Characterization of a Human Ovarian Adenocarcinoma Line, IGROV1, in Tissue Culture and in Nude Mice

Jean Bénard, Jacqueline Da Silva, Marie-Christine De Blois, Pierre Boyer, Pierre Duvillard, Eugenia Chiric, and Guy Riou

ABSTRACT

A cell line, IGROV1, originating from an ovarian carcinoma of a 47-year-old woman was established in tissue culture and in nude mice. Maintained in monolayer cultures, IGROV1 cells exhibited a 20-h doubling time and highly tumorigenic properties. The s.c. injection of 2 × 10^6 cultured cells into nude mice gave rise to fast growing tumors, while the i.p. route induced a peritoneal carcinomatosis with ascites which killed the animals in 2 months. The epithelial morphology of IGROV1 cells was retained during in vitro and in vivo passages, as judged by both the light and the electron microscopes. Two cytogenetic markers characterize IGROV1 cells: a paracentric inversion of chromosome 3, and a translocation between chromosomes 2 and 5. The constitutional karyotype of the patient was normal. These characteristics make the IGROV1 cell line a suitable experimental model for the treatment of human ovarian carcinomas and for biological studies of human solid tumors.

INTRODUCTION

Human epithelial ovarian cancers represent a major form of gynecological carcinomas. Their treatment consists primarily of surgery and chemotherapy. Adriamycin, cyclophosphamide, and cis-dichlorodiammineplatinum are combined in most clinical types of treatment. Although considered as a chemosensitive cancer, 60% of such treatments are ineffective, leading to the search for more effective drugs (4). Therefore it seems urgent to set up experimental models which can be used for exploring new modalities of treatment or for screening new agents.

Recently a human cell line, NIH:OVCAR-3, has been proposed as a model for the study of drug-resistant, androgen and estrogen receptor-positive ovarian carcinomas (10). In this paper we report the characterization of a human ovarian carcinoma line, the IGROV1 cell line. This cell line is drug resistant and hormone receptor negative, thus constituting another class of ovarian tumoral material of clinical relevance. The IGROV1 cell line exhibits an epithelial character, highly tumorigenic properties, and a low doubling time. It grows quickly in nude mice as a solid tumor or as an i.p. ascitic carcinomatosis, killing the mice rapidly. In addition there are consistent cytogenetic markers in which oncogenic rearrangements can occur. IGROV1 is therefore proposed as a model for experimental studies of human ovarian adenocarcinoma, including molecular and cell biology, preclinical pharmacology, and experimental therapeutics.

MATERIALS AND METHODS

Origin of the Tumor

The tumor was obtained from a 47-year-old woman suffering from a stage III ovarian cancer. Prior to surgery this patient had been thought to be suffering from a cervical carcinoma and had been treated by cobalt therapy of the cervix and vagina. Surgery revealed a primary ovarian cancer extending to the cervix and rectum. A tumoral specimen was taken from the right ovary, thus constituting previously untreated material. The histological diagnosis was of a glandular and polymorphous ovarian epithelium with multiple differentiations, endometroid for the major part of the tumor, with some serous clear cells and undifferentiated foci.

Culture Methods: Origin of the IGROV1 Cell Line

Within 10 min after surgery the tumor was divided into two parts and placed in RPMI 1640 (Eurobio Co., Paris, France) with penicillin (100 units/ml), dihydrostreptomyacin (100 μg/ml; Sarbach Laboratories, Challillon/Chalonnore, France), garamycin (10 μg/ml; Unilabo, Paris, France), and amphotericin B (5 μg/ml; Squibb, Paris, France); one part was used for heterotransplantation into nude mice and the other was used for tissue culture. For the latter the material was processed according to the two-step dissociation method of Slocum et al. (15). The tumor fragments, which had resisted mechanical dissociation, were exposed to 0.8% collagenase II and 0.002% DNease (Sigma Chemical Co., St. Louis, MO) in RPMI 1640 with 10% HIFBS3 (Eurobio) for 2 h at 37°C. The "mechanical" and "enzymatic" cellular suspensions were collected and washed successively in isotonic saline solution and in RPMI 1640. Finally 10^6 cells from each cellular suspension were introduced into Nuncion flasks (25 cm²; Nunc, Inter Med. Co., Roskilde, Denmark) with 5 ml of RPMI 1640 supplemented with 10% HIFBS to initiate in vitro growth of the cells.

