Establishment and Characterization of Cancer Cell Cultures and Xenografts Derived from Primary or Metastatic Mullerian Cancers

Claire F. Verschraegen, Wei Hu, Yu Du, John Mendoza, Janet Early, Michael Deavers, Ralph S. Freedman, Robert C. Bast, Jr., Andrzej P. Kudelka, John J. Kavanagh, and Beppino C. Giovanella


ABSTRACT

Purpose: The purpose of this study was to characterize cell cultures and xenografts derived from patients with ovarian cancer.

Experimental Design: Ninety specimens from 67 patients were plated in RPMI 1640 or inoculated in nude mice. Growth characteristics of cell cultures and xenografts were determined. Expression of receptors for estrogen, progesterone, androgen, epithelial growth factor, fibroblast growth factor, HER-2/erbB-2/c-neu proto-oncogene, and the P53 expression were characterized by immunocytochemistry in 28 cell cultures.

Results: Forty-nine percent of samples were cultured successfully in vitro. Ascitic and pleural effusion specimens were more likely to produce a cell culture or a xenograft than solid tissue specimens ($P < 0.005$). All of the cell cultures had an epithelial morphology, and 89% were aneuploid with a mean DNA index of 1.6 (range, 0.9–3.0). Of 54 and 61 specimens inoculated into nude mice i.p. and s.c., 15 (28%) and 18 (30%) produced a xenograft, respectively, with two-thirds of these xenografts being reproducibly tumorigenic. The median time to first passage was 21 weeks for cell cultures and 8–12 weeks for xenografts. Expression of epithelial growth factor receptor, HER-2/erbB-2/c-neu proto-oncogene, fibroblast growth factor receptor, estrogen, progesterone, and androgen was seen in 24, 21, 31, 17, 43, and 18%, respectively. P53 was overexpressed in 62% of cell cultures analyzed.

Conclusions: Ovarian cancer cells collected from effusions are easier to grow in vitro than in vivo. The only characteristic that may be associated with tumorigenicity was abnormal P53 expression. This panel of ovarian cancer materials provides useful models for biological or therapeutic studies.

INTRODUCTION

Ovarian cancer is the fifth most frequent cause of cancer death in women and the leading gynecological cause of cancer death in the United States. Less than 20% of women with stage III ovarian cancer survive 5 years (1). Ovarian cancers vary in histological appearance, cell biology, and the tumor markers they produce. Therefore, it is important to derive cell cultures and xenografts from various ovarian cancers to serve as models for investigation of the molecular machinery of ovarian cancer cells, and for the testing of new diagnostic or therapeutic modalities in vitro, or in animal models before clinical use (2). Ovarian cancers express or overexpress various receptors for sex steroids and growth factors (3, 4). Overexpression of growth factors on ovarian cancer cells, such as epidermal growth factor and hereregulin receptors (EGFR$^1$ and HER-2), appears to correlate with poor prognosis in patients (5–8). Overexpression of FGF and its receptor may accelerate neoplastic processes such as development of metastases and neangiogenesis (9). Additionally, high-level expression of abnormal P53 has been detected in ~50% of epithelial ovarian cancer specimens (10). Therefore, we studied the ovarian cell cultures we obtained for hormonal and growth factor receptor expression, which may have a clinical potential at this time because of the commercial availability of inhibitory drugs (11–13).

We sought to determine which type of tumor material from patients diagnosed with ovarian cancers would be developing into laboratory systems and which cellular characteristics could predict this development.

MATERIALS AND METHODS

Cell Cultures. Cell cultures were established from tumor samples taken from patients with Mullerian cancers (ovarian, fallopian, or peritoneal origin), after patient consent to incidental tissue procurement was obtained according to institutional guidelines. Tumor specimens were obtained from residual spec-

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1 The abbreviations used are: EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; ER, estrogen receptor; PR, progesterone receptor; AR, androgen receptor; FGFR, fibroblast growth factor receptor.
imens collected under aseptic conditions for therapeutic or diagnostic procedures. Approximately 1 g of tumor, or 1000 ml of ascites or pleural effusion was then processed in the laboratory in the following manner. Tumor cells were isolated with scissors in a sterile manner. Dissociated tumor cells were then resuspended in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS and glutamine (2 mM).

