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Detection of DNA copy number alterations in cancer by array comparative genomic hybridization

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Over the past few years, various reliable platforms for high-resolution detection of DNA copy number changes have become widely available. Together with optimized protocols for labeling and hybridization and algorithms for data analysis and representation, this has lead to a rapid increase in the application of this technology in the study of copy number variation in the human genome in normal cells and copy number imbalances in genetic diseases, including cancer. In this review, we briefly discuss specific technical issues relevant for array comparative genomic hybridization analysis in cancer tissues. We specifically focus on recent successes of array comparative genomic hybridization technology in the progress of our understanding of oncogenesis in a variety of cancer types. A third section highlights the potential of sensitive genome-wide detection of patterns of DNA imbalances or molecular portraits for class discovery and therapeutic stratification. ***Genet Med* 2007:9(9):574–584.**

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UNDERSTANDING CANCER THROUGH THE STUDY OF CANCER GENOMICS

Cancer results from a series of genetic and epigenetic alterations that allow cells to become independent of growth signals, to escape growth inhibitory and apoptotic signals, to acquire unlimited growth potential, and ultimately to invade neighboring tissues and metastasize to other organs.[1] In the past decades, research has been successful in identifying some of the genes controlling these cellular processes, thereby contributing to the unraveling of the genetic basis of cancer. A number of these genes emerged as key players with a central role in the tight control of cell cycle, apoptosis, and DNA repair. This multitude of genes controls networks of a restricted number of signaling pathways. Two such pathways, centering around the *RB1* and *TP53* genes, respectively, are assumed to be disrupted in virtually all cancer types. Other major signaling pathways perturbed in cancer include the RAS/MAP kinase (growth control), PI3/AKT (survival, apoptosis), TGFβ, and JAK/STAT pathway.[2] Cytogenetic and molecular genetic investigations provided insight into the plethora of mutations that disrupt the normal function of genes implicated in cancer. Proto-oncogenes are known to be activated through gain-of-function mutations at the base pair level or are over-expressed because of copy number gain, amplification, or translocation. Tumor suppressor genes can be inactivated by loss-of-function mutations, deletions affecting one (haplo-insufficiency) or both alleles, or epigenetic modifications. A particular class of oncogenic events most often (but not exclusively) observed in leukemias, lymphomas, and soft tissue tumors are chromosome rearrangements causing in frame fusion of parts of two genes leading to the formation of hybrid genes with particular oncogenic properties.[3]

Classical cytogenetics has been extremely important in identifying such recurrent rearrangements, in particular recurrent translocations, which helped to uncover the position and allowed the molecular cloning of the genes implicated in such proto-oncogene activation or gene fusion events.[3,4] This interplay between cytogenetic and molecular genetics allowed the generation of new tools for diagnosis and follow-up of the disease. Furthermore, the discovery of gene fusions represented the first step toward the unraveling of the pathogenesis of these particular cancers and, more importantly, for the development of molecular-targeted therapy.[5,6] Treatment of chronic myeloid leukemia with the tyrosine kinase inhibitor imatinib illustrates how the identification of such cancer genes and perturbed signaling pathways revealed targets for less toxic and more efficient molecular therapy.[7,8]

For some tumor entities, however, classical cytogenetics has been less successful because of the complexity of karyotypes or difficulty of culturing tumor cells in vitro. Chromosomal comparative genomic hybridization (CGH) opened the way for the study of DNA copy number alterations in such cancers. A particular advantage of this technique is that dividing cells are not required, allowing the study of archived material such as frozen biopsies or paraffin-embedded material. A large body of literature describes the findings by chromosomal CGH on such tumors, which contributed to the mapping of tumor suppressor genes and amplified proto-oncogenes and the identification of prognostically relevant genomic subclasses.[9,10] The role

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of low copy number gain in cancer is still poorly studied, but evidence is accumulating that gain of one single copy of a gene, chromosomal region, or entire chromosome can effectively contribute to the tumor phenotype.

Because the original CGH procedure used chromosomes as the target for assessing DNA copy number alterations, the resolution of such analyses remained limited to approximately 5 to 10 Mb.[11] The use of DNA clones spotted in array format on slides as targets for hybridization of normal and test DNA (array CGH) opened the way for a dramatic increase in resolution up to 30 kb[12] and more rapid and streamlined handling of assays, avoiding the tedious production of well-spread chromosome slides and subsequent karyotyping. As a result of these advantages, particularly the broader accessibility for laboratories to implement array CGH, this new technique has now become popular and widely applied, among other technologies for studying gains and losses in tumors.[13–16] This widespread application in tumor genetics, however, only followed several years after the first technical reports describing the technique.[13–15] This can be explained by the limited availability of good quality slides with a high density of probes. cDNA arrays were more readily available, but the rather low sensitivity makes averaging across multiple clones necessary for scoring gains and losses.[11] BAC clones were suitable alternatives as probes,[17] but quality of spotted slides could vary greatly because of changes in temperature, humidity, BAC DNA concentration, and damage to spotting pins. The quality of sample and control DNA, batch variation of Cot I DNA, labeling, and hybridization conditions are also important factors for success.[18] The availability of a 1-Mb BAC set and exchange of experimental information during various meetings[18] greatly triggered the dissemination of the technology. At the same time, the possibility of the use of SNP chips for assessment of DNA copy number alterations was discovered,[19–22] and improved high-density oligonucleotide slides were produced, further broadening the accessibility of array CGH platforms and leading to a rapidly increasing number of laboratories successfully implementing this new technology.[17,23–26]

