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***Invited review***

# Comprehensive genetic analysis of cancer cells

**Nicholas C. Popescu***

*Molecular Cytogenetics Section, Laboratory of Experimental Carcinogenesis, National Cancer Institute, Bethesda, Md, USA*

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## Abstract

Human cancer is viewed as a disorder of genes originating from the progeny of a single cell that has accumulated multiple genetic alterations. The genetic alterations include point mutation, chromosomal rearrangements and imbalances. Amplifications primarily involve oncogenes whose overexpression leads to growth deregulation, while deletions commonly target tumor suppressor genes that control cell cycle checkpoints and DNA repair mechanisms. With the advent of molecular cytogenetics procedures for global detection of genomic imbalances and for multicolor visualization of structural chromosome changes, as well as the completion of human genome mapping and the development of microarray technology for serial gene expression analysis of the entire genomes, a significant progress has been made in uncovering the molecular basis of cancer. The major challenge in cancer biology is to decipher the molecular anatomy of various cancers and to identify cancer-related genes that now comprise only a fraction of human genes. The complete genetic anatomy of specific cancers would allow a better understanding of the role of genetic alterations in carcinogenesis, provide diagnostic and prognostic markers and discriminate between cells at different stages of progression toward malignancy. This review highlights current technologies that are available to explore cancer cells and outlines their application to investigations in human hepatocellular carcinoma.

**Keywords**: cancer genetics - oncogenes - tumor suppressor genes - genomic alterations - molecular cytogenetics spectral karyotyping - comparative genomic hybridization - expression microarray

## Introduction

A new era in biological research and medicine is unfolding, after three decades of tremendous progress in molecular biology and genetics. This is an era of unprecedented opportunities and major challenges to study basic biology, to diagnose disease, as well as to develop new drugs and tailor therapeutics to specific disorders. The successful cultivation of human embryonic stem cells, the completion

*Correspondence to: Dr. Nicholas C. POPESCU
National Cancer Institute--37/3C05, 37 Convent Drive MSC 4255, Bethesda, Md 20892-4255, USA
Tel.: (301) 496-5688, Fax: (301) 496-0734

