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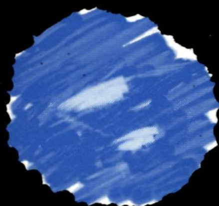

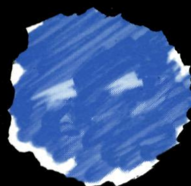

P.J. Poddighe

INTERPHASE CYTOGENETICS

IN

TUMOR PATHOLOGY

P.J. Poddighe

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IN

TUMOR PATHOLOGY

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de Medische Wetenschappen

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volgens besluit van het College van Decanen
in het openbaar te verdedigen op
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General Introduction

Genetic Predisposition of Cancer

It is generally accepted that cancer is a result of uncontrolled proliferation of cells with multiple genetic aberrations One pathway of tumorigenesis occurs at the somatic cell level. This means that somatic cells, which are genetically "normal" at constitution, transform into an abnormal or deregulated state and generate cancerous growth (Cairns, 1981). In 1914 Boveri postulated the hypothesis that specific chromosomal aberrations are associated with the development of cancer (Boveri, 1914). The first molecular proof for this observation was the detection of a t(9,22) chromosome, the so-called Philadelphia chromosome, in peripheral lymphocytes of patients with chronic myeloid leukemia (CML) (Nowell & Hungerford, 1960)

A second pathway of tumorigenesis may find its beginning at the germ cell level This implies that a genetic instability is brought about in all somatic cells at birth. This genetically predisposed form of tumorigenesis was first postulated by Knudson (1971) as the two-hit hypothesis, and assumes that a second genetic event at the homologue chromosome is necessary to transform the phenotypically normal somatic cell into a tumor cell

Recently, the process of tumorigenesis has been recognized as being a multistep process, in which several genetic changes are involved, rather than being the consequence of a single change in the DNA sequence of a somatic cell (Harris, 1991). Furthermore, since cells in many primary tumors show the same abnormal karyotype, the majority of human cancers are thought to develop from single transformed cells with a clonal unicellular origin (Fialkow, 1979) The classical cascade of events in carcinogenesis involves initiation, promotion, and tumor progression In tumor initiation, a carcinogen-induced genetic change gives a selective growth advantage to susceptible cells These initiated cells are less responsive to signals maintaining normal tissue architecture and regulating the homeostatic growth and maturation of cells During tumor promotion, unrestrained proliferation of cells increases the possibility of additional genetic changes These random somatic changes will then lead to cellular heterogeneity within malignant tissues (Heppner, 1984, Nicolson, 1987) During tumor progression, a further increase of genomic instability can cause other chromosomal aberrations, gene amplification, and altered gene regulation and expression (Sandberg, 1990)

Genes involved in Cancer

The molecular approach of cancer has led to the study and identification of several genes involved in tumor development and progression (Croce, 1991, Solomon et al, 1991) The two major groups of genes in tumorigenesis are (a) the proto-oncogenes, and (b) the

tumor suppressor genes

Proto-oncogenes were first identified as the cellular homologues of activated viral oncogenes that constitute a stable part of the genome of acutely transforming retrovirusses The general importance of cellular proto-oncogenes in malignancy is evident by their activation by means of retroviral transfection, point mutations, amplification or chromosomal translocations in both viral induced and naturally occurring malignancies They act dominantly in producing the malignant phenotype as a result of abnormal activation and/or aberrant expression of their gene product (Bishop, 1991) Many proto-oncogenes encode proteins that are involved in signaling pathways and are therefore thought to be critical to the control of cell proliferation and differentiation For example, proto-oncogenes that encode growth factors (e g c-sis), growth factor receptors (e g c-fms, c-kit), transducers of growth factor responses (e g c-src, c-ras, c-abl), and transcription factors (e g N-myc, c-jun) have all been described (Cantley et al, 1991)