Several good reviews on array CGH have been published.[16,27–30] In this review, we specifically discuss the present and future role of array CGH in the study of cancer genomes in relation to other emerging technologies. First, we discuss technical issues with particular relevance for array CGH in cancer. Next, we illustrate how array CGH is contributing to the identification of cancer genes and our understanding of molecular pathogenesis. Finally, we assess how tumor class discovery based on genomic molecular portraits can contribute to clinical management.

TECHNICAL CONSIDERATIONS RELATED TO ARRAY CGH ANALYSIS OF TUMOR SAMPLES

With array CGH procedures becoming more firmly established, DNA quality still remains a crucial factor in performing a successful array CGH experiment. In general, high molecular weight DNA can be easily obtained from fresh or frozen tumor material, but difference in quality can occur depending on the type of DNA isolation procedure. Therefore, when using kits for fast DNA isolation, an extra DNA purification step can be useful to further improve DNA quality. The issue of DNA quality is of particular importance for formalin-fixed paraffin-embedded tissue (FFPE) material. FFPE samples are of importance for cancer research, as they are often more readily available than frozen samples and thus represent an important source of archival tissue with long clinical follow-up. The major disadvantage to using FFPE samples is DNA degradation resulting from the type and duration of fixation. Procedures have been developed to obtain DNA of sufficient quality, but array CGH on such samples remains challenging and typically generates lower signal-to-noise ratios. Given the high cost of array CGH analysis, it is essential to reduce the number of failures to a minimum through assessing DNA quality before hybridization. Although a simple DNA gel electrophoresis can be performed for this evaluation, this may require too much valuable patient material and may also insufficiently predict samples compliancy for further analysis, indicating that parameters other than size are important for success. It has been demonstrated that DNA cross-linking because of the fixation procedure is also an important factor influencing DNA quality. Therefore, an optimized selection process was developed. This includes an improved DNA isolation protocol promoting DNA de–cross-linking, followed by multiplex PCR-based quality control to assess residual DNA cross-linking, resulting in a more precise estimation of archival samples suitable for array CGH analysis.[31] Recently, another study published guidelines for qualifying FFPE DNA samples for genotyping, loss of heterozygosity, and copy number analysis, including random amplified polymorphic DNA-PCR as a critical evaluation step.[32] In addition, commercial kits to determine the quality of genomic DNA isolated from FFPE tissue are becoming available. For example the BioScore screening and amplification kit (Enzo Life Sciences, Inc., NY) uses a novel whole-genome amplification method to predict sample performance. After this quality screening step, the amplified genomic DNA can be directly used for subsequent array analysis.

A second important issue in array CGH of cancer cells is contamination with normal cells. Spiking experiments, mixing various amounts of nontumoral DNA with a known glioblastoma xenograft sample containing known homozygous and heterozygous deletions, showed that array CGH of a sample with tumor cell percentage less than 50% agreed only poorly with genomic profiles obtained from 100% tumoral DNA, with both chromosomal gains and losses being difficult to recognize.[33] As a general guideline for tumor analysis, histological review should be performed on each tumor specimen to ensure sufficient tumor cell percentage and the absence of necrotic material.

It is also important to consider tumor ploidy when studying tumor genetics. In the case of near-triploid tumors, imbalances–in particular gains–may become more difficult to detect using standard thresholds. To resolve this problem, various ad-

vanced algorithms have been developed,[34] such as circular binary segmentation (CBS).[35] This algorithm translates noisy intensity measurements into regions of equal copy number and thus can be very useful in detecting the copy number transitions (Fig. 1). CBS can also greatly increase the detection rate for copy number alterations in samples with a percentage of normal cells up to approximately 10 to 20%. Recently, another algorithm was described that takes into account the nature of the noise in array CGH data to more accurately determine the regions of aberration and boundaries of breakpoints.[36]

