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REVIEW

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Tumor cytogenetics revisited: comparative genomic hybridization and spectral karyotyping

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Abstract Fluorescence in situ hybridization techniques allow the visualization and localization of DNA target sequences on the chromosomal and cellular level and have evolved as exceedingly valuable tools in basic chromosome research and cytogenetic diagnostics. Recent advances in molecular cytogenetic approaches, namely comparative genomic hybridization and spectral karyotyping, now allow tumor genomes to be surveyed for chromosomal aberrations in a single experiment and permit identification of tumor-specific chromosomal aberrations with unprecedented accuracy. Comparative genomic hybridization utilizes the hybridization of differentially labeled tumor and reference DNA to generate a map of DNA copy number changes in tumor genomes. Comparative genomic hybridization is an ideal tool for analyzing chromosomal imbalances in archived tumor material and for examining possible correlations between these findings and tumor phenotypes. Spectral karyotyping is based on the simultaneous hybridization of differentially labeled chromosome painting probes (24 in human), followed by spectral imaging that allows the unique display of all human (and other species) chromosomes in different colors. Spectral karyotyping greatly facilitates the characterization of numerical and structural chromosomal aberrations, therefore improving karyotype analysis considerably. We review these new molecular cytogenetic concepts, describe applications of comparative genomic hybridization and spectral karyotyping for the visualization of chromosomal aberrations as they relate to human malignancies and animal models thereof, and provide evidence that fluorescence in situ hybridization has developed as a robust and reliable technique which justifies its translation to cytogenetic diagnostics.

Key words Fluorescence in situ hybridization · Comparative genomic hybridization · Spectral karyotyping · Chromosome aberrations · Tumor progression

Abbreviations *CCD* Charge-coupled device · *CGH* Comparative genomic hybridization ·

dmin Double minute chromosome · *FISH* Fluorescence in situ hybridization · *hsr* Homogeneously staining region · *SKY* Spectral karyotyping

Introduction

The identification of recurrent chromosomal aberrations in hematological malignancies and solid tumors has rested mainly on karyotype analysis by means of chromosome banding techniques [1, 2]. Nonrandom chromosomal changes in malignancies often mirror events at the molecular level and provide entry points for gene identification strategies [3, 4]. Moreover, the identification of nonrandom tumor or tumor stage-specific aberrations is an integral constituent of diagnosis, differential diagnosis, prognostication, and therapy planning in human malignancies [5]. Despite the important role of conventional chromosome banding techniques in the assessment of karyotype changes, technical limitations often confound a comprehensive characterization of tumor genomes employing classical cytogenetic analysis alone [6]. The technical hurdles faced in banding studies of human malignancies and in particular in lymphomas and solid tumors are numerous: the mitotic index is often low, and the quality of metaphase chromosomes is frequently of such an inferior quality that high resolution analyses cannot be performed. In addition, the selective growth of certain subclones, or even normal cells, may not reflect tumor-specific rearrangements. The analysis of complex chromosomal rearrangements, homogeneously staining regions (hsr's) or double minute chromosomes (dmin's), that are common in tumor metaphases is not possible with banding techniques and leave the cytogeneticist with a disturbingly high number of marker chromosomes.

Molecular cytogenetic techniques utilizing fluorescence in situ hybridization (FISH) with chromosome-specific or chromosomal breakpoint-specific DNA probes facilitate the confirmation of presumed chromosomal aberrations with high sensitivity and specificity [7]. A particular advantage of FISH techniques is the possibility to study chromosomal aberrations also in nondividing cells, which is useful for the visualization of chromosomal aberrations directly in cytological preparations and tissue sections [8]. However, FISH analyses with locus-specific probes or chromosome-specific DNA libraries [9, 10] are restricted to the targeted chromosome or chromosomal subregion. Therefore, and in strong contrast to chromosome banding based karyotype analysis, FISH with locus-specific probes or whole chromosome paints has one severe shortcoming: while most valuable in the confirmation of previously characterized chromosomal aberrations, it cannot serve as an a priori screening test for chromosomal rearrangements [11]. This review focuses in particular on approaches that combine the sensitivity and specificity of FISH with the power of conventional cytogenetics, i.e., the screening of entire tumor genomes for chromosomal aberrations in a single experiment. These techniques include comparative genomic hybridization (CGH) and spectral karyotyping (SKY; Fig. 1). Each approach has distinct and specific advantages in the analyses of tumor chromosomes and genomes.

Comparative genomic hybridization

CGH is a technique that identifies and maps DNA copy number changes in tumor genomes in a single hybridization experiment [12, 13]. These changes are identified on karyotypically normal reference metaphase chromosomes. Tumor cell culture is not required. The results of CGH analyses are karyograms of tumor-specific DNA gains and losses. CGH has become a workhorse for tumor cytogenetics and has for some cancers already surpassed the number of cases analyzed by means of chromosome banding analyses.

Methodology

Comparative genomic hybridization is based on quantitative two color fluorescence in situ suppression hybridization (Fig. 2). Total genomic DNA from a tumor specimen is isolated following routine procedures. A reference, or control, DNA is extracted from an individual with a normal karyotype (46,XX or 46,XY). The two genomes are labeled differentially with, for example, fluorescein-dUTP (green fluorescence) for the tumor genome and rhodamine-dUTP (red fluorescence) for the reference genome in a standard nick translation reaction. The labeled genomes are then pooled and hybridized to reference human metaphase spreads (Fig. 2a–c). In order to suppress the cross-hybridization of highly repetitive sequences present in both genomes, an excess of unlabeled Cot1-fraction of human DNA (enriched in repetitive sequences) is included in the hybridization mixture. This step is necessary because the ubiquitous distribution of repetitive DNA would impair the evaluation of the single-copy sequences that are over- or underrepresented in the tumor genome.

The differences in fluorescence intensities along the chromosomes on the reference metaphase spread are a reflection of the copy number changes of corresponding sequences in the tumor DNA: if chromosomes or chromosomal subregions are present in identical copy numbers in both the reference and the tumor genome, the observed fluorescence is a blend of an equal contribution of red and green fluorescence. If chromosomes are lost or chromosomal subregions are deleted in the tumor genome, the resulting color is shifted to red. A gain of a certain chromosome in the tumor is reflected by a more intense green staining of the respective chromosome in the reference metaphase preparation. Increased supernumerary of DNA sequences in the tumor genome, for example, amplification of oncogenes, appear as intensely labeled green signals at the chromosomal map position

