Research Article

Current Opinion in Gynecology and Obstetrics Evidence for Predictive Markers of Platinum Effectiveness in Ovarian Carcinoma by Microarray- based Comparative Genomic Hybridization

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Abstract

Purpose: Seventy percent of all ovarian cancer (OvCa) diseases are diagnosed at an advanced stage due to poor screening methods. In general, the therapy consists of cyto-reductive surgery followed by first line platinum based chemo therapy, which frequently leads to a long-term response. A resistance against platinum therapy was detected in some cases by refractory recurrence within six months after the last donation. Identifying a marker predicting platinum effectiveness could be of great benefit for affected patients.

Patients and Methods: In this study we propose cyto-genetic procedures identifying markers of platinum resistance. Fresh tumor material was obtained from nine OvCa patients. After the last platinum-based chemotherapy, five patients had refractory recurrence within six month. DNA was isolated from tumor tissues for array-based comparative genomic hybridization (aCGH) analysis. Ten cultured metaphases per case were analyzed by spectral karyotyping (SKY) to detect repeated chromosome rearrangements. The obtained genomic data were correlated to the patients' clinical follow-up data.

Results: Eight chromosome aberrations correlated with recurrence later than six months were detected in tumors by SKY. Further seven chromosome aberrations correlated with a refractory recurrence. Non refractory tumors more frequently showed losses in 19q13.31-q13.42 by aCGH. Tumors with refractory recurrence more frequently showed gains in 17q21.32-17q24.3 and losses in 14q11.2, 9p22-p21.1, and 1p22.3-p22.1. OvCa showed separating cyto-genetic changes differentiated in platinum-refractory and non-refractory cases.

Conclusion: Our results suggest that aCGH is the most suitable strategy for this, due to a significantly higher resolution. The loss in 19q13 correlated with better prognosis in OvCa.

Keywords: Ovarian carcinoma; Platinum effectiveness; aCGH; Spectral karyotyping; Predictive markers; 19q13

Introduction

Although enormous efforts have been taken in recent years to understand chromosomal aberrations in ovarian cancer (OvCa), the effects on morbidity and mortality remain far from satisfactory. The overall survival rate could not be improved substantially during the last years and there is still no evidence based method of early detection in OvCa, not even in patients with a high risk gene constellation like the BRCA-mutations. As seen in the prostate, lung, colorectal, and OvCa screening trial, a screening by transvaginal ultrasound and examination of CA-125 in serum didn't reduce mortality in the OvCa patients [1]. Due to the lack of screening and the unspecific symptoms [2], the majority of the patients with ovarian carcinomas were diagnosed at advanced stages (FIGO IIIc-IV). The standard of care for OvCa is radical cyto-reductive surgery followed by platinum- and taxan-based chemotherapy regimen. By introducing the anti angiogenic drug bevacizumab some progress was achieved for the OvCa patients' outcome [3-5], but OvCa still remains as the deadliest gynecological malignancy. Patients with a platinum-refractory carcinoma defined as progression during or immediately after the chemotherapy within six months, show especially poor outcome. As the chemotherapy with platinum and taxan is still associated with a broad spectrum of serious adverse side effects affecting the general state of health, genetic markers for the prediction of the efficiency of these therapies might help to predict a platinum resistance of the tumor. Ultimately, this could lead to a more personalized therapy sparing inefficient treatment and offering other treatment options.

Our aim was to find frequent cyto-genetic markers that match platinum responsiveness. Thus, we used two strategies in order to detect recurrent markers. On the one hand, we were looking for marker chromosomes by spectral karyotyping (SKY) [6]. On the other hand, we were looking for recurrent imbalances with a higher resolution by using array-based comparative genomic hybridization (aCGH). In the past, we had used both techniques successfully to describe chromosome aberrations in cell lines and primary tumors of OvCa [7-9]. In order to find meaningful markers for the effectiveness of platinum therapy, the suitability of both techniques should be compared. In our study, we examined nine samples of ovarian carcinomas with primary-serous or serous-papillary OvCa. Five patients had a platinum-refractory disease recurring within 6 months after the end of their chemotherapy. The other group of patients had a longer recurrence free interval.

By comparing frequent chromosomal aberrations from the cell-based SKY analysis with the quantification of gains and losses by aCGH, we found specific differences in both groups. The results may be helpful to subgroup patients and can act as the basis for further studies with a larger group of patients.