Tumor heterogeneity is another factor that may complicate the interpretation of array CGH data. Furthermore, array CGH may not fully appreciate tumor heterogeneity as DNA copy number changes in a small number of cells will be diluted by the remainder of normal cells without copy number changes. Both the problem of normal contaminating cells and genetic heterogeneity of tumors can be addressed through the use of microdissection of clusters or individual tumor cells[37,38] followed by DNA amplification to produce sufficient DNA for labeling and hybridization (approximately 300 ng to 2 μg). Such DNA amplification procedures are also valuable for processing and analysis of small tumor samples, e.g., obtained through needle biopsies. The increasing demand for robust and unbiased whole-genome amplification methods has triggered the development of several genome-wide protocols that can roughly be divided in variations of PCR amplification (including degenerate oligonucleotide-primed PCR,[39] primer extension preamplification,[40] linker adaptor PCR,[41] and interspersed repetitive sequence PCR[42]) and isothermal DNA amplification using the ϕ29 bacteriophage DNA polymerase (including rolling circle amplification[43] and its derivative, multiple displacement amplification[44]). Although both PCR and isothermal amplification approaches generate several micrograms of amplified DNA from a small initial amount, the major challenge when combining whole-genome amplification techniques and high-resolution array CGH remains in obtaining an unbiased replication of all chromosomal regions. Several reports have already successfully used a combination of whole-genome amplification and array CGH analysis, with or without correction for amplification errors.[33,45–50] An elegant example illustrating the power of whole-genome amplification is the isolation of DNA samples from the histopathologically heterogeneous mixture of discrete foci of invasive carcinoma and premalignant high-grade prostatic intraepithelial neoplasia. Subsequent amplification using multiple-displacement amplification and hybridization onto BAC arrays identified particular chromosomal changes associated with the two disease stages.[51] An attractive novel method for whole-genome amplification is the Genomeplex technology (Sigma-Aldrich, UK), which uses random fragmentation and adaptor annealing via PCR to generate high-quality amplified DNA from a variety of sources (Fig. 2). Using this technology, it was recently shown that reliable genomic copy number profiles could be obtained even starting with a single cell.[52] A dedicated data

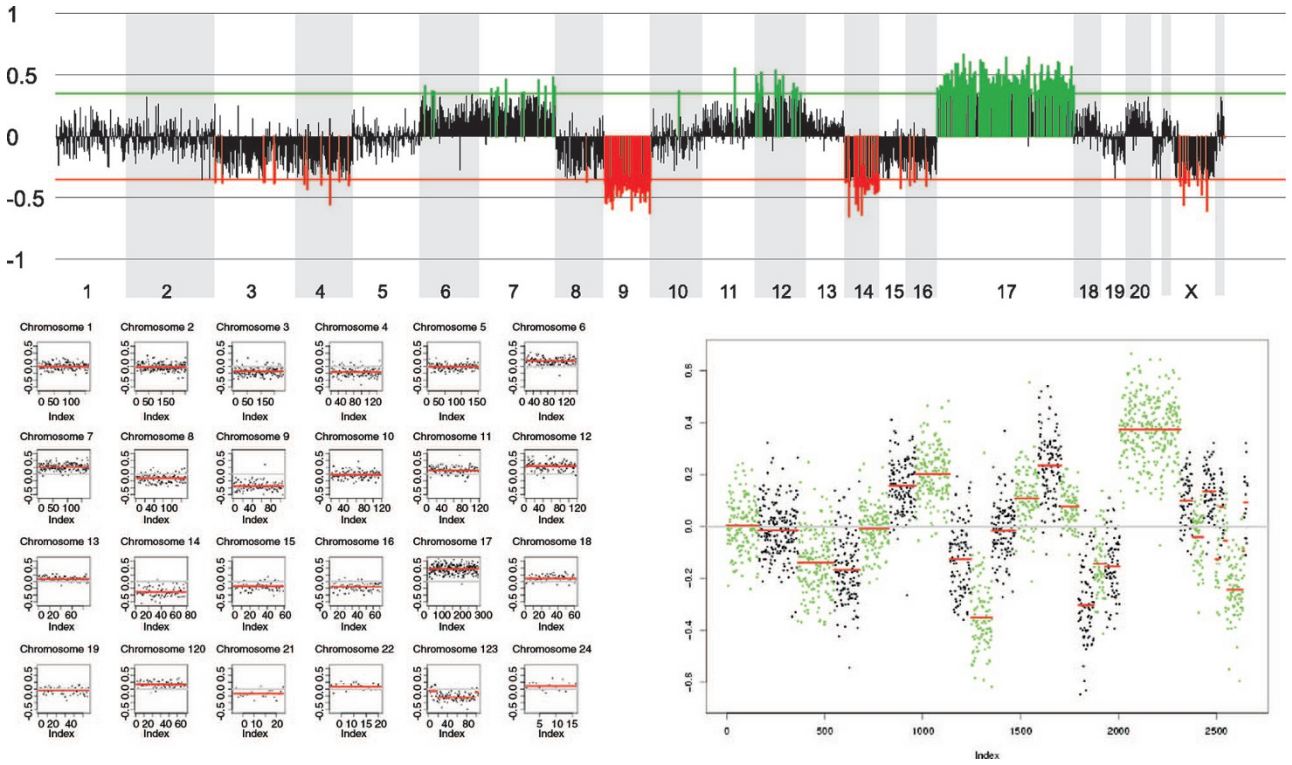

Fig. 1. Circular binary segmentation (CBS) as a tool for unbiased scoring of copy number alterations. Top, Array CGH copy number profile of a typical near-triploid neuroblastoma tumor showing numerical imbalances. The X-axis represents the chromosomes (alternating white and gray bars); the Y-axis represents the normalized $\log_2$ Cy3/Cy5 fluorescence intensity. Bottom, Application of CBS for facilitating unbiased scoring of DNA copy number alterations, in particular for presumed chromosomal imbalances with ratios below the standard threshold, e.g., in hyperploid cells, heterogeneous samples, and samples with normal contaminating cells.