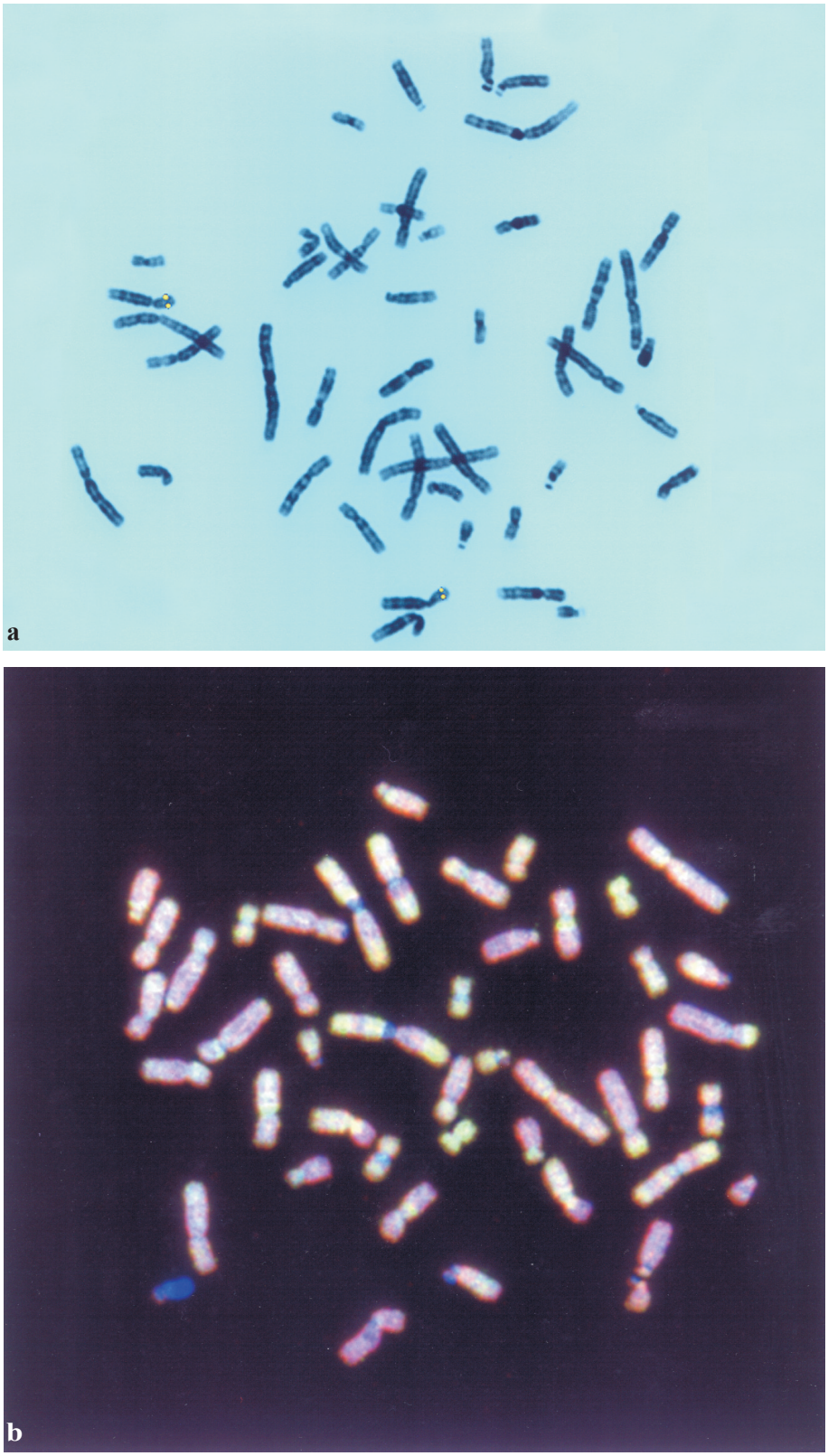


**Fig. 1** **(a)** Chromosomal localization of the DLC-1 gene by FISH. Digital image of a normal human metaphase exhibiting G-like banding of DAPI counterstained chromosomes. Both chromosomes 8 have fluorescent signal on the short arm at band 8p21. 3-22. (Reprinted with permission from *Cancer Research*, **58**: 2196-2199, 1998). **(b)** Digital three color image of a comparative genomic hybridization metaphase.Normal chromosomes were hybridized with genomic DNA from normal and hepatocellular carcinoma (HCC) tumor cells.

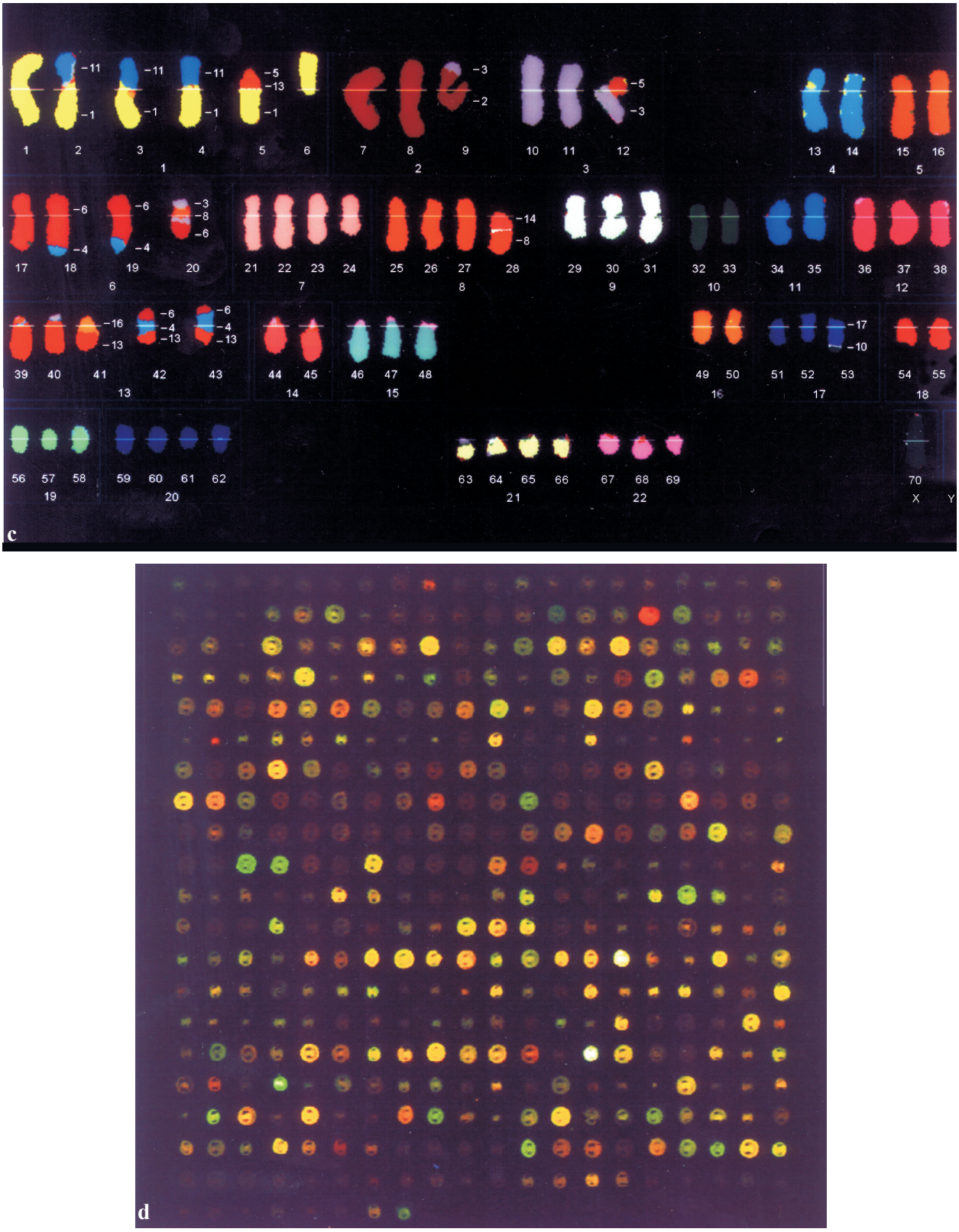


**Fig. 1** (cont'd) **(c)** A spectral karyotype of an aneuploid cell derived from a HCC cell line. Pairs of normal chromosomes have distinctive colors and several rearranged chromosomes can be readily distinguished by colors. **(d)** Gene expression cDNA microarray from a HCC cell line.

