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Array-based Comparative Genomic Hybridization and Its Application to Cancer Genomes and Human Genetics

Microarray comparative genomic hybridization (CGH) has proven to be a specific, sensitive, and rapid technique, with considerable advantages compared to other methods used for analysis of DNA copy number changes. Array CGH allows for the mapping of genomic copy number alterations at the sub-microspecific level, thereby directly linking disease phenotypes to gene dosage alterations. The whole human genome can be scanned for deletions and duplications at over 30,000 loci simultaneously by array CGH (~40 kb resolution). Array CGH can be used for analysis of DNA copy number aberrations that cause not only cancer and human genetic disease, but also normal human variation. This review gives the various array CGH platforms and their applications in cancer and human genetics. **(J Lung Cancer 2011;10(2):77-86)**

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INTRODUCTION

Conventional comparative genomic hybridization (CGH) is a molecular cytogenetic technique that is designed for identifying and mapping chromosomal segments with a copy number alteration of DNA sequences (1,2). Total genomic DNA is isolated from test and reference cell populations, differentially labeled, and hybridized to represent the genome that allows the binding of sequences at different genomic locations to be distinguished. Metaphase chromosomes in conventional CGH are used for the representation of the genome and the location of copy number variations between test and reference genomic DNA is mapped to the physical position on the chromosomes (3). Hybridization of highly repetitive sequences is typically suppressed by the inclusion of unlabeled Cot-1 DNA in the reaction.

Array-based CGH improves the resolution of the technique by substituting the hybridization target, the metaphase chromosome spread, with genomic segments spotted in an array format. Over the past several years, array CGH has demonstrated its value for analyzing DNA copy number variations. Detecting genetic aberrations and interpreting them within the context of broader knowledge facilitates identification of critical genes and pathways involved in biological processes and disease, and provides clinically relevant information. This article describes the various array CGH platforms and their application in identifying genetic alterations in cancer, genetic disease, and normal genomic variation.

CONVENTIONAL CGH

Conventional CGH has been used mainly for the identification of chromosomal regions that are recurrently lost or

gained in tumors (4), as well as for the diagnosis (5) and prognosis of cancer (6). Conventional CGH is an efficient technique in detecting numerical aberrations and can be used in the diagnosis of difficult cases. It has been reported that conventional CGH analysis demonstrates the presence of a partial duplication of a chromosomal segment (7), and allows the elucidation of more complex chromosomal alterations. Conventional CGH is also a reliable technique for detecting structural aberrations, and in specific cases, may be more efficient in diagnosing complex abnormalities than karyotyping (8). However, conventional CGH is unable to detect mosaicism, balanced chromosomal translocation, inversions, and whole-genome ploidy changes (9). The resolution of conventional CGH is also a major practical problem that limits its clinical application. Owing to the limitation of resolution of metaphase chromosomes, aberrations smaller than 5～10 Mb cannot be detected using conventional CGH (3). For detection of such abnormalities, a high-resolution technique is required. The microarray-based CGH technique combines the resolution of fluorescence *in situ* hybridization with the whole-genome screening capacity of conventional CGH, and holds great potential for the analysis of DNA copy number changes in clinical genetics (10).

MICROARRAY-BASED CGH

Low resolution, the main limitation of conventional CGH, is overcome (11). Chromosomes have largely been replaced by DNA microarrays containing elements that are mapped directly to the genome sequence (11,12). In array CGH, the detection of aberrations is in more detail, and it is possible to map the changes directly onto the genomic sequence. Differentially-labeled test and reference DNAs are hybridized to cloned fragments, which are spotted on a glass slide (Fig. 1). The DNA copy number aberrations are subsequently measured by detecting intensity differences in the hybridization patterns of