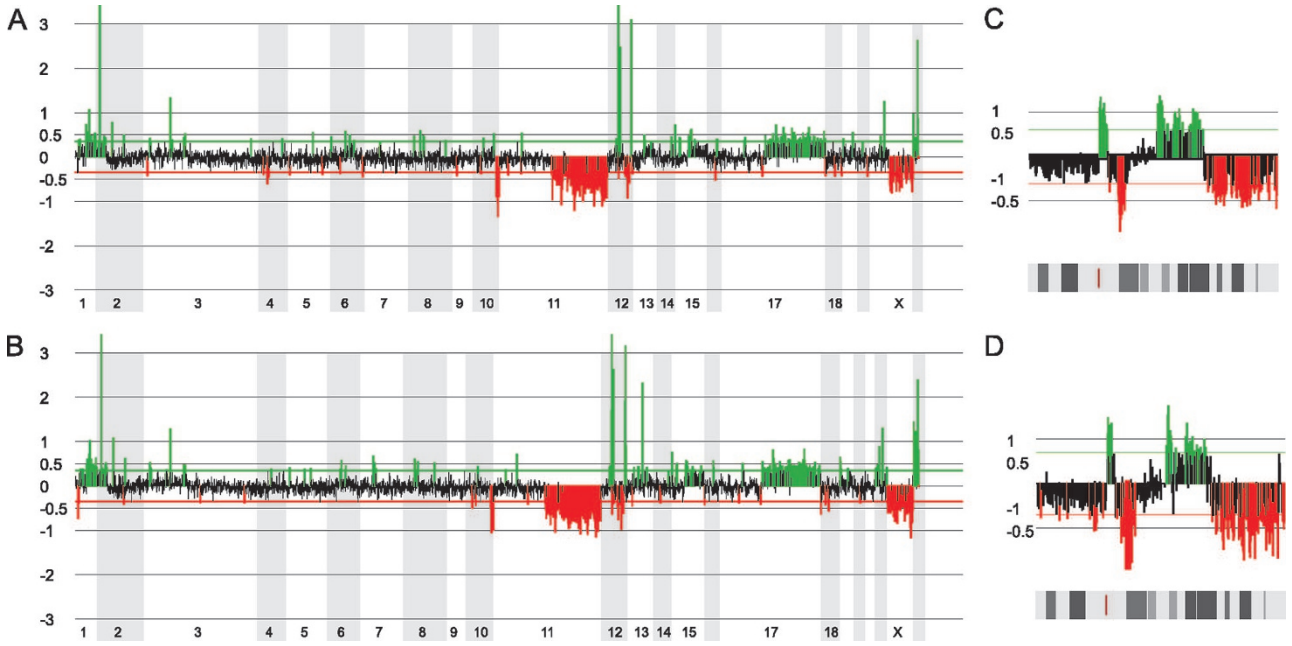

Fig. 2. Example of whole-genome amplification using Genomeplex technology (Sigma-Aldrich, UK). The X-axis represents the chromosomes (alternating white and gray bars); the Y-axis represents the normalized $\log^2$ Cy3/Cy5 fluorescence intensity. A, Array CGH result for the neuroblastoma cell line NGP starting from 500 ng DNA without prior amplification. B, Five-nanogram DNA amplified with Genomeplex technology. C, Detailed view of chromosome 17 in the Hodgkin lymphoma cell line HDLM2 starting from 500 ng DNA without prior amplification.[106] D, Five-nanogram DNA amplified with Genomeplex technology (Feys et al., personal communication).

analysis procedure allowed the detection of a single copy number aberration as small as 8.3 Mb, which is a remarkable increase in resolution compared with earlier experiments using multiple displacement amplification.[53] Such analyses of isolated cell islets, or even single cells, offer great opportunities to study intratumor heterogeneity, tumor progression through analysis of premalignant and malignant foci, or genomic changes in metastasized cells.

