Genomic analysis of genetic heterogeneity and evolution in high-grade serous ovarian carcinoma

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Abstract

Resistance to chemotherapy in ovarian cancer is poorly understood. Evolutionary models of cancer predict that, following treatment, resistance emerges either due to outgrowth of an intrinsically resistant sub-clone, or evolves in residual disease under the selective pressure of treatment. To investigate genetic evolution in high-grade serous (HGS) ovarian cancers we first analysed cell line series derived from three cases of HGS carcinoma before and after platinum resistance had developed (PEO1, PEO4 and PEO6, PEA1 and PEA2, and PEO14 and PEO23). Analysis with 24-colour fluorescence *in situ* hybridisation and SNP array comparative genomic hybridisation (CGH) showed mutually exclusive endoreduplication and loss of heterozygosity events in clones present at different timepoints in the same individual. This implies that platinum sensitive and resistant disease was not linearly related but shared a common ancestor at an early stage of tumour development. Array CGH analysis of six paired pre- and post-neoadjuvant treatment HGS samples from the CTCR-OV01 clinical study did not show extensive copy number differences, suggesting that one clone was strongly dominant at presentation. These data show that cisplatin resistance in HGS carcinoma develops from pre-existing minor clones but that enrichment for these clones is not apparent during short-term chemotherapy treatment.

Keywords

ovarian cancer; heterogeneity; evolution; chemotherapy

Introduction

Ovarian cancer is the leading cause of death from gynaecological malignancy, in spite of initially high response rates to chemotherapy treatment. The majority of cases are high-grade serous (HGS) histotype and present with late stage disease. Adjuvant or neoadjuvant treatment with platinum-based chemotherapy has response rates of 70–80 % (Herzog, 2004) but most patients relapse with chemotherapy-resistant disease, and five-year survival is less

Conflict of Interest

The authors declare no conflict of interest.

than 35 % (Herzog, 2004; Herzog and Pothuri, 2006). The molecular basis of acquired drug resistance remains poorly understood (reviewed in (Bast *et al.*, 2009).

Resistance to cisplatin and carboplatin therapy in ovarian cancers is, in part, determined by homologous recombination (HR) repair pathways (Bhattacharyya *et al.*, 2000). *BRCA*-null tumours have impaired repair of platinum induced DNA crosslinking and are preferentially sensitive to treatment with platinum-based chemotherapy. Intriguingly, it has been shown that some *BRCA*-null patients who acquire platinum resistance show reversion or compensating mutations at relapse, which restore BRCA1 or BRCA2 function (Sakai *et al.*, 2008; Swisher *et al.*, 2008). These reversions can also be induced *in vitro*, but it is unknown whether these secondary mutations in clinical samples arise *de novo* and are selected during treatment or are present at low frequency in minor sub-clones prior to exposure to chemotherapy.

Carcinogenesis is an evolutionary process in which genetic and epigenetic clonal populations are subject to natural selection, dependent both on their fitness to survive in their environment and rate of genetic drift (Nowell, 1976), reviewed in (Merlo *et al.*, 2006). Two potential models for the evolution of chemotherapy resistance in HGS ovarian carcinoma *in vivo* are therefore possible. First, genetically heterogenous clones may exist within the tumour mass prior to treatment, and populations with advantageous mutations are selected for survival and expansion following clearance of the dominant, sensitive clone by chemotherapy. Alternatively, if genetic heterogeneity is either not present or does not determine drug response, resistance could evolve progressively by mutation under the selective pressure of chemotherapy (Agarwal and Kaye, 2003). Progressive acquisition of mutations is most likely to occur in the dominant clone since the probability of a resistance mutation arising in a given sub-clone is dependent on its proportion in the population. The first model requires the presence of significant genetic heterogeneity within a tumour prior to treatment and may result in a relapsed genome that is non-linearly related to the majority clone at presentation. The second model predicts that the "relapsed" genome evolves from a presentation genome, most likely the dominant clone, with sequential accumulation of mutations and rearrangements conferring increased drug resistance.

Evidence for genetic heterogeneity prior to treatment and strong selective effects by chemotherapy has been shown in studies of sequential samples in acute lymphoblastic leukaemia (ALL), where relapsed disease has different copy number aberrations and point mutations than at presentation (Mullighan *et al.*, 2008; Zhu *et al.*, 1999). These apparently novel lesions can be detected at levels between 0.01 % and 1 % of pre-treatment ALL cells, using PCR assays specific for mutated alleles in the sub-clones (Zhu *et al.*, 1999). Specific resistance mutations in BCR-ABL can also be identified retrospectively in pre-treatment presentation samples of ALL that subsequently developed resistance to imatinib (Hofmann *et al.*, 2003).

It remains unclear whether similar mechanisms can explain the acquisition of drug resistance in solid tumours. HGS carcinoma has high rates of genomic instability (Bayani *et al.*, 2002) and somatic *TP53* mutation (Ahmed *et al.*, 2010; Kobel *et al.*, 2008), which could generate genetic heterogeneity. Intra-tumour genetic heterogeneity, has been shown at low resolution in ovarian and breast cancer (Khalique *et al.*, 2007; Torres *et al.*, 2007) using heterozygosity (LOH) analyses (25 loci) and classical comparative genomic hybridisation (CGH) (limited to detecting changes of ~ 20Mb). Interestingly however, classical CGH over-estimates divergence between sub-clones when compared to array CGH data (Liu *et al.*, 2009). Higher resolution investigation of genomic profiles from different metastatic sites in relapsed prostate cancer by array CGH showed a small number of sub-clonal changes present in different metastases (Liu *et al.*, 2009). Sequential studies before and after

chemotherapy treatment are limited and the most definitive study to date used deep sequencing of a single relapsed lobular breast carcinoma. This identified 6 point mutations that were present at frequencies of 1–13 %, in the sample taken at presentation nine years earlier (Shah *et al.*, 2009).

To determine which model of genetic evolution occurs under the selective pressure of chemotherapy in HGS ovarian carcinoma we used M-FISH and array CGH profiling to characterise three unique series of ovarian cancer cell lines established sequentially, before and after the development of platinum resistance, from three cases of HGS carcinoma in the early 1980's. In addition, to ask if genetic heterogeneity is altered *in vivo* during neo-adjuvant chemotherapy treatment we profiled six paired samples from the prospective CTCR-OV01 clinical study.