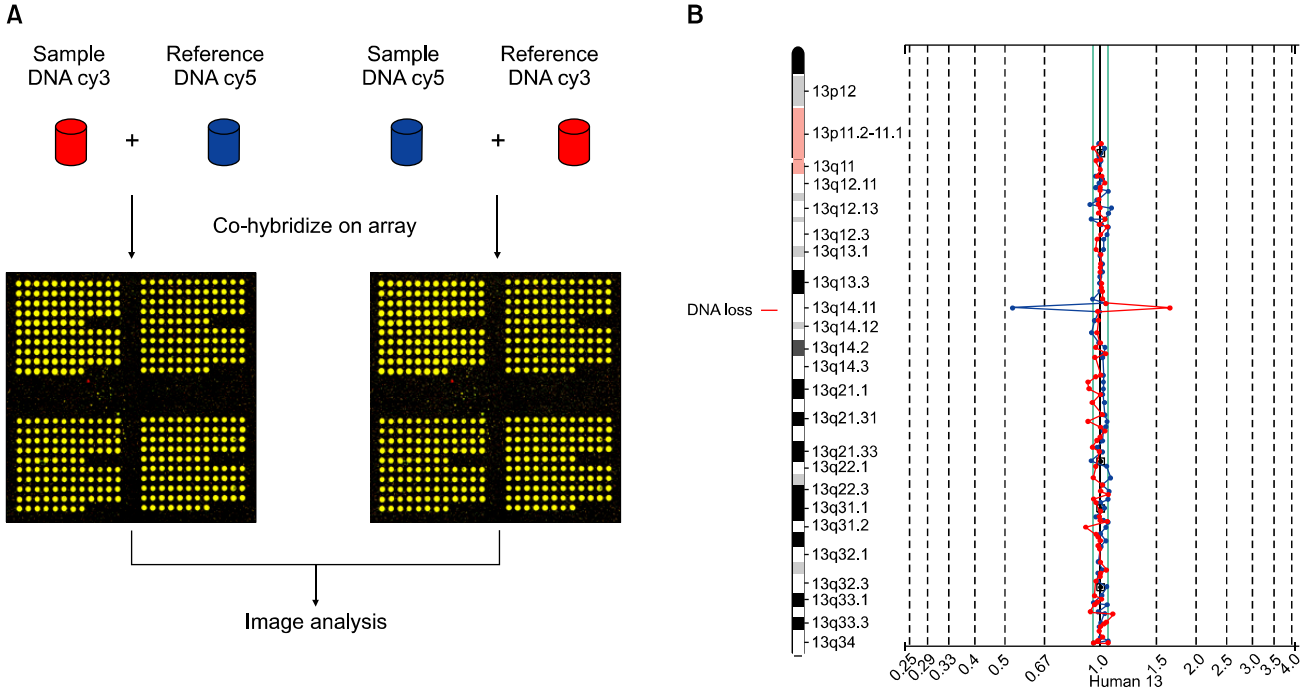

Fig. 1. Array comparative genomic hybridization. (A) Sample and reference DNAs are differentially labeled with fluorescent dyes (typically, cyanine-3 and cyanine-5), combined, and co-hybridized to cloned DNA fragments, which are spotted on a glass slide. The sample and reference competitively bind to the spots and fluorescence intensity ratios resulting from hybridization of both DNAs are reflected by their relative quantities. (B) To reduce the false-positive error rate, the two profiles of a dye-swap experiment are compared. Data are normalized so that the ratio is set to some standard value, typically 1.0 on a linear scale or 0.0 on a logarithmic scale. Each dot on the graph represents a clone spotted on the array. Blue values to the left and red values to the right of the '1' line indicate a loss of a genomic region, blue values to the right and red values to the left indicate a gain or amplification, and blue and red values at '1' indicate no copy number change. A value of 0.5 as seen in this figure indicates a homozygous deletion.

both DNAs. In array CGH, the resolution is determined by the distance between consecutive clones and the size of the cloned DNA fragments.

Array CGH has been used to identify segmental alterations in specific chromosomal regions initially associated with disease. Solinas-Toldo et al. (13) assessed the use of microarray technology to detect chromosomal imbalances for analysis of a panel of cell line and tumor genomes. This approach improved the resolution of conventional CGH from 10 Mb to the detection of segmental genomic alterations (SeGAs) of 75 ~130 kb in size by constructing an array which contained large-insert clones (LIC) spanning 13q14 and other regions.