Materials and Methods

Tumor Samples

The cells were harvested from fresh tumor samples of the ovaries of patients, who got a surgical therapy between 1998 and 2011 in our clinic of gynecology and obstetrics at the Christian-Albrecht's University of Kiel, Germany. The patients were followed up and were staged according to the FIGO classification. Furthermore, as a histopathologic examination of the removed tumor was performed and further clinical information, such as recurrence, survival and therapy data were collected. All samples originated from moderate to undifferentiated serous or serous-papillary ovarian carcinoma and the patients received an adjuvant platinum-based therapy. The tumor recurred within six month in five patients, and the tumor recurred later than six months in four patients. This research was approved by the Institutional Review Board of the University Medical Centre Schleswig-Holstein, Campus Kiel (AZ: B327/10) according to the Declaration of Helsinki. Written informed consent was obtained from all patients.

Tumor tissues from all patients were stored at -80°C storage and used for array CGH analysis. We used short-term culture generated from fresh tumor tissue for SKY. The cell cultures' identities were authenticated by short tandem repeats (STR) as previously described by our group [10]. The authentication is highly recommended as contamination is a major problem in cyto-genetics research [11]. We compared the STR patterns with data of the German collection of microorganisms and cell cultures (DSMZ, Braunschweig).

As the quality of the SKY analysis depends on the quality of metaphasis [12] the samples were selected by their clinical response to a platinum-based therapy and with a phase contrast microscope concerning their quality of metaphases.

SKY

We started our studies by performing SKY to get an overview of the chromosomal aberrations. For the hybridization we used the human spectral karyotyping

Figure 1: The metaphase-analysis of one sample of case 1 by 24-color fluorescence in situ hybridization. The metaphase on the upper left is DAPI counterstained. Immediately to the right is the same metaphase shown as a computed RGB-color image provided by the microscope equipped with spectrometer camerasystem Sky Vision II. At the bottom, a karyogram generated by analysis software Sky View 1.6 shows each chromosome as an inverted DAPI image next to its false colour defined by Sky View 1.6. The upper middle panel shows a SKY-ideogram created by NCBI SKY Database for the metaphase on the left. In the red box, a composite Sky -ideogram over all analysed metaphases of case 1 is shown. The numbers over the rearranged chromosomes in SKY-ideogram represents the number of metaphases containing that rearranged chromosome.

Figure 2: Overview of prominent, aberrant chromosomes according to the SKY analysis.

Figure 3: Heat map representing occurrence of chromosome changes detected by SKY in patients with early (refractory = before six months) and late (not refractory = after six months) recurrence after the last treatment with platinum.

Figure 4: Gains and losses of frequently found copy number variations identified by array-based comparative genomic hybridization relative to the platinum response of each case (refractory = before six month; not refractory = after six month). The heat map shows a significant distribution of gains and losses of genetic material relative to the platinum response.

Figure 5: Chromosomal imbalances in chromosome 19 by array-based comparative genome hybridization. In the left scale is information about imbalances related to chromosome position through ideogram on the very left. Losses oscillate the zero-line to the left and gains to the right. Green lines are representing refractory and grey lines the non-refractory cases. Colored bars on the left are represent significant losses of DNA. Colored bars on the right represent significant losses of heterozygosity (LOH). These are also shown by semi-transparent turkey background in separated single nucleotide polymorphism (SNP) panes of refractory cases only. Three cases have SNPs signals only on line 0 and 1 representing hemizygous LOH in region between 19q13.2 and 19qter.

kit from Applied Spectral Imaging Ltd. (Neckarhausen, Germany). We performed the 24-color FISH hybridization according to the manufacturers' instructions, including a washing protocol. After hybridization, we selected 10 metaphases for each patient by using a phase contrast, fluorescence microscope. The spectra emitted by hybridized fluorophores were sent through a triple band pass filter, interferometer and the cooled CCD-camera detected spectra for each pixel. We acquired the spectral images of the fluorophores using the Sky Vision II system and the Sky View software version 1.6, which were both provided by Applied Spectral Imaging. A corresponding common DAPI counter stain picture of each metaphase was used to define the regions of interest. SKY-ideograms were created by tools available at the NCBI CGH and SKY database until September 2016 (<https://www.ncbi.nlm.nih.gov/sky>) (Figure 1).

