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Chromosomal Alterations

Origin and Significance

With Contributions by

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Contents

Detection of Genetic Imbalances in Tumor Genomes by Fluorescence *in situ* Hybridization with Tumor Genomic DNA and Subregional DNA Probes

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1 Introduction

During the last decade strong evidence has been accumulated for a causal relationship between specific genetic alterations and the abnormal differentiation state and growth behaviour of malignant cells. Such alterations can be observed at different levels ranging from chromosome mutations to point mutations of certain genes and may arise in a given cell either spontaneously or due to the effect of mutagenic environmental factors.

The discovery of chromosome banding 25 years ago (Caspersson et al., 1968) has paved the way for comprehensive cytogenetic analyses of malignant cells. Such studies have led to the discovery of specific chromosome rearrangements, as well as complete or partial chromosome gains and chromosome losses consistently involved in the development of certain tumors. Detailed molecular analyses of specific chromosome breakpoints and chromosome segments present in aberrant copy numbers then have resulted in the discovery of genes, such as oncogenes and tumor suppressor genes, relevant for the malignant cell phenotype (for review see Rowley, 1990). In addition, the discovery of double minute chromosomes (DM) and homogeneously stained chromosome regions (HSR) has indicated the presence of amplified DNA segments in certain tumors (for review see Alitalo and Schwab, 1986). Tumor cytogeneticists may be considered as genetic cartographers. They are interested to map the sites where tumor genomes differ from normal ones. In order to fullfill their task to make maps, cytogeneticists have to solve two diagnostic tasks. Firstly, global assays are needed to search tumor genomes for any kinds of genetic alterations. Secondly,

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assays for fine mapping should be at hand to narrow down a specific genetic alteration detected by the global assay as precisely as possible and thus facilitate the hunt for tumor relevant genes. Up to now chromosome banding analyses have provided the most rapid and fruitful global assay to detect genetic alterations at the chromosome level. Unfortunately, the resolution of this approach is rather limited. Breakpoints can be located at best within the limits of a specific chromosome band (comprising a minimum of several Mbp DNA). Karyotyping of tumor cells remains often incomplete due to the presence of marker chromosomes with unknown genetic composition. Accordingly, specific gains and losses of chromosome material may be overlooked. The presence of DM and HSR can be easily recognized but the origin of amplified DNA sequences remains elusive. Finally, the lack of tumor metaphase spreads suitable for chromosome banding analyses has hampered the broad application of this approach, in particular in solid tumors.

In the following sections we will briefly describe most recent progress which will help to overcome some of the limitations of conventional cytogenetics. (i) Multiple color fluorescence *in situ* hybridization (FISH) (Nederlof et al., 1990, 1992; Ried et al., 1992a, Dauwerse et al., 1992, Lengauer et al., 1993) has provided a new tool for the fine mapping of genetic alterations with high resolution (in the kbp range rather than in the Mbp range) (e.g. Ried et al., 1992b) . However, in order to select DNA probes useful for the detailed analysis of a clinical or tumor cell sample, previous knowledge of the types of expected aberrations is required. (ii) Reverse chromosome painting with tumor genomic DNA, in particular the introduction of comparative genomic *in situ* hybridization (CGH), has provided a new global assay for the screening and precise chromosomal mapping of unbalanced genetic material even in cases where genomic DNA from a given tumor is the only material available for analysis (Kallioniemi et al., 1992, du Manoir et al., 1993, Joos et al., 1993).

2 Multiple color fluorescence *in situ* hybridization (FISH)

Nederlof et al., (1990) have described the possibility of combinatorial FISH for the simultaneous visualization of up to seven chromosome targets in different colors. For this purpose three DNA probes are labeled with haptens A, B, C individually, while four additional probes are labeled with various hapten combinations A+B, A+C, B+C, A+B+C. Following FISH the probes are detected with three different fluorochromes. Using whole chromosome paint probes, as well as centromeric repeat and cosmid probes, Ried et al., (1992) have applied combinatorial FISH and digital fluorescence microscopy. Using probe sets derived from multiple cosmid clones, these authors have demonstrated the possibility to achieve new types of specific staining patterns along individual chromosomes (see