ARRAY CGH AS A TOOL FOR CANCER GENE DISCOVERY AND UNDERSTANDING DISEASE PATHOGENESIS

Dissecting amplicons

In view of the knowledge that, in addition to mutations at the base pair level, larger genomic alterations contribute to the tumor phenotype by altering normal gene function, the search for such DNA imbalances has been an important part of ongoing cancer research, and array CGH has also been successful. From the beginning, array CGH studies have focused on the analysis of amplicons. Given the unprecedented resolution offered by array CGH, this method turned out to be very powerful for dissecting highly amplified regions in cancer cell genomes. The interest in such amplicons can be explained by the fact that these regions may harbor novel proto-oncogenes that, upon activation, contribute to the aggressiveness of cancer cells, response to therapy, and development of resistance. Because those genes or components of the pathways they control can represent druggable targets, their identification in recurrent amplified chromosomal segments has remained a major research aim (Fig. 3). The promise amplifications hold for targeted therapy has triggered a myriad of articles dissecting amplicons to identify driver oncogenes and the pathways they disturb.[54] It is impossible to review the entire existing literature on this topic, but we illustrate the possibilities of array CGH in the dissection of amplicons through a number of landmark articles and results from our own investigations in neuroblastoma. Amplicons in breast cancer are probably among the most intensively studied. Albertson et al.[55] reported that quantitative measurement of DNA copy numbers across amplified regions using array CGH facilitates identification of *ZNF217* and *CYP24* as oncogenes, based on mapping of amplicon boundaries and amplification maxima. Another example of a particular target gene identified through mapping of a 19q13 amplicon is the finding of the *IXL* gene in pancreatic cancer as a cell survival regulator.[56] Monni et al.[57] used a combination of molecular, genomic, and microarray technologies to identify target genes within the 17q23 amplicon, a common region of amplification in breast cancers linked with poor prognosis. Two common regions of amplification were defined, and further expression analyses enabled the selection of a small number of consistently over-expressed genes. Various other studies have focused on the mapping of breast cancer amplicons in cell lines and primary tumors.[58–63] In recent extensive studies, Neve et al.[64] and Chin et al.[65] report combined high-resolution DNA and expression profiling of 51 and 145 breast cancer cell lines and tumors, respectively. In tumors, nine recurrent amplicons on chromosomes 8, 11, 12, 17, and 20 were found, in keeping with previous studies[14,66–69] with an indication for preferential co-amplification for certain regions. Using an in-

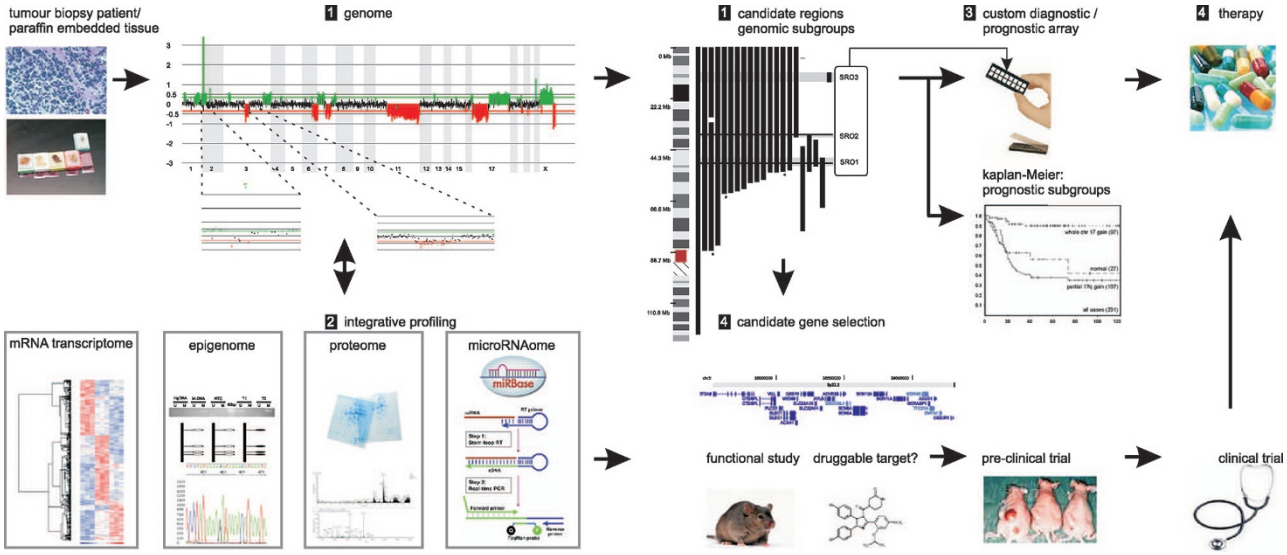

Fig. 3. Implementation of array CGH in cancer research and clinical management through integrated genomic profiling (1): combination of array CGH mapping of critical regions for losses, gains, and/or amplifications (2) and high throughput data from gene expression, proteome, and epigenetic profiling platforms (3) produce tools for prognostic assessment and class discovery (4) and lists of candidate genes for functional studies for understanding tumor biology and screening and testing of new therapeutic compounds.

tegrated approach, combining both expression and genomic data, a total of 66 over-expressed genes likely to be functionally important in these tumors was highlighted, a significant portion of which are high-priority therapeutic targets.[64,65] These findings hold promise for patients whose disease does not respond to current aggressive therapies.[65] For example, some of these genes reside in the HER2 amplicon and are co-activated with *HER2*. Manipulation of these genes could therefore trigger a synergistic therapeutic response, which could be beneficial for patients with a poor response to Herceptin.[70]

In addition to the classical amplicons encompassing larger genomic regions, which often encompass multiple genes, Holst et al.[71] reported a single-gene amplification of the *ESR1* gene, which encodes estrogen receptor alpha at 6q25 with further extended screening on tissue microarrays demonstrating *ESR1* amplification in 20.6% of breast cancers. Of particular interest, amplification was also detected in benign and precancerous breast diseases, which could point to *ESR1* amplification as a common mechanism in proliferative breast disease and a very early genetic alteration in a large subset of breast cancers.[71]