The cells remained in culture until sufficiently confluent for a first tissue culture passage. A cell culture was considered established if it could be carried through at least five in vitro passages. However, for establishment of a cell line, at least 15 passages were required.

When enough material was available, specimens were inoculated into Swiss BALB/c nude mice i.p. and/or s.c. to obtain xenografts. All of the animals were treated in accordance with the Stehlin Foundation Animal Committee guidelines. Established cell cultures suspended in RPMI 1640 supplemented with 10% FCS at a concentration of 10^6 cells/ml were also inoculated into nude mice. A xenograft was considered established if it could be carried through at least five in vivo passages.

**Immunocytochemistry.** Cell cultures were suspended in RPMI 1640 at a concentration of 5 × 10^5 cells/ml and cytospinned. After a 1-min cytopin at 750 rpm, the slides were fixed in cold acetone for 5 min at 20°C and stored at −20°C until processing.

Anti-EGFR (clone 528) mouse monoclonal antibody, anti-FGFR rabbit polyclonal antibody (C15), and monoclonal mouse antihuman ER (clone F10) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PR mouse monoclonal antibody (clone 1A6) was obtained from Nova Castra Laboratories Ltd. (Burlingame, CA). Mouse IgG1 and IgG2a were obtained from Dako (Carpinteria, CA). Monoclonal antibody to human AR (clone F39.4.1) was obtained from BioGenex, Inc. (San Ramon, CA). Monoclonal antibodies to wild-type and mutant P53 (PAB240) were obtained from Oncogene Research Products (Cambridge, MA), and to HER-2 (AB-E2 4001) from Neomarkers (Fremont, CA). Rabbit IgG1 was obtained from Vector Laboratories (Burlingame, CA). Immunocytochemical analysis was done using the avidin-biotin immunoperoxidase method (ABC kit; Vector Laboratories). Briefly, cell line slides were air-dried for 30 min. The slides were then rinsed in phosphate buffered saline. Endogenous peroxidase activity was blocked by incubating the slides in 3% H2O2 with methanol for 15 min. The slides were washed in PBS and incubated with 1% normal horse serum for (ER, AR, PR, HER-2, and EGFR), and 1% goat serum for FGFR for 30 min at room temperature to reduce nonspecific binding, and then the slides were incubated with anti-AR monoclonal antibody (1:60 dilution), anti-ER (1:25 dilution), anti-PR (1:40 dilution) monoclonal antibody, anti-FGFR and anti-EGFR antibodies (1:500 dilution), or anti-P53 and anti-HER-2 (1:100 dilution) for 1 to 2.5 h at room temperature or overnight at 4°C. After the slides were washed three times with PBS, they were incubated with the biotinylated second antibody (1:200 dilution) for 30 min at 37°C. The slides were then washed with PBS and incubated with biotin-avidin peroxidase conjugate at a dilution of 1:50 for 30 min at room temperature. After slides were then washed with PBS, they were

<table>
<thead>
<tr>
<th>Table 1 Characteristics of donor patients (n = 67)</th>
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<tbody>
<tr>
<td>Characteristic</td>
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<td>---------------------------------</td>
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<td>mainland age</td>
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<tr>
<td>Histology</td>
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<tr>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>Clear cell tumor</td>
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<tr>
<td>Endometrioid carcinoma</td>
</tr>
<tr>
<td>MMMT</td>
</tr>
<tr>
<td>Differentiation</td>
</tr>
<tr>
<td>Moderate to low grade</td>
</tr>
</tbody>
</table>

More than one cell culture may have been obtained per patient. Numbers reported in this table reflect patient numbers. aCell lines are ≥15 passages. bCell lines are ≥10 passages. cCell lines are ≥5 passages. dMMMT, malignant mixed Mullerian tumor.