also Lichter et al., 1990). Recently, combinatorial FISH has also been performed with up to seven DNA-probes directly labeled with three fluorochromes in all possible combinations (Popp et al., 1993). To further enhance the number of chromosome targets which can be distinguished multiple color chromosome painting has also been performed using chromosome specific library DNAs labeled with distinctly different hapten ratios (e.g. biotin/digoxigenin) (Nederlof et al., 1992, du Manoir et al., 1993). Using different ratios of three haptens for whole chromosome composite probe labeling, Dauwerse et al., (1992) have demonstrated the simultaneous visualization of twelve chromosomes in different colors. To test whether individual chromosomes can be discriminated solely on the basis of fluorescence ratios (FR), FISH hybridization experiments were carried out in our laboratory with chromosome specific libraries for chromosomes 1, 4, 8, 13 and 16 using the following hapten ratios (biotin/digoxigenin): chromosome 1 (4/1), chromosome 4 (1/4), chromosome 8 (1/1), chromosome 13 (biotin only), chromosome 16 (digoxigenin only). For each painted chromosome type the range of fluorescence ratio values (FR) observed in fourteen metaphase spreads did not overlap with the range of FR obtained for any other painted chromosome type. These results demonstrated that individual FRs can indeed be used as a single, reliable parameter to identify chromosomes. Furthermore, it can be predicted from these results that four spectrally separable fluorochromes in various proportions should suffice to distinguish all chromosomes of the human chromosome complement by fluorescence ratio measurements (du Manoir et al., 1993).

Using cosmid probes, the percentage of metaphase spreads showing all expected signals simultaneously was low. Considering a hybridization efficiency of 80% to the expected target by each individual cosmid clone, a series of 10 clones should yield complete hybridization patterns in only 11% of the hybridized metaphase spreads. In order to implement multiple color FISH as a tool for diagnostic applications, improved hybridization efficiencies are essential. Recently, we have demonstrated that Alu-PCR products selectively amplified from YAC clones provide very efficient probes for FISH (Lengauer et al., 1992a,b). The hybridization efficiency for many YACs was close to 100% both in metaphase spreads and interphase nuclei. Whole chromosome paint probes and Alu-PCR amplified sequences from numerous YAC clones (labeled either indirectly with different hapten combinations or directly with different fluorochrome combinations) can be combined as multiplex probes and used to achieve new, colored chromosome staining patterns, termed chromosomal bar codes (Lengauer et al., 1993). In contrast to conventional G- or R-bands, the chromosomal position, extent, individual color and relative signal intensity of each "bar" can be modified by varying probes and labeling procedures. Accordingly, this approach provides a high flexibility. Specific types of chromosomal bar coding can be applied to solve specific problems in clinical and tumor cytogenetics.

3 Comparative genomic *in situ* hybridization (CGH)

For CGH, test genomic DNA prepared from tumor specimens is chemically modified with certain haptens (e.g. with biotin). Control genomic DNA prepared from cells with normal chromosome complements (46,XY or 46,XX) is labeled with a different hapten (e.g. digoxigenin). Labeled test and control genomic DNA are mixed in defined proportions, e.g. 1:1, and used as a probe to paint chromosomes of metaphase spreads with normal chromosome complements in two colors (Kallioniemi et al., 1992, du Manoir et al., 1993). During CGH homologous chromosome specific DNA sequences, present in both test and control genomic DNAs, compete for the same normal target chromosomes. Hybridized test and control DNA sequences are detected by different fluorochromes, e.g. FITC and TRITC. The rationale of CGH is based on the expectation that the ratios of FITC/TRITC fluorescence intensities which can be measured for each chromosome segment reflect the relative copy number of the respective segment in the tumor genome as compared to the disomic state in the normal genome. The fluorescence ratio should decrease by a factor of 0.5 for monosomies and become zero for nullosomies. It should increase by a factor of 1.5 for trisomies, by a factor of 2 for tetrasomies, 2.5 for pentasomies, and so forth.

For quantitative fluorescence measurements we have used a Zeiss Axiophot microscope equipped with a 100 W mercury lamp and appropriate filter sets coupled with a cooled CCD (charge coupled device) camera (Photometrics, Tucson, AZ, USA) with the Kodak KAF 1400 chip (1317 x 1035 pixels) (for details see du Manoir et al., 1993). The optimal exposure time for each slide and filter set was chosen in order to avoid saturation values in all pixels, and to cover at least half total dynamic range of the camera. Exposure times and all optical settings of the microscope were kept constant for a whole series of image acquisitions. Identification of chromosomes was made on the basis of DAPI CCD-images. Digital images were processed either by the SAMBA 2005 image analyzer system (Alcatel-TITN Co., Grenoble, France) or the TCL Image software (Multihouse, Amsterdam, The Netherlands).

We have tested the feasibility of CGH using genomic test DNAs from a number of tumors, including genomic test DNAs prepared from cultured cells of a renal papillary carcinoma cell line as well as genomic DNAs prepared directly from primary tumor material. Significant differences of the fluorescence ratios could be measured for chromosome types present in different copy numbers in these test genomes. In addition, chromosome material involved in partial gains and losses of the different tumors could be mapped to their normal chromosome counterparts in normal metaphase spreads. An alternative and more simple evaluation procedure based on visual inspection of CCD-images of CGH-metaphase spreads also yielded consistent results from several, independent observers (du Manoir et al., 1993 and our unpublished data).