The second and perhaps more heterogeneous group of genes in tumorigenesis has been shown to have onco suppressor activity Loss of gene function is required to initiate or progress the disease However, both copies of such genes have to be inactivated and so the causal mutations are recessive at the cellular level The study of retinoblastoma has provided insight into the genetic mechanisms of these suppressor genes Knudson's model (1971) has been made more specific by the finding that the two target loci are the two copies of the chromosome 13q14-associated retinoblastoma (Rb) gene, and that the critical mutations serve to inactivate Rb function Mutation or loss of the p53 gene is the commonest alteration at the gene level yet found in many types of human cancers (Hollstein et al, 1991) The p53 protein is found in the nucleus and has many attributes of a transcription factor, including the ability to bind to a specific DNA sequence and the presence of a transcription-activating domain (Kern et al, 1991) Recently, the biological function of p53 has been described as a cell cycle control protein at the level of G1 to S phase transition, acting as a checkpoint control for DNA damage In tumor cells, that have lost the wild-type function of p53, show gene amplification, which can be considered as a form of genetic instability (Livingstone et al, 1992) These cells are more susceptible to the killing effect of DNA damaging agents because they replicate through the damage (Lane, 1992, Vogelstein and Kinzler, 1992)

Methods to Study Chromosomal Aberrations

Changes in the cellular DNA content and the proliferative fraction of malignancies are regarded as prognostic parameters For the study of genetic aberrations during tumor development and progression, several techniques can be applied Therefore, these techniques have found their way in tumor pathology, to improve the clinical diagnosis of malignancy, and to increase our understanding of the multistep process involved in tumorigenesis (Fearon & Vogelstein, 1990)

1. Cytometry

Cytometry may be defined as the quantitative measurement of individual cells and cellular constituents In recent years, cytometry is applied to an increasing extend in clinical pathology Flow cytometry (FCM) and image cytometry are the two major technical approaches in this respect Both techniques help to characterize biological properties of tumors Since these determinations can be performed in interphase nuclei, there is no need to culture cells and therefore every tissue type or cell population is accessible for analysis.

In image cytometry, cells or tissues are fixed onto glass slides and are analyzed for parameters, such as shape, size, texture, and DNA content, using an imaging system. Although the number of cells that can be analyzed is much less than in FCM, a few hundred cells per tumor, the procedure may generate much more information per cell

In FCM the total DNA content of a large population of cells can be quantitated The estimation of tumor cell ploidy has been described to be of prognostic significance (Dressler & Bartow, 1989) However, the technique is limited in the detection of a minimal DNA difference An increase in DNA content of about 4% can be seen as a split peak in heterogeneous cell populations (Vindelov et al, 1983) FCM includes the possibility of flow sorting, in which the malignant and the non-malignant cells can be separated by means of combination with immunohistochemical procedures For example, Beck et al (1990) have developed a FCM procedure to distinguish epithelial, primary tumor cells from non-epithelial cells in mixed tumor cell populations on the basis of their intermediate filament expression patterns

In general, in adult solid tumors, aneuploidy has been found to correlate with increasing tumor grade and stage and with worse prognosis, whereas a high S-phase fraction also in non-aneuploid TCCs predicts a more aggressive tumor behavior. FCM analyses of transitional cell carcinomas (TCCs) of the urinary bladder have shown that tumor stage is strongly correlated with the DNA ploidy. Low grade/low stage TCCs often appear to have a (near-) diploid DNA index, while the more advanced stages of TCC show more often an aneuploid DNA content (Tribukait, 1984, Smeets et al, 1987)

2 Karyotyping

The development of chromosome banding techniques some 25 years ago has provided an efficient tool for chromosome analyses (Caspersson et al 1968) Until recently, conventional karyotyping was practically the only way to obtain information on specific chromosomal aberrations in tumors So far, about 70% of the data concerning chromosomal abnormalities of cancer have come from studies on hematological malignancies, despite the fact that they comprise only 10% of all cancers (Mitelman et al, 1991) These karyotyping studies are used in determining diagnostic and prognostic parameters, since well established knowledge of chromosome aberrations in several hematological malignancies exists