Note: Cell lines were determined to be negative for wild-type AR, No. tested, ER, No. tested, P53, No. tested. EGFR, No. tested.
visualized with 0.1% diaminobenzidine solution and then counterstained with hematoxylin for 3 min. Control cell lines were obtained from the Stehlin Foundation for Cancer Research and included a colon (MCCN), lung (DOY), melanoma (BRO), and pancreatic (PANC-1) cancer. These cell lines were used as negative controls in the studies of hormonal receptors. Normal ovarian epithelial cells were cultured in vitro and used as control for the immunochemistry experiments (14).

**Semiquantitative Measurement.** Expression of ER, PR, AR, EGFR, HER-2, FGFR, and P53 was shown by brown staining. In each case, a total of 200 cells counted in different (three to four) fields at ×400 was assessed. Isotitibodies were used to control for labeling. The results were scored by percentage of stained cells with moderate intensity or higher. The percentage of stained tumor cells was scored as follows: none, <25%, between 25% and 75%, and >75%.

**DNA Index and Cell Cycle Analysis.** The DNA index and the distribution of cells in specific phases of the cell cycle were determined by standard flow cytometry methods, using an Epics Elite Analyzer (Coulter Corp., Hialeah, FL; Ref. 15).

**Verification of Species.** s.c. xenografts were tested for the presence of human isoenzymes lactate dehydrogenase-A,B gene expression and nucleotide position gene polymorphism as described previously (16).

**Statistical Analysis.** The statistical methods used to assess the establishment of cell cultures and xenografts were descriptive. \(\chi^2\) test of association has been computed to determine whether there is a relationship between in vitro growth and origin of the specimen. Concordance rate and \(k\) values for inter-rater agreement were calculated for different types of growth between cell cultures and xenografts originating from cell cultures or patient specimens (17). ANOVA was used to calculate differences in characteristics between samples (18).

**RESULTS**

### Characteristics of Patients.

Tumor samples were collected from 67 donor patients from 1995 to 1998. Table 1 shows the characteristics of the patients, and derived cell culture and xenografts. The median patient age was 58 years (range, 35–86 years). Forty-four patients had papillary serous adenocarcinomas, 16 had adenocarcinomas otherwise not specified, 2 had clear cell carcinomas, 2 had endometrioid carcinomas, and 3 had malignant mixed Mullerian tumors with high-grade carcinoma-

### Table 2. Cell cultures and nude mouse xenografts obtained directly from patients with ovarian cancer

<table>
<thead>
<tr>
<th>Samples</th>
<th>Cell cultures</th>
<th>n passage &lt;5</th>
<th>n passage ≥5 to &lt;15</th>
<th>n passage ≥15</th>
<th>s.c. xenografts</th>
<th>i.p. xenografts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascites</td>
<td>57% (38/67)</td>
<td>8</td>
<td>20</td>
<td>10</td>
<td>33% (14/42)</td>
<td>27% (11/41)</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>75% (3/4)</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>75% (3/4)</td>
<td>75% (3/4)</td>
</tr>
<tr>
<td>Solid tissue</td>
<td>16% (3/19)</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>7% (1/15)</td>
<td>11% (1/9)</td>
</tr>
</tbody>
</table>

### Table 3. Xenografts obtained from ovarian cancer cell cultures

<table>
<thead>
<tr>
<th>Origin of cell culture</th>
<th>s.c. xenografts</th>
<th>i.p. xenografts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascites</td>
<td>27% (9/33)</td>
<td>22% (5/23)</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>0% (0/3)</td>
<td>0% (0/2)</td>
</tr>
<tr>
<td>Solid tissue</td>
<td>0% (0/2)</td>
<td>0% (0/1)</td>
</tr>
</tbody>
</table>

### Establishment of Cell Cultures and Xenografts from Patient Specimens.

Table 2 shows the rate of successful culture for specimens isolated from ascites, pleural fluid, and solid tumor tissue.

### Establishment of Cell Cultures from Ascites, Pleural Effusion, and Solid Tissue.

Of 67 specimens of ascites obtained from 57 patients, 38 cell cultures were established (rate of establishment, 57%). Another cell culture was contaminated and, therefore, discarded. Multiple specimens were obtained from 9 patients. In 1 of these cases, there was discordance in the establishment of cell culture, in that 1 specimen developed into a cell culture, and the other did not. The median time to the first cell culture passage was 21 weeks (range, 1–60 weeks). Cultures were passed a median of 8 times (range, 1–22 passages).