Applying a chromosome-specific array to study breast cancer, SeGAs at multiple regions were detected on 20 chromosomes (11). Thus, higher-density arrays would probably reveal more complex chromosomal alterations in cancer genomes than has been previously appreciated. A number of bacterial artificial chromosome (BAC) clone-based contiguous arm arrays have been developed. These arrays provide complete coverage of the 1p, 3p, and 5p arms, which are frequently altered in a variety of cancers (14-17). These arrays have proven instrumental in fine-mapping SeGAs in oral squamous cell carcinoma, and in small-cell and non-small-cell lung carcinomas.

Although CGH studies using regional and chromosomal microarrays have yielded enormous information, these studies are naturally biased to specific areas of the genome and require *a priori* knowledge of regions of interest. Genome-wide arrays have been employed to overcome regional bias. The genome-wide approach using cDNA microarrays was originally used in gene expression profiling (12). The advantage of this technique is that high-level amplification and deletions can be directly correlated to changes in expression using the same platform (12,18). However, the low signal-to-noise ratio and variable signal intensities are major concerns with using cDNA clones as targets for detecting copy-number alterations (19). These results from the smaller target size of cDNA clones compared with genomic LICs due to a lack of intronic regions and the varying length of the cDNA target. A moving-average of clone intensities must be calculated in order to detect single copy changes, thereby reducing the resolving power of the arrays. In order to generate a strong signal, larger quantities (micrograms) of sample genomic DNA are required; thus, there is a limitation to the utility of cDNA array CGH (12).

BAC arrays were constructed for genome-wide SeGA profiling because LICs provide stronger signal intensities. An array with an average marker interval of 1.4 Mb was created using 2460 BAC and P1 clones (20). Such an array is able to detect high- and low-level genomic alterations, and to substantiate the utility of LIC-based genomic microarrays. The main advantages of genome-wide arrays are that LICs, such as BACs, provide robust targets for sensitive detection of hybridization signals, and BACs are not limited to loci annotated with genes. The size of the arrayed elements also provide a higher signal-to-noise ratio compared to platforms using smaller targets, as signal intensities increase as the complexity of the DNA spotted increases (21). The complexities of the genomic DNA and of the DNA in the array elements significantly affect signal intensities and thus play a dominant role in determining the genomic resolution of different array CGH technologies (22). Array elements made from genomic BAC clones (complexity ~100~200 kb) typically provide more intense signals than elements employing shorter sequences, such as cDNAs, polymerase chain reaction (PCR) products, and oligonucleotides due to a number of complex kinetic factors. The higher signals from the more complex array elements result in better measurement precision, allowing detection of single-copy transition boundaries and localization of copy number transitions to a fraction of the length of the array element in some circumstances (23). BAC-based arrays allow highly sensitive and reproducible detection of a wide range of copy number changes, including single copy number gains and losses, homozygous deletions, and high-level amplifications (21).

Oligonucleotide arrays are also used in copy-number detection. These arrays contain 25-mer oligonucleotides originally designed to detect human single-nucleotide polymorphisms (SNPs; 24,25). In a method known as whole-genome sampling assays, linker-mediated PCR is performed on the sample DNA to enrich for small XbaI restriction fragments throughout the genome in order to reduce sample complexity prior to hybridization (26). Although the reduced sample no longer represents the entire genome, this process decreases the probability of cross-hybridization to multiple short oligonucleotide targets on the array, effectively decreasing non-specific signals (24).