In the case of solid tumors, chromosome banding techniques are often hampered by technical difficulties, such as (1) inferior quality of metaphase chromosome spreads from

solid tumor cell samples, (2) low number of mitotic cells in primary tumors, (3) selective outgrowth of certain fast growing subpopulations, and (4) loss of genetic material, both due to culturing of the cells (Teyssier, 1989)

Also the stage at which changes become apparent differs between hematological malignancies and solid tumors The clinical and biological appearance of leukemia becomes apparent at an early stage of the disease, at which the primary karyotypic changes are observed Hematologic malignancies are often diagnosed at an early stage and are then characterized by single or few chromosomal abnormalities, most often reciprocal translocations that represent the primary chromosomal changes On the contrary, many of the solid tumors are diagnosed and studied at later stages, when they display complex and heterogeneous patterns of chromosomal aberrations, involving both structural and numerical changes, as well as polyploidization The primary chromosomal changes are masked by secondary changes related to tumor progression, ageing, metastasis, resistance to therapy etc (Nowell, 1986, Sandberg, 1990, Mitelman et al, 1991)

Cytogenetic analysis of TCCs of the urinary bladder has revealed several specific chromosomal changes, i e iso(5q), +7, -9, or -9q- and possibly 11p- (Ramaekers & Hopman, 1993) Whether there is a correlation between the cytogenetic changes and biological behavior of the tumor needs to be determined in more detail

3 Allelotyping

Another powerful tool to study genetic imbalances in genomic tumor DNA can be performed by restriction fragment length polymorphism (RFLP) analysis For this purpose, labelled polymorphic DNA probes are used as markers to identify individuals heterozygous for specific chromosomal loci Alleles present in tumor DNA from heterozygous individuals can be compared with those present in the corresponding constitutional DNA isolated from normal blood cells to detect allele loss or loss of heterozygosity (LOH) Such LOH in the vicinity of putative onco-suppressor gene loci has been used as support for Knudson's two hit hypothesis of carcinogenesis (Knudson, 1989), involving loss of gene function by homozygous inactivation of tumor suppressor genes Using RFLP analysis, allelic losses in the vicinity of onco-suppressor gene loci have now been observed in a high proportion of several human solid tumor types, such as breast cancer (17p, 17q, Sato et al, 1991), colon cancer (5q, 17p, 18q, Vogelstein et al, 1988), and bladder cancer (9q, 11p, 17p, Tsai et al, 1990) This approach requires however relatively large numbers of polymorphic DNA markers, which must then also be informative for the patient under investigation (Cavenee et al, 1983, Bishop, 1987) Although amplifications of specific DNA sequences can be detected, it is difficult to distinguish between two and three copies of a specific DNA segment Furthermore, since this analysis is done on Southern blots from tumor DNA, no information is obtained at the single-cell level Also contamination with DNA from non-neoplastic cells may hamper the interpretation Therefore, the analysis of genomic DNA from tumor samples for chromosomal gain and loss by this method remains laborious and is

unlikely to be applied in a routine setting

4 In situ Hybridization

In situ hybridization (ISH) may be defined as the method for a direct detection of specific nucleic acid sequences in cells and tissues in which simultaneously morphological analysis can be performed ISH was originally described in 1969 using ^{32}P-labeled probes (Pardue & Gall, 1969, John et al. 1969) Improvements in recombinant DNA technology and isotopic labelling procedures allowed the demonstration of single copy genes on metaphase chromosomes (Gerhard et al, 1981) Subsequent development of non-radioactive detection methods increased the safety and simplicity of the procedure while also equivalent sensitivity could be achieved without using radioactive labels (Hopman et al, 1988; Raap et al, 1990) Since chromosomes are generally organized into distinct domains in interphase nuclei, ISH can be applied to detect chromosome aberrations in non-mitotic cells. Therefore, this technique is often called "interphase cytogenetics" (Cremer et al, 1986). In particular for solid tumors interphase cytogenetics is of great importance, because it has the potential to link the more morphologically-orientated techniques to the more molecular techniques, such as RFLP analyses