of the human genome mapping and the development of microarray technology are among the most important scientific advancements, that will undoubtedly help to conquer many disorders, including cancer. Already, a group of scientists, from the National Institutes of Health, USA, recently reported sustained regression of metastatic renal-cell carcinoma, after nonmyeloablative allogenetic peripheral blood stem-cell transplantation, in patients who were refractory to conventional immunotherapy. The patients who had complete response remained in remission for 16 to 27 months [1]. The human genome information encoded in 38. 000 to 120. 000 genes will be unraveled and several potential applications arise. These range from a broad understanding of human evolution to predicting or preventing disease and helping the design of tailored therapy. New fields such as functional genomics and proteomics are emerging, to study genes and protein structure and interactions, or pharmacogenomics, a field with broad clinical use, for selecting treatments on the basis of patients genetic makeup.

## Modern molecular cytogenetics

Since the discovery of chromosome banding and the subsequent molecular color revolution of fluorescence *in situ* hybridization (FISH), cytogenetics has become indispensable in experimental and clinical research, especially in cancer [2, 3]. FISH-based methodologies greatly enhanced the resolution of detection of genetic abnormalities in cancer cells. This review highlights the application of modern technologies to explore cancer cells and attempts to show how the identification of recurrent cytogenetic alterations can lead to the isolation of novel genes involved in pathogenesis of cancer.

Human cancer is viewed as a genetic disorder originating from a single precursor cell by cumulative acquisition of multiple genetic alterations caused by diverse types of damage [4, 5]. The genetic alterations in cancer cells include point mutation, chromosomal rearrangements and genomic amplifications and deletions. Amplifications primarily involve oncogenes whose overexpression lead to growth deregulation, while deletions commonly affect tumor suppressor genes that control cell cycle checkpoints and DNA repair mechanisms [6-11]. The major challenge in cancer biology is to decipher the molecular anatomy of various cancers and to identify cancer-related genes that now comprise only a fraction of human genes. The complete genetic anatomy of specific cancers would allow a better understanding of the role of genetic alterations in carcinogenesis, provide diagnostic and prognostic markers and discriminate between cells at different stages of progression toward malignancy [12].

## Conventional fluorescence *in situ* hybridization

Polymerase chain reaction (PCR) and FISH had the highest impact in molecular cytogenetics as powerful tools for detecting genetic alterations in cancer cells. FISH derives its power from the unique ability to couple cytological and molecular information directly. A variety of FISH-based procedures and DNA probes has been developed that permits the analysis of the whole cell genome, individual chromosomes or chromosome bands, and specific loci. Using conventional FISH, many new genes have been precisely localized in normal and cancer cells. An image of gene localization by FISH is presented in Fig. 1a. For FISH mapping, chromosomes derived from normal peripheral lymphocytes cultures are hybridized with a cDNA or a genomic probe of the gene of interest. After hybridization, gene signal consisting of symmetrical fluorescent spots at the same sites on homologous chromosomes, is precisely localized on enhanced G-like bands induced by 4',6-diamidino-2-phenylindole (DAPI) counterstaining. To increase the sensitivity of conventional FISH, alternative procedures such as oligonucleotide-primed *in situ* DNA synthesis and direct incorporation of labeled nucleotides by PCR have been developed. Their use greatly improved the detection of small DNA probes, thus bringing the sensitivity of the *in situ* hybridization to the intragenic level [13].

Currently, by combining molecular cytogenetics procedures, a variety of genomic alterations can be detected and virtually any chromosomal rearrangement regardless of its complexity can be resolved. To link and integrate the physical and genetic maps of the human genome with

**Table 1** Summary of fragile histidine trial gene alterations in hepatocellular carcinoma tumors and tumor derived cell lines.

| Alterations | Frequency | Tumor samples |
|---|---|---|
| Chromosome 3p rearrangements | 10/13 | Cell lines |
| Unbalanced translocations near or within the gene | 5/10 | Cell lines |
| Decreased or lack of mRNA expression | 9/14 | Cell lines |
| Allelic deletion at the fifth intron of the gene | 10/34 | Primary tumors |
| Absence of protein expression | 5/10 | Primary tumors |
| | 10/14 | Cell lines |

cytogenetic maps and maps of chromosomal rearrangements in human diseases, the National Cancer Institute, USA, has launched an initiative: the Cancer Chromosome Aberration Project (CCAP). A high-resolution FISH mapping of colony-purified BAC clones spaced at 1-2 Mb intervals across the entire genome is near completion. This project will assist investigators around the world by allowing a convenient distribution of BAC probes. The CCAP Web site illustrates the goals and progress of this initiative. (http: //www.ncbi.nlm.nih.gov/CCAP/).