This new approach should become the method of choice in cases where genomic DNA from the suspected cells is the only material available for analysis and is less time consuming than other molecular genetic approaches presently used to search a genome for genetic imbalances. We expect that CGH will also become of great importance to identify genetic imbalances in patients suspicious for a chromosomal syndrome, where the origin of unbalanced chromosomal segments of chromosomes cannot be identified with conventional chromosome analyses.

In addition, the normal chromosome sites from which amplified DNA sequences present in tumor genomes originated could be mapped by CGH or even more simply by reverse chromosome painting of the tumor genomic DNA to normal metaphase chromosome spreads (Kallioniemi et al., 1992, Joos et al., 1993). As a model system, we have analyzed three tumor cell lines with amplification units including the proto-oncogene c-myc (Collins and Groudine 1982). The smallest amplification unit was about 90 kb present in 16-24 copies, the largest unit was >600 kb present in 16-32 copies. Specific signals that co-localized with a differently labeled c-myc probe on chromosome band 8q24 were obtained with genomic DNA from each cell line. In further experiments, genomic DNA derived from primary tumor material was used in the case of a male patient with glioblastoma multiforme. Signals were found both on band 7p13 and bands 12q13-q15. Notably, the signal on 12q13-q15 was consistently stronger (Joos et al., 1993). These results indicate co-amplification of sequences from both bands 7p13 and 12q13-q15. Southern blot analysis using a EGF-receptor gene (EGFR) probe that maps to 7p13 indicated the amplification of sequences from this gene. Several oncogenes map to 12q13-q15 providing candidate genes for a tumor associated proto-oncogene amplification. These candidate genes are presently under further investigation. This approach should find wide applications to study the origin of amplified sequences contained in double minutes or homogeneously stained regions.

Several limitations of CGH as compared to conventional banding analyses also need to be emphasized. CGH does not provide any information in which way chromosome segments involved in gains and losses are actually arranged in marker chromosomes of the test genome and balanced chromosome rearrangements cannot be detected at all. Finally, CGH reveals only changes present in the majority of cells of a given tumor. CGH analyses performed with tumor DNAs prepared from a series of individual tumors representing a distinct tumor type should lead to the identification of those chromosomal imbalancies, which are consistently involved and thus help to identify candidate chromosome segments for genes of major biological importance for the tumor type in question.

4 An integrated approach for chromosome analyses

The now available tools of classical and molecular cytogenetics range from procedures useful for the global screening of chromosomal changes to the analysis of individual genes. These tools need to be applied in a sequence which optimally fits the needs of each investigation. In an integrated approach the advantages of each method will complement the limitations of others. Wherever metaphase spreads from a clinical or tumor specimen are available, chromosome banding provides the method of choice for a comprehensive and rapid analysis of both balanced and unbalanced chromosome rearrangements at the single cell level. Its resolution, however, is limited and its results may not adequately reflect the clonal heterogeneity of the test specimen. In cases where chromosome banding is not applicable or provides insufficient results, CGH can now be used as an additional global and rapid screening test to detect genetic imbalances predominant in a test specimen. FISH and molecular genetic approaches provide the tools to confirm and study specific chromosome aberrations suggested by the results of banding analyses and/or CGH with high resolution. A rapidly increasing number of chromosome band specific DNA probes can be chosen, which optimally fit the needs of molecular cytogenetics. YAC-contigs from the human genome are growing quickly and it can be expected that in near future rather complete contigs will exist for each human chromosome (Bellané-Chantelot et al., 1992, Chumakov et al., 1992). This development will provide ample possibilities to select pools of YAC clones which yield a unique signal on each chromosome band with high reproducibility both on metaphase chromosomes and in interphase nuclei. Chromosomal bar codes can then be constructed which are optimized for a given diagnostic purpose at any resolution needed. Candidate chromosome regions suspicious for a gain or loss of genetic material identified by CGH can then be mapped in detail using appropriate sets of DNA-probes hybridized to metaphase spreads and/or interphase nuclei obtained from a tumor specimen. Finally, we expect that CGH and chromosomal bar coding will help to establish more detailed karyotype-phenotype correlations in chromosomal syndromes with unbalanced rearrangements.