Clonogenic Assays-Chemosensitivity Tests

Clonogenic assays were undertaken using both enzymatic and mechanical cell suspensions. They were also carried out with IGROV1 subpassages generated from either cell cultures or nude mice heterotransplantations. Tumor cells were cultured according to the two-layer agar assay of Hamburger et al. (9). The materials used for these experiments were agar (Bacto-Agar; Difco Co., Detroit, Ml), McCoy's 5A, CMRL 1066, HIFBS, and horse serum (Eurobio). The methyl cellulose/soft agar assay was also performed as described by Slocum et al. (14); briefly 1 ml of 1.05% methyl cellulose (EM Premium; Dow Chemical Co., Midland, MI), containing 5 × 10^6 tumor cells in RPMI 1640, was poured onto a lower layer of 2.5 ml of 0.5% methyl cellulose/soft agar assay of Hamburger et al. (9). The materials used for these experiments were agar (Bacto-Agar; Difco Co., Detroit, MI), McCoy's 5A, CMRL 1066, HIFBS, and horse serum (Eurobio). The methyl cellulose/soft agar assay was also performed as described by Hamburger et al. (9). The materials used for these experiments were agar (Bacto-Agar; Difco Co., Detroit, MI), containing 5 × 10^6 tumor cells in RPMI 1640, was poured onto a lower layer of 2.5 ml of 0.5% methyl cellulose (EM Premium; Dow Chemical Co., Midland, MI), containing 5 × 10^6 tumor cells in RPMI 1640, was poured onto a lower layer of 2.5 ml of 0.5% methyl cellulose. This was done in order to obtain a lower layer of 2.5 ml of 0.5% methyl cellulose. This was done in order to obtain a lower layer of 2.5 ml of 0.5% methyl cellulose. This was done in order to obtain a lower layer of 2.5 ml of 0.5% methyl cellulose. This was done in order to obtain a lower layer of 2.5 ml of 0.5% methyl cellulose.

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1 This work was supported by a grant from the Institut Gustave Roussy, Villejuif (Contrat de Recherche Clinique 82D8).
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: HIFBS, heat-inactivated fetal bovine serum; CE, cloning efficiency.
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inverted microscope in order to detect and count cellular clumps. The cells were incubated in a humidified atmosphere of 7.5% CO₂ in air at 37°C. CE was measured 10 to 20 days later as the ratio between the cells were incubated in a humidified atmosphere of 7.5% CO₂ in air at 37°C, CE was measured 10 to 20 days later as the ratio between the number of colonies (containing more than 50 cells) and the number of viable cells plated.

In order to test the chemosensitivity of IGROV1 to Adriamycin, cis-platinum, and 4-hydroperoxycyclophosphamide, Asta Z 7557 (Asta Werke AG, Bielefeld 14, West Germany), a compound analogous to the active metabolite of cyclophosphamide, cells were grown in RPMI 1640-10% HIFBS and allowed to reach the exponential growth phase (3 x 10⁶ cells/25 cm² flask). The medium was replaced by RPMI 1640 without HIFBS. In this medium the cells were exposed to each drug for 1 h at 37°C, at final concentrations (in µg/ml) which include the one-tenth of the average maximal peak of plasma concentration achieved in patients (1): cis-platinum, 0.2; Adriamycin, 0.04; Asta Z 7557, 3.0. The cells were then harvested in trypsin/EDTA, counted, and cultured by the two-layer agar assay of Hamburger et al. (9). The sensitivity indexes of these in vitro assays were quantified by measuring the areas under linear survival-drug concentration curves (after 1 h exposure) with 0.1 µg/ml as the upper boundary concentration (1). Under these conditions the cells were considered to be sensitive to a drug in vitro if the sensitivity indexes were less than the following relative area units: 5.3 for Asta Z; 7.3 for Adriamycin; and 11.4 for cis-platinum. The colonies were counted at a magnification of 28, using a Leitz Diavert invertoscope (Leitz, Wetzlar, West Germany) equipped with a reticulum previously calibrated for a standard colony size.