Results

Cell lines established at different timepoints have different responses to cisplatin

The PEO/PEA series of ovarian cancer cell lines were established as spontaneously immortalised polyclonal lines from sequential samples from three separate cases of HGS ovarian cancer (Figure 1) (Langdon *et al.*, 1988). The cell line pairs PEA1/2 and PEO14/23 were established prior to treatment and at platinum-resistant relapse six and seven months later respectively. The PEO1/4/6 cell series has recently been shown to arise from an individual with a *BRCA2* germline mutation (Sakai *et al.*, 2009). We confirmed that the hereditary mutation in PEO1 and the secondary mutation in PEO4/6 restoring BRCA function were present in our stocks of these lines (data not shown). PEO1 was cultured from relapsed disease 22 months after combination chemotherapy with cisplatin and the patient was successfully re-treated with platinum-based agents. Relapse more than 12 months after initial treatment is clinically defined as platinum-sensitive disease (Markman *et al.*, 1991). PEO4 was derived from further progressive disease 10 months later, prior to re-treatment with high dose cisplatin. PEO6 was derived 3 months later, after failure to respond to high dose cisplatin.

We first confirmed that that the matched series of PEO1/4/6, PEO14/23 and PEA1/A2 each arose from a unique individual using STR genotyping (data not shown). To confirm that these lines were consistent with HGS histotype and not from endometrioid, clear cell or low-grade serous tumours we tested for mutations commonly associated with these subtypes in *KRAS* (Exon 2), *BRAF* (exon 15), *CTNNB1* (exon 3), *PIK3CA* (exons 10 and 21). All cell lines had pathogenic *TP53* mutations (Supplementary Table 1) but no other mutations, consistent with HGS histotype (Ahmed *et al.*, 2010). We then carried out *in vitro* cisplatin sensitivity assays to confirm platinum response was comparable with their clinical presentation. Pre-treatment cell lines, PEA1 and PEO14, were cisplatin sensitive with 40 ±27 % and 69 ±6 % viability respectively when treated with 6μM cisplatin (Supplementary Figure 1). PEO1, which was established at platinum-sensitive relapse, showed the same degree of sensitivity to cisplatin as PEA1 and PEO14 with 62 ± 20 % viability. The lines established from treatment-resistant relapsed disease, PEA2, PEO23 and PEO4/6, were resistant to cisplatin *in vitro* with an average viability of 97 % (range 93-102 %) (Supplementary Figure 1).

Since *BRCA1/BRCA2* mutation is associated with platinum sensitivity in hereditary ovarian cancer, and reversion of mutations is a mechanism of resistance in BRCA mutation carriers, we sequenced *BRCA1* and *BRCA2* in the pre-treatment lines PEO14 and PEA1. Both of these platinum sensitive lines had wild-type *BRCA1* and *BRCA2*, confirming that they were not derived from BRCA mutation carriers and therefore excluding compensating or reversion BRCA mutations as the mechanism of resistance in these lines (data not shown).

Cell lines established at different disease stages had extensive karyotypic divergence

We next examined the consensus karyotypes of each cell line series to look for evidence of sequential genetic change. PEA1/A2, PEO14/23 and PEO1/4/6 were tetraploid, triploid and diploid respectively. M-FISH (Table 1 and Supplementary Figure 2) identified striking differences between the chemotherapy sensitive and resistant lines as PEA1 and PEA2 had only 2/37 derivative chromosomes in common. In addition, one chromosome had junctions between chromosomes 7, 8 and 6 in both lines but had undergone further unique rearrangement in both PEA1 (joined to chromosome 11) and PEA2 (two versions joined to chromosomes 6 and 4). Chromosome painting of chromosomes 5, 8 and 11 confirmed these observations, thus excluding mis-classification artefacts of M-FISH as a cause of the differences (Supplementary Figure 3a).

PEO14 and PEO23 shared only 2/18 derivative chromosomes with a common rearrangement of der(5)t(3;5;10) and a del(9). The der(5)t(3;5;10) was confirmed by chromosome painting of 3 and 5 (Supplementary Figure 3b) and by FISH (Supplementary Figure 3c).

PEO1 and PEO4 shared 6/28 derivatives, plus five chromosomes that had one junction in common but had undergone further rearrangement in one or other line (Table 1 and Supplementary Figure 2). In contrast, PEO4 and PEO6 were virtually identical, with 15/17 derivatives present in both lines (Table 1 and Supplementary Figures 2 and 3d). PEO4 and PEO6 were independently established 3 months apart (Figure 1) and have been grown for 61 and 17 passages in culture respectively.

As the M-FISH data suggested that sensitive and resistant cell lines were highly genetically divergent, we next used high-resolution array CGH on the Illumina 1M-duo SNP array platform to characterise these differences in detail. All of the cell lines showed multiple copy number aberrations, consistent with an origin of HGS ovarian carcinoma (Etemadmoghadam *et al.*, 2009). Extensive differences between the sensitive and resistant lines for each patient were confirmed, as was the high similarity between paired resistant lines PEO4 and PEO6 (Figure 2). When compared to each of their platinum-sensitive earlier lines, PEO4, PEA2 and PEO23 showed copy number differences on at least 20 chromosomes (Figure 2a-c) involving 13 %, 37 % and 24 % of the genome respectively. In contrast, PEO4 and PEO6 only differed on chromosomes 2, 4, 6 and 20 (Figure 2d) at only 4 % of the genome.