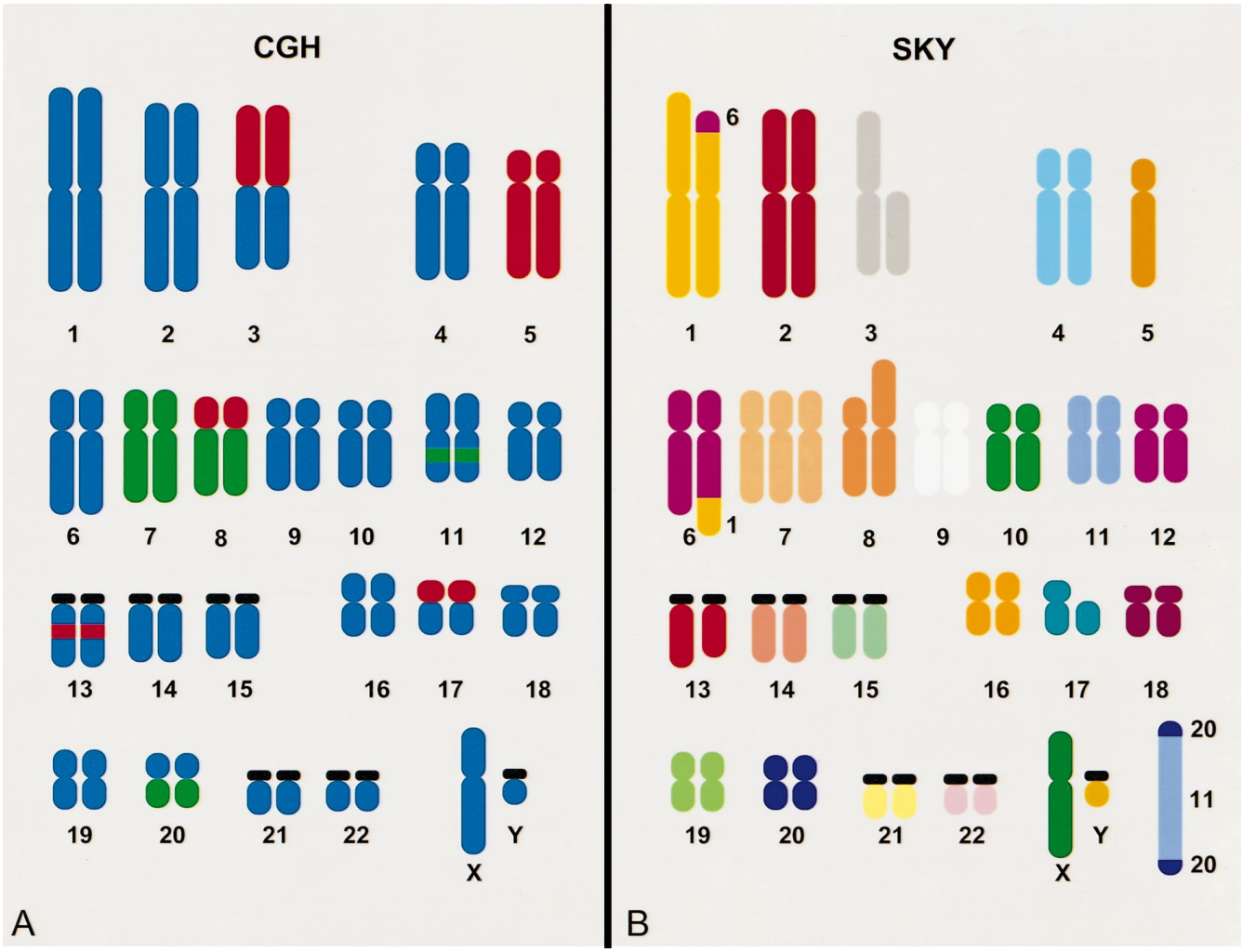


Fig. 1 Schematic presentation of CGH (A) and SKY (**B**). Both techniques have the distinct advantage that the entire test genome can be analyzed in a single experiment. **A** The simultaneous hybridization of differentially labeled tumor DNA (*green fluorescence*) and reference DNA (*red fluorescence*) to normal metaphase chromosomes allows identification and determination of the chromosomal mapping position of DNA copy number changes in tumor genomes. *Blue*, regions not affected by copy number changes; *red*, copy number decreases, indicating chromosome loss or chromosomal deletion; *green*, gain of DNA sequences in the tumor. For instance, chromosomes 1 and 2 are not subject to DNA copy number changes in this hypothetical case, however, the short arm of chromosome 3 is lost, and chromosome 7 is gained. A gene amplification was mapped to the long arm of chromosome 11, and a chromosomal band on 13q is lost. **B** SKY allows simultaneous visualization of all human chromosomes in different colors. The cartoon presents metaphase chromosomes from the same hypothetical tumor genome as in **A**. For instance, the loss of chromosome arm 3p in **A** (*red color* in the CGH experiment) is the consequence of a deletion of the short arm as determined by SKY. The trisomy 7 corresponds to the gain detected by CGH. Isochromosome formations are common patterns in human tumor cells and are caused by the fusion of, for example, the long arms of chromosome 8 with the consequence of the loss of the short arms, as depicted in the case shown here. The CGH pattern of such an isochromosome formation is presented in **A**, where copy number decreases on 8p that are accompanied by a gain of 8q are identified by the color shift. Balanced chromosomal aberrations, such as a reciprocal translocation between chromosomes 1 and 6 (**B**) do not affect the copy number. Therefore they are not visible by CGH. The gene amplification mapped by CGH to a chromosomal region on 11q is visible in the SKY karyotype as a hsr flanked by chromosomal material originating from chromosome 20

of the amplified oncogene on normal metaphase chromosomes (Fig. 2d).

The quantitative measurement of fluorescence intensity values based on digital image analysis is crucial for precise CGH analysis [14, 15]. This analysis includes image acquisition of the fluorescein and rhodamine fluorescence with a charge-coupled device (CCD) camera and fluorochrome specific optical filters. Using specialized software, the result of the measurement of the fluorescence values can be visualized by means of a look-up table where pseudocolors refer to gains or losses in the tumor genome (Fig. 2e). The final step in the quantitative fluorescence measurement employs the calculation of average ratio profiles along the axis of each individual chromosome based on data from at least five metaphase spreads. Ratios of 1 indicate equal copy numbers of the respective chromosomes in the tumor and reference genome, ratios of 0.5 a deletion of one homologous chromosome, and ratios of

1.5 a trisomy in a diploid tumor (Fig. 2f). Gene amplifications can be mapped to reference metaphase chromosomes according to peak fluorescence ratios of more than 2.5 (e.g., on chromosome 8q; Fig. 2c–f).

The validity of CGH for delineating complex genetic changes in tumor genomes has been carefully established by comparing CGH results with those of chromosome banding analyses [13]. Another independent study to verify the results of CGH analysis has been described by Schröck et al. [16] with a series of human gliomas. In this sample collection banding was often impossible due to inferior spreading of the metaphase chromosomes and the frequent observation of dmin chromosomes. By means of interphase cytogenetics with yeast artificial chromosome clones for chromosomal subregions that revealed gains and losses after CGH, the presence of all imbalances could be confirmed in interphase nuclei prepared from tissue sections, i.e., ratios of 1.5 after CGH were in accordance with three signals in interphase nuclei. In addition, a DNA amplification that was mapped to chromosome 4 by CGH was shown to be present in dmin chromosomes of this tumor after FISH with a chromosome 4 specific DNA library to metaphase chromosome preparations.

Also, the amplification of the *EGFR* gene determined by DNA fingerprint analysis [17] resulted in peak fluorescence ratio values on chromosomal map position 7p12, known to harbor the gene encoding this growth factor receptor. Several groups have analyzed tumor specimen by CGH and studies for loss of heterozygosity [18, 19]. In many instances the results are in accordance. Discrepancies, however, can be explained by the lower spatial resolution of CGH or by genetic mechanisms that result in loss of heterozygosity but not in reduced copy number, such as mitotic recombination and endoreduplication [20]. In aneuploid tumor genomes, on the other hand, reduced copy numbers can be detected by CGH that are not accompanied by loss of heterozygosity because the loss of two chromosomes in a tetraploid tumor would clearly result in a relative copy number decrease; however, the two parental alleles could still be maintained.