Cell Subcultures. After a confluent monolayer was formed subculturing was performed by trypsinization for 10 min at 37°C in 7.5% CO₂ [0.25% trypsin/0.02% EDTA/10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid buffer, pH 7.4; Grand Island Biological Co., Grand Island, NY]. The resulting suspension was washed twice in RPMI 1640 and centrifuged at 100 x g; the final pellet was resuspended in RPMI 1640 supplemented with 10% HIFBS and seeded in a Nuncolon flask (25 cm²). When confluence was achieved the cells were transferred at a transfer factor of 1/4. When necessary for morphological studies the cells were grown on coverslips placed in the bottom of a 35-mm plastic Petri dish (Nunc).

Heterotransplantations Into Nude Mice

Six to 8-week-old female nude mice (SPF Swiss nude mice; origin, K. Hansen, NIH, Bethesda, MD) were provided by the Animal Experimentation Branch of the Institut Gustave Roussy (Dr. C. Gosses) (7). Heterotransplantations were achieved by s.c. injection into the two flanks of the animals as either 3- x 3- x 3-mm tumor fragments or cell suspensions from the tumor. In other experiments cell suspensions were injected i.p. The animals were inspected weekly for the development of tumors.

Cell Freezing. The tumor tissues from the patient or from different heterotransplantation passages were dissociated as mentioned previously (15). These cellular suspensions or those from the different passages of the in vitro cell line were frozen in RPMI 1640 supplemented with 10% HIFBS and 10% dimethyl sulfoxide (Eastman Co., Rochester, NY), at a final concentration of 10⁶ cells/ml. They were stored in liquid nitrogen after a progressive shift of temperature. The cell cultures were periodically checked for the absence of Mycoplasma contamination according to the procedures of Bonissol et al. (2) and Uitendaal et al. (16).

Morphological Studies

Pathological procedures were routinely performed on tumors, using the classical techniques of fixation, paraffin embedding, and staining. For electron microscopic studies 1-mm³ fragments of tumors from nude mice were fixed in 2% glutaraldehyde in phosphate buffer (pH 7.3), postfixed in 1% osmium tetroxide, and dehydrated in ethanol and propylene oxide by successive washings. The samples were embedded in Epon.

Aggregates of cells and colonies were processed in the same way in 1-ml conic tubes in order to avoid cellular loss during successive handling. For monolayers grown in Nuncolon flasks (25 cm²), the inclusion was performed directly in the culture flask using the above technique without propylene oxide. After Epon polymerization the flasks were broken to give access to the monolayer material which adhered to the Epon block. Semithin (3 µm) and ultrathin (700 Å) sections were obtained with an LKB ultramicrotome (LKB, Bromma, Sweden). The semithin sections were placed on slides, covered with coverslips, and observed under a phase-contrast Photomicroscope Zeiss (Carl Zeiss, Oberkochen, RFA). The ultrathin sections were placed on grids, stained with uranyl acetate and lead citrate, examined, and photographed using a Philips EM 300 electron microscope.

Cytogenetic Studies

Exponentially growing IGROV1 cells from the 5th and 20th passages were treated with colchicine at a concentration of 0.5 µg/ml in RPMI 1640 supplemented with 10% HIFBS for 20 min at 37°C. The cells were harvested by trypsinization (0.05% trypsin/EDTA). After washing, centrifugation, and resuspension the tumor cells were submitted to hypotonic shock in 0.75 M KCl. The cells were then fixed three times in Carnoy’s solution at 0°C before spreading on slides kept at 4°C. G- and R-banding of the chromosomes were obtained by trypsin and heat denaturation, as described previously (5, 13). The chromosomes were stained with Giemsa solution (Giemsa; RAL, Villeneuve, France).

Constitutional karyotype was performed on peripheral lymphocytes of the patient 5 months after the last chemotherapeutic treatment cycle. The technical treatment of the lymphocytes was as usual: incubation with Colcemid (0.5 µg/ml) for 2 h at 37°C; hypotonic shock in AB human serum/RPMI 1640 (1/5,v/v); fixation with acetic acid/methanol (1/3, v/v); and trypsin denaturation. The chromosomes were stained with Giemsa.