Sensitive and resistant sub-clones were not linearly related

The genetic differences within the PEO1/4/6, PEO14/23 and PEA1/A2 series suggested that the later, platinum-resistant cells were not direct descendants of the earlier lines, but had diverged independently from a common ancestor. PEA1 and PEA2 were pseudo-tetraploid with pairs of derivative chromosomes (Supplementary Figure 2), suggesting that each has undergone genome duplication. However, the derivative chromosomes that show duplication, and were therefore present before the duplication event, were different for each line. PEA1 had duplications of der(1)t(1;6), der(3)t(3;6), der(4)t(4;5), der(6)t(10;6) and der(15)t(15;4), while PEA2 had duplications of der(4)t(4;11), der(7)t(7;18;8), der(10)t(6;10) and der(11)t(11;3). This suggested that the genetic divergence of these sensitive and resistant populations started prior to genome duplication. PEO14 contained three derivatives of chromosome 3 but no normal copies of this chromosome, while PEO23, derived seven months later, contained two normal copies of chromosome 3 and none of the derivatives present in PEO14. Chromosome painting of chromosomes 3, 5 and 7 confirmed this M-FISH data (Supplementary Figure 3b). Similarly, PEO1 contained no normal copies of chromosome 1, 9, 11 or 17, while PEO4 contained one apparently normal copy of each (Supplementary Figures 2 and 3d).

We then focussed on LOH events to further confirm the non-linear relationship within cell line series. Genotyping calls from the SNP array data showed that all of the series had chromosome regions where the pre-treatment lines showed LOH while the relapse lines had both maternal and paternal sequences. PEA1 had LOH on regions of chromosomes 5, 7, 12 and 16 that were heterozygous in PEA2 (Supplementary Figure 4), while PEO14 had a single region of LOH on chromosome 1 that was heterozygous in PEO23. PEO1 showed LOH on regions of chromosomes 1–5, 8, 12 and 21, all of which were heterozygous in PEO4. PEO4 had regions of LOH on chromosomes 6 and 20 that were heterozygous in PEO6, possibly indicating further divergence even in late stage disease.

Tumour cell survival of initial treatment was not necessarily genetically determined

Given the changes seen between sensitive and resistant disease in PEO14/23, PEA1/2 and PEO1/4 and the lack of significant differences in the short interval between PEO4/6, we then asked if array CGH changes were seen in 12 tumour samples taken before and after neoadjuvant chemotherapy from six cases of HGS (Table 2). These samples were obtained from the CTCR-OV01 clinical study, which was designed to identify sequential genomic change during neoadjuvant chemotherapy (Ahmed *et al.*, 2007; Swanton *et al.*, 2007; Swanton *et al.*, 2009). We confirmed that five of the six pre-treatment samples were wild-type for *BRCA1* and *BRCA2* confirming that these were sporadic cases and therefore that restoration of BRCA function through reversion or compensation would not be a mechanism of platinum resistance in these tumours. The remaining pre-treatment sample (case 08) had insufficient DNA available for sequencing.

Array CGH was performed using Agilent's CGH 44k Microarray. The tumours showed evidence of genomic instability with multiple copy number aberrations in each sample consistent with HGS origin. Of the six pairs of primary tumour samples taken before and after treatment, three showed the same pattern of copy number aberrations in the pre-treatment biopsy and post-treatment debulking surgery samples (cases OV01-08, 14 and 41). The remaining three cases (OV01-11, 12 and 17) showed 2–5 differences in debulking surgical samples compared to initial biopsy (Figure 3 and Table 3). Eight of these were relatively small, less than 6 Mb, and located at the telomeres, possibly indicating a common artefact (Figure 3d). However, there was an increase of 38 Mb of 4q31.2-q35.2 and Xq at debulking surgery in case 11 and gain of the whole of chromosome X in case 12 (Figure 3d). Pre- and post-treatment paired samples did not show the magnitude of divergence seen between the PEO1/4, PEO14/23 and PEA1/2 series, although this comparison was not on an array CGH platform that provided LOH information.

Early events in carcinogenesis

HGS cancer is a genetically unstable disease and shows considerable variation in the genetic aberrations present between patients (Kobel *et al.*, 2008). This has made it difficult to identify the key oncogenes and tumour suppressor genes driving HGS cancer development, and to distinguish between driver and passenger mutations. Given the divergence within each cell line series we asked which genomic aberrations were common across all time points for matched cell lines, in order to identify early genomic changes that were present in the putative ancestral genome.

PEO1/4/6 shared 15 homozygous deletions, ranging in size from 0.8 kb to 2.9 Mb. Four of these were in regions containing known deletion polymorphisms (listed as structural variations on the UCSC genome browser, hg18), and of the remaining 11 only six affected genes. However, three of these included tumour suppressor genes *p16INK4a/ARF* (Kamb *et al.*, 1994), *WWOX* (Paige *et al.*, 2001), and *NF2* (*merlin*) (Curto and McClatchey, 2008; Trofatter *et al.*, 1993), two affected *RAB32* (Schulmann *et al.*, 2005) and *GNG7* (Shibata *et*

al., 1998), both of which are down-regulated in other tumour types, and one included KIAA0195, a gene of unknown function (Table 4). PEO14/23 did not have homozygous deletion of any known tumour suppressor gene, however both lines showed homozygous loss of *JARID2*, which represses expression of the oncogene cyclin D1 (Toyoda *et al.*, 2003) and *ezrin*, which interacts with NF2 (Curto and McClatchey, 2008).

Interestingly, while 10 homozygous deletions were common to all cell lines within a set, only one was found in one cell line but not the other from the same series (data not shown). This suggests that homozygous deletions occur early and clones containing them are maintained by strong selection. PEA1 and PEA2 did not contain any common non-polymorphic homozygous deletions affecting genes. However, PEA1 showed heterozygous deletion of known tumour suppressor gene *FAT*, which has progressed to homozygous deletion in PEA2 (data not shown), as a result of loss of the majority of 4q, consistent with two-hit tumour suppressor gene inactivation.

Discussion

HGS ovarian cancer is a genetically unstable disease with tumours showing highly rearranged karyotypes with numerical and structural chromosome aberrations (Bayani *et al.*, 2002). The underlying cause of instability in many cases is likely to be linked to an extremely high frequency of *Tp53* mutation, 97 %, in HGS cancers (Ahmed *et al.*, 2010) and frequent germline and somatic DNA repair defects (Turner *et al.*, 2004). This genetic instability has the potential to generate significant genetic diversity. Phenotypic and transcriptional heterogeneity within solid tumours are frequently reported (Kang *et al.*, 2003; McAlpine *et al.*, 2008; Ruijter *et al.*, 1996). Whether or not there is a genetic basis for these differences, or whether they are a consequence of differentiation hierarchies and stemness within tumours has not been investigated.