Several non-radioactive methods have been developed for in situ DNA-DNA hybrid detection A chromosome-specific DNA probe can chemically be modified with 2-acetylaminofluorene (Landegent et al, 1984) or mercury-(II)-acetate (Hopman et al, 1986), or enzymatically be modified by a nick translation reaction using deoxyuridine triphosphate labeled with biotin (Langer et al, 1981), digoxygenin (Kessler, 1990), or fluorescein (Wiegant et al, 1992) These haptens can be subsequently detected by affinity-cytochemical techniques, using specific monoclonal or polyclonal antibodies Therefore, fluorochromes, such as FITC, TRITC, Texas Red, or AMCA, as well as enzymes, such as horse radish peroxidase or alkaline phosphatase, can be applied (for review see Raap et al, 1989) By using multiple-labeled probes the number of probes that can be detected simultaneously has been extended (Nederlof et al. 1990)

The clinical application of ISH depends on the availability of probes that bind specifically to regions of genetic or cytogenetic interest The probes now in use fall into three general classes

(1) Probes for repeated DNA sequences specific for only one chromosome type All human chromosomes have been shown to carry DNA sequences that are tandemly repeated several hundred to several thousand times in the centromeric region. Most of these sequences are of the alpha-satellite (tandemly repeats of about 171 bp monomers) or the satellite-III (5 bp monomers) families The specificity of probes for these regions comes from the fact that there is significant variation in the repeated monomers among chromosome types Chromosome specific repeat sequence probes have now been isolated and cloned for human chromosomes 1, 3-12, 15-20, X, and Y These probes are used primarily for chromosome counting, since their target sequences can be detected as dot-like signals,

reflecting the number of the individual target chromosomes.

(2) Whole chromosome probes which comprise elements with DNA sequence homology along the length of a target chromosome. These probes, typically made from chromosome-specific recombinant DNA libraries, are useful for analysis of both structural and numerical aberrations in metaphase spreads The phage DNA chromosome libraries were produced from flow-sorted chromosomes (Pinkel, 1988). Subcloning of the phage inserts into plasmids reduces the amount of vector DNA associated with each human insert. These probes are referred to as "chromosome painting" probes, since they are used to stain (paint) whole chromosomes

(3) Probes for specific loci. These are typically single copy gene probes, homologous to specific targets ranging in size from 15 to >500 kb, so that the signals are bright and reliable DNA sequences cloned into large insert phages, cosmids, or yeast artificial chromosomes have proven to be useful as specific locus probes (Tkachuk et al, 1991).

The application of these probes in tumor pathology for the detection of numerical and structural chromosome aberrations in both metaphase spreads and interphase nuclei will be discussed in Chapter 1

Scope of this Thesis

The general purpose of this study was the application of nonradioactive ISH techniques to the analysis of interphase nuclei of single cell suspensions and tissue sections from hematological malignancies and TCC of the urinary bladder Furthermore, procedures were developed to facilitate the detection of chromosomal abnormalities in these tumors

Genetic alterations in these cancers have been identified by classical karyotyping procedures However, in case the tumor cells are cultured to obtain more and better metaphase spreads as compared to the direct analysis, a potential danger exists in the loss of genetic material and the selection of certain fast-growing subpopulations In diagnostic pathology the FCM analysis of these lesions has become an accepted screening method for the determination of the DNA content and proliferative capacity of malignant cells in solid tumors These parameters can be used as prognostic and diagnostic indicators

