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Cytogenetic evolution of human ovarian cell lines associated with chemoresistance and loss of tumorigenicity

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Abstract. In order to identify genomic changes associated with a resistant phenotype acquisition, we used comparative genomic hybridization (CGH) to compare a human ovarian cell line, Igrov1, and four derived subcell lines, resistant to vincristine and presenting a reversion of malignant properties. Multicolor FISH (Multiplex-FISH and Spectral Karyotype) and conventional FISH are also used to elucidate the karyotype of parental cell line. The drug-resistant subcell lines displayed many chromosomal abnormalities suggesting the implication of different pathways leading to a multidrug resistance phenotype. However, these cell lines shared two common rearrangements: an unbalanced translocation der(8)t(8;13)(p22;q?) and a deletion of the 11p. These chromosomal imbalances could reflected the acquisition of the chemoresistance (der(8)) or the loss of tumorigenicity properties (del(11p)).

Colour figure can be viewed on http://www.esacp.org/acp/2003/25-3/struski.htm.

1. Introduction

The development of resistance to chemotherapeutic agents is a limiting factor in the management of neoplastic diseases and results in treatment failure.

The multidrug-resistance (MDR) is a cross-resistance toward several cytostatic agents showing homology of structure or mechanism of action [25]. *In vitro* studies on cell lines provide interesting models for the characterization of the biologic mechanisms mediating this phenomenon. Several multidrug resistance mechanisms have been proposed, e.g., increased drug efflux, enhanced intracellular drug detoxification, alterations in nuclear targets, modifications of DNA repair systems, and apoptotic ways [1,21,25].

Because of complexity and heterogeneity of tumors and cell lines karyotypes, identification of recurrent and specific chromosomal changes may be facilitated by comparative genomic hybridization (CGH) [12]. Indeed, several studies have used CGH as a tool to identify loci associated with chemoresistance [9,15,16,18, 22,23,26,27,30].

We studied a previously described human ovarian adenocarcinoma cell line, Igrov1, and its derived subcell lines, which acquired a vincristine-resistance and a loss of tumorigenicity properties [2]. The parental

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Igrov-1 karyotype was resolved by a "multi-technique approach": karyotype, classical FISH, multiplex FISH (M-FISH), spectral karyotyping (SKY), and CGH. The chromosomal study of interest genes in the resistant subcell lines was performed by a CGH approach.

2. Materials and methods

2.1. Cell line

The human ovarian adenocarcinoma cell line, Igrov1, was obtained from a patient treated at the Gustave Roussy Institute with her previous consent (Jean Bénard, Villejuif, France) [3]. Igrov1 was cultured in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (Sigma, Saint Louis, USA) supplemented with 20% fetal calf serum (Boehringer Mannheim, Meylan, France). Four resistant sublines, derived from the parental Igrov1 cell line and obtained by increasing concentrations to vincristine (VCR), have been previously described [2,28,29]. Briefly, the cells were exposed continuously or discontinuously (for 24 h at each passage during the exponential phase of growth) to vincristine. Discontinuous exposure gave rise to a OV1/VCR subline resistant to a 2 μg/ml for 24 h (OV1/VCR.discont.) while continuous exposure led to a OV1/VCR subline resistant to 0.2 μg/ml (OV1/VCR.cont). Two sublines derived from both VCR resistant cells were serially subcultured for more than 100 generations in a drug free culture medium to give OV1/VCR.cont.st. and OV1/VCR.discont.st. (st. for starvation). We precise that the sublines derived from the same cell.

2.2. Karyotype

R-banding karyotype was obtained as previously described for cytogenetic techniques [6]. Karyotypic abnormalities were described according to the International System for Human Cytogenetic Nomenclature [20].

2.3. SKY analysis

The SKY hybridization and analysis protocol were realized according to instructions and in collaboration with Applied Spectral Imaging (SKYPaint mixture, Migdal HaEmek, Israel) to study Igrov1 cell line. Spectral images were captured with a SD200 SpectraCube system (Applied Spectral Imaging) mounted on a Zeiss Axioplan-2 microscope (Carl-Zeiss-Jena, Germany). A mathematical classification algorithm was applied to discriminate differentially labeled chromosomes based on their emission spectrum. The SkyView automatic spectral karyotyping software combined the Dapi-banded image with multicolor SKY information and chromosome ideograms.