We have used a combination of cell line model systems and paired primary tumour samples to investigate genome evolution, and the role of genetic heterogeneity in response to chemotherapy in HGS ovarian carcinoma. We observed an extensive and non-linear genetic divergence between treatment-sensitive and treatment-resistant clones cultured from the same individual. However, neo-adjuvant treatment with 3–6 cycles of chemotherapy, even when a clinical response was observed, did not provide significant enrichment for a substantially different genetic sub-clone.

The extensive non-linear divergence of sensitive and resistant sub-clones that we observed in the three ovarian cancer cases from which the PEO/PEA lines were derived suggests profound intra-tumour genetic heterogeneity, consistent for a genomically unstable tumour. The extent and type of the differences between the early and late cell lines suggests that they are only very distantly related and have evolved in parallel rather than as a direct progression. Genetically divergent sub-clones that are intrinsically resistant to platinum-based treatment may therefore exist within a tumour at the time of presentation and before treatment begins.

It has recently been shown that a mechanism of cisplatin resistance in PEO4/6 is restoration of a functional BRCA2 repair pathway. PEO1 has a germline BRCA2 mutation (Sakai *et al.*, 2009) and our array CGH data confirms loss of the wild-type allele. In PEO4 this region is still present as a single copy, but a second mutation has occurred which restores the wild-type amino acid sequence (Sakai *et al.*, 2009). Our data shows extensive differences between PEO4 and PEO1 confirming that the resistant PEO4 sub-clone in this case is most likely to have existed prior to treatment, paralleling what has been observed for resistant cells in leukaemia (Hofmann *et al.*, 2003; Mullighan *et al.*, 2008; Zhu *et al.*, 1999).

Interestingly, we observed more genomic complexity at presentation than at relapse. There are three possible explanations for this. Firstly, the relapse genome may have had a slower growth rate and gone through fewer cell divisions. Secondly, intrinsically resistant cells may be genomically more stable. This possibility is particularly intriguing given the recent evidence showing reversion mutations in *BRCA1*/*BRCA2* as a mechanism of resistance (Sakai *et al.*, 2009; Sakai *et al.*, 2008; Swisher *et al.*, 2008). BRCA defects may be associated with genomic instability through an increased incidence of double-strand breaks (Bhattacharyya *et al.*, 2000) and therefore restoration of this repair pathway may stabilise the genomes of resistant sub-clones. Thirdly, it is possible that cells revert to a more constrained genome under the pressure of chemotherapy. Although this seems less likely given our evidence for a non-linear relationship between sensitive and resistant genomes.

While our results showed large genetic differences between cell lines established at different timepoints during disease course, there were very few differences between profiles before and during chemotherapy treatment in the same patient. Although the array CGH platform used to profile clinical samples was approximately one fifth the resolution of the 1M platform used to profile the cell lines, the heterogeneity observed in the cell lines was not biased towards either large or small changes. The array CGH data should therefore be representative of the overall picture of genomic similarity in the clinical samples.

Comparison of primary tumour samples pre-treatment and post three cycles of neo-adjuvant chemotherapy suggested that much of the residual disease at debulking surgery was not a genetically different sub-clone. This may be because disease has survived the initial cycles of treatment for some environmental reason, such as accessibility to drug or tumour-matrix interaction, or there may be refractory disease present without significant genetic differences as detectable by array CGH. In the cell line system PEO1, which was established after treatment with platinum-based chemotherapy, is still sensitive to cisplatin *in vitro*, suggesting that this first relapse arose due to failure to clear all sensitive disease. Three neo-adjuvant cycles of chemotherapy may therefore be too short an interval to remove all of the treatment-sensitive disease present. Cell line responses to *in vitro* treatment with cisplatin were consistent with clinical response at the timepoint when they were derived. This confirms that the sensitivity of these clones to cisplatin is genetically determined, and therefore that clinical relapse with resistant disease in these cases was not due to tumour inaccessibility to drug.

The inability of high-resolution array CGH to detect the effects of selection during initial treatment suggests that one clone is strongly dominant at the time of presentation. Minor clones identified in leukaemia (Zhu *et al.*, 1999) and breast cancer (Shah *et al.*, 2009) are only present as up to 1 % and 13 % of the population respectively and were detected by PCR or deep sequencing approaches. Given these data we could speculate that a significant, potentially up to 97 %, reduction in the dominant clone would be needed before minor subclones could be detected by array CGH, which outputs the consensus genome of a large number of cells. RECIST criteria require a 30 % reduction in target lesions to classify patients as achieving partial clinical response. This would be insufficient to identify minor populations with intrinsic resistance.

Although array CGH fails to identify genetically divergent sub-clones within primary tumours the magnitude of the changes observed in the cell line system argues against culture artefacts as an explanation. In spite of genomic instability cell line genomes can be relatively stable through passages in culture (Roschke *et al.*, 2002). The high similarity of the PEO4 and PEO6 genomes suggests a low rate of change in culture in this system. The few differences between PEO4 and PEO6 could have arisen in culture although the number is similar to the small number of differences seen between the CTCR-OV01 samples taken at

relatively short time intervals. Concordance between matched pairs of cell lines established from a single patient at a single timepoint is common, having been observed in a more recently established matched cell line set (Ouellet *et al.*, 2008). Although any individual rearrangement might have arisen in culture, overall the cell line genomes are likely to represent a genuine, although not necessarily dominant, clone from the primary tumour.

As a consequence of the extensive divergence between presentation and relapse disease, the few rearrangements that are common are potentially enriched for the very earliest events in carcinogenesis. There is a striking enrichment for known and candidate tumour suppressor genes in the common deletions between PEO1 and PEO4, including homozygous deletions of *p16INK4a*/*ARF*, *NF2*, and *WWOX*. These will lead to deregulated proliferation, reduced apoptosis and loss of contact-dependent inhibition of proliferation. These changes therefore confer many of the 'Hallmarks of Cancer' (Hanahan and Weinberg, 2000) and consequently may well be what defines these cells as tumour cells. In turn, this suggests that early homozygous deletions identified in the other pairs may harbour previously unidentified tumour suppressor genes.