As mentioned before in this chapter, interphase cytogenetics using chromosome-specific repetitive DNA probes may be applied as an alternative approach for these two classical techniques, especially in the case of solid tumors

The aim of the study described in this thesis was to use mainly probes for repetitive DNA sequences in combination with ISH, and to make this approach suitable for application in a more or less routine setting The findings of the ISH methods were compared with those obtained by other techniques, such as conventional karyotyping and RFLP analyses

Chapter 1 reviews the techniques of interphase cytogenetics and its clinical applications and results in tumor pathology

In the first part of the thesis the ISH technique is applied to interphase cells of hematological tumors since these tumor cells could be easily analyzed at the single cell level, and routine karyotyping could be applied to virtually all cases In Chapter 2 a panel of centromere associated DNA probes has been applied to detect numerical chromosome aberrations in interphase nuclei These findings were correlated to conventional karyotyping data Chapter 3 describes a new application of ISH on agar cultures, where a numerical chromosome aberration can be used as a marker for progenitor cells In Chapter 4 this new method has been applied to investigate in vitro cultured progenitor cells and single cells of patients with AML in order to illustrate the usefulness of the technique to study behaviour of AML during preleukemic phase active disease, remission, and under in vitro culture conditions

The second part of the thesis demonstrates the usefulness of interphase cytogenetics for the detection of chromosomal aberrations in solid cancers Therefore, the transitional cell carcinoma of the urinary bladder was chosen as a model While cytogenetic analyses of these malignancies have been used to provide a basic knowledge of genetic alterations in bladder cancer ISH procedures have been applied to further screen for numerical (Chapter 5) and structural (Chapter 6) chromosomal aberrations Chapter 6 describes the application of the ISH technique to concentrate on structural chromosome 1 aberrations Finally Chapter 7 compares the interphase cytogenetic technique on frozen tissue sections to RFLP analysis of the same tumors

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Interphase Cytogenetics of Tumors

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Journal of Pathology (1992) 166: 215-224

The prognosis of malignant or premalignant lesions is in many cases correlated with the quantitative and structural aberrations in the genomic content of the disease. Therefore, techniques such as flow cytometry (FCM), karyotyping and molecular techniques have been developed for the detection and characterization of such genetic changes, which may be central to the initiation and progression of neoplasms.

The FCM technique quantitates the total DNA content of a tumor cell population, but it gives no information with respect to specific chromosome aberrations and has limitations in the detection of minor quantitative DNA changes.

Chromosome analysis of cancer cells by karyotyping (metaphase cytogenetics), on the other hand, facilitates the identification of small deviations in chromosome content and chromosome structure. However, karyotyping is often only possible after tissue culturing, which may result in a selective growth of cells with the highest mitotic index and loss of chromosomal material. Furthermore, such analyses are often hampered by the small number of recognizable metaphases, the lack of chromosome spreading, poor banding quality, and a condensed or fuzzy appearance of the chromosomes.

The multiple molecular techniques, such as DNA sequencing, Southern and Northern blotting, RFLP analysis and PCR. make it possible to study genes, their copy number, structure and the regulation of their expression. These techniques have identified different genes involved in cancers, like proto-oncogenes and tumor suppressor genes. Although the sensitivity of these molecular techniques is high, partially as a result of the large amount of starting material, no information is obtained on the single cell level, and heterogeneity within a population of cells is often difficult to detect.

The non-radioactive in situ hybridization (ISH) procedure has been developed to overcome the limitations of FCM, karyotyping and molecular genetics. The use of chromosome specific DNA probes in combination with the ISH technique enables the detection of numerical and structural chromosome aberrations in both metaphase spreads and interphase nuclei. The term "interphase cytogenetics" refers to the cytogenetic analysis by means of ISH applied to non-mitotic cells, and was originally introduced by Cremer et al (1986).