2.4. M-FISH analysis

The M-FISH protocol and the analysis were realized in collaboration with MetaSystems (24Xcyte, Altlussheim, Germany) to explore the Igrov1 cell line. Separate fluorochrome images were captured using a fluorescence microscope (Axioplan-2, Zeiss) equipped with a motorized filter wheel and specific filter sets for Dapi, DEAC, FITC, Spectrum Orange, Texas Red and Cy5 (Chroma Technology, USA). The resultant images were analyzed using the Isis/M-FISH software (MetaSystems).

2.5. CGH analysis

CGH was performed according to the method described by Kallioniemi et al. [11] and Lichter et al. [19]. The parental cell line, Igrov1 DNA, was labeled, by nick translation, with Texas Red-dUTP (Spectrum Red direct labeled total human genomic DNA, Vysis, Downers Grove, USA) and the four resistant cell lines, OV1/VCR DNAs, were labeled with FITC-dUTP (Spectrum Green, Vysis). For image acquisition, we used a fluorescence DMRB microscope (Leica, Switzerland) with 3 separate band pass filters (Leica) for Dapi, FITC and Texas Red. The data from all individual fluorescence ratios (FR) were calculated by an appropriate software (ISIS: option CGH: MetaSystems, Altlussheim, Germany) and, using standard reference intervals [13,14], under- or over-represented chromosomal regions were identified.

2.6. FISH analysis

FISH was carried out using telomere specific probes for the chromosomes 3 (3p: D3S4559 and 3q: D3S1272) and 18 (18p: 52M11 and 18q: D18S1390) (Chromoprobe-T, Cytocell, UK), and two alpha satellite specific probes for the chromosomes X (DXZ1) and 18 (D18Z1) (Oncor, Gaithersburg, MD). Whole chromosome painting probes (WCP) have been also used (Cambio, Cambridge, UK; Oncor). Probes were labeled with digoxigenin or biotin and revealed by

Fig. 1. R-banded karyotype of a representative near-tetraploid metaphase of Igrov1. The karyotype was interpreted as 85–92,XXX,der(X)t(X;18)(p11;p11),t(2;5)(q3?6;q2?3)x2,der(2)t(2;20)(q3?7;?),−3,der(3)inv(3)(p13p25)del (3) (p14) x2 , − 6, ?der (7) (p22) , der(9) t(9; 18) (q34; q2 ?2) x2, der(13)t(13;20)(q34;q12)x2,−18,+20, del(20)(q12)x2,−21.

FITC or CY3, respectively. Hybridizations were performed according to manufacturer's protocol and the slides were counterstained with Dapi. Metaphases were analyzed under fluorescence DMRB microscope (Leica).

3. Results

Colour figure can be viewed on http://www.esacp.org/acp/2003/25-3/struski.htm.

3.1. Characterization of the Igrov1 parental cell line

3.1.1. Karyotype

Igrov1 karyotype has been previously established in 1985 [3]. We observed that the modal chromosome number was near-tetraploid and the karyotype was first interpreted as 85–92,XXX,del(X)(p21p22), t(2; 5) (q3? 6; q2? 3) x2 , −3,der(3)inv(3)(p13p25)del(3) (p14)x2,−6,?der(7)(p22), add(13)(q34)x2,−18,+20, del(20)(q12)x2,−21 [cp25] (Fig. 1).

We precise that all cell lines have been frozen in 1985 and defrosted for this work. So, there is no karyotypic evolution between the two studies.

3.1.2. SKY and M-FISH

We have had the opportunity to compare SKY and M-FISH at the time of a demonstration. So, we have analyzed the parental cell line, but we have not had the occasion to explore the sub-lines.