RESULTS

Growth of IGROV1 Cells. Primary cells obtained from enzymatic and mechanical cell suspensions (15) were tested for their ability to grow in agar. After 15 days of culture in semisolid agar, the enzymatic cells presented an 0.3% CE while the mechanical cells exhibited a 10-fold lower CE. In the methyl cellulose/soft agar system the CE of the enzymatic cells was only 0.07%, indicating a more stringent selection of the clonogenic population. After two s.c. heterotransplantations in nude mice, the primary tumor gave rise to a CE of 0.15%. In contrast after one passage in nude mice the enzymatic cell showed an 0.5% CE. Therefore the enzymatic clonogenic cell population was chosen, as the more proliferative material, to be used for the establishment of the cell line in vitro and in vivo. After 30 passages in liquid medium the CEs in agar and in methyl cellulose were 0.35% and 0.1%, respectively.

The growth curve of IGROV1 in liquid medium is shown in Chart 1. A 24-h lag period was followed by an exponential growth phase before reaching a plateau. The beginning of the plateau corresponded to a cell density of 1.5 x 10⁶ cells/ml. In the exponential growth phase the average doubling times at the 10th, 30th, and 45th passages were estimated at 22, 17, and 19 h, respectively. Beginning at the 5th passage when IGROV1 cells had almost become confluent, adhering cells began to pile up forming foci, many of which became loose and floated freely in the culture medium as clusters of varying sizes. When cloned in methyl cellulose at the 10th and 30th passages, the pooled colonies gave rise to monolayers and floating clusters with characteristics identical to those of the parental line. No cellular growth crisis has been observed during the establishment of
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Chart 1. Growth curve of IGROV1 (40th passage). Each point represents the mean of three dishes. Bars, SD.

IGROV1. Thus far IGROV1 cells have undergone 60 passages, corresponding to about 200 generations.

Morphological Studies. The primary tumor of the patient was an ovarian carcinoma with multiple differentiations, endometrioid glands and in colonies. Glycogen secretion and agyrophil granules, possibly of endocrine origin, were also detected (Fig. 3). Following criteria: presence of cellular junctions; microvilli, and abundant secretions. Calcific formations, a characteristic feature of ovarian adenocarcinomas, were observed in floating aggregates, and the colonies obtained by agar culture corresponding to about 200 generations.

When the line was first established islands of polygonal epithelial cells were surrounded by unorganized epithelial cells. No fibroblasts could be seen. After 10 passages a uniform monolayer consisting of polygonal epithelial cells appeared.

Morphological studies were carried out on the monolayer, floating aggregates, and the colonies obtained by agar culture (Figs. 2 to 5). The epithelial nature of these cellular materials was assessed under light and electron microscopes according to the following criteria: presence of cellular junctions; microvilli, and abundant secretions. Calcific formations, a characteristic feature of ovarian adenocarcinomas, were observed in floating aggregates and in colonies. Glycogen secretion and agyrophil granules, possibly of endocrine origin, were also detected (Fig. 3b).

Cells grown in vitro and injected into nude mice either s.c. or i.p. induced the development of tumors with morphologies similar to that of the patient’s primary tumor. The s.c. tumor had an acinus structure (Fig. 7a), the basal membrane being oriented toward the outside of the acinus. Cellular epithelial islands were surrounded by collagen and conjunctive cells which formed stream-like structures around the tumor cells. Outside the tumor a papillary formation could be observed. Electron microscopy revealed a cellular polymorphism characterized by at least two types of cells (Fig. 7b): small cells with clear cytoplasm, a round nucleus, and very little heterochromatin; large cells containing a cytoplasm rich in organelles (endoplasmic reticulum, mitochondria, and dense granules) and a polyglobular nucleus. These large cells exhibited abundant microvilli.

Cells injected i.p. gave rise to disseminated carcinomatoses associated with abundant ascites. The s.c. xenografts of nude mice had the same morphological characteristics as those of the patient’s solid tumor. In the ascitic fluid there were clusters of cells similar to the floating aggregates observed in vitro (Fig. 6). These aggregates exhibited a cystic structure usually empty but in rare cases full of cells similar to those surrounding the cyst. At the ultrastructural level a cellular morphology and polymorphism similar to those of the s.c. tumor were also detected. It should be noticed that the material grown in nude mice was more markedly polymorphous than was the in vitro grown tissue.