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References

Alitalo K, Schwab M (1986) Oncogene amplification in tumor cells. In: Advances in Cancer Research. Academic Press, Orlando, Florida

Bellané-Chantelot C, Lacroix B, Ougen P, Billault A, Beaufils S, Bertrand S, Georges I, Glibert F, Gros I, Lucotte G, Susini L, Codani J-J, Gesnouin P, Pook S, Vaysseix G, Lu-Kuo J, Ried T, Ward D, Chumaskov I, Le Paslier D, Barrillot E, Cohen D (1992) Mapping the whole human genome ba fingerprinting yeast artificial chromosomes. Cell 70: 1059-1068

Caspersson T, Farber S, Foley GE, Kudynowski J, Modest EJ, Simonsson E, Wagh U, Zech L (1968) Chemical differentiation along metaphase chromosomes. Exp Cell Res 49: 219-226

Chumakov, I., Rigault, P., Guillou, S., Ougen, P., Billaut, A., Guasconi, G., Gervy, P., LeGall, I., Soularue, P., Grinas, L., Bougueleret, L., Bellanne-Chantelot, C., Lacroix, B., Barillot, E., Gesnouin, P., Pook, S., Vaysseix, G., Frelat, G., Schmitz, A., Sambucy, J.-L., Bosch, A., Estivill, X., Weissenbach, J., Vignal, A., Riethman, H., Cox, D., Patterson, D., Gardiner, K., Hattori, M., Sakaki, Y., Ichikawa, H., Ohki, M., Le Paslier, D., Heilig, H., Antonarakis, S., and Cohen, D. (1992) Continuum of overlapping clones spanning the entire human chromosome 21q. Nature 359: 380-387

Collins S, Groudine M (1982) Amplification of endogeneous myc-related DNA sequences in a human myeloid leukaemia cell line. Nature 298: 679-681

Dauwerse JG, Wiegant J, Raap AK, Breuning MH, van Ommen GJB (1992) Multiple colors by fluorescence *in situ* hybridization using ratio-labelled DNA probes create a molecular karyotype. Hum Mol Genet. 1: 593-598.

du Manoir S, Speicher MR, Joos S, Schröck E, Popp S, Döhner H, Kovacs G, Robert-Nicoud M, Lichter P, Cremer T (1993) Detection of complete and partial chromosome gains and losses by comparative genomic *in situ* hybridization. Hum Genet. In Press

Joos S, Scherthan H, Speicher MR, Schlegel J, Cremer T, Lichter P (1993) Detection of amplified DNA sequences by reserve chromosome painting using genomic tumor DNA as probe. Hum Genet. In Press

Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D (1992) Comparative genomic *in situ* hybridization for molecular cytogenetic analysis of solid tumors. Science 258: 818-821

Lengauer C, Green ED, Cremer T (1992a) Fluorescence *in situ* hybridization of YAC Clones after Alu-PCR Amplification. Genomics 13:286-289

Lengauer C, Riethman HC, Speicher MR, Taniwaki M, Konecki D, Green ED, Becher R, Olson MV, Cremer T (1992b) Metaphase and interphase cytogenetics with Alu-PCR amplified YAC clones containing the BCR-gene and the protooncogenes c-raf-1, c-fms, c-erbB-2. Cancer Res 52: 2590-2596

Lengauer C, Speicher MR, Popp S, Jauch A, Taniwaki M, Ramaiah Nagaraja M, Riethman HC, Donis-Keller H, D'Urso M, Schlessinger D, Cremer T (1993)

Chromosomal bar codes constructed by fluorescence *in situ* hybridization with multiple ALU-PCR products of multiple YAC clones. *Submitted*

Lichter P, Tang CJC, Call K, Hermanson G, Evans GA, Housman D, Ward DC (1990) High-resolution mapping of human chromosome 11 by *in situ* hybridization with cosmid clones. Science 247:64-68

Nederlof PM, van der Flier S, Wiegant J, Raap AK, Tanke HJ, Ploem JS, van der Ploeg M (1990) Multiple fluorescence *in situ* hybridization. Cytometry 11: 126-131

Nederlof PM, van der Flier S, Vrolijk J, Tanke HJ, Raap AK (1992) Fluorescence ratio measurements of double-labeled probes for multiple *in situ* hybridization by digital imaging microscopy. Cytometry 13: 839-845

Ried T, Baldini A, Rand TC, Ward DC (1992a) Simultaneous visualization of seven different DNA probes by *in situ* hybridization using combinatorial fluorescence and digital imaging microscopy. Proc Natl Acad Sci USA 89: 1388-1392

Ried T, Lengauer C, Cremer T, Wiegant J, Raap AK, van der Ploeg M, Groitl P, Lipp M (1992b) Specific metaphase and interphase detection of the breakpoint region in 8q24 of Burkitt lymphoma cells by triple-color fluorescence *in situ* hybridization. Genes Chrom Cancer 4:1-6

Rowley JD (1990) Molecular cytogenetics: Rosetta Stone for understanding Cancer-twenty-ninth GHA Clowes memorial award lecture. Cancer Research 50:3816-3825