The importance of screening for amplicons in our understanding of tumor biology was recently illustrated by the detection of *MDM4* amplification in retinoblastoma providing an explanation for suppression of *TP53* activation in these tumors, by allowing them to escape from apoptotic signaling upon *RB1* inactivation.[72]

Neuroblastoma (NB), a childhood embryonal tumor, was one of the first tumors in which high-level DNA amplification was discovered.[73] *MYCN* amplification was present in a specific subset of high-stage tumors with poor prognosis, and mapping studies indicated that *MYCN* was the only consistently amplified gene across all examined amplicons.[74–76] Our research group has studied neuroblastoma using molecular cytogenetic techniques and, more recently, array CGH. A series of 100 NB primary tumors and 29 cell lines were analyzed. This and other studies showed that, in addition to the frequently amplified *MYCN* proto-oncogene, other rare amplicons can also occur. Such observations further illustrate the genetic heterogeneity in these tumors. The occurrence of known amplicons, including *CCND1* and *MDM2*, point at common oncogenic (progression) pathways in tumors. Previously undetected amplicons may also be found.[77–79] Because of the possible prognostic impact of such findings, using sufficiently high-density arrays both for research and prognostic purposes is important (in keeping with the above mentioned *ESR1* amplification in breast cancer).

In acute myeloid leukemia, amplicons are less frequently observed but, again, typically occur in aggressive forms. An example of the use of array CGH in the study for such amplicons was published by Baldus et al.,[80] who identified *APP*, *ETS2*, and *ERG* as over-expressed genes in chromosome 21-derived amplicons. In a genome-wide array CGH study by Rucker et al.,[81] a more general role of proto-oncogene activation in acute myelogenous leukemia (AML) pathogenesis was suggested. As already indicated, fusion genes are important (but often not sufficient) genetic events driving oncogenesis. Although such additional genetic defects may accompany gene fusions such as *PAX5* deletions in *ETV6/RUNX*-positive childhood acute lymphoblastic leukemias (ALLs),[82] over-expression of the fusion product because of amplification can also increase aggressiveness of the tumor cell[83] or lead to therapy resistance, as illustrated by *BCR/ABL1* amplifications in imatinib-treated chronic myelogenous leukemias.[84]

Despite our increasing knowledge of target genes within amplicons, the mechanisms leading to amplicon formation remain largely unresolved.[54] The power of array CGH to unravel these mechanisms was shown in the study of a neuroblastoma

cell line with amplified sequences in the absence of more common *MYCN* amplification. This study also illustrated that the combination of DNA array data and (M)-FISH analysis on metaphase slides allowed interpretation of array CGH data in the context of genomic position and complete characterization of a complex rearrangement leading to amplicon formation. The detection of a reciprocal t(8;16) translocation with breakpoints near co-amplified *MYC* (8q24) and *ATBF1* (16q22.3-q23.1) genes suggested that this translocation might have triggered amplicon formation through a complex process of translocation-excision-deletion-amplification mechanism leading to nonsyntenic amplification of *MYC* and *ATBF1*.[85]

MAPPING DELETIONS IN SEARCH FOR TUMOR SUPPRESSOR GENES

Array CGH has been extensively applied for fine mapping of deletions and identification of putatively involved tumor suppressor genes. Such studies have contributed to refinement of known critically deleted regions and have also enabled the detection of new regions of recurrent losses (Fig. 3). Clearly, the use of high- or even ultra high-resolution DNA arrays greatly facilitates accurate breakpoint mapping and, most importantly, allows the identification of small (homozygous) deletions.[86–96]

In our analysis of neuroblastoma tumors and cell lines, a 1-Mb BAC array was supplemented with tiling paths for recurrently deleted regions (1p, 3p, and 11q) and gains (17q). This approach resulted in a significantly improved delineation of the critical deleted regions on 3p, among others, through the detection of small interstitial deletions in neuroblastoma cell lines. In total, three regions of loss were delineated that were also known to be involved in more common epithelial neoplasms, thus providing support for the relevance of our findings in neuroblastoma.[97–100] Stallings et al.[91] detected recurrent deletions of a region on 9p encompassing the *PTPRD* locus, indicating a possible tumor suppressor function for this gene in neuroblastoma. Remarkably, shortly after this discovery, the same locus was found to be deleted in cutaneous squamous cell carcinomas.[90] The finding of common genetic defects in both embryonal tumors, such as neuroblastoma and epithelial cancers, strongly suggests that similar molecular pathways are involved in these seemingly unrelated tumors. In Wilms tumor, another embryonal tumor of childhood, an unexpected small intrachromosomal deletion on the X-chromosome was detected, pointing to a role for the *WTX* gene in non-*WT1* mutant tumors.[101] As discussed previously, the full malignant phenotype of cancer cells results from multiple genetic defects. Keeping this in mind, Mullighan et al.[82] set out to search for previously unnoticed submicroscopic lesions in childhood ALL. This search uncovered the presence of mono-allelic deletions targeting the *PAX5* gene in as many as 30% of all investigated ALL samples. Moreover, further investigation of non-deleted cases showed point mutations and cryptic translocations resulting in haplo-insufficiency for this gene. Furthermore, deletions affecting many other genes involved in pathways controlling B-cell development were found, firmly establishing the link between cancer and cellular differentiation.[82,102] Yet another example of the power of such high-resolution scanning of the cancer genome for deletions is the detection of a novel recurrent del (p12p13) in T-cell ALL (T-ALL),[11] resulting in loss of a negative regulatory region upstream of *LMO2* and consequent activation of the *LMO2* promoter, representing a novel activation mechanism of *LMO2* in pediatric T-ALL.[103] Furthermore, genome-wide SNP array technology in AML showed that approximately 20% of normal karyotype samples displayed previously unnoticed uniparental disomy for particular chromosome segments.[104] Subsequent analysis identified homozygous mutations at distinct loci, indicating that isodisomy can play a role in rendering a leukemic cell homozygous for an existing mutation.[105] Homozygous deletions are of particular interest in mapping tumor suppressor loci, as the affected region is relatively small, thus reducing the number of candidate tumor suppressor genes for further functional testing. Homozygous deletions were found through integrative genomic and gene-expression analyses in B-cell non-Hodgkin lymphoma (B-NHL) and allowed the localization of 20 homozygous deletions at seven chromosome regions in 48 cell lines derived from patients with different B-NHLs, thereby identifying several new tumor suppressor gene targets.[106] This and many other examples of small homozygous deletions have now been described as a result of the increased resolution of current array CGH platforms.[86–89,91–95,107,108]