Our results support the model that pre-existing intra-tumour genetic heterogeneity is a major factor in determining relapse with resistant disease. Our study does not address the degree of heterogeneity or the proportions of divergent cells that may be present. The presence of a strongly dominant clone makes finding genetically different sub-clones at a single timepoint difficult and future studies may benefit from the use of microdissection or flow sorting techniques in order to sample different populations. Alternatively, analysis of relapse disease to identify relapse-specific lesions that can be retrospectively identified at low prevalence in matched presentation disease may prove more effective (Mullighan *et al.*, 2008; Zhu *et al.*, 1999). The increase in resolution and the range of aberrations that can be detected due to high-throughput sequencing technologies will be essential in examining the full extent of heterogeneity within tumours.

There are important clinical implications of genetic heterogeneity within tumours. The identification of early events and the order in which genetic aberrations occur is extremely difficult due to large numbers of passenger mutations. By profiling apparent divergent populations sequentially we may improve the identification of early initiating events. Quantifying heterogeneity remains a major challenge and future studies will need to address the problems associated with sample collection, including obtaining samples from end-stage disease. Identifying different genetic sub-clones would allow therapy either to be targeted at a common change or personalised to combine therapies targeting different subpopulations.

Materials and methods

Cell culture

Cell lines were grown in RPMI-1640 + 10 % FBS + 1x Penicillin/Streptomycin (all Invitrogen, Paisley, UK) at 37C, 5% CO_2. Cell lines were thawed at passage numbers 34 (PEA1), 31 (PEA2), 71 (PEO1), 61 (PEO4), 17 (PEO6) and 20 (PEO14 and PEO23). STR typing was carried out using the AmpFLSTR Identifiler PCR amplification kit (Applied Biosystems, Warrington, UK). Mutation screening was carried out according to the methods of Ahmed *et al.* (2010). Genomic DNA for array CGH was extracted from a confluent T75 using the Gentre Puregene cell kit for 1-2×10^7 cells (Qiagen, Crawley, UK) according to manufacturer's instructions into 150ul nuclease-free water.

Cisplatin sensitivity assay

Cells were plated at a density of 3000/well in 96-well plates and allowed to adhere in normal growth medium. The following day fresh medium with a range of concentrations of cisplatin

(Sigma-Aldrich, Poole, UK) (0uM, 1.5uM, 3uM, 6uM) was added. After 48 hrs, cell viability was assayed using a Cell Titre Glo Assay (Promega, Southampton, UK) according to manufacturer's instructions.

M-FISH

Metaphases were prepared as previously described in (Alsop *et al.*, 2006). M-FISH was carried out using the 24XCyte Human mFISH Painting Kit (MetaSystems, Altlussheim, Germany) according to manufacturer's instructions. At least 10 metaphases for each line were captured using a Zeiss Axioplan epifluorescent microscope equipped with camera VAC-30054 (MetaSystems) and analysed using MetaSystems' Isis software. An analysis of the heterogeneity present between metaphases for each line is included in Supplementary Figure 2.

Primary tumour samples

Primary tumours were obtained from the prospective randomised study, CTCR-OV01, conducted in the Department of Oncology, University of Cambridge under an ethically approved protocol (Ahmed *et al.*, 2007). All samples were confirmed as HGS by histopathological examination and were late-stage (III or IV). Samples were taken before and after treatment and snap frozen. Sections from all post-treatment samples were hematoxylin-eosin stained to evaluate the percentage of tumour cells and genomic DNA isolated using proteinase-K treatment and phenol chloroform purification. Pre-treatment needle biopsies were too small to allow sectioning as well as DNA extraction and were processed using TriReagent (Invitrogen) according to manufacturer's instructions in order to obtain RNA from the same specimens for other studies (Ahmed *et al.*, 2007; Swanton *et al.*, 2007; Swanton *et al.*, 2009). Following exclusion of patients with non-serous ovarian cancer (4), severe treatment toxicity (3), sample collection at only one timepoint (5), insufficient biopsy material for array CGH (24), low tumour cellularity (4) and poor quality DNA (2) we were left with six pairs of samples.

Array CGH

Array CGH on primary tumour samples was performed using the Agilent 60-mer oligonucleotide CGH 44k Microarray (Agilent Technologies, Wokingham, UK) which gives an average spatial resolution of 35kb. Array CGH on cell line samples was carried out on the Illumina Human1M-Duo BeadChip (Illumina, Chesterford, UK), giving an average resolution of 7kb. Experimental details and analysis parameters are given in the Supplementary Methods. The array data has been deposited in GEO with accession numbers GSE18453 (primary tumour samples) and GSE18461 (cell line samples).

Resequencing

Sequencing of p53, KRAS, BRAF, CTNNB1 and PIK3CA to confirm that cell lines were of HGS histotype was carried out according to the method of Ahmed et al. (2010). Details of BRCA1 and BRCA2 sequencing and primer sets used for both cell lines and primary tumour samples are given in Supplementary Methods. Sequence traces were analysed in Seqscape v2.6 (Applied Biosystems, Warrington, UK) and using the UCSC genome browser (http:// genome.ucsc.edu/cgi-bin/hgGateway). Any mutations called by the software were visually checked to eliminate sequencing artefacts and checked against dbSNP build 130 to remove any normal polymorphisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

Agarwal R, Kaye SB. Ovarian cancer: strategies for overcoming resistance to chemotherapy. Nat Rev Cancer. 2003; 3:502–16. [PubMed: 12835670]

Ahmed AA, Etemadmoghadam D, Temple J, Lynch AG, Riad M, Sharma R, et al. Driver mutations in TP53 are ubiquitous in high grade serous carcinoma of the ovary. J Pathol. 2010; 221:49–56. [PubMed: 20229506]

Ahmed AA, Mills AD, Ibrahim AE, Temple J, Blenkiron C, Vias M, et al. The Extracellular Matrix Protein TGFBI Induces Microtubule Stabilization and Sensitizes Ovarian Cancers to Paclitaxel. Cancer Cell. 2007; 12:514–527. [PubMed: 18068629]