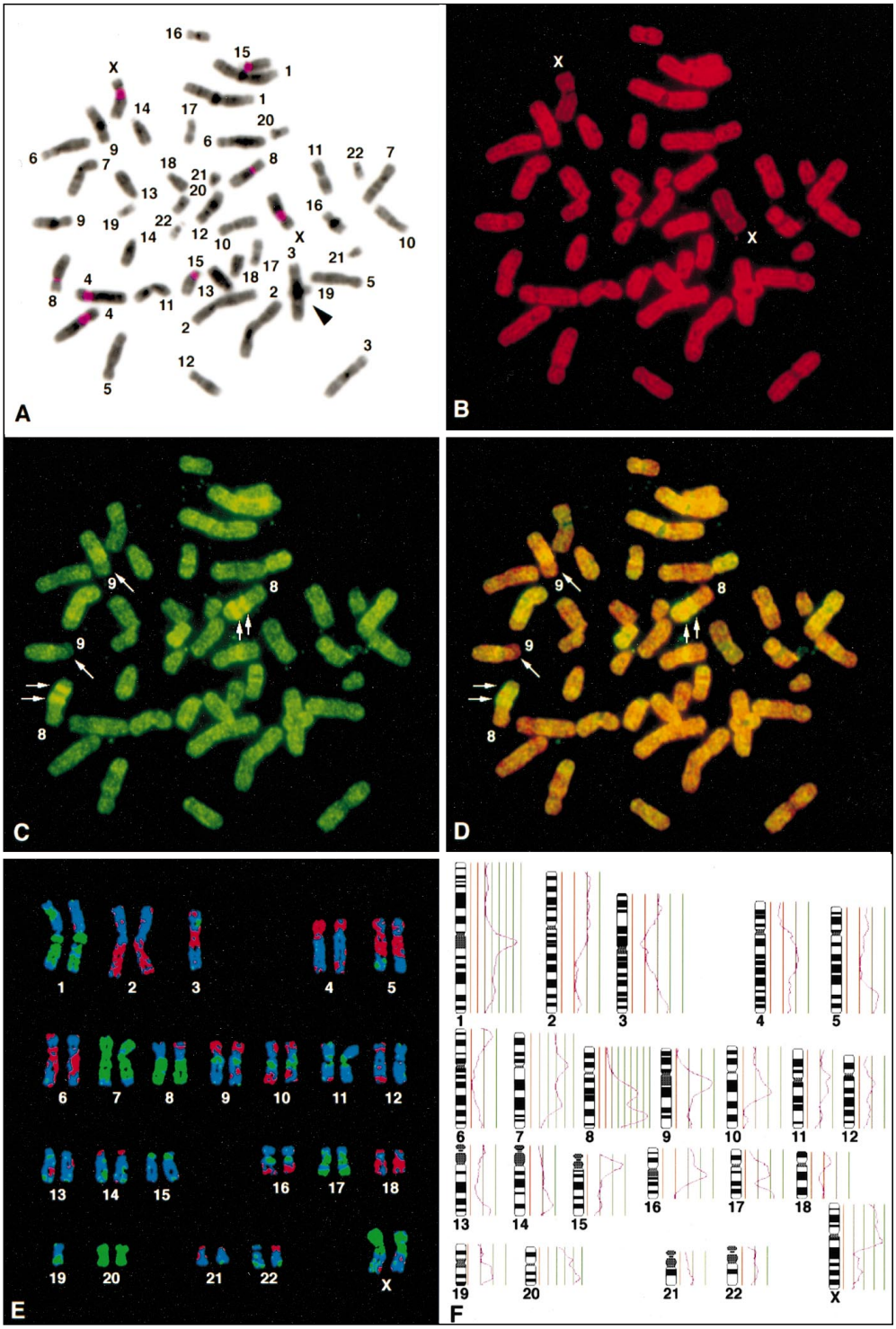
CGH remains, of course, a cytogenetic technique, and its sensitivity in terms of spatial resolution is limited mainly by the degree of chromosome condensation on the reference metaphase chromosomes, but it is also influenced by the copy number change. The resolution limit for low copy number increases or decreases is estimated to be in the range of 10 Mbp; however, amplicons as small as 50 kb can be visualized if the copy number change is high.

Applications

The importance of comparative genomic hybridization as a screening test for chromosomal aberrations in tumor genomes can be easily deduced from the experimental concept. Only genomic tumor DNA and metaphase preparations from a karyotypically normal donor are required for this cytogenetic test. This circumvents one of the thorny issues in solid tumor cytogenetics, i.e., the preparations of high-quality tumor metaphase spreads suitable for chromosome banding analyses [6]. In addition, and most importantly, tumor DNA extracted from archived, formalin-fixed paraffin blocks can be used for the analysis as well [21–23]. This particular feature does not only allow identification of chromosomal aberrations retrospectively, and consequently facilitates the search for correlations between cytogenetic findings and clinical course. CGH can also be applied to the identification of chromosomal aberrations of previously stained and histologically defined regions of solid tumor tissue sections, including premalignant lesions, and is therefore an ideal tool for studying chromosomal aberrations during tumor progression [24–26]. Taken together, it has been possible to establish a comprehensive phenotype/genotype correlation in solid tumors and namely solid tumor progression.

Since its introduction in 1992 CGH has been applied to a broad variety of test specimens. While CGH may also be useful for karyotype analysis in the clinical cytogenetic laboratory, for example, for detecting partial de-

Fig. 2A–F Example of the CGH analysis of the breast cancer cell line SKBR3. **A** The normal metaphase chromosomes were stained with DAPI and displayed in an inverted mode to generate a G-banding pattern. This pattern is the basis for chromosome identification (see *numbers*). The centromeres of chromosomes 4, 8, 15, and X were hybridized with a Cy5-conjugated centromere-specific repeat probe. This cohybridization facilitates chromosome identification considerably. *Arrow*, overlap of chromosomes 3 and 19. These two chromosomes are therefore excluded from the quantitative evaluation and occur only once in the ratio image (**E**). **B** Visualization of the reference genome-specific *red fluorescence* (rhodamine). Note that all chromosomes are labeled homogeneously except for the X-chromosomes. The weaker staining reflects the fact that the reference DNA was prepared from a male donor, which consequently results in a "monosomy" for the X-chromosome. **C** Visualization of the *green fluorescence* (fluorescein), specific for the tumor genome. Note the different fluorescence intensities on the normal metaphase chromosomes, for example, peak green intensities on the long arm of chromosome 8q (*arrows*), indicating the chromosomal map position of amplifications and reduced intensities on chromosome arm 9p. **D** Electronic merging of the red and green fluorescence images in **B** and **C**. *Arrows*, some of the regions subject to copy number increases (note two discrete amplification sites on chromosome 8q) or decreases (e.g., on the short arm of chromosome 9). **E** Ratio image of the hybridization shown in **B**, **C**. Karyotype display of a ratio image. The evaluation is based on a quantitation of the fluorescence intensities. *Blue*, regions not affected by copy number changes in the tumor (compare also to the cartoon in Fig. 1A); *red*, loss of genetic material; *green*, regions overrepresented in the tumor genome. One copy of chromosomes 3 and 19 each was excluded from the analysis due to overlap (see *arrowhead* in **A**). **F** Based on at least five analyzed metaphases, an average ratio profile is computed for all chromosomes. The evaluation of average ratio profiles forms the basis for mapping copy number changes in tumor genomes. In the breast cancer cell line (SKBR3) shown here, DNA gains were observed on chromosomes and chromosome arms 1q, 7, 8q, 10q, 17q, 19q, 20, and Xp. Losses were mapped to 2q, part of chromosome 3, 4p, 5q, 6q, 8p, 9p, 10q, and 16q. Peak fluorescence values that indicate DNA amplifications were identified on chromosomal bands 8q21 and 8q24