About twenty years ago, the technique of in situ hybridization was developed using radioactive probes (Pardue & Gall, 1969; John et al. 1969). The radioactive ISH has, nowadays, reached a very high sensitivity. The detection of DNA sequences of single copy genes (Harper et al, 1981), viral genomes (Brigati et al, 1982; Burns et al, 1986), or mRNA molecules in individual cells (Coglan et al, 1985) or tissue slides (Angerer et al, 1985; Burns et al, 1987; McGee et al, 1987; Denijn et al, 1990) has been described.

The radioactive procedure has the disadvantages of long exposure times for detection, biological hazard and limited spatial resolution. Therefore, modifications of nucleic acids with haptens or other labels, which allow a non-radioactive detection (e.g. by fluoro-

chromomes, enzymes, or colloidal gold particles) have been developed (Raap et al, 1989). Moreover, the non-radioactive ISH allows the detection of two, three, or even more different DNA targets as well as mRNAs by using differently labelled probes followed by different immunocytochemical detection systems (Landegent et al, 1985; Hopman et al, 1986, Nederlof et al, 1989, 1990; Lichter et al, 1990). Also the combination of immunocytochemistry and in situ hybridization is feasible (Denijn et al, 1990).

Methodological Aspects

To employ the ISH procedure in Pathology practice and to apply it to single cell suspensions paraffin sections and frozen sections of solid tumors, the next general methodological outline is followed

1) selection of probes; 2) modification of the probe by non-isotopic, enzymatic or chemical labelling; 3) fixation of the biological material, and pretreatment of tissue material on the slides for ISH; 4) specific hybridization of the modified probe with denatured target DNA, and 5) immunocytochemical detection of the hybridized probe

DNA Probes

The eukaryotic DNA contains many repeated base-pair sequences, with highly repetitive DNA (satellite DNA) usually localized near centromeres. Some satellite DNAs have a heptanucleotide sequence repeated more than ten thousand times. Another category of repetitive DNA sequences is represented by the genes for rRNA and histones A third category comprises single-copy DNA, consisting of sequences that occur only once (or a few times) in a haploid genome (Jelinek & Schmid, 1982)

Several types of DNA probes can be used to detect numerical and structural chromosome aberrations

DNA probes recognizing highly repetitive sequences, mostly in the centromeric and telomeric region (Table 1), are now routinely applicable in pathology practice (Figures 1 and 2), also since their number is constant during the entire cell cycle.

Probes recognizing entire chromosomes or large single copy genes are also being developed In order to obtain cyto-staining of individual human chromosomes using commercially available genomic DNA libraries, a suppression of hybridization signals from ubiquitous repeated sequences, such as the Alu and KpnI elements, is necessary. This can be achieved by using total human DNA in a reannealing procedure, and is therefore also referred to as Chromosome In Situ Suppression (CISS) hybridization (Cremer et al, 1988). This principle is also used to facilitate the selective hybridization of unique sequence subsets from cosmid DNA clones in ISH experiments (Landegent et al, 1987; Fuscoe et al 1989) As a result, a more or less homogeneous hybridization signal on individual human chromosomes from pter to qter can be obtained (Figure 1E), which can also be recognized in the interphase nuclei as chromosome domains (Figure 1F and I) Large single copy genes

Table 1. Chromosome specific repetitive DNA probes

Chromosome	Name Probe	Insert (kb)	Repetitive Sequence	Reference
1	pUC1 77	1 77	sat.III	Cooke et al, 1979
1p36	p1-79	0 90	unknown	Buroker et al, 1987
3	pα3 5	2 10	alphoid	Waye et al, 1989
6	p308	3 00	alphoid	Jabs et al, 1984
7	p7t1	0 68	alphoid	Waye et al, 1987
8	D8Z2	2 55	alphoid	Donlon et al, 1986
9	pHUR98	0 16	sat III	Moyzis et al, 1987
10	D10Z1	0 95	alphoid	Devilee et al, 1988
11	pLC11A	0 85	alphoid	Waye et al, 1987
12	pα12H8	1 35	alphoid	Looijenga et al, 1990
15	D15Z1	1 80	sat III	Higgins et al, 1985
16	pHUR195	1 20	sat II	Moyzis et al. 1987
17	p17H8	2 70	alphoid	Waye et al, 1986
18	L1 84	0 68	alphoid	Devilee et al, 1986
X	pBAMX5	2 00	alphoid	Willard et al, 1983
Y	DYZ5	2 10	sat III	Cooke et al, 1982