Heterotransplantations into Nude Mice. The s.c. heterotransplantation of 3- x 3- x 3-mm fragments of the tumor into eight nude mice led to the formation of palpable tumors in all animals within 15 days after the inoculation. With cells from the 10th in vitro passage and using approximately the same amount of solid fragments, the score of takes and the time needed to obtain palpable material were identical.

When 10⁷ cells from the 10th in vitro passage were injected into the peritoneum of mice, abdominal tumors appeared within 40 to 50 days, with formation of abundant ascites before the death of the animals. After six passages in nude mice, i.p. injections gave 100% abdominal takes. After the formation of ascites extensive carcinomatosis associated with large intraabdominal tumoral masses was observed in the sacrificed mice. Some of these masses adhered to the diaphragm, others to the wall of the abdomen or to the small intestine. All the i.p.-injected mice died. The average survival time of 15 mice was 62 days (range, 42 to 68 days).

Cytogenetic Studies. Fifty mitotic cells of the 5th and 20th passages were analyzed and their chromosomes were photographed, identified, and counted, indicating the presence of two cellular clones, one pseudodiploid with 46 chromosomes and the other hypotetraploid with 92 chromosomes. As reported previously the number of tetraploid cell increased as a function of the number of subcultures.

As shown in Fig. 8 full karyotype showed two chromosomal markers: a paracentric inversion involving the short arm of chromosome 3 with the breakpoints located in 3p13 and 3p25; a translocation between chromosomes 2 and 5, t(2;5) (q33;q22).

Other anomalies were detected inconsistently in different mitoses. Independently of the number of passages and of the modal distribution of the chromosomes, all the cells exhibited simultaneously these two chromosomal markers. Thus these markers characterize the IGROV1 ovarian cell line: 46,XX,inv(9)(p13p25); t(2;5)(q33;q22). The translocation t(6;14), previously reported in an ovarian cystadenocarcinoma (17), has not been found. The constitutional karyotype of the patient was obtained using peripheral lymphocytes. It did not reveal any cytogenetic abnormalities.

Drug Sensitivity of IGROV1 Cells. Using agar/agar colonyforming assays, the effects of Adriamycin, cis-platinum, and Asta Z were studied on IGROV1 cells of the 46th passage. The results are shown in Chart 2. From these curves and using 0.1 μg/ml as the upper boundary drug concentration for measurement of area under curve, we calculated the sensitivity indexes obtained with these three drugs. They were found to be equal to 7.7 for Adriamycin, 6.3 for cis-platinum, and 10 for Asta Z. When one considers the sensitivity indexes estimated by Alberts et al. (1) for these drugs, these data indicate that IGROV1 cells are sensitive to cis-platinum, are resistant to Asta Z, and show an intermediate drug response to Adriamycin. It should be noticed that IGROV1 cells was originated from the tumor of a patient not treated by chemotherapy.
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Despite the malignancy of ovarian human cancers and the difficulties encountered in their treatment, very few cell lines have been extensively studied and proposed as models (19). New cellular human ovarian models are needed to extend our knowledge of the biology and treatment of gynecological cancers. The cellular human ovarian models are needed to extend our knowledge of the biology and treatment of gynecological cancers. The best models should be those that can be studied both in vitro and in vivo. The NIH:OVCAR-3 cell line has recently been proposed as the first model for papillary ovarian adenocarcinoma (10). In this paper we describe a new ovarian cell line, IGROV1. This cell line was obtained from the solid primary tumor of a patient with polymorphous and moderately differentiated ovarian carcinoma. The IGROV1 cell line does not exhibit hormonal receptors, is sensitive in vitro to cis-platinum, is resistant to Asta Z, and presents an intermediate drug response to Adriamycin.