letions or supernumerary of chromosomes [27], the field that benefits most from CGH is clearly solid tumor cytogenetics. CGH has been applied to map chromosomal copy number changes in virtually all human cancers, including common tumors such as lung [18, 28], breast [29–31], colon [24, 32], brain [16, 33–35], head and neck [36, 37], prostate [38–41], hematological malignancies [42, 43], ovary [44], bladder [45], and uterine cervix [25, 26]. While it is impossible to summarize all data in this review, especially those that describe chromosomal aberrations in rather rare tumors, it has become clear that the molecular cytogenetic analysis of various tumor entities revealed a highly characteristic pattern of chromosomal gains and losses, i.e., a blueprint of chromosomal aberrations. This means that both the number of chromosomal aberrations as a measure for crude genetic insta-

bility and the genomic distribution of these aberrations define certain tumor types. For instance, a gain of chromosome 7 and a loss of chromosome 10 seem to be the landmark aberrations of glioblastomas [16, 33]. These chromosomes, on the other hand, are only rarely involved in, for example, breast cancer [29, 30], where acquisition of additional copies of chromosomes 1, 8, 17, and 20 are specific aberrations, accompanied by a loss of chromosomal arms 13q and 17p or the chromosomal mapping position of the retinoblastoma and p53 tumor suppressor genes. Also, numerous new chromosomal loci that are subject to high-level copy number increases (amplifications) have been identified, again in a tumor-specific distribution.

The chromosomal mapping of commonly deleted or amplified regions offers entry points for the search for a gene (or genes) involved in growth control in certain tumor types. The analysis of breast cancers by CGH, for instance, has identified a high number of hitherto unknown amplifications sites on chromosomes 17q and 20q [29]. Based on the identification of recurrent amplification sites a subsequent high-resolution physical map of these regions has been generated [46, 47]. The molecular analysis of these regions by means of cloning and transcript identification strategies is now well under way and will help to identify genes whose overexpression is important in breast carcinogenesis.

The first series of CGH studies, due to the scarcity of cytogenetic data, focused on the description of a karyogram of gains and losses for certain tumor entities. In the recent past the focus has shifted from a mere description of the status quo of chromosomal aberrations in a particular tumor type towards the use of CGH to understand chromosomal aspects of tumor progression and the dynamics of chromosomal aberrations that occur during (and cause) the multistep process of carcinogenesis. For instance, an elegant study performed by Visakorpi and colleagues [40, 41] compared the pattern of chromosomal aberrations in primary carcinomas of the prostate gland and in tumors that developed resistance to pharmaceutical castration. The authors identified an amplification site on the short arm of the X-chromosome in therapy-resistant tumors. A search for candidate genes unraveled the androgen receptor gene as coincident with the amplification locus. Subsequent FISH and molecular analyses have clearly proven that the androgen receptor gene is indeed the target for amplification. From a tumor perspective this makes perfect sense. The sustenance of rapid growth of the tumor cells, inherently dependent on androgen, required the amplification of the receptor to utilize even small amounts of this hormone after chemically or surgically induced hormone depletion.

CGH has been used to complement immunohistochemical and DNA-ploidy measurement data and histomorphology to establish a phenotype/genotype correlation in solid tumor progression [24, 25]. In a recent report we have attempted to generate a comprehensive picture of genomic and chromosomal aberrations during defined stages of colorectal carcinogenesis. A combination of CGH, DNA-ploidy measurements, the detection of proliferation markers, and the expression of tumor suppressor genes was applied to analyze normal epithelium, low- and high-grade adenomas and invasive carcinomas of the colon. In these studies DNA for CGH analyses was extracted from histologically characterized tissue sections. The analysis revealed a sequence of genetic events that occur at defined steps in colon carcinogenesis [24]. While the gain of chromosomes 7 and 20 were clearly identified as early chromosomal changes, the acquisition of additional copies of chromosome 8q and 13q as well as the loss of chromosomes 8p and 18q occur rather late in tumor progression. The latter changes coincide with the transition from high-grade adenomas to invasive carcinomas and are accompanied by apparently mutant p53 expression, high proliferative activity, and crude DNA aneuploidy as measured on Feulgen-stained interphase cells of the tissue section used also for CGH.

An even clearer picture emerged when CGH was applied to identify chromosomal aberrations during the genesis of cervical carcinomas [25, 26]. Cervical carcinoma is the second most common carcinoma in women worldwide. Despite the tremendous contribution to morbidity and mortality, conventional cytogenetic analysis based on chromosome banding analyses had failed to identify recurrent chromosomal aberrations that could have been used for subsequent positional cloning endeavors [48]. CGH performed with DNA extracted from normal cervical epithelium, different stages of dysplasia, and invasive carcinomas unveiled a distinct chromosomal abnormality at the transition from preinvasive severe dysplasia to invasive disease (Fig. 3). The gain of chromosome 3q (more precisely chromosomal bands 3q26–28) was observed only sporadically in severe dysplasia; however, this chromosomal aberration was identified in nine of ten invasive carcinomas. In many cases the gains were high-level copy number increases, lending further weight to the importance of genes on this chromosome for progression of cervical tumors. It is conceivable that the gain of chromosome 3q defines the genetic aberration that is required for the transformation of cervical epithelium in human papilloma virus infected dysplasias. If so, this genetic marker could become useful as a predictor for the potential of cervical dysplastic lesions to progress.

It is also clear that the knowledge of stage-specific chromosomal aberrations in cervical carcinogenesis can possibly be used to complement cytology based staging of PAP smears. This is even more likely because, by means of interphase cytogenetics using a specific probe for the amplified region of chromosome 3q, the copy number status of this chromosome can be studied directly on routine cytological preparations, i.e., together with and complementing phenotype analysis. An example of a hybridization of DNA-probes directly to routine cytological preparations is shown in Fig. 4. This translation of CGH results to routine cytological preparations provides clear evidence of the usefulness of an initial screening test for chromosomal aberrations in solid tumor progres-