Alsop AE, Teschendorff AE, Edwards PA. Distribution of breakpoints on chromosome 18 in breast, colorectal, and pancreatic carcinoma cell lines. Cancer Genet Cytogenet. 2006; 164:97–109. [PubMed: 16434311]

Bast RC Jr. Hennessy B, Mills GB. The biology of ovarian cancer: new opportunities for translation. Nat Rev Cancer. 2009; 9:415–28. [PubMed: 19461667]

Bayani J, Brenton JD, Macgregor PF, Beheshti B, Albert M, Nallainathan D, et al. Parallel analysis of sporadic primary ovarian carcinomas by spectral karyotyping, comparative genomic hybridization, and expression microarrays. Cancer Res. 2002; 62:3466–76. [PubMed: 12067990]

Bhattacharyya A, Ear US, Koller BH, Weichselbaum RR, Bishop DK. The breast cancer susceptibility gene BRCA1 is required for subnuclear assembly of Rad51 and survival following treatment with the DNA cross-linking agent cisplatin. J Biol Chem. 2000; 275:23899–903. [PubMed: 10843985]

Curto M, McClatchey AI. Nf2/Merlin: a coordinator of receptor signalling and intercellular contact. Br J Cancer. 2008; 98:256–62. [PubMed: 17971776]

Etemadmoghadam D, deFazio A, Beroukhim R, Mermel C, George J, Getz G, et al. Integrated genome-wide DNA copy number and expression analysis identifies distinct mechanisms of primary chemoresistance in ovarian carcinomas. Clin Cancer Res. 2009; 15:1417–27. [PubMed: 19193619]

Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000; 100:57–70. [PubMed: 10647931]

Herzog TJ. Recurrent ovarian cancer: how important is it to treat to disease progression? Clin Cancer Res. 2004; 10:7439–49. [PubMed: 15569973]

Herzog TJ, Pothuri B. Ovarian cancer: a focus on management of recurrent disease. Nat Clin Pract Oncol. 2006; 3:604–11. [PubMed: 17080178]

Hofmann WK, Komor M, Wassmann B, Jones LC, Gschaidmeier H, Hoelzer D, et al. Presence of the BCR-ABL mutation Glu255Lys prior to STI571 (imatinib) treatment in patients with Ph+ acute lymphoblastic leukemia. Blood. 2003; 102:659–61. [PubMed: 12663457]

Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, et al. A cell cycle regulator potentially involved in genesis of many tumor types. Science. 1994; 264:436–40. [PubMed: 8153634]

Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordon-Cardo C, et al. A multigenic program mediating breast cancer metastasis to bone. Cancer Cell. 2003; 3:537–49. [PubMed: 12842083]

Khaique L, Ayhan A, Weale ME, Jacobs IJ, Ramus SJ, Gayther SA. Genetic intra-tumour heterogeneity in epithelial ovarian cancer and its implications for molecular diagnosis of tumours. J Pathol. 2007; 211:286–95. [PubMed: 17154249]

Kobel M, Huntsman D, Gilks CB. Critical molecular abnormalities in high-grade serious carcinoma of the ovary. Expert Rev Mol Med. 2008; 10:e22. [PubMed: 18671886]

Langdon SP, Lawrie SS, Hay FG, Hawkes MM, McDonald A, Hayward IP, et al. Characterization and properties of nine human ovarian adenocarcinoma cell lines. Cancer Res. 1988; 48:6166–72. [PubMed: 3167863]

Liu W, Laitinen S, Khan S, Vihinen M, Kowalski J, Yu G, et al. Copy number analysis indicates monoclonal origin of lethal metastatic prostate cancer. Nat Med. 2009; 15:559–65. [PubMed: 19363497]

Markman M, Rothman R, Hakes T, Reichman B, Hoskins W, Rubin S, et al. Second-line platinum therapy in patients with ovarian cancer previously treated with cisplatin. J Clin Oncol. 1991; 9:389–93. [PubMed: 1999708]

McAlpine JN, Eisenkop SM, Spirtos NM. Tumor heterogeneity in ovarian cancer as demonstrated by in vitro chemoresistance assays. Gynecol Oncol. 2008; 110:360–4. [PubMed: 18632143]

Merlo LM, Pepper JW, Reid BJ, Maley CC. Cancer as an evolutionary and ecological process. Nat Rev Cancer. 2006; 6:924–35. [PubMed: 17109012]

Mullighan CG, Phillips LA, Su X, Ma J, Miller CB, Shurtleff SA, et al. Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia. Science. 2008; 322:1377–80. [PubMed: 19039135]

Nowell PC. The clonal evolution of tumor cell populations. Science. 1976; 194:23–8. [PubMed: 959840]

Ouellet V, Zietarska M, Portelance L, Lafontaine J, Madore J, Puiffe ML, et al. Characterization of three new serous epithelial ovarian cancer cell lines. BMC Cancer. 2008; 8:152. [PubMed: 18507860]

Paige AJ, Taylor KJ, Taylor C, Hillier SG, Farrington S, Scott D, et al. WWOX: a candidate tumor suppressor gene involved in multiple tumor types. Proc Natl Acad Sci U S A. 2001; 98:11417–22. [PubMed: 11572989]

Roschke AV, Stover K, Tonon G, Schaffer AA, Kirsch IR. Stable karyotypes in epithelial cancer cell lines despite high rates of ongoing structural and numerical chromosomal instability. Neoplasia. 2002; 4:19–31. [PubMed: 11922387]

Ruijter ET, van de, Kaa CA, Schalken JA, Debruyne FM, Ruiter DJ. Histological grade heterogeneity in multifocal prostate cancer. Biological and clinical implications. J Pathol. 1996; 180:295–9. [PubMed: 8958808]

Sakai W, Swisher EM, Jacquemont C, Chandramohan KV, Couch FJ, Langdon SP, et al. Functional restoration of BRCA2 protein by secondary BRCA2 mutations in BRCA2-mutated ovarian carcinoma. Cancer Res. 2009; 69:6381–6. [PubMed: 19654294]

Sakai W, Swisher EM, Karlan BY, Agarwal MK, Higgins J, Friedman C, et al. Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. Nature. 2008; 451:1116–20. [PubMed: 18264087]

