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Review

Large-scale genomic analysis of ovarian carcinomas

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ABSTRACT

Epithelial ovarian cancers are typified by frequent genomic aberrations that have been difficult to unravel. Recently, high-resolution array technologies have provided the first glimpse of the remarkable complexity of these aberrations with some ovarian cancers containing hundreds of copy number breakpoints, micro-deletions and amplifications. Many of these alterations contain cancer-related genes suggesting that the majority is disease-associated and not just the product of random genomic instability. Future developments such as next-generation sequencing and integrated analysis of data from multiple array platforms on large numbers of samples are poised to revolutionise our understanding of this complex disease.

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1. Short history of ovarian genomics

The first karyotypes of ovarian cancer in the literature were published in the 1960s (e.g. Yamada et al., 1966), an era when cell culture was in its infancy and chromosomes 6–12 were still indistinguishable. Nonetheless, it was clear that epithelial ovarian cancer showed highly aneuploid cells with multiple marker chromosomes and high heterogeneity both within and between individual cases. Improvement in cytogenetic techniques and innovations such as flow cytometry and fluorescence in situ hybridisation enabled increasingly more detailed interrogation of ovarian cancer genetics. By 1990, over 150 ovarian cancer karyotypes had been published (Mitelman et al., 2008), but the complexity of the ovarian

carcinoma genome was such that conventional karyotyping was still unable to identify the key translocations (Mitelman et al., 1997; Taetle et al., 1999) in the way that it had been successful in identifying the Philadelphia chromosome as the key driver of chronic myeloid leukemia (CML).

The development of comparative genomic hybridisation (CGH) in 1992 opened a new window into ovarian genomics (Kallioniemi et al., 1992). By 2000, the most common amplifications and deletions were well documented, such as gains of 8q, 3q, 1q and 20q and losses of 4q, 5q, 8p, 22q, 18q and 17p. These findings are exemplified by the Progenetix database (www.progenetix.net) (Baudis and Cleary, 2001), which collates CGH and karyotype information for multiple cancer types (Figure 1a). Amplifications targeting known oncogenes

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such as *MYC* and *ERBB2* (Baker et al., 1990; Slamon et al., 1989) were identified in a proportion of cases. However, despite the increase in resolution provided by this technique, the amplicons could still not be refined sufficiently to unequivocally identify many of the specific gene targets.

In parallel with the categorization of numerical and structural chromosome aberrations, studies of loss of heterozygosity (LOH) were also conducted. Interest in LOH analysis stemmed from the “two-hit” hypothesis (Knudson, 1971) for the inactivation of tumor suppressor genes (TSGs), epitomized by mutations in genes like *RB1* (Cavenee et al., 1983). Mapping regions of LOH were predicted to flag the location of novel TSGs. Initially, LOH studies employed restriction fragment length polymorphisms (RFLPs) detected by Southern hybridisation but this method was extremely laborious and there were few RFLPs available. Within a few years, PCR amplifiable microsatellite markers overtook RFLP analysis as the LOH detection method of choice but despite the increased resolution, most analyses still had little power to resolve minimal regions of LOH. Over 70 studies were reported by 2002 (reviewed by Liu and Ganesan, 2002); the most common regions of LOH were chromosomes 17, 22q, 6q, 11q, 18q and 13q. These studies often found a very high frequency of LOH that differed from

the reported frequencies of copy number loss, due to the presence of copy number neutral LOH. In addition, copy number gains, which create allelic imbalance, could also be mistaken for LOH. These difficulties made it difficult to pinpoint the TSGs targeted by microsatellite based LOH analyses. The lack of success of LOH analyses to identify specific gene targets led some investigators to question the validity of the whole approach (Tomlinson et al., 2002).

By the year 2000, the most common genomic aberrations in ovarian carcinoma had been well annotated, however identification of specific target genes remained problematic as epithelial ovarian cancer is cytogenetically complex and genetically unstable, with multiple aneuploidies and translocations. A further complication is the fact that the disease is heterogeneous, with multiple malignant histopathological subtypes, including serous (40%), endometrioid (24%), mucinous (6%) and clear cell (26%) types (Gilks et al., 2008). The different subtypes are likely to have different aetiologies and develop through distinct developmental pathways. For example, ovarian tumors of the endometrioid subtype are more likely to harbor mutations in *PTEN* and *PIK3CA* (Campbell et al., 2004; Obata et al., 1998), whereas tumors of the mucinous subtype are more likely to harbor *KRAS* mutations

Figure 1 – Comparison of CGH data with 500K SNP array data. (A) Frequency data from 528 ovarian tumors showing most frequent gains (green) and losses (red) from Progenetix (Baudis and Cleary, 2001). (B) Frequency data from 31 ovarian tumors by 500K SNP array (Gorringe et al., 2007). The overall frequency of gains and losses is broadly similar between the two data sets. Note the additional ability to detect allelic imbalance gives much additional information over losses alone (e.g. chr13, chr22).