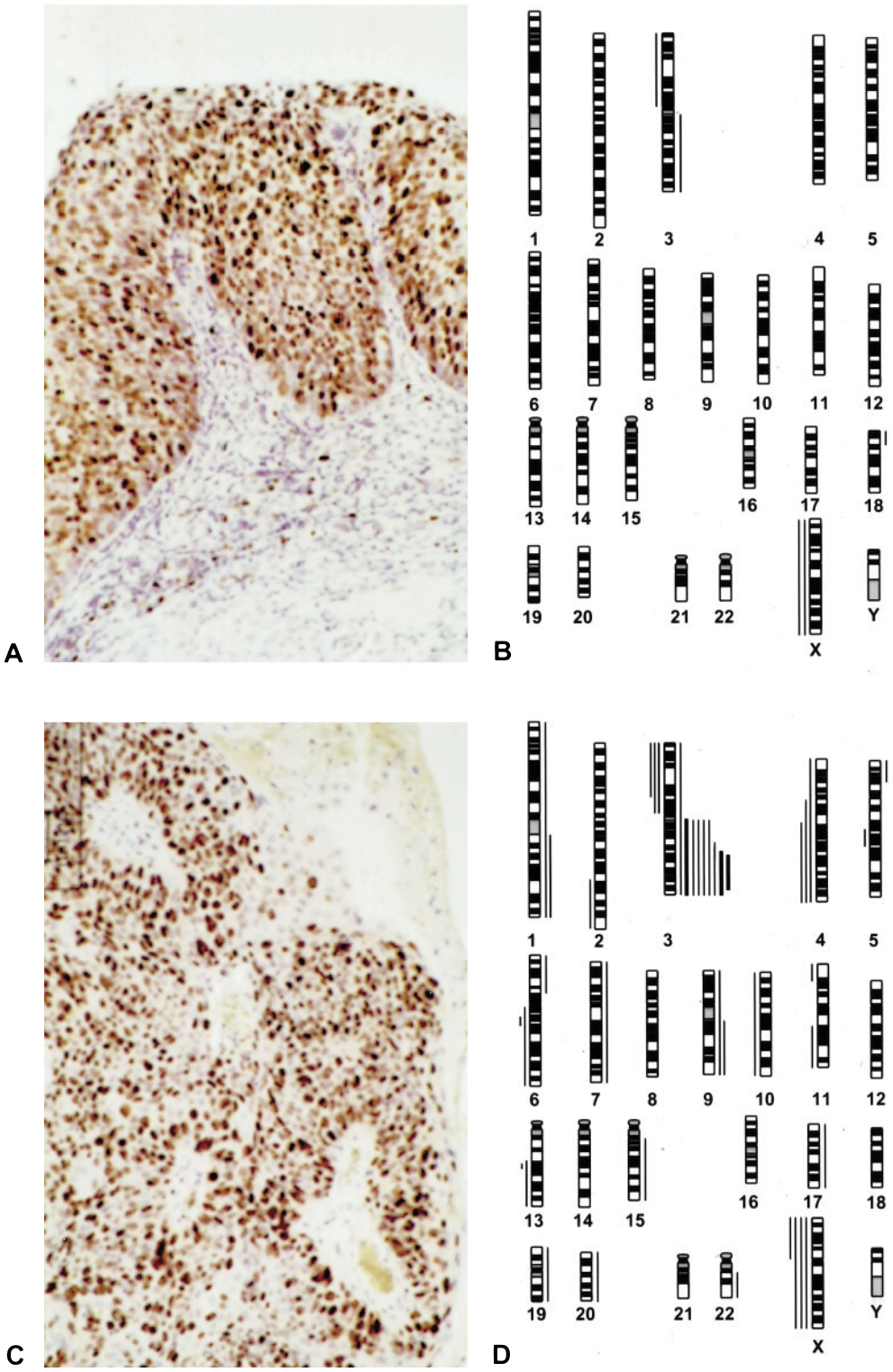


Fig. 3A–D Application of CGH to establish correlations between the microscopic phenotype and the genotype during cervical tumorigenesis. **A** A tissue section diagnosed as cervical severe dysplasia is shown after staining with an antibody directed against the Ki-67 antigen. This tissue was used for microdissection, DNA extraction, and CGH analysis. Twenty dysplastic lesions were investigated by CGH. **B** The karyogram of gains and losses is the preferred way to summarize the analysis of a high number of cases from a certain tumor. Chromosome ideograms are presented schematically. *Lines on the left of the ideograms*, a loss of chromosomal material in a particular tumor; *lines on the right*, a gain in a specific case; *solid squares*, *bars*, high-level copy number increases (amplifications). In the series of cervical dysplastic lesions summarized here chromosome arm 3q was gained in one case, accompanied by a reduced copy number for chromosome arm 3p. **C** Representative tissue section from an invasive cervical carcinoma (stage I). CGH analyses were performed from microdissected material in ten cases. **D** The karyogram of gains and losses indicates a nonrandom pattern of chromosomal aberrations. The gain of chromosome arm 3q occurs in virtually all invasive carcinomas. The results indicate that the acquisition of additional copies of 3q coincides with the transition from preinvasive dysplastic lesions to invasive carcinoma. This chromosomal marker might become useful for diagnostics and prognostication

sion because its application to diagnostically relevant questions can be achieved readily. The interphase analysis can be performed using either fluorescence or colorimetric detection formats. Automated or computer-assisted interphase spot counting devices are available [49] and might become useful for high throughput analysis of PAP smears after in situ hybridization.

Improvements in CGH analysis can be envisioned by matrix-based DNA or RNA hybridization techniques that could potentially replace the need for chromosome preparations. An increasing number of reports have shown that quantitative analysis of hybridizations to nucleic acids immobilized on, for example, DNA chips is in principle possible [50–52]. It is not yet clear to what extent or

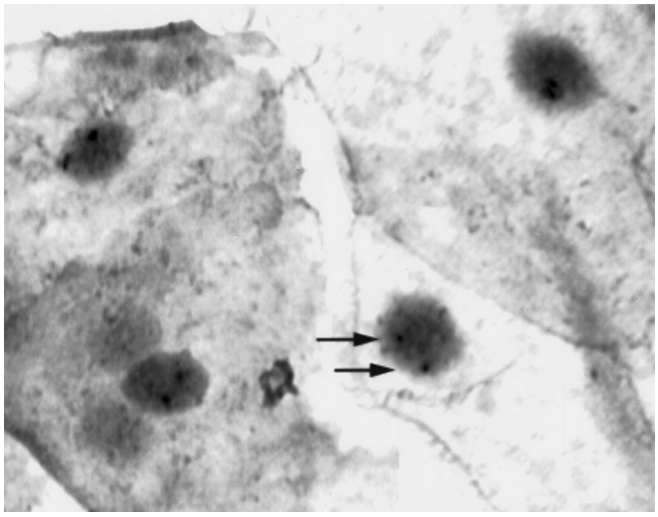


Fig. 4 Interphase cytogenetics on a routine cytological preparation from normal cervical epithelium (PAP smear). Based on the identification of chromosome 3q as a genetic marker for progression in cervical carcinomas, a genomic clone specific for chromosome band 3q27 was isolated. The in situ hybridization to cytological preparations can be visualized in either a fluorescent detection format or, as shown here, after colorimetric visualization. Two spots appear in the majority of the cells, indicating normal copy numbers for chromosome band 3q27 (*arrows*). The automated enumeration of hybridization signals should allow for a high throughput analysis of cytological preparations after in situ hybridization. Therefore cytology based staging can be complemented and improved with a pertinent genetic marker

when related technology will allow the generation of a comprehensive screening test of genetic aberrations in tumor genomes on a routine basis. It is conceivable that in the not so distant future a set of tumor or tumor stage-specific oncogenes and tumor suppressor genes plotted on a matrix will become the standard for the evaluation of the stage-specific expression status. However, and this should not be underestimated, FISH is the only practicable technique so far that allows identification of genetic markers directly in whole tumors cells, thereby maintaining the wealth of information that phenotypic changes provide.

The application of CGH to identify chromosomal aberrations in human leukemias, lymphomas, and solid tumors has added significantly to our understanding of nonrandom, tumor and tumor stage specific genetic changes and will therefore guide positional cloning efforts. Using interphase cytogenetics in certain tumors with DNA probes specific for chromosomal regions, CGH data can be translated directly to improve diagnostics and diagnostic staging and may therefore help to devise more carefully adapted therapeutic regimens.

Spectral karyotyping

Despite all the advantages and the particular elegance of comparative genomic hybridization one should not overlook its limitations. CGH allows only those chromosomal aberrations to be identified that result in DNA copy number changes. For instance, a chromosomal aberration such as the Philadelphia chromosome – arguably an important event in the transformation of hematological cells of myeloid origin [53] – would remain undetected by CGH. Also, the chromosomal mechanisms by which individual cells generate copy number changes, for example, duplications, isochromosome formations, dmin's, hsr's, and others, remain elusive. Lastly, at the present stage of technology development, CGH generates an average of the most common aberrations in tumor genomes, disregarding important features such as clonal heterogeneity, which provides tumors with the genetic diversity to react more flexibly to environmental changes. FISH, using the plethora of available probe sets is an important technique for analyzing chromosomal aberrations on a single cell level. However, a targeted analysis of, for example, the deletion status of a tumor suppressor gene, leaves the rest of the genome unanalyzed.