Schulmann K, Mori Y, Croog V, Yin J, Olaru A, Sterian A, et al. Molecular phenotype of inflammatory bowel disease-associated neoplasms with microsatellite instability. Gastroenterology. 2005; 129:74–85. [PubMed: 16012936]

Shah SP, Morin RD, Khattra J, Prentice L, Pugh T, Burleigh A, et al. Mutational evolution in a lobular breast tumour profiled at single nucleotide resolution. Nature. 2009; 461:809–13. [PubMed: 19812674]

Shibata K, Mori M, Tanaka S, Kitano S, Akiyoshi T. Identification and cloning of human G-protein gamma 7, down-regulated in pancreatic cancer. Biochem Biophys Res Commun. 1998; 246:205–9. [PubMed: 9600093]

Swanton C, Marani M, Pardo O, Warne PH, Kelly G, Sahai E, et al. Regulators of mitotic arrest and ceramide metabolism are determinants of sensitivity to paclitaxel and other chemotherapeutic drugs. Cancer Cell. 2007; 11:498–512. [PubMed: 17560332]

Swanton C, Nicke B, Schuett M, Eklund AC, Ng C, Li Q, et al. Chromosomal instability determines taxane response. Proc Natl Acad Sci U S A. 2009; 106:8671–6. [PubMed: 19458043]

Swisher EM, Sakai W, Karlan BY, Wurz K, Urban N, Taniguchi T. Secondary BRCA1 mutations in BRCA1-mutated ovarian carcinomas with platinum resistance. Cancer Res. 2008; 68:2581–6. [PubMed: 18413725]

Torres L, Ribeiro FR, Pandis N, Andersen JA, Heim S, Teixeira MR. Intratumor genomic heterogeneity in breast cancer with clonal divergence between primary carcinomas and lymph node metastases. Breast Cancer Res Treat. 2007; 102:143–55. [PubMed: 16906480]

Toyoda M, Shirato H, Nakajima K, Kojima M, Takahashi M, Kubota M, et al. jumonji downregulates cardiac cell proliferation by repressing cyclin D1 expression. Dev Cell. 2003; 5:85–97. [PubMed: 12852854]

Trofatter JA, MacCollin MM, Rutter JL, Murrell JR, Duyao MP, Parry DM, et al. A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. Cell. 1993; 75:826. [PubMed: 8242753]

Turner N, Tutt A, Ashworth A. Hallmarks of 'BRCAness' in sporadic cancers. Nat Rev Cancer. 2004; 4:814–9. [PubMed: 15510162]

Zhu YM, Foroni L, McQuaker IG, Papaioannou M, Haynes A, Russell HH. Mechanisms of relapse in acute leukaemia: involvement of p53 mutated subclones in disease progression in acute lymphoblastic leukaemia. Br J Cancer. 1999; 79:1151–7. [PubMed: 10098750]

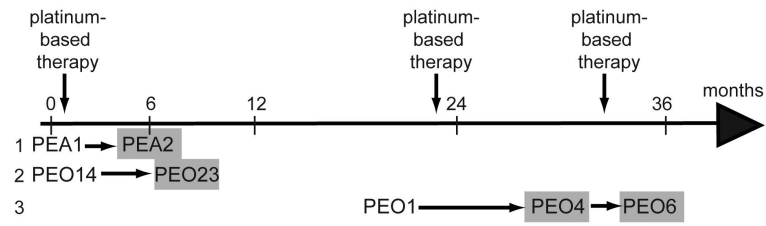**Figure 1.**
Experimental design: Cell lines from 3 individuals established at different timepoints through disease progression were analysed. All patients received platinum-based chemotherapy. Grey boxes indicate platinum-resistant cell lines.
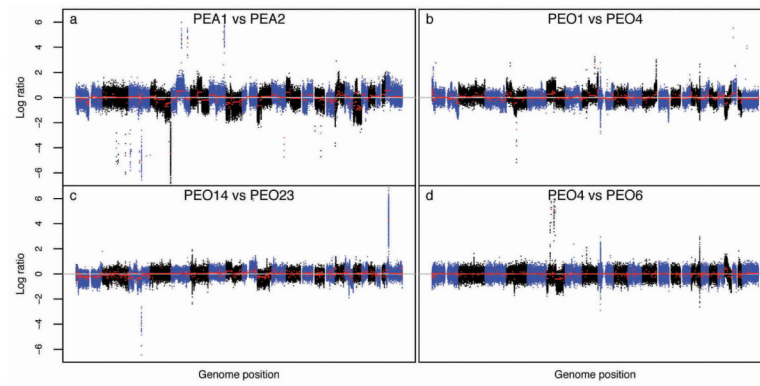
Figure 2.
Probe-by-probe subtraction plots of the array CGH profiles for matched cell line pairs. (a) PEA2 – PEA1 (b) PEO4 – PEO1 and (c) PEO23 – PEO14 show differences (log$_2$ ratio ≠ 0) on the majority of chromosomes. (d) PEO6 – PEO4 show genuine differences on chromosomes 4, 6 and 20 plus artefacts caused by homozygous deletions on chromosomes 9 and 16. All subtraction plots show increased noise around some telomeres and centromeres.
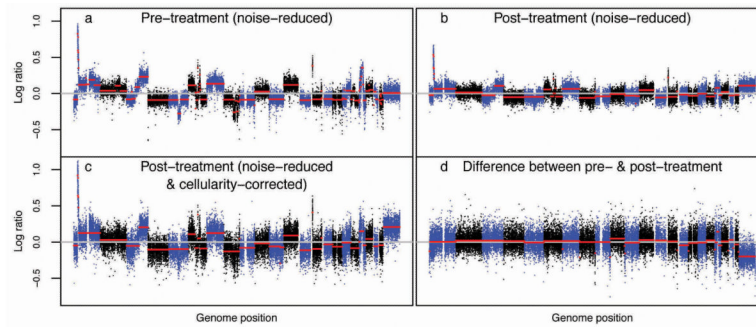
Figure 3.
Array CGH profiles for patient OVO12 at (a) presentation and (b) debulking surgery. (c) Log$_2$ ratio values of the debulking surgery profile (b) were scaled to match the range in the presentation array (a) to account for the difference in cellularity. (d) Difference in log ratio on a probe-by-probe basis between profiles of the two timepoints after correcting for cellularity. Alternate chromosomes are shown in different colours. The majority of the genome shows no difference between the samples but the negative difference in the log$_2$ ratio for the X chromosome indicates gain in the debulking surgery sample. Several chromosomes, e.g. 1p, 7p, show differences in a very small number of markers immediately adjacent to the telomere or centromere, possibly as a common artefact due to increased noise in these regions.