The representative oligonucleotide microarray analysis (ROMA) method reduces the complexity of the genomic DNA sample to ~2.5% of the genome via the BglII restriction enzyme and

linker-mediated PCR amplification (27). Using an oligonucleotide microarray consisting of 85,000 target probes of 70-mers designed to detect the genomic 'representations' created by selective amplification of restriction fragments, ROMA can detect SeGAs at an average theoretical resolution of 30 kb, assuming an even distribution of *Bgl*II sites throughout the human genome. The identification of sub-megabase copy-number alterations by this method in breast cancer cell lines and tumors illustrates the need for high-resolution analysis of cancer genomics. Brennan et al. (28) have recently demonstrated that labeling of total genomic DNA can be used to detect single-copy alterations on an oligonucleotide array without a genomic reduction step. However, microgram quantities of starting material are required, which may limit the technique to large tumors and cell lines.

The marker-based genome-wide arrays, albeit only representing up to 10～15% of the genome, have been instrumental in identifying large (typically ＞1～2 Mb) variations in somatic genetic changes in tumors, as well as large-scale copy number variations in the human population (29,30).

Ishkanian and colleagues (31) published the first sub-megabase resolution tiling set (SMRT) array that contiguously covered the human genome in a tiling path manner. The resolution of the array was increased beyond the size of a single BAC clone by using overlapping clones and gains and losses of regions as small as 40～80 kb are detectable. Like other LIC-based approaches, the SMRT array yields high signal-to-noise ratios due to the hybridization sensitivity of the BACs to their corresponding genome targets. A major advantage of using a tiling-path array is in detecting small gains and losses of genes, since marker-based genomic microarrays inherently have a large number of gaps due to the distance between target probes, reducing the likelihood of detecting novel microalterations. That is, the probability of missing a small genetic alteration is inversely proportional to the genome coverage or representation of the detection strategy (19). The tiling path array offers a much greater probability of detecting small-sized alterations (eg, 40 kb) than marker-based genomic arrays. The SMRT array, a tiling-path array, is composed of 32,433 overlapping BAC clones spotted in triplicate on two microarray slides (31). The SMRT re-array (SMRTr) contains a more selective set of clones, representing 83% of the original collection, eliminating unnecessary redundancy while maintaining tiling path coverage. The ～27,000 clones can be spotted on to a single slide in duplicate, reducing the cost and time of analysis.

Microarray-based CGH has some advantages for analysis of gene copy changes. Array CGH does not require dividing cells, as does karyotyping, and enables the analysis of the whole genome in a single experiment. It is also characterized by high resolution, which is its major advantage with respect to conventional CGH (32). Array CGH can be a highly specific technique with a generally low number of false-positive signals, and in most cases, no false-negative signals (33,34). Not only the high specificity, but also the sensitivity of array CGH is also very high (35). Standard resolution varies between 1 and 5 Mb, but can be increased up to approximately 40 kb by supplementing the array with extra clones (32). Another advantage is that array CGH is a fast technique because part of the procedure is semi-automated (34). The amount of clones on the slide, as well as the form of clone amplification, is important to the final sensitivity and quality of the analysis. The use of genomic LICs, such as BACs and P1-derived artificial chromosomes (PACs), provide sufficiently intense signals to detect single-copy changes and to locate aberration boundaries accurately. Arrays using cDNA yield a high spatial resolution, but the number of cDNAs is limited by the genes encoded on the chromosomes (12); they have low sensitivity because of cross-hybridization. The arrays with short, 25～70 mer oligonucleotides allow rapid, cost-effective, and easy processing, but have low sensitivity for detecting single-copy changes (36).

The main disadvantage of array CGH is its limited ability to detect mosaicism and in its inability to detect aberrations that do not result in copy number changes. The level of mosaicism that can be detected is dependent on the sensitivity and spatial resolution of the clones. Rearrangements present in approximately 50% of the cells are the detection limit (37). In addition to this technical drawback, practical aspects, like background signals and clone quality, play a role in the limitations of array CGH (38).

APPLICATIONS OF ARRAY

1) Array CGH applications to cancer genomes

Cancer is a disease characterized by genomic instability. Many types of genetic instability result in production of point

mutations, chromosomal rearrangements, DNA dosage abnormalities, altered microsatellite sequences, and epigenetic changes. DNA dosage alterations that occur in somatic cells are frequent contributors to cancer. Identifying segmental genomic alterations and their genes will yield molecular targets for diagnostics and therapy.