Therefore the cytogeneticist would like to add to the methodological spectrum an approach that allows the visualization of all human chromosomes in different colors. The goal of increasing the number of chromosomal targets that can be discerned simultaneously, i.e., the multiplicity of FISH experiments has long been perceived [54–59]. The scarcity of suitable probe labeling and fluorescence detection formats, however, makes this a nontrivial task. This is due mainly to the nature of the fluorochromes itself. In many instances the emission spectra of fluorochromes overlap [60]. Therefore it is difficult to discern an ever-increasing number of fluorochromes using conventional, fluorochrome-specific optical filters, and color karyotyping was not possible until recently when Speicher and colleagues [61] reported the FISH-based discernment of all human chromosome using sequential exposures with six different optical filters. We have developed a novel approach for the visualization of FISH experiments. In strong contrast to conventional epifluorescence filter technology, we have explored the possibility of using spectral imaging to distinguish multiple and overlapping fluorochromes simultaneously, and hence achieved the goal of color karyotyping human (and other species) chromosomes [62–65].

Methodology

Spectral imaging utilizes a combination of epifluorescence microscopy, CCD imaging, and Fourier spectroscopy to measure, at all sample points of an image, the entire fluorescence spectrum [66, 67]. The technique therefore combines spectroscopy, i.e., the measurement of the full spectrum, with high-resolution imaging, i.e., the acquisition of this information for an entire image. The spectral image is generated by passing the emission light beam through a collection optics and than through a Sagnac interferometer where an optical path difference is produced. The light beam reaches the detector, and the interferogram is measured for every pixel of the CCD

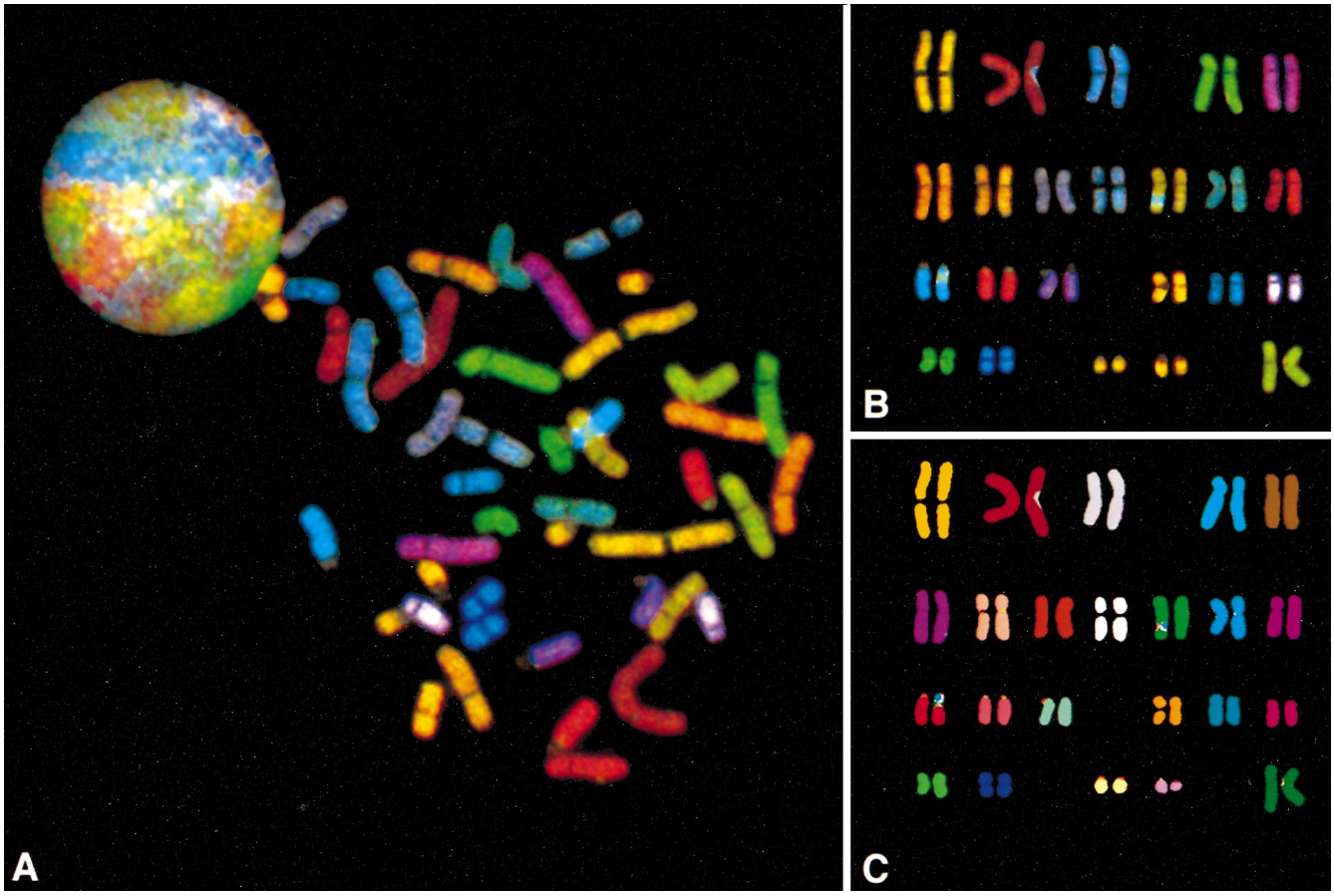


Fig. 5A–C SKY for human chromosome analysis. **A** Simultaneous visualization of all human chromosomes in different colors after hybridization with 24 combinatorially labeled chromosome painting probes (normal human metaphase preparation, 46,XX). Note that the spherical interphase nucleus (*upper left corner*) is also stained entirely with the combined chromosome painting probes. **B** Karyotype arrangement of the metaphase in **A**. **C** A spectra-based classification algorithm allows unambiguous identification of all pixels in the image that have the same or similar spectra. All pixels with the same spectrum are assigned the same pseudocolor. This feature provides the basis for color karyotyping human chromosomes. Overlapping chromosomal regions show a difference in classification colors due to the merging of the contributing fluorescence spectra

camera. By applying a Fourier transformation the spectrum of the emission light can be computed from the interferogram. As a result the full spectral information is available for each pixel of the image. Subsequently a specific color is assigned to all pixels in the image that have identical spectra, resulting in the spectral classification of all chromosomes.

The application of spectral imaging to the field of cytogenetic research and diagnostics is termed SKY. Below we describe some of these applications to chromosome analysis both in human malignancies and as in animal models of certain tumors, and we suggest how cytogenetic diagnostics might be performed in the near future.

Application of spectral karyotyping

Spectral karyotyping of human chromosomes is based on the simultaneous hybridization of a 24-chromosome-specific probe pool. Chromosome specific probe pools, or chromosome painting probes, can be generated from flow-sorted human chromosomes [68] or by chromosome microdissection [69]. In order to produce a chromosome-specific spectrum after hybridization each chromosome library was labeled either with a single fluorochrome or with specific combinations of two or three fluorochromes, allowing us to increase the number of discernible targets beyond the number of fluorochromes that are suitable for DNA labeling. Using combinatorial labeling with five different fluorochromes, 31 different targets can be distinguished. The hybridization was visualized using spectral imaging through a single optical filter that allowed the excitation of all fluorochromes and the measurement of their emission spectra without the need to change from one fluorochrome-specific optical filter to another. Figure 5 shows SKY of normal human chromosomes.