MAPPING GAINS IN THE SEARCH FOR DOSAGE-SENSITIVE PROTO-ONCOGENES

Mapping of gains in genomic cancer research has thus far received little attention, but evidence is accumulating that such gains leading to a subtle increase in gene expression can indeed contribute to the cancer phenotype. Indirect evidence comes from developmental genetics, in which dosage effects resulting from single copy number changes (often deletions but sometimes duplications) are known to affect normal development. Given the involvement of developmental genes in cancer, similar effects at the cellular level can be expected in tumor genetics. In cancer cells, gains of chromosomes are a known recurrent defect. Trisomy can result in an extra copy of an activated proto-oncogene (e.g., *MET*[109] and the *MLL* tandem duplication[110]), but in most instances, the underlying pathogenetic mechanism and genes contributing to the tumor phenotype remain elusive. The longstanding and much debated hypothesis for a role of numerical aneuploidy in cancer development has found support through the observation that familial cancer may occur in individuals affected by *BUB1* mutations, leading to random gains and losses in somatic cells.[111] A specific example for the importance of low copy gain of proto-oncogenes was provided by an array CGH study of T-ALL that reported a small duplication leading to increased expression of the *MYB* gene, disturbing normal differentiation of hematopoietic progenitor cells in a subset of T-ALL.[112] Furthermore, identification of genes targeted by gains in a specific tumor can provide clues for other tumor entities, as shown in advanced serous

epithelial ovarian cancers and breast cancers.[113] High-density array CGH analysis identified a common 1.1-Mb region of copy number gain at 1q22 in approximately half of the advanced serous epithelial ovarian cancers and subsequently in breast cancers, leading to the identification of *RAB25* small GTPase as a mediator of aggressiveness in both ovarian and breast cancers.[113] In aggressive neuroblastomas, 17q gain is the most frequent genomic imbalance, suggesting that dosage-sensitive genes may be implicated. To refine the large region of common gain (35 Mb), we performed array CGH with a chromosome 17 BAC tiling path array. In total, 52 different breakpoints were mapped at the resolution of a BAC, but no interstitial duplications, such as those detected in T-ALL, were found. We therefore looked for clusters of over-expressed genes using an integrated genomic approach, including gene expression data from both primary tumors (favorable vs. unfavorable) and normal fetal neuroblasts obtained through laser capture microdissection from fetal adrenal glands.[114] Using an in-house–developed tool for positional gene enrichment analysis (PGE), we were able to identify regions that were significantly over-represented within a list of genes that are more highly expressed in neuroblastomas with 17q gain versus normal neuroblasts genes[114] (De Preter, unpublished data). This PGE analysis revealed two regions that coincided with the boundaries obtained by mapping data for 17q gain using a chromosome 17 BAC tiling path array, pointing to several candidate dosage-sensitive genes on chromosome 17 in neuroblastoma (Vandesompele et al., personal communication). Recently, it was shown that combined alterations in several genes may have a synergistic effect with a transforming capacity that cannot be obtained by any of the single gene defects.[115] Likewise, it is possible that increase in dosage for several 17q genes leads to a synergistic effect. A possible test for such a hypothesis is combined knockdown of sets of dosage-sensitive genes in the critical region of gain. Similar integration of genome-wide copy number and expression data is a strategy to identify novel candidate genes (Fig. 3). Examples of this are found in B-cell lymphoma,[106] lung cancer,[116] glioblastoma,[117] small cell lung cancer,[118] non-small cell lung cancer,[119] oral squamous cell carcinoma,[120] glioblastoma,[121] melanoma,[122] and testicular germ cell neoplasm.[123]