Four malignant pleural fluid specimens were obtained from 3 patients. A cell culture was established from 3 specimens from 2 patients (rate of establishment, 75%). The time to first passage was 6 weeks for the specimen from 1 patient, and 21 and 26 weeks for the 2 specimens from the second patient. Highest passage was 22 (range, 12–22 passages). Three different specimens (pleural effusion and ascites) were obtained from both patients. All of the specimens grew into cell cultures.

Nineteen solid tumor specimens were obtained from 18 patients during a therapeutic tumor reductive procedure. Only 3 cell cultures were established from these specimens (rate of establishment, 16%). One of the cell cultures came from a patient from whom an ascitic specimen was also obtained that yielded a cell culture as well. The time to first passage for these 3 cell cultures was 6, 26, and 30 weeks. It took 30 weeks to establish the cell culture from the solid tissue and ascitic specimen from the same patient. Highest passage was 7 (range, 3–7 passages). Nine of these patients also donated an ascitic sample, which grew in vitro in 6 cases, but only 1 of the 9 corresponding solid tumor samples developed into a cell line.

### Establishment of Xenografts.

Forty-one specimens of ascites were inoculated i.p. into nude mice. Eleven specimens from 9 patients developed into a xenograft (rate of take, 27%), and of these, 7 were established (≥5 passages; passage range, 5–25). It took a median of 8 weeks for the first in vivo passage (range, 4–47 weeks).

Forty-two specimens of ascites were inoculated s.c. Fourteen xenografts developed from specimens from 12 patients (rate of take, 33%), but only 8 were established (≥5 passages; range, 7–18 passages). The median time to the first passage was 12 weeks (range, 4–30 weeks). In 2 cases s.c. xenografts were obtained when no cell cultures could be established.

All of the pleural effusions that yielded a cell culture also developed into i.p. and s.c. xenografts in nude mice. The highest
passages were 25 and 15 for i.p. xenografts, and 3 and 18 for s.c. xenografts.

One i.p. and 1 s.c xenograft developed from 2 specimens of solid tissue that yielded a cell culture.

Establishment of Xenografts from Cell Lines. Secondary xenografts were also grown from established cell lines (Table 3). It took 1–35 weeks (median, 21 weeks) for these xenografts to develop. Except for 1 cell culture, all of the lines that grew i.p. also grew s.c. Of 23 such cell cultures inoculated i.p., 5 (22%) developed into an i.p. secondary xenograft after 8 weeks. Three of these 5 xenografts were well established (≥5 passages; range, 7–10 passages), and were derived from patients other than the ones whose original specimen became a well-established i.p. primary xenograft. In 3 of these 5 cases, a primary xenograft had developed from the original patient sample after i.p. inoculation, but not all 3 of these primary xenografts were well established.

Of 33 cell cultures inoculated s.c., 9 (27%) secondary xenografts were obtained, 4 of which were well established (≥5 passages; passage range, 5–9). Two were from specimens that did not produce a primary xenograft when the original tumor had been inoculated in the nude mouse.

Agreement between Types of Growths. Table 4 shows the concordance rates and the κ values for the measures of agreement between the growth of tumor cells (a) from ascites samples plated in vitro and inoculated in vivo; (b) as s.c. and i.p. xenografts; and (c) as xenografts from original patient specimens and from established cell cultures of the same patients.

Summary. Of 90 original specimens, 44 cell cultures (49%) were grown from 35 patients. Of these 44 cell cultures, 35 cell cultures were passed 5 times or more (range, 5–22 passages). Cell cultures were derived more frequently from effusions than from solid tumors (P < 0.005). This difference may be related to the lack of anchorage dependence of cells floating freely in effusions, perhaps because of the absence of CD44 (19). All of the ovarian cell cultures had an epithelial morphology, and most grew in a fashion reminiscent of papillary growth, with the formation of empty areas resembling cysts (Fig. 1). Of the 35 established cell cultures, 28 were studied in detail. The mean DNA index was 1.6 (range, 0.9–3.0), and 89% (25 of 28) of the cell cultures were aneuploid. The median percentage of these cells in S phase in vitro was 28% (range, 4.7–46%). There was no statistical difference in the rate of take in nude mouse by DNA index, aneuploidy, or S phase.