## Spectral karyotyping

Multifluor or spectral karyotyping (SKY) and comparative genomic hybridization (CGH), two FISH-based procedures, raised the genetic analysis of normal and abnormal cells to a new level. SKY allows the identification of structural and numerical alterations on a global basis; the origin of complex rearrangements can be accurately determined and the breakpoints of translocations precisely localized [14, 15]. CGH on the other hand permits

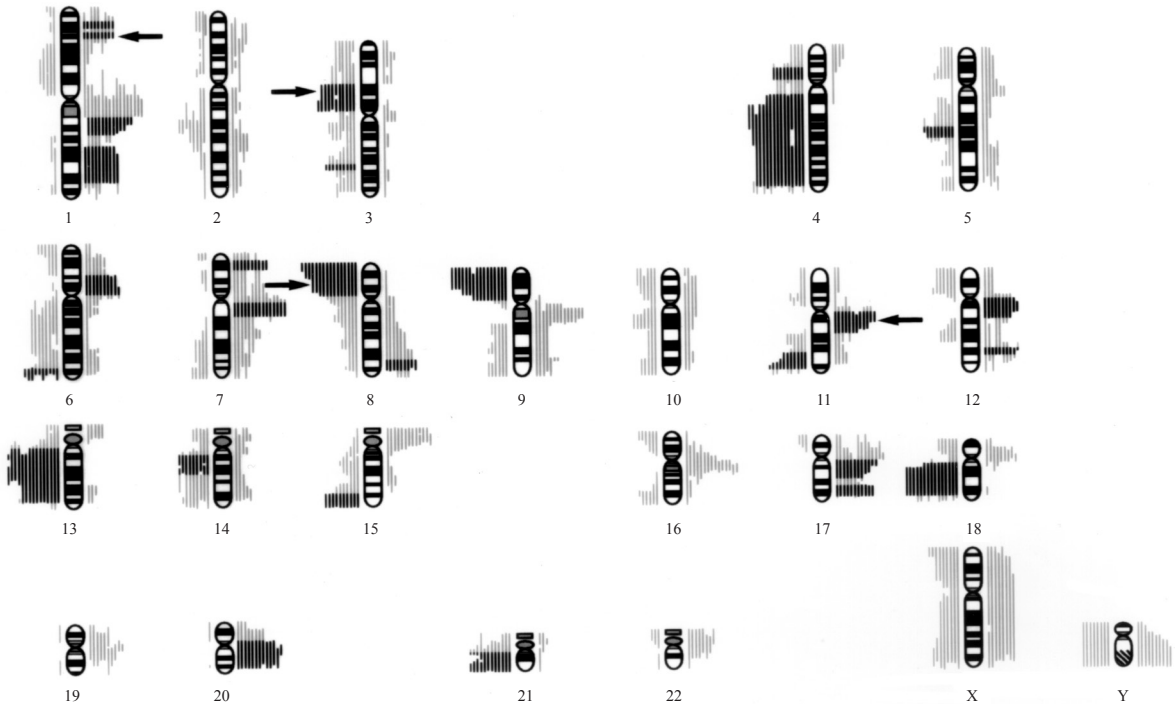


**Fig. 2 Summary of genetic imbalances identified in eighteen hepatocellular carcinoma cel lines.** Vertical lines to the left and to the right of chromosomes ideogram represent loss and gain of DNA copy-number, respectively. Bold lines represent regions of minimal overlap detected in 50% or more of the cell lines. (Reprinted with permission from *Hepatology*, **29**: 1208-1214, 1999).

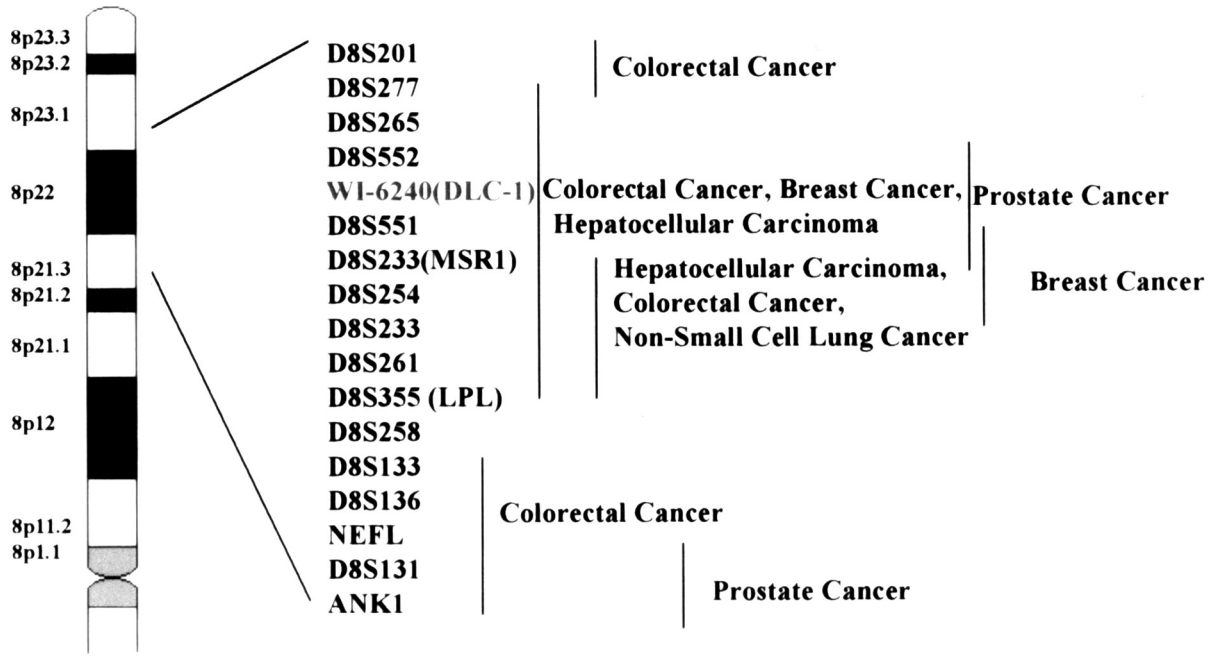


**Fig. 3** Ideogram of the short arm of chromosome 8 and representation of common region of deletions in liver, breast, colon, lung and prostate cancers, overlapping with the location of DLC-1 gene.