Array CGH provides a powerful entry point for studies involving cancer due to its ability to analyze DNA from a wide variety of specimens. High-resolution analysis of tumor genomes is needed for the discovery of genes involved in the disease. This is evident for two main reasons (19). First, high-resolution array CGH has the ability to refine known consensus regions of alterations. This is important as it allows researchers to narrow their focus to smaller areas of the genome. Second, high-resolution analysis has a greater probability of detecting small novel alterations that may be important for the disease, but may be missed by lower-resolution techniques. As array-based CGH continues to increase in resolution, cancer-causing genes will continue to become easier to discover.

Tumor genomes have different types of genetic instability. When genomic phenotypes in cancer have a wide range, array CGH will provide significant information on the locations of important cancer genes for some sets of specimens, whereas it will also be uninformative for others. Copy number profiles of cell populations reveal past genomic instability that leads to the clonal expansion of a cell population with a relatively stable genome, or at least stable within its selective environment (22). Some tumors appear very stable *in vivo*, with primary tumors and recurrences having nearly identical copy number profiles even though there are many years between them (39,40). Ongoing genomic instability results in heterogeneity that is not detectable by CGH and is best assessed by techniques that examine individual cells (41).

Amplification of oncogenes and deletion of tumor suppressors are common events in cancer progression. Gains of genomic material can be associated with an increase in dosage of proliferation-enhancing genes (eg, proto-oncogenes), and losses point to loci of tumor suppressor genes.

Array CGH is a molecular cytogenetic technique particularly useful in detecting and mapping DNA copy number alterations (42). In the past decade, array CGH has been successfully used in detecting genomic amplification and deletions of many types of tumors. Information of copy number aberrations can have clinical use in diagnosis, and in some case provide useful prognostic information. Comparison of tumor samples representing different stages of tumor development, such as premalignant or *in situ* lesions, invasive cancers, and metastatic diseases, has demonstrated that the overall copy number changes increase during tumor progression (43-45). An association of DNA copy number aberrations with prognosis has been found for a variety of tumor types, including lymphomas (46,47), chronic lymphocytic leukemia (48), gastric cancer (49), prostate cancer (50), and breast cancer (51,52). An important and well-known characteristic of most cancer cells is the increase in genetic instability during disease progression. Martinez-Climent et al. (46) used a 2400-clone array with a 1.4 Mb resolution to study follicular center lymphoma (FCL) cell lines and paired biopsies from patients who have transformed from FCL to the more aggressive diffuse large B-cell lymphoma (DLBCL). The genomic profiles were then compared with their corresponding gene expression data for FCL to DLBCL transformation from another study (47). An increase in genomic complexity was observed in association with transformation. Weiss et al. (49) have used a 1.4 Mb interval maker-based array to compare gastric hyperplastic polyps containing intraepithelial neoplasia (dysplasia) to gastric adenomas. The hyperplastic polyps contained numbers of SeGAs per sample, comparable to the adenomas. However, both the polyps and adenomas had fewer SeGAs per sample when compared with gastric carcinoma profiles. A breast cancer study by Nyante et al. (52) used a 1.4 Mb interval array to examine the relationship between a lobular carcinoma *in situ* (LCIS) sample, a ductal carcinoma *in situ* (DCIS) sample, and an invasive lobular cancer (ILC) sample from a single patient. That study suggests that LCIS and ILC share a genomic signature distinct from the DCIS samples. The presence of this clonality can progress to an invasive cancer.

In addition to tumor progression, the identification of specific genetic aberrations associated with patient outcomes has been the goal in a number of aCGH studies (45,53-55). The high number of copy number aberrations have been linked with poor patient prognosis for gastric cancer (56), mantle cell lymphoma (57), and bladder cancer (58). Amplification at 5q31-q35 in ovarian cancer has been linked with poor prognosis, whereas losses at 4p16 are associated with a favorable outcome. In

breast cancer, copy number profiling has identified distinct loci of copy number aberrations associated with different clinicopathologic features, such as tumor grade, estrogen receptor (ER) status, TP53 mutation, gene expression subtype, and overall survival (59-61). Detection of these copy number aberrations may define important genes contributing to breast cancer development and progression, and may provide a basis for improving patient prognosis with breast cancer (60).