Xenografts grew from the specimens of 18 patients. The rate of take after i.p. and s.c. inoculations of original tumors in nude mice was 28% (15 of 54) and 30% (18 of 61), respectively, but were well established (≥5 in vivo passages) from 20% of the tested specimens (Fig. 2). All of the s.c. tumors were tested for human isoenzymes. Only the tumors of human origin (true xenografts) were kept. In 9 cases, murine tumors developed and were discarded. Once a xenograft was established, it was pos-

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Table 4  Concordance rates and kappa values (κ) between types of growth

<table>
<thead>
<tr>
<th>Type of growth</th>
<th>n</th>
<th>Both grew</th>
<th>None grew</th>
<th>Concordance rate</th>
<th>κ</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.p. Xg/Cell culture, from ascites specimens</td>
<td>41</td>
<td>11</td>
<td>13</td>
<td>59%</td>
<td>0.29</td>
</tr>
<tr>
<td>s.c. Xg/Cell culture, from ascites specimens</td>
<td>42</td>
<td>11</td>
<td>9</td>
<td>48%</td>
<td>0.09</td>
</tr>
<tr>
<td>i.p. Xg/s.c. Xg, from any specimens</td>
<td>39</td>
<td>7</td>
<td>22</td>
<td>74%</td>
<td>0.4</td>
</tr>
<tr>
<td>s.c. Xg from cell line/s.c. Xg from specimen of same patient</td>
<td>27</td>
<td>5</td>
<td>15</td>
<td>74%</td>
<td>0.42</td>
</tr>
<tr>
<td>i.p. Xg from cell line/i.p. Xg from specimen of same patient</td>
<td>13</td>
<td>2</td>
<td>8</td>
<td>77%</td>
<td>0.46</td>
</tr>
</tbody>
</table>

*a* Xg, xenograft.

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Fig. 1  Phase-contrast photomicrograph of live culture of human ovarian papillary serous carcinomas. The cells show an epithelial morphology and grow in a fashion reminiscent of papillary growth with the formation of empty areas resembling cysts.

Fig. 2  Photomicrograph of a histological section of ovarian carcinoma from nude mice xenografts.

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sible to derive a cell line from all of the grafts, typically in <2 weeks.

Expression of Cellular Receptors in the Cell Cultures Derived from Ovarian Cancers. The expression of EGFR, HER-2, FGFR, AR, ER, and P53 in the cell cultures obtained from primary or metastatic tumors was assessed by immunocytochemical staining with antibodies against each receptor and P53 (Fig. 3). Biomarkers were considered expressed if they were present on at least 25% of the cells. EGFR was expressed in 5 of 21 selected cell cultures (24%) and FGFR in 4 of 13 cultures (31%). HER-2 was expressed on the membrane of 3 of 14 cell cultures (21%). Lung cancer, melanoma, and colon cell lines were used for controls of hormonal receptor staining. Seventeen percent of cell cultures showed high staining for ER. High PR expression was observed in 43% of cell cultures and high AR expression in 18%. P53 was highly overexpressed in 13 of 21 cell cultures (62%; Table 5). In normal ovarian epithelial cells, only EGFR was highly expressed in all 6 of the controls (6). HER-2 was focally expressed in the cytoplasm in 4 of 6 controls. Expression of P53 was uncommon and weak. Expression of ER and PR was not seen.

Comparison of Expression of Cellular Receptors and P53 in Tumorigenic and Nontumorigenic Cell Lines. To investigate whether the tumorigenicity in nude mice was associated with increased expression of EGFR, FGFR, HER-2, ER, PR, AR, or P53, the expression of these cellular receptors and of P53 was determined in tumorigenic and nontumorigenic cell lines. Five of 8 (63%) tumorigenic cell cultures expressed P53, whereas only 6 of 13 (46%) nontumorigenic cell cultures expressed P53. However, because of the small sample size, there was no significant difference (P > 0.05; Table 6). ER, PR, AR, FGFR, HER-2, and EGFR were not expressed differentially in tumorigenic and nontumorigenic cell lines (Fig. 4).