the mapping of the DNA copy number on the entire tumor genome [15]. Regions of recurrent translocations or amplification identified by SKY or CGH can be microdissected, amplified, microclone libraries can be constructed, and probes localized on extended chromosomes or chromatin fibers, for construction of high resolution physical maps that are critical for positional cloning and gene identification [13].

Chromosomal abnormalities are the hallmark of cancer cells. Recurring and highly consistent structural and numerical alterations have been identified in a large number of leukemias, lymphomas, and solid tumors [8]. The identification of recurrent genetic alterations and the isolation of molecular markers have clinical applications in diagnosis and prognosis of neoplasia and in detection of minimal residual disease that are essential for designing the most effective therapeutic approach. Chromosome banding has been used to recognize chromosomal abnormalities in cancer cells and other disorders. However, chromosome banding resolution has limits, as many abnormalities cannot be resolved. A particular chromosome band is identified by its position on a normal chromosome. Cryptic or hidden changes involving small chromosome segments that appear abnormal do not always have a characteristic band to be accurately identified. Balanced or unbalanced translocations and numerical deviations can readily be identified by painting with whole chromosome probes. However, until recently, only limited combinations of spectrally non overlapping fluorochromes could be visualized simultaneously. Now, SKY allows the simultaneous visualization of all normal chromosomes in a different color. A cocktail of 24 chromosome probes, each labeled with different fluorochromes or combinations of fluorochromes, is hybridized on metaphases prepared from the test sample. Images are generated and recorded, using a combination of epifluorescence microscopy images taken by a sensitive CCD camera and Fourier spectroscopy [14, 16]. A SKY karyotype shown in Fig. 1c, illustrates identification of each chromosome pair by color and the rearrangements among several chromosomes. Over 300 cases of SKY analyses have been reported and their number is growing rapidly [16].

## Comparative genomic hybridization

CGH, that allows positional identification of gains and losses of DNA sequences in the entire

tumor genome, is based on competitive FISH of differentially labeled total genomic DNA, from appropriate control tissue and tumor DNA, to normal chromosome spreads. The ratio of the fluorescence intensities, generated by two different fluorochromes incorporated into test and control DNA, is used to differentiate chromosome regions with a normal genomic content, from those showing over/or underrepresentation of sequences [15]. The spatial resolution of CGH mapping is approximately 10 Mbp for low-copy-number gains and losses and close to 2Mbp for high-copy-number amplifications [17]. This analytic approach has already been applied over a wide range of tumor specimens, cell lines and archival materials to define chromosomal imbalances. CGH studies of solid tumors such as prostate carcinoma, breast tumors, small-cell lung cancer, squamous head and neck, renal, hepatocellular carcinomas and gliomas, have been repeatedly reported. [18, 19]. A metaphase from a normal human cell after CGH is presented in Fig. 1b. Green and red areas, respectively, reflect regions of gain and loss of DNA sequences in tumor DNA, compared to normal DNA. Special computer software is required to quantify the ratio of signal generated by the two fluorochromes and to map regions of DNA-copy number overrepresentation and underrepresentation on G-band ideograms.

## Comparative genomic hybridization, tissue and cDNA microarray

Shortly after CGH procedure was firmly established, new methodologies based on the same principle have been developed [20 - 23]. CGH in a DNA-chip format, tissue microarrays and cDNA microarrays have been devised for screening the genome of cancer cells and other disorders. On CGH microarray an array of genes instead of metaphase chromosomes are hybridized with probes of interest, thus overcoming the limitation of the conventional CGH and greatly increasing the level of resolution [21]. The ability of CGH microarray to measure copy number with high accuracy in human genome and to analyze clinical samples was demonstrated with chromosome 20 aberrations in breast cancer [21]. The use of array CGH is not limited to cancer cells as it has general clinical applications for disease gene identification and genetic aberrations [21]. While conventional CGH and CGH microarrays can comprehensively screen one specimen at a time, using tissue microarrays, hundreds of specimens from patients in different stages of the disease can be surveyed for cancer gene candidates [22]. Tissue biopsies from as many as 1000 cylindrical samples are placed on microarray membrane and hybridized with one or more