Specific genetic aberrations discovered by array CGH have also been linked to differential responses to various cancer therapies (45,62,63). Multiple different genetic aberrations associated with chemoresistance have been studied in ovarian cancer (62). It has been suggested that losses of 13q32.1 and 8p21.1 are the most reliable markers of treatment-resistant disease. In breast cancer, losses of 11p15.5-p15.4, 1p36.33, 11q13.1, and 11p11.2 have been found significantly more often in the recurrence group after tamoxifen treatment (63). These loci might harbor genes associated with treatment resistance and tumor progression of breast cancer.

All later stage tumors had a greater average number of SeGAs per sample than their precursors. Although many of these secondary genetic changes may be responsible for disease maintenance and proliferation, the majority are not likely to contribute to cancer pathogenesis and are probably a consequence of random events (19). The increased genomic stability of tumor precursors improves the likelihood that a SeGA discovered may be necessary for the disease. Furthermore, discovering localized regions that contain these genes might be easier, as they may not be 'masked' by the gross genetic alterations occurring at later stages.

If a gene is a target of selection within a region of a copy number increase, it should be overexpressed in tumors in which it is found at an elevated copy number. But, overexpression does not distinguish it from other genes in the aberrant region that may not contribute to tumor development because expression of 40～60% of all genes may be elevated (18,64). That a gene is always overexpressed when at increased copy number, and is sometimes overexpressed when not present at increased copy number, supports its functional role in cancer (22). Genes that drive copy number gains may also be altered by mutation (65). The decrease in expression due to the decrease in copy number is sufficient for the gene to be significant to the tumor in some cases. But, in the classic case of tumor suppressor genes, function is totally abrogated by deletion of all copies of a gene, deletion of one copy, and mutation or epigenetic alteration of the other (66), or alteration of one copy and replacement of the other by a duplicate of the altered copy. The latter type of aberration results in loss of heterozygosity, not copy number change, and is not detectable by array CGH. Developing SNP profiling technologies may be able to provide additional information concerning these events, perhaps eventually providing information on heterozygosity and dosage for some types of specimens (25,67). Candidate genes within recurrent regions of loss can be assessed for expression changes and examined to determine if the remaining copies are mutated or methylated (66).

2) Identification of segmental copy number changes in genetic diseases

Array CGH was shown to be a specific and sensitive approach in detecting submicroscopic aberrations, such as Prader-Willi syndrome and Angelman syndrome (33). Several studies using both genome-wide and chromosome-specific arrays support the finding that submicroscopic chromosomal abnormalities can be readily detected with array CGH (68-70). Microarray CGH is also useful in determining the critical region of specific disease. Veltman et al. (71) reported a series of 20 patients with congenital aural atresia, for whom the detailed extent and nature of the deletions were studied by a chromosome-specific array. A common genomic region of 5 Mb on chromosome 18q22.3-q23 was found to be deleted in all patients. Rauen et al. (72) used a genome-wide array to detect a small deletion covering the 12q21.2-12q22 chromosomal region. High-resolution array CGH is a potential approach to the analysis of phenotype-genotype correlations (32).

3) Array CGH as a diagnostic tool and its application in clinical settings

The correlation of cytogenetic aberrations with disease outcomes has already proven to be reliable and is utilized in guiding treatment. Detection of changes in tumor DNA, such as loss of heterozygosity, gene amplification, and microsatellite instability, have become useful markers associated with malignant development. Historically, technologic developments, such as fluorescence *in situ* hybridization and comparative genomic hybridization, greatly increase the resolution power of the

cytogenetic approach with concomitant benefits. Array CGH has the potential to improve this resolution by orders of magnitude at a reasonable cost and offers the capacity to be a high throughput approach.