DISCUSSION

The ease with which cultures derived from cancer cells can be established in vitro depends on the cancer type. For example, breast cancer cell lines are notoriously difficult to establish, and the rate of take of human breast cancer in nude mice is <10%, with 65% of these xenografts overexpressing HER-2. On the
ovarian cancers are not known to be easily grown in vitro. This could be related to the heterogeneity of early cell cultures. However, a few cell cultures were growing fast in vitro. Eleven cell cultures were passed ≥15 times in vitro, and per our definition could be called cell lines.

About 30% of the original ovarian cancer specimens were tumorogenic as shown by the production of xenografts in nude mice (Tables 2 and 3). However, only 20% could be reproducibly established (≥5 passages in vivo; Ref. 29). If a cell culture did not develop from a given specimen, the same specimen did not grow i.p., i.e., at an orthotopic site, but some did grow s.c. Therefore, the concordance between in vitro and in vivo growth was moderate for specimens growing as i.p. xenograft, and poor for the ones growing s.c. Differences in growth conditions were probably responsible for selection of different clones (30). The concordance between the development of a xenograft from a cell culture derived from a given specimen and the development of a xenograft from the same original patient specimen was better (Table 4). However, the ease of growth was not always similar for a given specimen growing in different conditions. Furthermore, specimens originating from effusion usually adapted more easily to in vitro and in vivo systems than solid tissue specimens, which may require a matrix to grow. Interestingly, multiple specimens from a same patient would usually take the same number of weeks until the first in vitro passage. Although the interpatient variability was considerable, the intrapatient variability was minimal.

Many alterations in cell cycle control occur in cancer, including the production of peptide growth factors and the overexpression of receptors, usually through the activation of oncogenes and loss of tumor suppressor genes. These anomalies are potentially useful targets for diagnostic and therapeutic strategies in clinical oncology. A number of clinical responses or stabilization of disease has been reported after the administration of hormonal agents to patients with ovarian cancer (31), and the level of ER and PR may correlate with the response to hormonal manipulation (32). Depending on the method of detection (immunostaining versus biochemical test), ERs have been found in 25–75% of all ovarian cancers (33). Whereas none of the normal controls expressed hormone receptors, 17% and 43% of our cell cultures had a high level of ER and PR, respectively. ER expression was less than expected. The lower rate of ER expression could be attributed to an in vitro selection of receptor-negative cells (34). Functional ERs may also play an important role in the prevention of clonal progression of ovarian cancer by protecting P53 from being deactivated by human double min-2 (35). ERs may not always be functional because of ER-α mutations or ER-β deletion (36, 37).

Therapeutic androgen manipulations in patients with ovarian cancer have not induced remission (38). However, AR is expressed in ovarian cancers (39) and may interact with EGFR (7, 40). Ilekis et al. (7) demonstrated that EGFR levels significantly correlated to AR levels in ovarian cancers. In our study, 18% of the cell cultures expressed high levels of AR. No correlation was found with EGFR expression, and neither AR
nor EGFR were expressed differentially in tumorigenic and nontumorigenic cell lines.

EGFR expression has been found in the majority of normal epithelial ovarian cells (6). However, overexpression of EGFR or HER-2 is associated with aggressive disease (41–45), and the FGF family promotes mitogenesis, angiogenesis, and chemotaxis, thereby playing an important role in tissue development, differentiation, and repair. High levels of expression of EGFR, HER-2, and FGFR were found in 24%, 21%, and 31% of cell cultures, respectively. Hence, drugs and/or biological response modifiers able to interfere with the growth factor-mediated control of cell proliferation may be useful in ovarian cancer therapy.

About 50% of ovarian cancers have been found to overexpress mutant P53 (46, 47), which agrees with the 62% noted in our study. We used American Type Culture Collection cell line 2774 as a positive control (25). P53-overexpressing cell cultures are yet to be determined. This panel of cell lines and xenografts may provide clinically relevant models for investigation of the biological characteristics of ovarian cancers, which in turn may lead to the discovery of new therapies for these now incurable tumors.

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REFERENCES


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