can be detected in the interphase nucleus if the target covers about 10 30 kb of unique sequences The application of these probes in pathology is still in a developmental stage, and the number of papers is limited (Cremer et al, 1988, Pinket et al, 1988, Arnoldus et al, 1991)

Probe modifications

To detect the molecular hybrids of probe and target sequences, a reporter molecule has to be introduced into the DNA probe This can be done by the enzymatic incorporation of biotin, digoxygenin, BrdUTP, and recently FITC-dUTP, or by a chemical modification with e g an acetylaminofluorene (AAF) group, mercury ions, or a sulphone group (for review see Raap et al, 1989)

Recently, also the chemical synthesis of oligonucleotide probes containing functional groups (i e primary aliphatic amines or sulfhydryl groups) has been described, thus in principle allowing the application of nucleotide oligomers in non-radioactive ISH by coupling haptens such as biotin or reporter molecules like fluorochromes or enzymes (Raap et al, 1989) These modified oligonucleotide probes will become of great importance as their automated synthesis makes them available to laboratories not familiar with DNA recombinant technology

Detection Systems

In most cases the haptens, or the modified probe, are detected by affinity cytochemical techniques, using monoclonal or polyclonal antibodies directed to the introduced haptens.

Indirect immunocytochemical procedures use fluorochromes such as fluorescein (FITC), rhodamin (TRITC), Texas Red, or AMCA (amino-methylcoumarin acetic acid). For routine light microscopy enzymes such as peroxidase with or without amplification, alkaline phosphatase, or the avidin/biotinylated-anti-avidin system are applied, resulting in a precipitation of enzyme substrates with different colours.

The simultaneous hybridization of two or more differently labeled probes (e g. biotin, digoxygenin and AAF) can be visualized using fluorochromes, such as FITC, TRITC and AMCA In principle, the peroxidase and alkaline phosphatase can also be applied in precipitation reactions to detect two different nucleic acids by routine light microscopy (Herrington et al, 1989) The localization and resolution of the signals is however not so good as compared with the use of fluorochromes. In Figures 1 and 2 we show some examples of the use of fluorochromes or enzymatic detection systems

Processing of Biological Material for ISH in Pathology

In Pathology, fresh tumor material obtained after surgery or by fine-needle aspiration biopsies is divided for different diagnostic approaches, such as immunocytochemistry, histo- or cytological diagnosis, flow cytometry, or in situ hybridization. Most of the fixatives used in pathology, such as ethanol 70% in flow cytometry, or formaldehyde in some immuno-histological procedures, do not hamper an ISH reaction To accomplish a good penetration of the DNA probes and subsequently for the antibodies, proteolytic enzymes such as proteinase K and pepsin are applied in different ISH protocols, to reverse the effect of the fixative, thus increasing the accessibility of the target DNAs (Hopman et al, 1988a, Raap et al, 1990) by removing the cytoplasm and part of the nuclear proteins (Hopman et al, 1988b) In general, to obtain optimal hybridization signals, cells with a higher DNA content and larger nuclei need a milder pepsin pretreatment than cells with smaller nuclei. For sections of routinely processed tumor material a higher concentration of pepsin has to be used (Hopman et al, 1991) As a result of sectioning of the nuclei in paraffin blocks or frozen material, an underestimation of the real copy number can be the result. Therefore, we correlated chromosome copy numbers as detected in single cell suspensions isolated from fresh tumor material with the number as detected in paraffin sections of the same tumor Comparison of the ISH data shows that an aneuploidy can easily be detected (Figure 2) The surplus value of ISH on paraffin sections as compared to ISH on isolated tumor cells can be summarized as follows 1) chromosome heterogeneity (e g tetraploidization) can be detected within a tumor, 2) focal tumor cell areas with chromosome aberrations can be recognized in the sections and be correlated with the histologic appearance; 3) discrimination between stromal, inflammatory cells and tumor cells is possible, 4) no selection of cells occurs as a result of the cell disaggregation procedure