aCGH

The DNA was extracted with the Gentra Puregene core kit (Qiagen, Hilden, Germany) according to the manufacturer's Gentra Puregene Handbook Third Edition from April 2010. We used Sure Print G3 Cancer CGH+SNP Arrays from Agilent Technologies (Waldbronn, Germany) in a 2x400k and 4x180k format. For restriction incubation with Alu1 and RSA1 0.5 µg DNA was used, labeling with Cyanine 3-dUTP for reference DNA (Agilent Male_dbSNP141) and Cyanine 5-dUTP for tumor DNA and hybridized on the array following protocol Version 7.4 for Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis. The hybridization procedure was performed in a rotating oven at 67°C for 24 hours. The washing of the arrays followed the same protocol as described above using an ozone-free instrument from SciGene (Sunnyvale, California). After washing the array was scanned in a Sure Scan Microarray Dx Scanner from Agilent Technologies. The raw data were generated by Feature Extraction Software Version 12.0.3 (Agilent). The analysis was made with CytoGenomics Analysis Software Version 3.0.6.6 (Agilent). The genetic map was based on GRCh37/hg19.

Statistics

All calculations were performed with Statistica Ver.6 software (Stat Soft. Inc. 2001, Hamburg, Germany). In order to test the significance of chromosome aberrations grouping with the platinum effect, we performed a t-test of paired samples. The hypothesis that gains or losses were dependent on the platinum effect was tested by the Pearson Chi²-test and Fischer's exact test. Due to the low

case number, we also tested dependence by analysis of variance (ANOVA). For correlations between chromosome aberrations and the platinum effect, we calculated a survival analysis by Kaplan-Meier and verified it by a Log-Rank-test.

Results

Ten metaphases per patient were analyzed by a 24-color fluorescence *in situ* hybridization. By summarizing all ninety Sky-ideograms we could detect repeated chromosomal aberrations. We defined the occurrence of changes in at least four of nine cases as repeated chromosomal aberrations (Figure 2).

The recurrent chromosomal aberrations mainly originated from chromosomes 1, 3, 7, 10 and the X-chromosome. The most prominent aberration der(3)del(3)(p21), was counted 17 times among seven of nine cases. Represented in only six cases der(7)del(7)(q11) was also detected 17-times. der(10)del(10)(q22) and the der(X)del(X)(p11) were detected 14 times each in seven and six cases, respectively and der(1)del(1)(p31) was only detected 11 times in five cases (Figure 2). In opposite to the definition of repeated chromosome aberrations we used for the overview of all ovarian cancer cases, we found only up to three repeated chromosome aberrations in refractory and non-refractory sub classes. Thus, we found new aberrations in patients with a recurrence free interval of more than six months. These were der(10)del(10)(q), der(3)del(3)(p21q21), der(6)del(3)(q14), der(12)del(12)(p12), der(2)del(2)(q32), der(11)del(11)(p12q23), der(11)del(11)(q23) and der(8)del(8)(q13). In contrast, patients whose tumours were refractory to platinum therapy, showed a different pattern of aberrant chromosome changes. These patients most frequently had der(12)del(12)(q23), der(21)dup(21)(q11qter), der(3)del(3)(q11), der(1)del(1)(p11), der(9)del(9)(p21) and der(4)del(4)(q12) (Figure 3).

The SKY analysis was able to detect significant chromosomal modifications ($p < 0.00001$, t-test for paired samples) between the chromosomal aberrations as shown in figure 3, and the effectiveness of platinum therapy. However, dependency could not be confirmed by chi²-test or ANOVA.

The aCGH showed a variety of high resolution modifications and could be allocated to cases with recurrences before and after six months, as shown in figure 4.