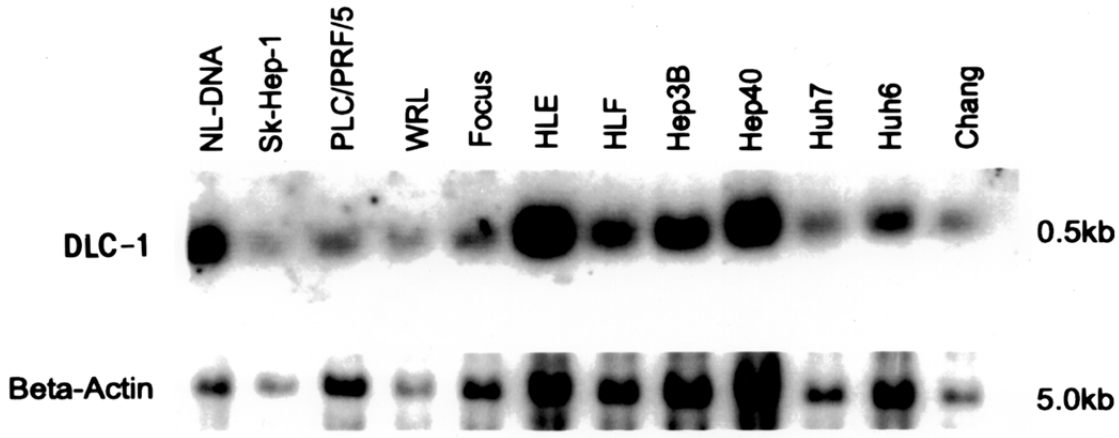


**Fig. 4 Genomic deletions of the DLC-1 gene in HCC cell lines**. Southern blot analysis of human HCC cell lines. Nine cell showed reduction of DNA intensity of different degrees compared with human normal liver genomic DNA. (Reprinted with permission from *Cancer Research*, **58**: 2196-2199, 1998).

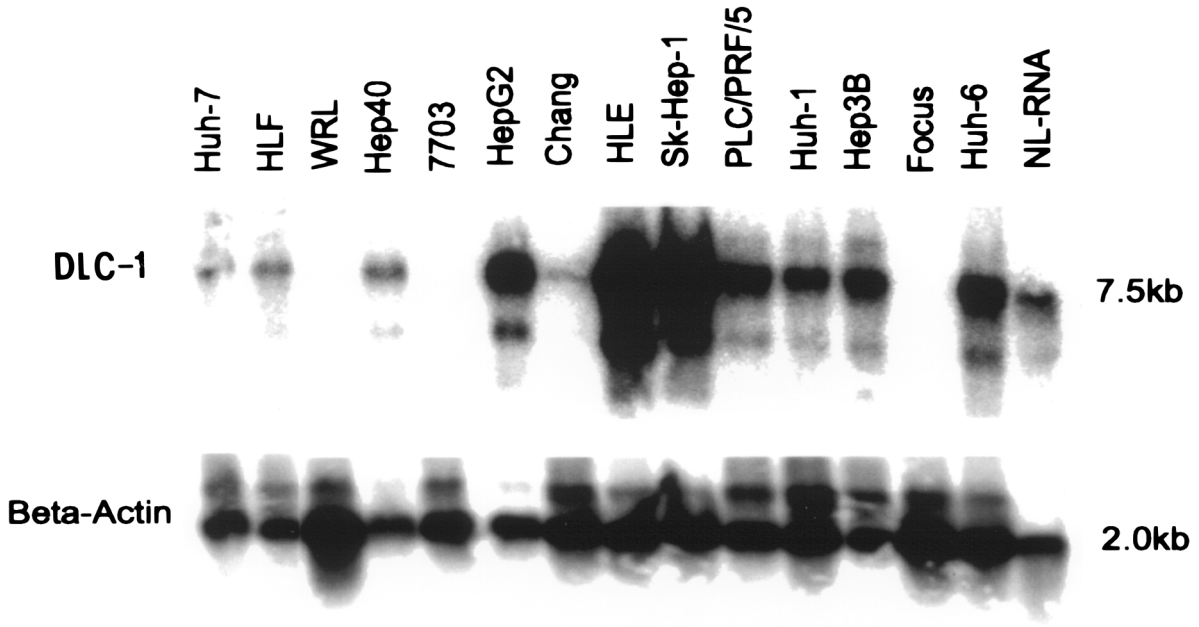


**Fig. 5 mRNA expression of the DLC-1 gene in hepatocellular carcinoma cell lines**. This gene is expressed as a 7. 5-kb major transcript and a 4. 5-kb minor transcript. mRNA of the DLC-1was not detected in four cell lines. NL is the control sample from normal liver cells. (Reprinted with permission from *Cancer Research*, **58**: 2196-2199, 1998).

genes of diagnostic, prognostic and therapeutic importance. In the original description of this procedure, six gene amplification, p53 and estrogen receptor expression were visualized in many breast cancer biopsies, thereby demonstrating the utility and the power of this procedure [22].