Although standard cytogenetic analysis and chromosomal CGH for DNA-based diagnosis has wide application in a clinical setting, it suffers from low resolution and is not precisely linked to sequence-based map information. Array CGH offers the opportunity to globally profile segmental copy number imbalances at an unprecedented resolution in constitutional or tumor DNA samples, thus serving as a diagnostic and investigative tool (73).

Disease-specific arrays have been constructed for cancer diagnosis. These arrays are enriched for the coverage of multiple cancer gene loci, facilitating simultaneous assessment of gains and losses of tumor suppressor and oncogenes in a variety of cancers (48,74). Diagnostic arrays have been designed for the diagnosis of congenital anomalies, developmental delay, and mental retardation (68,75,76), as well as the detection of chromosomal aberrations in embryos (77-79). In order for array CGH to have a more prominent role in clinical diagnosis, many factors, such as cost, standardization of protocol, robustness of arrays, and user acceptance, need to be addressed (73).

4) Application to normal genomic variations

Array CGH measurements using BAC arrays immediately reveal copy number polymorphisms. Fifty-five unrelated individuals were examined to quantify genetic variation using an array of 2632 LICs (29). Overall, 255 loci across the human genome contained genomic imbalances, and on average, each person had 12.4 variant ratios. Twenty-four variants were present in $>10\%$ of the participants and 6 of these large-scale copy number variations (LCVs) were present in 20% of the individuals. Among the total number of LCVs, greater than one-half (142) harbored_genes. Strikingly, 14 LCVs were located near loci associated with cancer or genetic diseases, suggesting that certain individuals may have higher susceptibility to disease than others. Polymorphisms have also been detected using the ROMA approach to CGH. Twenty individuals were analyzed and a total of 221 copy number differences represented 76 unique copy number polymorphisms (30). On average, each individual differed by 11 variations, with an average length of 465 kb. The copy number variations contain genes that have been implicated in neurologic function, regulation of cell growth, regulation of metabolism, and several genes known to be associated with disease.

Array CGH has established the prevalence of copy number polymorphisms in the human genome, although the understanding of this normal variation is incomplete. Redon at al. (80) have constructed a first-generation copy number variation map of the human genome from four populations with ancestry in Europe, Africa, or Asia (the HapMap collection). A total of 1,447 copy number variable regions (CNVRs), which can encompass overlapping or adjacent gain or losses, covering 360 megabases (12% of the genome) were identified. These CNVRs encompassed more nucleotide content per genome than SNPs, underscoring the importance of copy number variation in genetic diversity and evolution. Measurement noise has restricted detection to polymorphisms that involve genomic segments of many kilobases or larger. More comprehensive studies using whole-genome tiling path arrays are necessary to enumerate and identify all such LCVs in the human population.

Array CGH technology has been used in interspecies comparisons. In a comparison of the human genome against four great ape genomes, using an LIC array of 2,460 BACs, 63 sites of DNA copy number variation between the human and great apes were identified (81). A significant number of these sites existed in interstitial euchromatin. Using a cDNA array CGH approach, over 29,000 human genes among human, bonobo, chimpanzee, gorilla, and orangutan were compared leading to the identification of >800 genes that gave genetic signatures unique to a specific hominoid lineage (82). There was a more pronounced difference between copy number increases and decreases in humans, and a number of genes amplified are thought to be involved in the structure and function of the brain (73).

CONCLUSION

In the past decade, genomic arrays have been successfully used to detect genomic amplification and deletions in all types of human tumors. Array CGH can also be used for the identification of yet unidentified abnormalities, as well as the screening of known submicroscopic aberrations in human genetic diseases (33). Array CGH has elucidated amplifications

and deletions that represent the critical steps in the tumorigenesis of many tumors. Detection of smaller amplifications and deletions has improved significantly with the introduction of spotted high density BAC arrays, or high density oligonucleotide arrays to detect SNPs. The higher resolution data that is produced with these devices is ongoing. The continuing technical advances and growing databases of disease-specific profiles will broaden the use of array CGH in both research and clinical settings.

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