The both methods, realized onto Igrov1, confirmed the translocation t(2;5) as well as the loss of one copy of the chromosomes 3, 6, 18 and 21, and the pentasomy of the chromosome 20. SKY and M-FISH identified the add(13) as a translocation der(13)t(13;20). They also revealed three others unsuspected translocations: der(2)t(2;20), der(9)t(9;18) and der(X)t(X;18).

3.1.3. CGH

The CGH hybridization of a green-labeled normal DNA with the red-labeled Igrov1 DNA, revealed losses of the whole chromosomes 3, 6 and 21, and loss of the q-arm of chromosome 18. Gain of chromosome 20 was also detected. Only the partial loss of chromosome 18 was unsuspected at the cytogenetic level.

3.1.4. FISH

The 18 and X alpha satellite probes hybridized the centromeres of the normal chromosomes 18 and chromosomes X, respectively. The der(X)t(X;18) was hybridized by the X alpha satellite probe. The q-telomere specific probe of chromosome 18 hybridized to the two der(9) and to one chromosome 18. The 18 p-telomere specific probe hybridized to the three chromosomes 18 and to the der(X).

The telomere specific probe of chromosome 3 hybridized to the p- and the q-telomeres of the normal and the two der(3) chromosomes.

3.1.5. Revised karyotype

Thanks to the CGH, SKY and M-FISH results, refined by FISH analysis, we observed more rearrangements than those observed on karyotype. The revised Igrov1 karyotype included the translocations t(X;18), t(2;20), t(9;18) and t(13;20), and it was finally interpreted as 85-92,XXX,der(X)t(X;18)(p11;p11),t(2;5)(q3?6;q2?3)x2, der(2)t(2;20)(q3?7;?),−3,der(3) inv(3)(p13p25) del(3) (p14)x2,−6,?der(7)(p22),der(9)t(9;18)(q34;q2?2) x2, der(13) t(13;20) (q34;q12)x2,−18,+20, *del(20)(q12)x2*,−21 [cp25].

The karyotype including FISH and CGH results was: 85-92,XXX, der(X)t(X;18)(p11;p11). Ish der(X)(wcpX+, wcp18+, DXZ1+, 52M11+), t(2;5) (q3?6; q2?3)x2. ish t(2;5)(wcp2+, wcp5+; wcp5+, wcp2+) x2, der(2)t(2;20)(q3?7;?). ish der(2)(wcp2+, wcp20+), −3. ish3(wcp3x3). rev ish dim(3), der(3)inv(3)(p13p25)del(3)(p14)x2. ish der(3)(wcp3+, D3S4559+, D3S1272+)x2, −6. ish 6(wcp6x3). rev ish dim(6), ?der(7)(p22). ish der(7)(wcp7+), der(9)t(9;18) (q34; q2?2)x2. ish der(9)(wcp9+, wcp18+, D18S1390+)x2, der(13)t(13;20) (q34;q12)x2. ish der(13)(wcp13+, wcp20+)x2, −18. ish18(wcp18x3, D18Z1x3, 52M11x3, D18S1390x1). rev ish dim(18q), +20. ish20 (wcp20x5). rev ish enh(20), del(20)(q12)x2. ish del(20) (wcp20+)x2, −21. ish21(wcp21x3). rev ish dim(21).

3.2. Characterization of the vincristine-resistant cell lines

3.2.1. Cell lines

Bénard et al. [2] and Teyssier et al. [28,29] have previously reported the characteristics of the Igrov1-derivated sublines (OV1/VCR). The four OV1/VCR have been described as expressing a multidrug-resistant phenotype, associated with an overexpression of the *MDR1* gene, without amplification of the locus. The sublines also displayed a typical reversion of *in vivo* and *in vitro* malignant properties.

All results and characteristics of OV1/VCR sublines are summarized in Table 1.

3.2.2. Karyotypes

Karyotypes of the four OV1/VCR cell lines presented common abnormalities to the parental cell line Igrov1, as t(2;5), der(3), −6, del(20) and −21. The four sublines also displayed differences compared Igrov1 as losses, gains and supplementary rearrangements. These karyotypes being complex, they were difficult to interpret (Table 1). However, we identified a der(8)t(8;13).