(Enomoto et al., 1991) and 9p LOH (Feltmate et al., 2005). The serous subtypes appear to comprise two distinct entities with low-grade serous tumors being characterised by the possession of KRAS and BRAF mutations (Haas et al., 1999; Tothill et al., 2008) while high-grade serous tumors commonly show loss of BRCA1 (Press et al., 2008) and TP53 mutation. These complexities have made rationalisation of copy number and LOH data difficult.

The finding of frequent loss of BRCA1 in ovarian carcinomas has suggested that many “sporadic” ovarian tumors may in fact have a strong inherited basis (Press et al., 2008). This factor adds additional complications to ovarian carcinoma analyses as there is only limited knowledge of the somatic genetic alterations of BRCA1/2-related ovarian cancer. Recent studies have found that tumors arising in women with germline BRCA1/2 mutations may acquire a different repertoire of genetic alterations (Press et al., 2008; Ramus et al., 2003). In particular these tumors may have an increased frequency of copy number neutral LOH (Walsh et al., 2008) that is not reflected in any increase in copy number changes as assessed by CGH (Ramus et al., 2003).

Recent studies have applied CGH to attempt to identify genetic alterations that correlate with resistance to chemotherapy and patient survival (Bernardini et al., 2005; Birrer et al., 2007; Hauptmann et al., 2002; Kim et al., 2007; Osterberg et al., 2005). These studies identified dozens of significant regions, for example Bernardini et al. identified 13 regions that showed different copy number between chemo-resistant and chemo-sensitive tumors using a BAC array CGH (aCGH) platform. The studies were rarely consistent, however, with only three regions replicated: 21q21 loss and 17p11 loss were both associated with resistance or poor survival in two studies and 13q21–22 gain was associated with resistance or poor survival in three studies. This variation between studies appears to be almost a hallmark of ovarian cancer, and may arise due to the heterogeneity of the disease, which means that these relatively small studies (17–63 tumors) will find different results depending on the mix of cases present. The different platforms used, from metaphase CGH to 22K oligonucleotide aCGH, may also have contributed to the inconsistency.

2. New technologies, new hope

The perennial problem of unravelling the cytogenetic complexities of epithelial ovarian cancer may soon be resolved with the development of high-resolution array platforms (Table 1). At the highest resolution, oligonucleotide aCGH can enable whole genome tiling, although these require running multiple arrays and are therefore relatively expensive. Lower density oligonucleotide aCGH platforms with resolutions of just a few kilobases are now readily available at a reasonable cost. High-density SNP arrays, which were originally designed for use in allele association studies, are now being applied to somatic cancer genetics and have the advantage of being able to simultaneously capture copy number and LOH information and importantly can identify copy number neutral LOH (Figures 1 and 2). These technologies increase the resolution of copy number and LOH aberrations to a level that enables near-complete characterisation of the genome.

Recent studies have determined that alterations as small as 25 kb are readily detectable in ovarian carcinoma and have included known cancer-related genes (Figure 2, Gorringe et al., 2007). Small alterations can even be detected using DNA extracted from formalin-fixed paraffin-embedded tissue (Jacobs et al., 2007). These small copy number alterations were found to be enriched for cancer genes, based on the Cancer Gene Census (Futreal et al., 2004). Examples include a 350 kb amplification of FGFR1 and three homozygous deletions in RB1 all smaller than 220 kb. Unbiased detection of such small regions would have been impossible with earlier techniques, and suggests that the increased resolution has a genuine likelihood of identifying novel cancer genes.