The applications of SKY for visualizing chromosomal aberrations involved in human diseases are manifold. Chromosome banding based karyotype analysis is performed routinely in the prenatal and postnatal cytogenetic laboratory. The benefits of SKY in this field include (a) the identification of subtle chromosomal aberrations such as the translocation of telomeric chromatin that is difficult

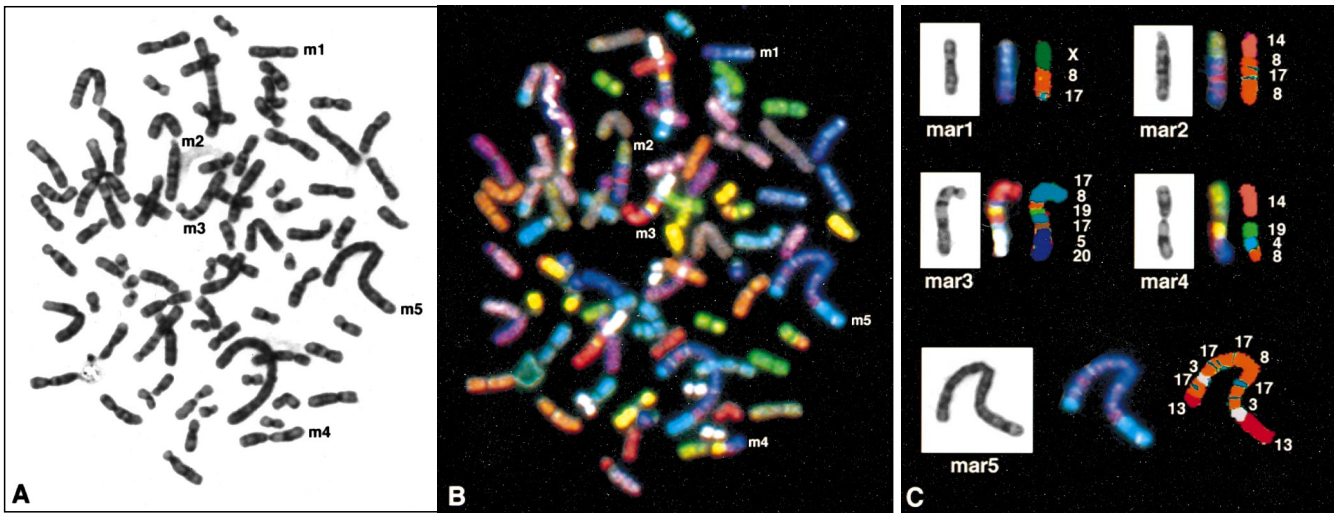


Fig. 6A–C Molecular cytogenetic analysis of the breast cancer cell line SKBR3 by conventional G-banding analysis and SKY. **A** G-banding of metaphase chromosomes prepared from the breast cancer cell line. Note that the aneuploid breast cancer genome contains multiple abnormal chromosomes, few of which can be unambiguously analyzed using chromosomal banding analysis alone. Several marker chromosomes (e.g., mar1–mar5) are present. One of the marker chromosomes (mar5) contains stretches of material with almost no banding pattern, termed homogeneously staining regions (hsr's). Hsr's are the cytogenetic correlate of (onco)-gene amplification. **B** SKY of the same previously G-banded metaphase spread of this tumor allows visualization of all chromosomes in different colors. Numerous chromosomes are involved in rearrangement events. The origin of chromosomal material in the marker chromosomes can be identified. SKY complements CGH analyses by identifying also structural chromosomal aberrations with no effect on the copy number as well as elucidating the structure and mechanisms of chromosomal aberrations. **C** Classification of selected marker chromosomes from the breast cancer cell line shown in **A**, **B**. Note that chromosomal breakpoints can be mapped with high accuracy when the banding and SKY results are combined. Marker 1 contains material derived from the X chromosome, and chromosomes 8 and 17. The breakpoint on the X chromosome occurs at chromosomal band Xq21. A second marker chromosome is derived from chromosomes 8 and 14, with interspersed chromosome 17 sequences. The giant marker 5 was described as containing an hsr. SKY identified the coamplification of chromosome 8 and 17 sequences, which is consistent with the CGH results. The hsr is framed by material originating from chromosomes 3 and 13

to detect using banding alone and (b) the identification of small markers that remain elusive after banding. In a recently conducted study of cases with unidentified constitutional chromosome abnormalities SKY was able to refine karyotype interpretation in the majority of the cases [70]. SKY, in combination with chromosome banding analysis might also enable the automation of karyotype analysis in the clinical cytogenetic laboratory, where the majority of the karyotypes are actually normal. However, the need to complement karyotype analysis with SKY is even more obvious in tumor cytogenetics. This is due to certain characteristic features of metaphase chromosomes from malignant cells. In many instances the mitotic index is low. As a consequence the few cells that are available would preferably be analyzed as comprehensively as possible. Also, tumor metaphase preparations, in particular those established from solid tumors and lymphomas, are often of poor quality, which precludes high-resolution banding analysis.

The matter becomes even more complicated because tumor karyotypes are often highly rearranged. This shuffling of chromosomal segments makes it extremely difficult to identify the origin of translocated chromatin because the sequence of chromosomal bands is obscured. This problem could be overcome by adding color information that unambiguously identifies the origin of rearranged chromosomal material. Indeed, it has been shown that the combination of banding and SKY allows one to identify marker chromosomes and also chromosomal breakpoints with higher accuracy than in the past [62, 65]. Lastly, SKY has been used to characterize chromosomal structures such as dmin's, hsr's, and other cytogenetic reflections of oncogene amplification whose origin could not be deduced by banding methods [62, 65]. An example of the application of SKY to the chromosome analysis in solid tumors is presented in Fig. 6. Here SKY was applied to visualize chromosomal aberrations, including giant marker chromosomes, in the breast cancer cell line SKBR3. Virtually all chromosomes are involved in translocation events. The spectra-based classification ascertains the origin of all marker chromosomes unambiguously.

SKY (or any other FISH-based multicolor karyotyping technique) will not replace chromosome banding analyses. Chromosome banding provides, depending on the band resolution, 400–800 landmarks along the chromosomes. It is not likely that the wealth of this information can be routinely obtained by any other approach. SKY must therefore be understood as an approach that complements banding-based analysis by specifically filling in where banding is particularly difficult, such as in the identification of subtle translocations and insertions and the reconstruction of complex chromosomal aberrations and tumor-specific cytogenetic features such as hsr's.

It is likely that the percentage of unidentified chromosomal material in solid tumor cytogenetics will be re-