Serial analysis of gene expression and cDNA microarrays undoubtedly will have a more profound impact than other major technologies in biomedical research and medical practice. The generation of complete gene transcription profile of normal and cancerous cells or cells infected with oncogenic viruses will define a new era in cancer pathology. Classifications of cancers will provide a new clinical tool for an accurate diagnosis and prognosis of the disease and should lead to the identification of new targets for therapeutic drugs. Review articles and a practical guide to DNA microarray analysis have recently been published [24 - 26]. As in other FISH procedures, cDNA copied from mRNA of tumor and normal cells labeled with a red and green fluorescent dye respectively, are mixed in equal amounts, hybridized to an array of thousand of genes, and scanned using a computer-controlled inverted fluorescence confocal microscope, equipped with a triple set laser illumination system, that can asses red, green and yellow emissions. Red and green indicates higher gene expression in tumor cells and normal cells, respectively, while yellow reflects the same level of expression in both cells (Fig. 1d). Special computer software integrate and generate quantitative assessment for all target genes. Recent data obtained with cDNA micro array technology exceeded the expectations of the scientific community. Clinical diagnosis of acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) is often difficult. Based on gene expression profile for more than 6800 genes, 50 genes exhibited a different level of expression in AML and ALL, thereby clearly separating the diseases [27]. The gene expression pattern generated by a " lymphochip" of 18.000 genes showed distinctive types of diffuse large B-cell lymphoma with different disease outcome [28]. A subset of melanomas was identified by mathematical analysis of gene expression, raising the possibility that melanoma may not be a disease lacking discernible entities, as it is generally believed [29]. Based on the expression of the estrogen receptor, breast cancers are divided into two groups. Global gene expression of breast

cancers lacking the receptor, that are usually more aggressive, revealed that these tumors consist of two groups, one of which elicits high expression of the ErbB-2 oncogene [30]. Amplification or overexpression of ErbB-2 in breast cancer serves as a prognostic criteria for the recurrence of the disease, a phenomenon that led to the development of targeted therapy [31 - 33]. Possibly, this group of breast tumors are most responsive to treatment with herceptine alone or in combination with chemotherapy.

The existing arsenal of molecular cytogenetic and microarray procedures permits a complete dissection of genetic alterations in cancer cells. For example, the notoriously complex cytogenetic constitution of the cervical carcinoma HeLa cell line was definitively established by a combined SKY, CGH and FISH characterization. Cryptic rearrangements, undetectable by other techniques, showed that papillomavirus-18 integration triggered genomic instability, a critical step in tumor development [34]. A complete genetic analysis of cancer cells should include molecular cytogenetic profile, SKY, CGH and FISH, for interpreting microarray data. This trend became obvious in recent reports [35].

## Molecular genetic characterization of liver cancer

In the following, I will describe applications of molecular cytogenetics in combination with molecular biology, in our investigations aimed at identifying recurrent chromosomal alterations and isolating new genes, that are relevant to neoplastic development and may serve as markers for early detection of neoplasia, as well as targets for therapy. A successful utilization of advanced molecular cytogenetic procedures, for multicolor SKY, CGH and high resolution FISH, resulted in identification of recurrent genetic alterations and lead us to isolate new genes in solid tumors and hematological malignancies. Only a part of our work with human hepatocellular carcinoma (HCC) will be briefly discussed.

HCC is one of the most common cancers worldwide and accounts for 90% of all liver carcinomas. It is also one of the most common visceral malignant tumors, and is known to have a poor prognosis [36, 37]. HCC is closely related to oncogenic viruses and chemical carcinogens that can cause genetic damage. The cumulative literature cytogenetic data based on analysis of 8 adenocarcinomas of the liver comprises only two recurrent breakpoints on the short arm of chromosome 1 [8].

## Recurrent chromosome translocation and gene alterations

We examined a number of HCC, and non-random chromosomal alterations and recurrent breakpoints in unbalanced rearrangements were identified, by G-banding and chromosome painting and, more recently, by SKY analysis (Fig. 1c). Chromosome rearrangements involved several chromosomes. The most common changes, thus far identified, were deletions and unbalanced translocations of chromosomes 1 and 3, with the breakpoints clustered at regions 1p36 and 3p14-21, close to the loci of p73, and fragile histidine triad gene (FHIT) tumor suppressor genes, respectively [38]. This was the first report, describing unbalanced translocations of chromosomes 1 and 3, with the breakpoints nonrandomly involving these loci in HCC. The molecular consequences of unbalanced changes are unknown. As in balanced alterations, activation of protooncogenes or formation of oncogenic chimeric genes whose products are often transcriptional factors may occur in the breakpoints of unbalanced rearrangements [9]. Unlike in balanced abnormalities, loss of tumor suppressor genes may also result from unbalanced alterations. Cytogenetic abnormalities of chromosome 1 are common in HCC and several lines of evidence implicate alterations of specific sites on the short arm in pathogenesis of this neoplasia [39]. Region 1p36, a region of fragility and recombination, is suspected to harbor multiple tumor suppressor genes. We detected loss of p73, a tumor suppressor gene, closely related to p53, due to unbalanced translocations with the breakpoints close to the p73 locus, at region 1p36. Since several oncogenes and, at least, two cell senescence genes are located on chromosome 1, it has been postulated that structural alterations and consequential imbalance of chromosome 1 may be critical for gene dosage in certain types of cancer [40], that may include liver cancer.