The increase in resolution is also reflected in the number of copy number breakpoints detected. Cytogenetic analysis has identified a median of 1 breakpoint per ovarian cancer case (range 0–32, Taetle et al., 1999), while metaphase CGH studies have typically found 10–20 breakpoints (range 0–62, e.g. Hu et al., 2003). This is contrasted with the median of 95 breakpoints (range 0–818), that we detected using a 500K SNP array (Gorringe et al., 2007). It is likely that more breakpoints arising from even smaller changes exist, as the frequency distribution of the gains and losses detected suggested that the effective resolution of the technology (25 kb) was still limiting (Figure 2).

The resolution of LOH detection also increases with high-density platforms. A recent study of lung cancer found that with an increase in resolution from 399 microsatellite markers to 10,000 SNP markers the minimum LOH region detected fell from 17.5 Mb to 1.6 Mb (Janne et al., 2004). In our 500K SNP array analysis, the smallest LOH region detected in 31 ovarian cancer samples without any concomitant copy number breakpoints was 137 kb. As well as vastly increasing the resolution of LOH detection over microsatellite markers, SNP arrays can also distinguish between “true” LOH (i.e. LOH in which one allele is completely absent) and allelic imbalance (AI) where amplification of one allele (within a region that is still heterozygous) can give the impression that the other allele has been lost. This quantitative analysis assists in narrowing regions of LOH and more accurately defining true LOH, which is more likely to target a tumor suppressor gene than allelic imbalance attributable to a copy number gain.

3. Future developments

While it appears overtly that much progress has been made in the last 10 years, epithelial ovarian cancer has yet to be fully characterised. The heterogeneity of the disease, both between patients and within a single tumor, combined with the genetic instability that leads to such deranged chromosome complements, have defied full description. Studies of large numbers of tumors using multiple high-resolution techniques combined with sophisticated bioinformatics analysis will be required to decipher ovarian cancer.

It is hard to envisage that higher resolution arrays than currently available (>2000K probes) would be of much additional benefit, especially since small alterations (<5 kb) may be better detected through next-generation sequencing technologies. Massively parallel sequencing has multiple applications that will impact ovarian cancer genomics.

Table 1 – Genome-wide technologies for copy number analysis.

Platform	Resolution (probe sets)	Advantages/disadvantages	OVCA examples
24-colour FISH ^{a,b} (M-FISH, SKY)		Detects balanced translocations but labour intensive. Analysis is cell-by-cell. Gives absolute copy number.	Bayani et al. (2002), Rao et al. (2002)
Cytogenetic CGH ^{b,c}	10 Mb	As for all remaining platforms, gives copy number relative to a control and unable to resolve certain rearrangements, such as determining translocation partners.	Kiechle et al. (2001), Osterberg et al. (2005), Partheen et al. (2004), Sonoda et al. (1997)
cDNA aCGH ^c	Dependent on gene density (10–30K)	Poor quality, unevenly spaced data but easy to integrate with expression.	Brown et al. (2006)
BAC aCGH ^c	2–4 Mb (1.3K), 300 kb (32K)	Good quality but relatively low resolution.	Bernardini et al. (2005), Kim et al. (2007), Lambros et al. (2005)
Affymetrix SNP ^{c,d,e}	76 kb (100K, 2 arrays) (Greshock et al., 2007), 13–25 kb (500K, 2 arrays) (Gorringe et al., 2007; Greshock et al., 2007; Gunnarsson et al., 2008), ~10 kb (1800K)	SNP-only platforms (50K, 250K) unevenly spaced. Also detect LOH/allelic imbalance. Single fluorescent label.	Gorringe et al. (2007), Jacobs et al. (2007), Walsh et al. (2008)
Illumina ^{c,e} SNP oligonucleotide	25 kb (317K) (Gunnarsson et al., 2008), <25 kb (620K), <<25 kb (1100K)	Uneven probe spacing. Also detect LOH	
Oligonucleotide CGH ^{c,e} e.g. Agilent, NimbleGen	16–25 kb (185K) (Greshock et al., 2007; Gunnarsson et al., 2008), <16 kb (244K), 14 kb (1500K, 4 arrays) (Greshock et al., 2007), <14 kb (2100K, 1 array)	Lower resolution arrays have uneven probe spacing. Good signal:noise ratio. Most expensive array based technology.	
Massively parallel sequencing ^f – short read e.g. Illumina (Solexa), Applied Biosystems (SOLiD)	Paired-end sequencing for copy number (15 kb window), rearrangements (base pair) (Campbell et al., 2008), single-end sequencing for whole genome (Ley et al., 2008) or targeted resequencing (Hodges et al., 2007), including multiplexing of samples (Craig et al., 2008)	Good dynamic range and potentially single base-pair resolution of copy number. Can determine rearrangement partners. Bioinformatically challenging. Expensive.	
Massively parallel sequencing ^f – long read e.g. Roche (454 FLX)	Whole genome or targeted resequencing (Albert et al., 2007; Dahl et al., 2007; Thomas et al., 2006), including multiplexing of samples (Binladen et al., 2007)	400 bp reads. Not validated yet for copy number due to fewer reads per run but base-pair resolution of small alterations and rearrangements. Expensive.	