3.2.3. FISH

FISH analysis allowed to observed loss of der(X) t(X;18), t(2;20), and one copy of der(9)t(9;18), in the four sublines, except for OV1/VCR.discont.st. which lost the two der(9). The two der(13)t(13;20) were conserved in both OV1/VCR.cont.

Two chromosomal rearrangements were common to the four sublines. We observed a deletion of the 11 p-arm in OV1/VCRcont.st., and in both OV1/VCR.discont. The OV1/VCR.cont. cell line presented an unbalanced translocation der(1)t(1;11)(p3?5;p12). We also confirmed the der(8)t(8;13)(p22;?), observed in the karyotype, in the four OV1/VCR lines (Fig. 2).

3.2.4. CGH

CGH hybridizations of a red-labeled normal DNA with green-labeled OV1/VCR DNA, and CGH hybridizations of red-labeled Igrov1 DNA with green-labeled OV1/VCR DNA, supplied supplementary informations on the chromosomal changes associated to each drug-resistant cell line (Table 1).

OV1/VCR.cont. and OV1/VCR.cont.st. cell lines presented common unbalances: losses of 5, 13p13–q21.3 and 14, and gains of 7 and 8. OV1/VCR.cont. showed gain of chromosome 1, and OV1/VCR.cont.st., gain of chromosome 12 and loss of 11p region.

OV1/VCR.discont. and OV1/VCR.discont.st. cell lines shared losses of 4, 9, 10, 11p, 13p13–q21.3, 14, and gains of 1p21–q44 and 7. OV1/VCR.discont. displayed loss of chromosome 2 and gain of 15q22–q26. OV1/VCR.discont.st. showed gains of chromosome 8 and 18q11–q21.1.

Table 1

Karyotype, CGH and FISH results of OV1/VCR sublines. The cellular properties and chromosomal changes compared to Igrov1 are presented. “no” means absence. The gray areas and the bold types mean that the chromosomal abnormality was conserved (compared to Igrov1 cell line)

	IGROV1	**OV1/VCR.cont.**	**OV1/VCR.cont.st.**	**OV1/VCR.discont.**	**OV1/VCR.discont.st.**
Drug Exposition	no drug	0,2 μg/ml of vincristine	0,2 μg/ml of vincristine, then drug free culture	2 μg/ml of vincristine	2 μg/ml of vincristine then drug free culture
Multidrug-resistance phenotype	no	MDR phenotype overexpression of MDR1 without locus amplification	MDR phenotype overexpression of MDR1 without locus amplification	MDR phenotype overexpression of MDR1 without locus amplification	MDR phenotype overexpression of MDR1 without locus amplification
Malignancy	yes	reverse malignant properties	reverse malignant properties	reverse malignant properties	reverse malignant properties
	85-92 chromosomes	86-173 chromosomes	86-173 chromosomes	82-95 chromosomes	82-95 chromosomes
	der(X)t(X;18) t(2;5)x2	no	no	no	no
	der(2)t(2;20)	no	no	no	no
	-3	no	no		no
Karyotype	**der(3)inv(3)del(3)x2**				
	-6				
	der(9)t(9;18)x2	x1	x1	x1	no
	der(13)t(13;20)x2			no	x1
	-18	no	no	no	gain
	+20	no	no	no	no
	del(20)(q12)x2				
	-21				
		supplementary chromosomal changes :			
		+der(1)t(1;11)(p3?5;p12) [91%], -5, +7, + der(8)t(8;13)(p22;?), del(11)(p12) [9%], -13, -14, -17, +3mar	der(1)t(1;11)(p3?5;p12) [2%], -5, +7, +der(8)t(8;13)(p22;?), del(11)(p12) [98%], +12, -13, -14	+1p21-q44, -2, -4, +7, der(8)t(8;13)(p22;?), -9, -10, del(11)(p12), -der(13)x2, -14, +der(?)t(?;15), -17, +5mar	+1p21-q44, -4, +7, +der(8)t(8;13)(p22;?), -9, -10, del(11)(p12), -der(13), -14, -17, der(18), +4mar
FISH	18 and X centromeres 3 and 18 telomeres	wcp 1, 2, 3, 6, 11, 18, 20	wcp 1, 2, 6, 8, 11, 18, 20	wcp 1, 2, 8, 11, 13, 14, 15, 18, 20, X	wcp 1, 2, 11, 18, 20
CGH	**dim (3, 6, 18q, 21), enh (20)**	dim (5, **6**, 13p13q21.3, 14, **21**) enh (1, 7, 8)	dim (5, **6**, 11p, 13p13q21.3, 14, **21**) enh (7, 8, 12)	dim(2, **3**, 4, **6**, 9, 10, 11p, 13p13q21.3, 14, **21**) enh(1p21q44, 7, 15q22q26)	dim(4, **6**, 9, 10, 11p, 13p13q21.3, 14, **21**) enh(1p21q44, 7, 8, 18q11q21.1)