Table 1

Derivative chromosomes identified by spectral karyotyping in the consensus karyotype of matched ovarian cancer cell lines

Cell line	Derivative chromosomes
PEO1 (unique)	der(1)t(1;5), del(1), der(3)t(3;8), der(4)t(4;10), der(8)t(8;1), der(9)t(9;1), der(10)t(10;3), der(11)t(11;12), der(12)t(11;12), dup(15) der(17)t(17;6)
PEO1 & PEO4/PEO6 (shared)	der(7)t(8;7;8), der(16)t(16;4), der(19)t(19;7), der(22)t(22;20), der(X)t(X;22;X) [*]
PEO1 (part-shared with PEO4/PEO6)	der(2)t(6;2), der(5)t(1;5;9), der(9)t(3;9;1), der(10)t(10;17;7), der(11)t(2;11)
PEO4/PEO6 (part-shared with PEO1)	der(2)t(6;2;20), der(5)t(5;9), der(9)t(9;3), der(10)t(10;17), der(11)t(2;11;17) [**]
PEO4/PEO6 (unique)	der(1)t(1;17), der(3)t(X;3), del(4), i(8)(q10), del(14), der(14)t(14;X) [*], der(21)t(13;21)
PEO6 (unique)	del(6)
PEA1 (unique)	der(1)t(1;6), der(1)t(1;5), der(3)t(3;6), der(3)t(1;3), der(4)t(4;5), der(6)t(6;10), der(7)t(7;15), der(8)t(8;14), der(8)t(8;22), der(8)t(8;6), der(9)t(X;9), der(11)t(11;5), der(12)t(16;12), der(15)t(15;4), der(17)t(13;17), der(17)t(22;17), der (X), t(9;X)
PEA1 & PEA2 (shared)	i(5)(p10), del(18)
PEA1 (part-shared with PEA2)	der(7)t(11;7;8;6)
PEA2 (part-shared with PEA1)	der(7)t(6;7;8;6), der(7)t(4;7;8;6)
PEA2 (unique)	del(1), del(3), der(4)t(4;11), der(4)t(22;4), der(5)t(2;5), del(6), der(7)t(7;18;8), der(8)t(11;8), der(10)t(6;10), der(11)t(11;3), dup(14), der(16)t(6;16;6), der(16)t(16;13), der(19)t(19;7), der(22)t(13;22)
PEO14 (unique)	der(1)t(17;1), der(1)t(1;8), der(1)t(1;5), der(3)t(X;3), der(3)t(3;7), der(5)t(5;2), del(7), del(10), i(11)(q10), der(11)t(19;11), der(12)(?), del(18), der(21)t(21;18)
PEO14 & PEO23 (shared)	der(5)t(5;3;10), del(9)
PEO23	der (1)t(1;7), der(2)t(2;9), i(13)(q10)

[*] Not present in PEO6

[**] present as der(11)t(2;11;17) in PEO4

Table 2

Clinical data for 6 ovarian cancer cases with paired samples taken pre- and post-neo-adjuvant treatment

Patient	Age	Treatment	Cycles	CA125 pre	CA125 post	Response	PFS (months)	OS (months)
CTCR-OV01-08	56	P	3	14282	1291	Responder	> 60	> 60
CTCR-OV01-11	51	C	3	679	39	Responder	47	> 60
CTCR-OV01-12	73	P	3	877	854	Non-responder	24	25
CTCR-OV01-14	68	P->C+P	3+3	750	1537	Non-responder	11	15
CTCR-OV01-17	61	C	3	913	235	Responder	11	16
CTCR-OV01-41	75	P	3	3399	531	Responder	12	26

P: paclitaxel; C: carboplatin; CA125 pre: CA125 level before neoadjuvant chemotherapy; CA125 post: CA125 level after 3 cycles of neo-adjuvant chemotherapy; Responder/Non-responder: response as evaluated according to GCIG criteria; PFS: progression-free survival; OS: overall survival.

Table 3

Differences between presentation and debulking surgery samples as identified by segmentation of a probe-by-probe subtraction of two array CGH profiles

Patient	chrom	loc.start	loc.end	Size (kb)	num.mark	Change at surgery
11	4	153366202	191259440	37893	380	decrease
11	9	132490800	138284700	5794	138	increase
11	16	87238098	87698143	460	17	increase
11	17	48539	4746522	4698	165	increase
11	X	65677977	154405100	88727	1074	increase
12	8	181530	391354	210	4	increase
12	8	143211215	146250765	3040	95	increase
12	9	135113605	138284700	3171	85	increase
12	11	186966	2745315	2558	86	increase
12	X	2693677	154405100	151711	1813	increase
17	X	153116320	154405100	1289	42	increase
17	X	2693677	139604640	136911	1596	increase

Table 4

Common non-polymorphic homozygous deletions between matched cell line pairs

Cell line pair	Chr	Start (bp)	End (bp)	Size (kb)	Genes involved (RefSeq)
PEO1 & PEO4	6	146912623	146917015	4.4	RAB32 (intronic)
PEO1 & PEO4	9	209911332	23837615	2926.3	Multiple including p16-INK4a and ARF
PEO1 & PEO4	16	76706245	77427046	720.8	WWOX
PEO1 & PEO4	17	70973977	71003489	29.5	KIAA0195
PEO1 & PEO4	19	2463038	2463876	0.8	GNG7 (3'UTR)
PEO1 & PEO4	22	28327011	28334929	7.9	NF2
PEO14 & PEO23	5	155414747	155421495	6.7	SGCD (intronic)
PEO14 & PEO23	6	39177246	39180659	3.4	C6orf64
PEO14 & PEO23	6	159134798	159237500	102.7	EZR, OSTCL