The second recurrent site of translocation involves the region where FHIT gene is located. FHIT is a candidate tumor suppressor gene, encompasses the fragile site FRA3B, a region with the highest fragility in human genome, and is altered in a large number of human cancers, particularly those of epithelial cell origin and associated with known carcinogenic agents [41-44]. HCC is closely related to carcinogenic agents such as: hepatitis B and C virus infections, dietary aflatoxin, alcohol consumption, and exposure to chemical carcinogens. The FHIT gene is expressed in normal hepatic cells and is not expressed or is abnormally expressed in cultured tumor cells derived from HCC [45]. The summary of FHIT gene alterations in HCC cell lines and primary tumors is shown in Table 1. Our detection of chromosome 3p rearrangements, a decrease or absence of FHIT mRNA expression, intragenic deletions and absence of protein expression suggest that FHIT alterations are pathologically relevant to HCC [45]. Damage at FHIT locus appears to be a very early event in lungs of smokers. In lung adenocarcinoma from smoking subjects, loss of heterozygosity (LOH) at FHIT locus was considerably higher, due to the interaction of tobacco carcinogenes and FRA3B [42, 46]. Fragile sites are preferential targets for chemical carcinogens and oncogenic viruses [47]. Chemical carcinogens, such as aflatoxin B1, alcohol, hepatitis B virus (HBV), integration at fragile site (FRA3B) may initiate a background of genetic instability, early in the process of hepatocarcinogenesis, having critical consequences in pathogenesis of HCC. The large number of malignancies with FHIT gene alterations, comparable to or higher than p53 gene mutations, underline the importance of this gene to the development of human cancer. In liver cancer, as well as in other cancers, FITH gene changes should be used as a marker for early detection.

## Genomic imbalances

By CGH, we demonstrated a distinct pattern of multiple recurrent amplifications and deletions, that includes alterations frequently seen in other neoplasias, as well as changes potentially specific for HCC [48]. A normal metaphase spread displaying a pattern of red and green areas as a result of CGH with normal DNA and tumor DNA is presented in Fig. 1b. The survey of all changes identified in 18 cell lines is graphically represented in Fig. 2. Loss and gain of genetic material are represented, respectively, by lines on the left and right of the chromosome ideograms. Although chromosome imbalances involved multiple regions, a nonrandom pattern of copy-number alterations localized at specific sites emerged from the analysis. Twenty-eight recurrent chromosome imbalances were identified in nine or more of the tumor cell lines. The prevailing gains and losses are highlighted in Fig. 2 as minimal overlapping regions, while arrows indicate regions where our investigations focused to isolate novel genes or to find alterations of known genes. Seventeen genomic imbalances, that are novel in HCC, were detected, thus extending significantly the map of genetic changes and providing a starting point for the isolation of new genes relevant in pathogenesis of liver neoplasia. The majority of the overrepresented sites correspond with the location of protooncogenes and a half of the underrepresented regions overlap with sites of tumor suppressor genes [48]. The analysis of individual and combined CGH profiles, based on HBV status and the geographic origin of the primary tumors, showed a similar pattern of chromosome imbalances. Currently, in our laboratory, gene expression analysis of HCC cell lines, using cDNA microarrays of 6500 genes, is in progress. The comparison of SKY karyotypes, CGH and gene expression profiles will provide ample insights in the genetics of HCC, and should lead to the isolation of novel genes or novel HCC classification.

## Genomic imbalances and gene isolation

From a recurrent region of deletions on the short arm of chromosome 8 (Fig. 2), using representational difference analysis, a polymerase chain reaction-based subtractive hybridization, a new gene frequently deleted in liver cancer and hence named DLC-1 was isolated [49]. cDNA and protein sequences of the gene show a significant homology with rat RhoGAP gene, which encodes a GTPase activating protein, that can switch active GTP-bound Rho proteins to the inactive GDP-bound ones. Rho proteins synergistically function as

downregulators in Ras signaling, similar to NF1 gene which encodes GTP-ase activating protein for Ras protein [50-52]. DLC-1 gene was localized on chromosome 8p21.3-22 in a region of recurrent deletions in several cancers [49], (Fig. 1a and Fig. 3). Recurrent loss of DNA copy-number sequences have been detected by CGH in several solid tumors [18, 19] and recently loss of 8p was found to be associated with tumor metastasis in HCC [53]. Furthermore, LOH at chromosome 8p22-21 is a recurrent alteration in HCC, non-small cell lung carcinoma (NSCLC), colorectal cancer (CRC), and prostate cancer. A fragment from 8p22-21.3, from a commonly deleted region in HCC, CRC and NSCLC, was isolated [54]. Recurrent deletions of the short arm of chromosome 8 support the relevance of this alterations in cancer development. DLC-1 is the first candidate tumor suppressor gene involved in 8p deletions. Recently, another candidate tumor suppressor gene FEZ1 has been localized at this site [55]. We detected deletion and aberrant expression of DLC-1 gene in HCC primary tumors and tumor cell lines (Figs. 4 and 5), as well as in breast, lung and colorectal carcinoma, suggesting that DLC-1 may be a multiple tumor suppressor gene. The number of bonified tumor suppressor genes is relatively small [7]. Recently, we obtained evidence that DLC-1 elicits tumor suppressor activity. A consistent association between gain on the long arm of chromosome 8, closely related to MYC gene amplification and loss on the short arm, was identified in HCC (Fig. 2) as well as in NSCLC and node-negative breast carcinomas [18, 19]. This raises the possibility that DLC-1 may interfere with MYC oncogenic activity in liver cancer and other solid tumors [56]. The isolation of DLC-1 gene relates to methods of screening and diagnosis of a genetic predisposition to liver cancer and other cancer types and may lead to an improved gene therapy utilizing recombinant DNA technologies.

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