CROSS-SPECIES ONCOGENOMICS AND ANIMAL MODELS

Although comparisons of genomic alterations between human and animal models have been criticized, some results now support the notion that such cross-species analysis can yield additional power to mapping studies. Two recent articles demonstrate the usefulness of comparative oncogenomics using mouse models with a defined genetic background to identify driver genes in oncogenesis and metastasis in human tumors. Integration of high-resolution copy number profiles of mouse tumor models and human tumors resulted in the discovery of the *NEDD9* gene as a metastasis promoting gene in melanoma, whereas *cIAP1* and *YAP* were identified as oncogenes in hepatocellular carcinoma.[124,125] Further evidence illustrating the usefulness of cross-species comparisons comes from genomic analysis of the *MYCN* neuroblastoma mouse model, which revealed similar imbalances for syntenic chromosomal regions in mice and humans.[126] Further study of the mouse model indicates that the development of these tumors is driven by similar genomic alterations and could therefore facilitate the identification of causal genes. Major advantages of tumors obtained from model organisms are that virtually unlimited amounts of material can be obtained and different stages in tumor development can be studied. This was first illustrated in a study by Hodgson et al.,[127] who showed that array CGH on murine carcinomas that develop in the pancreatic islets of transgenic mice could narrow down critical regions of interest and identify candidate oncogenes and tumor suppressor genes. Similar articles demonstrate the use of a murine lymphoma model,[128] a mouse lung model,[129] a mouse melanoma model,[130] and mouse pheochromocytoma cell lines.[131]

ARRAY CGH FROM BENCH TO BEDSIDE

Beside the clues individual chromosome aberrations can provide toward cancer gene identification, the overall patterns of genomic aberrations (molecular portraits) also have intrinsic value and can provide a genomic framework for studying disease progression, tumor classification, and prognostic stratification. Tumor class discovery can become the starting point for a better comprehension of tumor biology and the development of different therapeutic strategies (Fig. 3). For example, using hierarchical clustering of oligonucleotide array data, it was recently shown that primary and secondary glioblastoma can be distinguished based on genomic aberrations. Moreover, two previously unappreciated genetically distinct cohorts were present within secondary glioblastoma.[132] Similarly, clustering of neuroblastoma BAC array CGH data showed that only approximately 80% of neuroblastoma can be assigned to the three major clinical-genetic subgroups, indicating that genomic profiling could identify novel subgroups with possible clinical relevance.[79]

High-resolution genomic profiles generated by array CGH can also be directly useful in clinical practice. This was illustrated in lymphoma, in which diagnostic accuracy distinguishing Burkitts lymphoma from diffuse large B-cell lymphoma is crucial to prevent both over- and under-treatment. Using global gene expression and genomic profiling, Hummel et al.[133] showed that Burkitt's lymphoma has a characteristic genetic signature that distinguishes it from cases of diffuse large B-cell lymphoma, allowing improved diagnostics and therapy choice. Targeted BAC/PAC diagnostic arrays are already constructed for B-cell chronic lymphocytic leukemia and t(11;14)-positive mantle cell lymphomas.[134,135]

Perhaps the most important challenge lies in assessing the prognostic power of molecular portraits for certain tumor entities, with or without taking into account other high-throughput clinically relevant information, such as coding gene and miRNA expression and methylation/chromatin modification profiles (Fig. 3). This approach was illustrated in a recent study

of 148 primary breast cancers on a BAC platform consisting of a limited number of cancer-related loci. Even with this small number of probes, a molecular taxonomy of breast cancer samples could be generated with relevance toward survival. The presence of genomic molecular portraits predictive for clinical behavior has been described in a variety of tumor types, including neuroblastoma,[79] gastric cancer,[136] lymphoma,[137] prostate cancer,[138] muscle-invasive bladder cancers,[139] and hereditary breast cancer.[140] However, for many entities, larger numbers of samples are required to find signatures predictive for survival using array CGH. Therefore, a first step toward prognostic array CGH-based classification is collecting data from large series of clinically well-documented, homogenously treated patient tumor samples. Furthermore, it was shown that array CGH could also be of importance in differentiating (histologically) similar tumor entities, such as gastrointestinal stromal tumors versus leiomyosarcomas,[141] CD4+CD65+ hematodermic neoplasm versus cutaneous myelomonocytic leukemia,[142] and primary versus secondary glioblastoma.[132]

These data indicate the immediate clinical use of microarrays in tumor classification and differentiation. With the cost of array CGH analyses decreasing, it can be expected that such tests will become more widely available and will perhaps become an integral part of diagnostic tumor work-up more quickly than we anticipated.

FUTURE PERSPECTIVES

Array CGH is making a significant contribution to our further understanding of tumor biology. Increasing resolution of arrays now enables analysis of genomic regions even up to the single base pair level, indicating that complete characterization of numerous genes is coming within range. Similar results are being obtained using novel sequencing technologies, yielding equally exciting information and new insights. Understanding cancer will, however, require equally challenging profiling of transcriptome, miRNAome, epigenome, and proteome to fully comprehend complex tumor behavior. In addition, subtle regulatory variation, patient-specific susceptibility, tumor environment, and immunological response are other aspects currently recognized as contributing to the complex and heterogeneous tumor phenotype.

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