There are a number of useful databases for various genetic data at the present time, including:

a Mitelman Database of Chromosome Aberrations in Cancer <http://cgap.nci.nih.gov/Chromosomes/Mitelman> (Mitelman et al., 2008).

b NCI and NCBI's SKY/M-FISH and CGH Database (2001), <http://www.ncbi.nlm.nih.gov/sky/skyweb.cgi> (Knutsen et al., 2005).

c Progenetix CGH database (Baudis and Cleary, 2001).

d The Cancer Genome Project (CGP) <http://www.sanger.ac.uk/genetics/CGP/> which includes Catalogue of Somatic Mutations in Cancer (COSMIC) (Forbes et al., 2006) and CGP LOH and copy number analysis.

e The Cancer Genome Atlas (TCGA) <http://cancergenome.nih.gov/>.

f Next-generation sequencing applications are expanding at a rapid rate; this information is by no means exhaustive.

Base-pair resolution of structural chromosome alterations has been demonstrated for cancer cell lines (Bignell et al., 2007; Campbell et al., 2008), showing remarkable complexity of copy number breakpoints and identification of fusion genes. This technology also gives quantitative measurement of copy number and LOH information comparable to SNP 1800K arrays. In combination with cDNA sequencing (Bainbridge et al., 2006; Sugarbaker et al., 2008) this technology has the potential to identify novel fusion genes and gene amplifications which might be exploited for targeted therapeutics as

has been demonstrated for ERBB2 (Trastuzumab) and BCR-ABL (Imatinib). This application is particularly relevant for ovarian carcinoma because the structural complexity of the genome renders case-by-case evaluation of all chromosome breakpoints near-impossible by traditional cloning methods. Next-generation sequencing will soon supersede the conventional Sanger method as it offers the potential to detect every somatic coding gene mutation in a tumor (Dahl et al., 2007).

The capacity for large-scale sequencing efforts to characterise hundreds of tumors is some time off yet. In the

Figure 2 – SNP array data enables high-resolution analysis of copy number and LOH. (A) Chromosome 17 plot (p-ter at left to q-ter at right) for a mucinous ovarian tumor showing a small amplification at ERBB2. LOH accompanied by copy number loss can be distinguished from copy number neutral LOH and allelic imbalance caused by copy number gain. (B) Frequency plot of small copy number alterations from [Gorringe et al. \(2007\)](#). Assuming these alterations would show an approximately normal distribution, it is clear that the detection of alterations less than 50 kb is compromised by the resolution of the platform.

meantime, collaborations such as The Cancer Genome Atlas (TCGA) are undertaking integrated analysis of large numbers (500) of ovarian tumors by combining targeted sequencing of hundreds of genes with multiple array platforms including gene and microRNA expression, methylation, SNP and CGH arrays. This study, by analysing the same tumor material for each platform, has great potential to integrate the somatic genetics with expression information in order to identify the genes targeted by copy number and LOH alterations.

Perhaps the biggest challenge ahead is not the acquisition of exquisitely detailed genomic information but making sense of it. There are very significant challenges in handling the terabytes of data per sample as well as determining the most appropriate bioinformatic and statistical tests to translate this data into meaningful biology. Sometimes this data may present us with unexpected challenges such as the previously unrecognised copy number polymorphisms (CNP, Redon et al., 2006). These can masquerade as somatic alterations and must be taken into account when analysing tumor data (Gorringe and Campbell, 2008). At present this is an underappreciated difficulty but is likely to be resolved within a few years as increasing numbers of normal variations are published and populate CNP databases.

Finally, discoveries made through detailed genomic analysis must undergo functional validation and cross-comparison with other large tumor cohorts. Thus, while there have been startling advances made in the last few years the current situation is perhaps best summarised by Winston Churchill “Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning.”

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