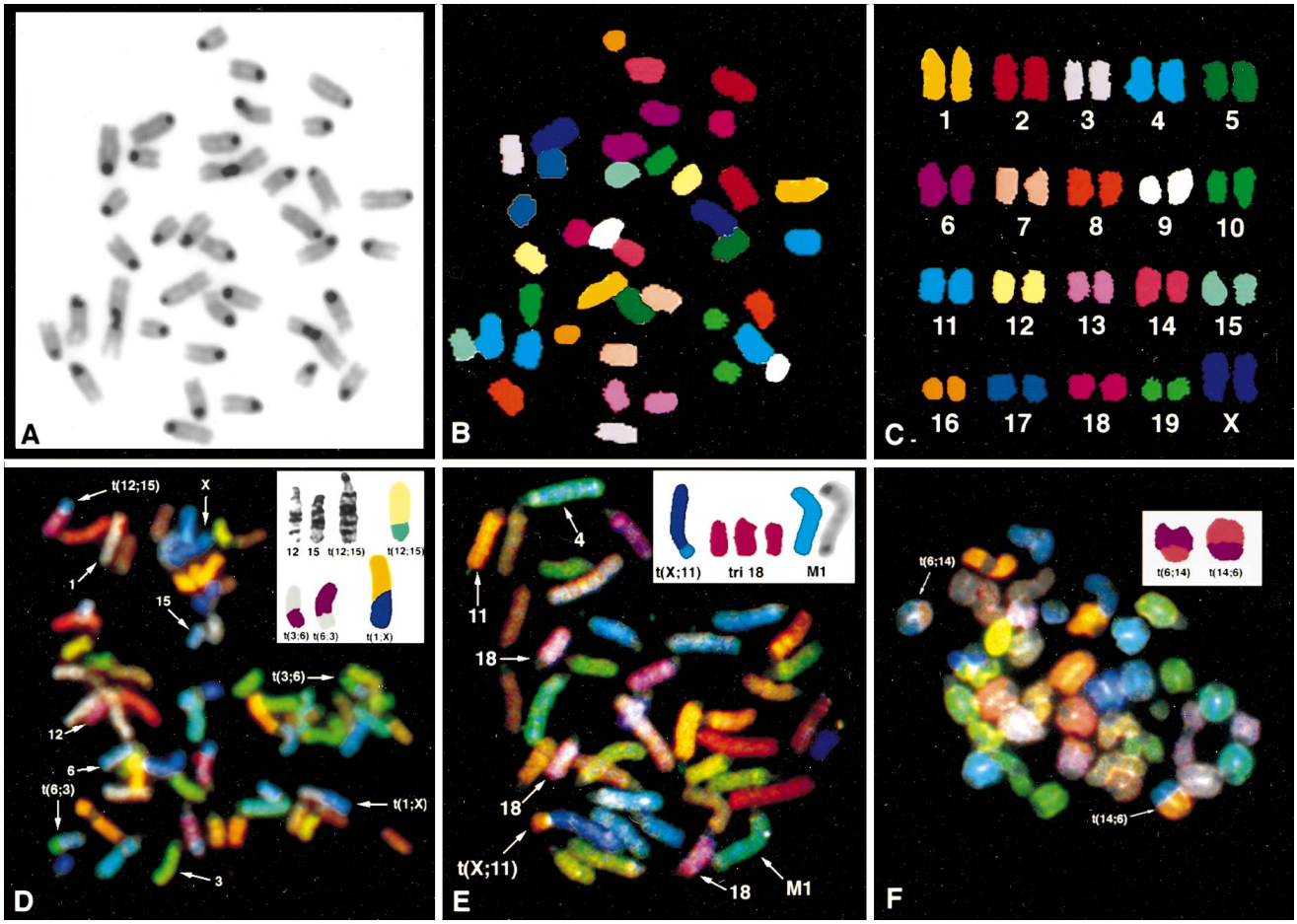


Fig. 7A–F Spectral karyotyping of mouse chromosomes. Examples of SKY for visualizing all mouse chromosomes in different colors after hybridization with 20 combinatorially labeled chromosome painting probes. **A** Normal mouse metaphase preparation (strain FVB). DAPI staining. Note that all mouse chromosomes are of similar size and shape. **B** Same metaphase cell after hybridization of a probe cocktail containing differentially labeled mouse chromosome painting probes. **C** Spectra-based classification allows color karyotyping of mouse chromosomes. **D** Application of SKY to identify chromosomal aberrations in murine models of carcinogenesis. The experiment shows the hybridization of mouse metaphase chromosomes prepared from chemically induced plasmocytomas. The mouse translocation T(12;15) is the hallmark of pristane-induced plasmocytomas in BALB/c mice. Note that additional aberrations can be identified readily. **E** Analysis of a mammary gland adenocarcinoma prepared from tumors that arise in transgenic mice that carry the c-*myc* oncogene under the control of the MMTV-promotor. Trisomy 18 and other aberrations were detected. **F** Mice deficient for tumor suppressor genes or genes involved in cell cycle control are ideal tools to study the role of these genes in the maintenance of chromosomal integrity. The example presents the SKY analysis of a thymoma that developed in knock-out mutants for the ataxia-telangiectasia (Atm) gene. The involvement of mouse chromosomes 12 and 14 suggests the involvement of T-cell receptor gene mutations in tumorigenesis in ATM-deficient mice. (Reproduced [63] with the kind permission of Nature Genetics)

duced dramatically. A refined chromosome analysis will identify additional recurrent chromosomal aberrations which provides critical information of diagnostic and prognostic importance. Furthermore SKY will define entry points for positional cloning endeavors for the ultimate goal of identifying a gene (or genes) involved in a particular tumor. Refined karyotype analysis will also enhance our understanding of the mechanism of chromosomal aberrations and shed light on the cellular mechanism of oncogene activation and tumor suppressor gene inactivation in particular and chromosomal consequences of malignant transformation in general.

While undoubtedly useful, the analysis of chromosomal changes in human tumor material remains a descriptive technique. To understand the specific effects of tumor suppressor gene inactivation, oncogene activation, and the chromosomal damage after carcinogen exposure, the researcher can take advantage of animal models of human carcinogenesis. Murine models, in particular mouse models, are extremely valuable tools for reproducing in vivo certain aspects of human carcinogenesis. Mouse models have been established for a multitude of tumors [71]. As for human tumors, karyotype analysis serves as a first screening test for chromosomal aberrations that indicate alterations of genetic pathways during carcinogenesis. Mouse chromosome karyotyping, however, is an art mastered only by few. Mouse chromo-

somes are similar in size a parameter that is used for human chromosome identification, and similar in shape, i.e., all acrocentric. Consequently the identification of aberrant mouse chromosomes in model systems can be extremely arduous, and data on recurrent chromosomal aberrations in mouse tumors are rare. We have therefore developed SKY for the differential color display of all mouse chromosomes [63].

Figure 7 demonstrates the potential of SKY for delineating chromosomal aberrations in murine models of carcinogenesis. The benefits are numerous: (a) Tumors in mouse models can be studied at an earlier stage of carcinogenesis than in human, with the potential of detecting tumor-initiating aberrations rather than secondary chromosomal changes that are often present in advanced stage human tumors. Primary cytogenetic changes can then be compared to aberrations that occur in human tumorigenesis and might become helpful in identifying stage-specific chromosomal aberrations. Furthermore, the consequences of the sequential activation of oncogenes on the induction of genetic instability can be established because chromosomal aberrations can be analyzed in a defined time course (Fig. 7d–f). (b) The chromosomal effects of tumor suppressor gene inactivation can be elegantly studied in mouse knock-out mutants and is extremely helpful in identifying recurring chromosomal aberrations and assessing the biological and genetic similarity of mouse models to human tumors with similar mutations [72]. For instance, the remarkably increased genetic instability in mice that are deficient in functional p53 [73] can be analyzed with enhanced precision. (c) The mutagenic potential of chemicals and drugs can be tested by exposing mice, thus providing a "mammalian Ames test." The same obviously holds true for radiation exposure, where SKY could be established as a biological dosimeter to monitor short and long-term effects of ionizing radiation.

Conclusion

Cytogenetic research has witnessed a remarkable shift based on the fusion of conventional cytogenetic techniques with molecular approaches such as DNA cloning and in situ hybridization. In particular the emergence of comparative genomic hybridization and multicolor karyotyping now qualifies molecular cytogenetics to do what chromosome banding has done so far, i.e., analyze the entire chromosome complement in a single experiment as an initial screening test for genetic rearrangements. The advances in probe generation, hybridization technology, fluorescence microscopy, and digital and spectral imaging conclude the existence of karyotype analysis as a black and white technique and open the gate for a blossoming, second spring. "The colorizing of cytogenetics: is it ready for prime time?" was the title of a review article published a few years ago by our mentor, David Ledbetter [74]. While the title and in particular the question mark were quite germane at that time, we hope to have provided ample evidence that the prime time of "colored" cytogenetics has come indeed.

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