The morphology and ultrastructure of IGROV1 cells both in vitro and in nude mice resemble those of the primary tumor. A point of interest is the induction of peritoneal carcinomatosis in nude mice after the i.p. injection of IGROV1 cells. The mouse peritoneum is of course of great interest in human ovarian cancer studies because of its similarity with the site where the disease occurs in women. A transplantable murine ovarian tumor had been proposed previously for such studies (11). On the other hand a phase I study of Adriamycin administration i.p. in patients has shown that this route has an important therapeutic advantage in terms of local drug concentration (12). After i.p. injection of IGROV1 cells in nude mice, we observed an extensive solid carcinomatosis associated with ascites leading to the death of the animals. This suggests that our cell line can be used to evaluate the effectiveness of new treatments on the basis of mean life survival instead of reduction of tumoral volume. Experiments are under way to obtain pure ascitic material without solid tumor formation.

IGROV1 cells show consistent and stable chromosomal markers: inv(3)p13p25; t(2;5)(q33;q22). These markers are always present, even when hyperdiploidy appears as a function of the number of in vitro passages. Chromosome 1 abnormalities seem to be the most frequently reported in ovarian adenocarcinomas (18) but translocation t(6;14) has also been described. Neither these abnormalities nor the presence of homogeneous staining regions and double minute chromosomes were detected in the IGROV1 cell line. Very few data are available concerning the presence or the expression of activated oncogenes in human ovarian cancers. Only recently has a study shown that a somatic activation of a Kirsten ras gene was present in a human ovarian cystadenocarcinoma (6). Experiments are in progress to evaluate the transforming capability of IGROV1 DNA on NHI 3T3 cells in order to reveal another oncogene activation in the ras family. The chromosomal markers are very probably involved in the presence or expression of oncogenes. raf 1 is located on chromosome 3 where the breakpoint of the paraacentric inversion 3p13-3p25 occurs (3). In addition when one considers the t(2;5)(q33;q22), it seems likely that the fms oncogene may be involved in this translocation. As a matter of fact c-fms is located on 5q34 (8) which is very close to 5q22, one of the breakpoints of the translocated chromosomal fragment on human chromosome 5. Experiments are in progress to analyze possible genomic rearrangements and variations in the expression of these two oncogenes.

With all these characteristics the IGROV1 cell line may contribute to the improvement of our knowledge of the biology and treatment of human ovarian cancers.

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Fig. 1. Histological studies performed on the primary tumor. Light microscopy showing the morphology of the specimen. H & E, × 200.
Fig. 2. IGROV1 cells growing as a monolayer (passage 13). a, coverslip culture. Toluidine blue, × 400. b, coverslip culture. Best carmine, × 1,000. c, horizontal semithin section. Phase-contrast, × 450. d, ultrathin section of the monolayer showing microvilli, cell-junction complexes (arrows), and glycogen-rich cytoplasm. × 4,800.
Fig. 3. IGROV1 cells growing as colonies in semisolid medium (after 12 days of culture). a, semithin section. Phase-contrast, × 450. The cystic structure and calcic formations are indicated (arrows). b, ultrathin section showing microvilli, cell junctions (arrows), and clear cytoplasm. × 4,800.
Fig. 4. IGROV1 cell aggregates floating in the culture medium over the cellular monolayer (passage 13). Semithin section. Phase-contrast, × 450.
Fig. 5. IGROV1 floating cell aggregates grown during five passages in RPMI-10% HIFBS. a, semithin section. × 450. A cystic cavity is present inside the aggregates. b, ultrathin section. × 4,000. The apical poles of the cells, with numerous microvilli and junction complexes, are outwardly oriented (arrows). The basal pole of the cell, inside the cystic cavity, shows amplified, folded over basal laminae (double arrows and inset, × 18,360).
Fig. 6. Ascitic effusion from IGROV1 tumors growing in nude mice after i.p. xenografts. Semithin section showing the polymorphism of cellular aggregates and the presence of calcic formations in the inner cavity of some of them (arrows). Phase-contrast, × 400.
Fig. 7. IGROV1 tumors growing in nude mice after s.c. xenografts. a, semi thin section showing the glandular structure. Phase-contrast, × 285. b, ultrathin section. Upper left, a cell with clear cytoplasm and a round nucleus with little heterochromatin; middle and lower right, several large polygonal cells covered with microvilli containing a cytoplasm filled with organelles, and one cell with a heterochromatin-rich nucleus. × 4,800.
Fig. 8. G-banding of IGROV1 cells from the 20th passage. Large arrows, consistent and stable cytogenetic markers; small arrows, inconstant markers.

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