tumors), 15q (5 of 12 tumors), 20q (6 of 12 tumors), and 22q (7 of 12 tumors). Common gains of whole chromosome copies were seen for chromosome 19 (6 of 12 tumors) and chromosomes 7, 8, and 17 (5 of the 12 DGs each). Although losses were generally rare, loss from chromosome arm 13q was seen in 58% (7 of 12 tumors) of the cases.

ESTs. The four ESTs all revealed copy number changes (average, 6 copy number changes/case; range, 3–12 copy number changes/case). Gain of 12p and gain from 1q were seen in three of four ESTs, whereas gains from 3p, 11q, and Xp and loss from 18q were each found in two tumors.

ITs. Compared with the DGs and ESTs, fewer changes were detected in the nine ITs (average, 1.4 changes/case; range, 0–4 changes/case). No changes were observed in four of the ITs (12T, 22T, 44T, and 46T). Gain of all or parts of 1p, 16p, 19, and 22q was each detected in two of five ITs with changes, whereas the other alterations were detected in one tumor each (Table 1).

V. M. Abeler, J. Kærn, and C. G. Tropé, unpublished observations.
SRO in OGCT. Among the 13 DGs and ESTs with gains from chromosome 12, 2 tumors (40T and 49T; both DGs) showed gain of the entire chromosome 12, whereas 11 tumors (69%) showed gain of parts of chromosome 12 (9T, 14T, 15T, 16T, 19T, 33T, 35T, 43T, 48T, 52T, and 55T), with 12p and 12q23-ter as minimal common regions of gain. Nine additional SROs were found, corresponding to gains of 1p35-ter, 3p21, 9q33-ter, 11q12–13, 16p12-ter, 20q, 21q22, and 22q13 and loss of 13q21–22.

DISCUSSION

Artificial copy number changes in CGH experiments have been observed at 1p33-ter, 16p, 17p, 19, and 22 (29, 31). In this study, we applied a modification of the original CGH protocol using a mixture of fluorochrome-labeled nucleotides (fluorochrome-dUTP and -dCTP) during nick translation (25, 29), which ensures a reduction of false positive signals in these “problem areas.” The strict threshold levels chosen for scoring gains (1.25) and losses (0.75) should further reduce the risk of overinterpretation. Furthermore, the facts that the analysis of each tumor profile was performed relative to the profile of the negative control in the same experiment and that repeated analyses of selected tumors gave the same results speak strongly in favor of the view that the detected 1p, 16p, 17p, 19p, and 22 gains in OGCTs are real. The CGH detection level is determined by the degree of amplification and the size of the amplicon or deletion, as well as the

Fig. 1. Schematic diagram of the gains (bars to the right) and losses (bars to the left) identified in the 21 OGCTs showing aberrations.

Fig. 2. Chromosome regions commonly altered in OGCTs. Color images of the chromosomes and CGH ratio profiles are shown. a, examples of common alterations detected in DGs and ESTs. Tumor 48T (far left) showed gain of the entire chromosome 8 and of 12pter-q13 and 12q23-qter, tumor 15T showed gain of 21q, and tumor 40T showed loss from 13q. b, examples of common alterations detected in ITs. Tumor 34T showed gains from 1p and 22q.
percentage of tumor cells containing the alteration in question. With the precautions taken in the present study, it is more likely that some alterations may have remained unrecognized than that false positives were accepted.

Very little is known about the genetic alterations behind germ cell tumorigenesis in the ovaries. Since 1982, 20 reports describing cytogenetic banding analysis of a total of 50 OGCTs have been published (Table 1). Thirteen tumors revealed normal karyotypes, whereas 37 had clonal chromosome aberrations. Among the tumors with structural changes (n = 20), i(12p) was present in DGs (4 of 4 tumors), ESTs (1 of 2 tumors), mixed GCTs (4 of 10 tumors), and in a metastasis with mature teratoma differentiation from an IT, but not in any of the 3 pure ITs. However, gain of the entire chromosome 12 was reported in two ITs (10, 14). In the present CGH study, gain of chromosome 12 or 12p sequences was found in the majority of DGs and ESTs (70%). The observed gain of 12p may reflect the presence of an i(12p) but might also be the result of other alterations. Because some of the tumors with gain from chromosome 12 have gain of 12p only, whereas others have gain of 12q only (Fig. 1), it is reasonable to suppose that two target areas exist along this chromosome. Gain of 12p was also reported in most DGs and ESTs in the recent CGH study by Riopel et al. (7). In summary, our findings and those of Riopel et al. (7) suggest that gain of 12p is equally frequent in adult and pediatric cases: in our series, 9 of 11 tumors with 12p gain were from patients older than 15 years, whereas 10 of 14 tumors with 12p gain reported by Riopel et al. (7) were from patients younger than 15 years. Finally, none of these two CGH studies has shown gain of 12p in IT.