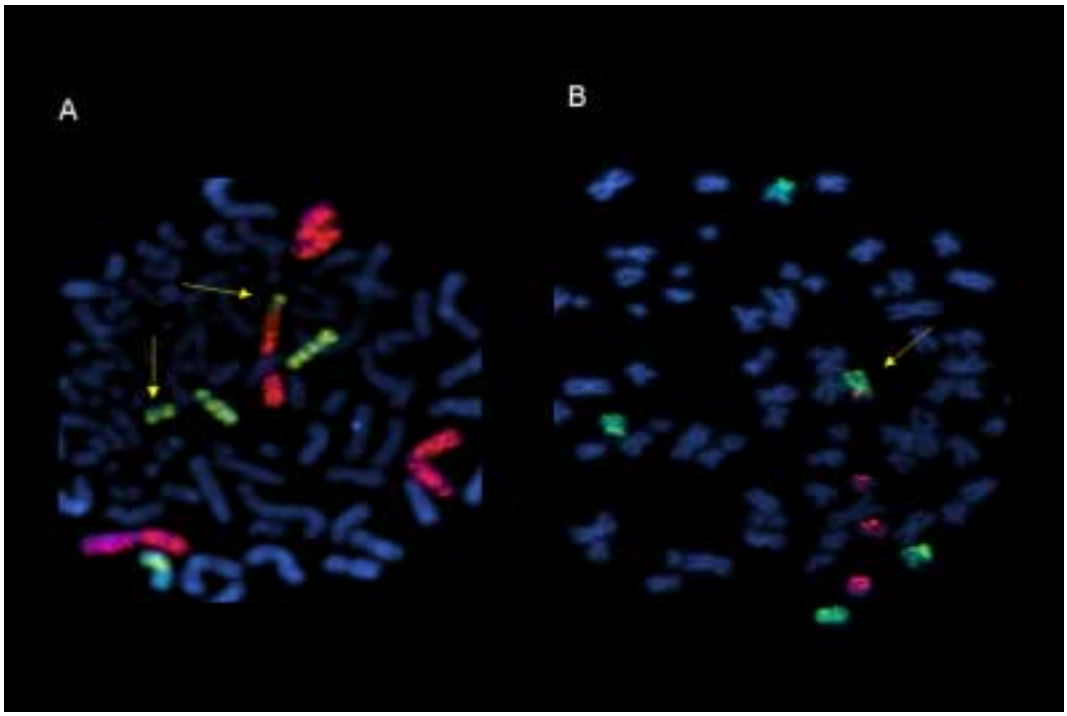

Fig. 2. Partial FISH results on the OV1/VCR cell lines. Hybridizations with WCP1 and WCP11 probes are shown, in red and green respectively, on the OV1/VCR.cont. cell line show the der(1)t(1;11) (arrow) (A). The der(8)t(8;13) is also presented (B) with hybridization of WCP8 (green) and WCP13 (red) probes. This figure can be viewed on http://www.esacp.org/acp/2003/25-3/struski.htm.