A better response to platinum frequently showed losses of genetic material in 19q13.31-19q13.42 (chr19_44015445-49686504; chr19_49839271-54711745). An insufficient response to platinum therapy correlated with gains in 17q21.32-q24.3 (chr17:_46748246-46771949; chr17:_61901897-61920213; chr17:_64420296-64477350; chr17:_67537847-67762983), and losses in 14q11 (chr14_20421677-20443785), 9p22.2-p21.1 (chr9_17931052-33115833) and in 1p22.3-22.1 (chr1_85921263-94426863) (Figure 4). All imbalances listed in figure 4 showed significant correlations with the platinum effect ($p < 0.023$, t-test for paired samples), but could not be confirmed by a χ^2 -test. However, losses in chr1_85921263-94426863, chr19_44015445-49107085, chr19_49147465-49686504, chr19_49839271-50128550, chr19_50185754-50335249, and chr19_50398880-54647399 correlate significantly ($p < 0.012$) with the platinum effect by variance analysis ANOVA. Losses in chr19_50335249-50398880 were correlated with a better overall survival by Kaplan-Meier (Log-Rank-Test: $p = 0.01149$).

Thus, the prominent detection of der(7)del(7)(q11) or der(1)del(1)(p31) by SKY confirmed deletions in chromosome 7q or 1p by aCGH. The strength of our study is the direct comparison of two types of genetic analysis in ex vivo ovarian cancer tissue, and we are sure to overcome the major limitations of the study with larger number of cases.

Discussion

It is known, that the effectivity of cytostatic drugs in cancer treatment depends on the genetic regulation of its antagonists [13]. Thus, it is important to get information about relevant regulative DNA-loci, and gene-doses-effects. The best would be, if those loci could use to predict the effectivity of drugs. Resistance to platinum-based chemotherapy is a significant clinical problem since patients with acquired resistance show a poor outcome in OvCa [14]. Developing a predictive tool to separate patients with platinum-resistant vs. platinum-sensitive tumours would be of potential advantage to disease management. We employed two techniques, SKY and aCGH, to identify genetic alterations as potential markers for response to platinum therapy. Genetic modifications of chromosome 19 in OvCa cells were identified at an early stage of cyto-genetics research [15] and are now known to occur in about 20% of the ovarian carcinomas being one of the most frequently reported chromosome with changes in copy number variations [16]. Of all human chromosomes, chromosome 19 contains the highest

density of genes and this fact results in a huge biological and evolutionary significance [17]. Recent analyses of The Cancer Genome Atlas project database showed that amplified somatic focal copy number variation in chromosome 19 is an indicator for poor prognosis [18]. Loss of 19q13.3/4 is correlated with longer disease-free survival as it contained the KLK locus (Kallikrein tissue, protein family of secreted serine proteases) [19]. Bayani et al. examined 81 chemotherapy naïve ovarian carcinomas by three-colour fluorescence *in situ* hybridization and immune histochemistry. They detected 29.6% losses in 19q13. Their results corresponded with those of our group (Figure 4) since we found a third of tumors (33%; 3/9 tumors) had losses in 19q13. Contrary to our data, they saw gains 53% more frequently that correlated with tumor grade, but did not necessarily led to protein over expression [19]. We were able to correlate the appearance of gains in 17q21.32-q24.1, and losses in 14q11, 9p22.2-pter and 1p21.3-22.2 with an insufficient response to first-line platinum-based chemotherapy (Figure 4). The loss of 19q13.33 (chr19_50335249-50398880) led to an increased survival with a significant (log rank test: $p = 0.01149$) differentiation. This region involved several genes. For example, *PNKP* (polynucleotide kinase 3'-phosphatase) is involved in DNA repair processes and *AKT1S1* is involved in cell growth and survival pathways of *TORC1*. Both proteins are known to be involved in essential processes of DNA repair or cell survival. Possibly, not only the doses of alleles affect the genetic function. As shown in figure 5, the loss of maternal or paternal alleles could have influences. Not compensated remaining affected gene copies could have an effect in response of cytotoxic treatments.

However, a very informative result is the assessment of both cyto-genetics techniques we used in order to find predictable markers for the effectiveness of platinum. The SKY technique involved a 24 color whole chromosome painting based on cultured tumor cells to be able to investigate metaphases. All analyses using the SKY technique requires a preceding cell culture and successful metaphase preparation. Furthermore, SKY is a single-cell analysis which is not problematic when all cells are homogeneous. The SKY technique had proven its clinical function as reviewed by Imataka and Arisaka [20]. They showed that, when analyzed cells are representative for the disease, SKY identifies important chromosomal rearrangements. Imataka and Arisaka reviewed indications for optimized detection of accessory and derivative chromosomes, insertions, complicated translocations and other cyto-genetics over-