Gains of chromosome 12 or i(12p) are not restricted to GCTs but have also been observed in different types of benign ovarian tumors (32–36), as well as in carcinomas (37–40) and in one mixed mesodermal tumor of the ovary (5). The pathogenetically essential outcome of the gain of chromosome 12 and i(12p) remains unknown.

Other recurrent imbalances found in more than 30% of the DGs or in at least two of the four ESTs studied were gain of all or parts of 1p, 1q, 3p, 6p, 7, 8, 11q, 12q, 15q, 17, 19, 20q, 21q, and 22q and losses from 13q and 18q. Gains from 1q, 3p, 6p, 7, 8, 15, 17, 20, 21, 22, and Xp and losses from 13q and 18q have been described previously in more than two ESTs, DGs, and/or mixed GCTs (Table 1; Ref. 7). To our knowledge, the gains at 1p, 11q, and 12q and the gain of chromosome 19 that we have seen have not previously been associated with OGCTs and suggest novel target areas in the development of these tumors. The most frequent change in DGs and ESTs was gain of 12p. Gains from 12q and 21q and loss of 13q were found in DGs at comparable frequencies, with 12q23–qter, 21q, and 13q21–22 being identified as SROs. The target genes affected by these changes remain unknown.

Three DGs (4T, 9T, and 40T) were from patients who had gonadoblastoma together with DG. In tumor 4T, the only change was loss of copy number of chromosome X, probably reflecting the known constitutional XY karyotype of this patient. The tumor DNA (XY) was hybridized with normal female reference DNA (XX) onto normal (XX) metaphase chromosomes. Tumors 9T and 40T, on the other hand, showed several acquired genetic changes that in both tumors included gains of parts of chromosomes 3, 6, 7, 12, 15, 19, and 20 and losses from chromosomes 4, 5, and 13. None of the other DGs (zero of nine DGs) showed gain of 3p21 or loss of 5p, suggesting that these changes might be specifically associated with gonadoblastoma or DG originating from a gonadoblastoma. We do not know the constitutional genotypes of these cases, but the CGH data indicate an XX sex chromosome complement in case 9T and XY in case 40T.

Pure IT components showed fewer alterations than did the DGs and ESTs, with one to four changes in five of the nine ITs examined. The number of changes we detected in ITs seems to be higher than that seen in previous CGH findings (7), where gain of chromosome 14 was the only change in one of six ITs studied. On the other hand, the imbalance profiles detected by us are in agreement with published karyotypes showing aberrations in 11 of 20 ITs, 8 of which had numerical changes only, and 3 of which had both structural and numerical changes (Table 1). We did not find any changes that were specific for the IT subgroup, although 16p gain seemed to be more frequent in ITs than in other OGCTs.

A comparison of the processes of tumorigenesis for TGCT and OGCT reveals striking similarities as well as some interesting differences. An early genetic event in the genesis of TGCT is polyploidization of a dysplastic germ cell precursor, resulting in a near-tetraploid carcinoma in situ (41). Nonrandom losses and gains of chromosome regions accompany the further evolution of the carcinoma in situ into a seminoma and then into a nonseminoma, which typically are hyperplloid and hypotriplloid lesions, respectively. An isochromosome for 12p is very common in primary TGCTs, and CGH studies have also revealed recurrent gains of all or parts of chromosomes and chromosome arms 1q, 7, 8, 12, and 21 as well as losses from 13q (42–45). With the exception of gonadoblastoma, precursor lesions such as carcinoma in situ have not been found in OGCTs in patients with abnormal sexual development. However, ploidy measurements by image analysis have revealed that most DGs and ESTs have cell populations in the triploid to tetraploid range (30). In contrast, the ITs of females are usually diploid, although aneuploidy has been detected in some grade 3 lesions (30, 46). The presence of i(12p) in both male and female GCTs and the similarities in other, presumably secondary, chromosomal imbalances (+7, +8, +12, +21, and −13) and ploidy indicate that these tumors evolve through some of the same pathogenetic mechanisms in both sexes. However, it seems that ITs develop through a different pathway than that of other malignant OGCTs, inasmuch as these tumors typically are diploid and do not have i(12p) or other imbalances of chromosome 12.

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**REFERENCES**


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