4. Discussion

4.1. Characterization of the Igrov1 parental cell line

The 24-color FISH methods, M-FISH and SKY, allowed to identify complex and cryptic translocations in a tumor karyotype, by the pattern of color distribution along aberrant chromosomes. The both methods have presented the same sensibility in this study. The value of the CGH has been to estimate the unbalanced or balanced nature of the regions implicated in the chromosomal rearrangements. In this present study, CGH discovered loss of the q-arm of chromosome 18 (in the Igrov1 genome), this imbalance was impossible to guess without this technique. Finally, classical FISH provided precision on concerned regions.

Thus, this study illustrates the complementarity of three approaches of hybridizations in the description of a highly abnormal karyotype. These analysis help to give a more comprehensive analysis of the types of rearrangements, which were present in an aberrant genome, the identity of the chromosomes involved and the specific chromosomal regions of net gain or loss.

4.2. Characterization of the drug-resistant cell lines, OV1/VCR

An understanding of the genetic mechanisms that underlie tumor cell sensitivity and resistance to chemotherapeutic agents is important from a biological as well as clinical standpoint. Previous reports have demonstrated the potential use of the CGH in the detection of regions of the genome implicated in chemoresistance [9,15,16,18,22,23,26,27,30].

Igrov1 cells were induced to become resistant *in vitro* to vincristine (VCR) by continuous and discontinuous stepwise exposure to the drug. The resultant resistant sublines, OV1/VCR, presented two major characteristics: the acquisition of a MDR phenotype and a marked loss of tumorigenicity properties.

OV1/VCR drug-resistant cell lines displayed a larger number of chromosomal abnormalities. The mechanism of drug resistance in ovarian cancer is multifactor, and accumulation of multiple genetic changes suggests implication of different pathways leading to chemoresistance acquisition [5,16]. However, besides the chromosomal markers of Igrov1, the resistant sublines revealed two common cytogenetic rearrangements: an

unbalanced translocation der(8)t(8;13)(p22;q?) and a deletion of the short arm of one chromosome 11, in OV1/VCR.cont.st. and in both OV1/VCR.discont. cell lines, the OV1/VCR.cont. presenting an unbalanced translocation der(1)t(1;11)(p3?5;p12).

Previous studies [7,24] presented a correlation between tumorigenic phenotypes and region 11p, suggesting that this chromosome carries a gene that controls tumorigenic expression. Horikawa et al. [10] observed in D98-OR cell line that the loss of one chromosome 11 with the duplication of another is associated with a reduced tumorigenicity in nude mice. Coleman et al. [4] mapped a locus within chromosome 11p11.2–p12 that suppresses the tumorigenic potential of some rat liver tumor cell lines. Wilms tumor 1 gene (WT1) may be implicated. Hill et al. [8] presented a loss of tumorigenicity in a methotrexate-resistant CEM cell line. This subline displayed a complex t(11;22), involving 11p14, suggesting the presence of a gene responsible of the loss of tumorigenicity.

The der(8)t(8;13) present in the four resistant cell lines could also attest of a common property. The chromosomal regions must be more explored and determinated to permit an identification of implicated genes. However, we can precise that chromosome 13 contains some genes of which the function has been already described in chemoresistance acquisition: the retinoblastoma 1 gene (RB1, 13q14.1–14.2) and the breast cancer 2 gene (BRCA2, 13q12.3), both tumor suppressor genes; the thioredoxin-dependent peroxide reductase gene (TDPX1, 13q12), antioxidant gene [16]; the MOAT-B gene (13q32) belonging to ATP-binding cassette subfamily [17].

The 8p22 region contains a putative tumor suppressor gene, LZTS1, and tumor necrosis factor receptor superfamily members (TRAILR1, TRAILR2, TRAILR3 and TRAILR4), mediating or inhibiting apoptosis way.

In summary, we present evidence that some cell lines could be drug-resistant and display a loss of malignancy characteristics, these both mechanisms seem to be independent. The implication of genes on chromosomes 8, 11 and 13, should be more explored by complementary molecular studies, such as quantitative PCR or CGH DNA arrays.

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