representations resulting from duplications in order to improve diagnosis. The SKY technique shows significant advantages in combination with conventional banding methods like G-banding, especially in the continuative analysis of genetic diseases in children. One of the first descriptions of primary tumors by SKY was published in 2000 [21] at follicular thyroid carcinoma revealing a manageable single balanced translocation. However, analysis by SKY is more complicated when the tumor cell population is heterogeneous such as in OvCa. An enormous effort is necessary to carve out tumor relevant chromosome rearrangements by analyzing single cells as shown in Figure 1. In principle, the more different the single cells described, the more complex the recording of the overall results. In 43 publications using SKY for analysis of different carcinomas between 1998 and 2016, only 13 prepared primary tumors, whereas the remaining used more easily establishing cell lines. One of the first complex aberrations in primary and established cell lines was described by Squire et al. for head and neck cancer [22]. Leone et al. [23], pursued approaches similar to ours, identifying recurrent breaking points of translocations in patients with glioblastoma multi forme using the SKY technique. Furthermore, they found significant expression profiles of genes close to the breaking points with aCGH and expression arrays correlating them with the time of survival. They favored the combination with spectral karyotyping to discover unidentified translocations and the associated rearrangements of genes. The group was able to describe six genes located at breakpoint regions, showing a high correlation with patients' outcome [23] finally by higher resolution molecular techniques. The description of complex rearrangements found by SKY disclosed a further limitations: the breakpoint descriptions were based on the banding resolution of chromosomes was limited a half a mega base to ten mega bases of DNA, which is not sufficient to identify the involved genes directly. Moreover, the SKY technique is also inadequate when chromosomes are spread sub optimal, leading to with fatal consequences in interpretation [12]. The initiation of aCGH overcame these resolution limitations. Since the innovation of CGH resolution, many authors [24-29] use SKY only to validate more productive aCGH results. A further advantage of aCGH is the more informative results concerning the genomic heterogeneity of tumor cells [30]. Using the SNP analysis involved in aCGH, it is possible to illuminate chromosome regions with loss of heterozygosity. This information may be important for judgment of gene relevance in cancer. In contrast to SKY analyses, aCGH is able to give a more detailed view of genetic modifications.

The SKY results alone are not powerful enough for a direct translational approach. The aCGH could be used to identify the frequency and summarized influence of changes in different copy number variations without extensively reprocessing single-cell analyses. The copy number variations detected by aCGH represent the mean of the total heterogeneity in all tumor cells. Some, but not all rearrangements detected by SKY were congruent with the imbalances we found by aCGH. One limitation of the aCGH approach is how to interpret repetitive sequences [31]. The array CGH itself is limited in its ability to analyze genetic regions close to the centromere and, of course, it is unable to reveal genetic variations that do not lead to a change in copy number, such as translocations and inversions [32]. In addition, it has to be clear that an alteration in copy number variation is not stringently followed by the increased or decreased expression of the encoded proteins. In addition differential methylation and regulation by micro RNAs are important regulators of transcription [33,34]. Using other established methods such as immune histochemistry, this limitation can be overcome.

Our study has limitations, such as a small number of cases, but since we wanted to compare two different techniques to detect genetic alterations that correlate with response to platinum therapy, we were able to show different chromosomal patterns. However, these results need to be shown in a larger cohort of patients. Another limitation is the fact that the classical definition of platinum sensitivity as well as resistance will be adjusted in the near future. The strength of our research is the perception that the two employed techniques were compared simultaneously in the same patients avoiding inter individual differences. Due to the enormous effort necessary for SKY in heterogeneous solid tumors, we prefer aCGH as a tool to find markers for the effectiveness of platinum therapy in OvCa. We are confident that this strategy could be used to detect indicative genetic markers of the effectiveness of platinum therapy or postulated course of disease by analyzing an enlarged number of patients.

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All procedures involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Declaration in Helsinki and its later amendments or comparable ethical standards (B372/10Kiel 2011). Informed consent was obtained from all individual participants included in the study. The patients' data were pseudonymous as defined by the German data protection act. We thank the institute of pathology of Christian Albrechts University Kiel for appropriation of pathologic appraisal.

Conflict of interest

This study was supported by University Kiel only with financial resources for research and teaching. This study did not receive further support or funding. No author has to be come into conflict with any interests of this study.

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