Pitfalls and Criteria for Evaluation

The estimation of chromosome ploidy in single cells as well as in sections may be incorrect for different reasons. Cells in which the chromosome copy number is underestimated can always be detected as a result of inefficient hybridization and colocalization of spots. An overestimation of ISH signal numbers can be made when the morphology of the cells is disrupted. Furthermore, the overlap of nuclei, the inhomogeneous fluorescent intensity of ISH signals, minor binding sides, and spots in paired arrangement (split spots), can interfere with the estimation of the real chromosome copy number of a cell.

Applications

The probes and methods described above have been applied to several types of human malignancies. In the following a summary of the most relevant data is given.

Hematological tumors

In hematological oncology chromosome banding techniques play an important role in determining diagnosis and prognosis since well established knowledge of chromosome aberrations in hematological malignancies exist. Therefore, it is to be expected that also interphase cytogenetics will be implemented rapidly for diagnosis and monitoring of leukaemia and lymphomas.

Recently, some studies were published, comparing the interphase cytogenetic procedure using non-radioactive ISH with conventional cytogenetic analyses of cell lines derived from solid tumors, in neoplastic cells from bone marrow and peripheral blood (Nederlof et al, 1989; Anastasi et al, 1990; Kibbelaar et al, 1991; Poddighe et al, 1991). These studies demonstrated that repetitive DNA probes can be applied routinely in a ISH procedure and are useful in addition to conventional cytogenetic techniques for the detection of both numerical as structural chromosome aberrations on interphase nuclei and metaphase spreads.

With the ISH method it is also feasible to detect low numbers of host cells in sex-mismatched bone marrow transplant recipients (Van Dekken et al, 1989). Figure 1-O shows results of a double-target ISH experiment using an X and Y specific probe, in which the appearance of a male cell in a female population, with a sensitivity in the order of 0.01%, can be detected. As compared to karyotyping this simple and fast method has furthermore the advantage that it does not require metaphase spreads, but can be applied on interphase nuclei.

Although centromeric DNA probes are already useful to detect numerical chromosome aberrations, it is also possible to detect structural aberrations with these centromeric probes (Nederlof et al, 1989; Poddighe et al, 1991). We demonstrated that the use of double-target ISH experiments on interphase nuclei of bone marrow cells (Figure 1K-N) can characterize

marker chromosomes and translocations between chromosomes.

To monitor patients for residual disease for which one or more cytogenetic hallmarks are known, interphase cytogenetics is also exquisitely suited. For example, chronic myelogenous leukaemia (CML) is genetically characterized by a fusion of the bcr and abl genes on chromosomes 22 and 9, respectively. This translocation can be detected by e.g. Southern blot analysis and/or other molecular genetic approaches such as in vitro amplification by polymerase chain reaction (PCR). However, these techniques give no information about individual cells. A two-color fluorescence ISH approach with probes recognizing the bcr and abl oncogenes was applied to demonstrate the bcr-abl fusion in interphase nuclei of individual blood and bone marrow cells (Tkachuk et al, 1990; Arnoldus et al, 1990).

Urological tumors

Bladder cancer. In order to detect numerical chromosome aberrations in transitional cell carcinomas (TCCs), several studies have used the hybridization of centromere specific DNA probes for chromosome 1, 7, 9, 11, 15, 17, and 18 (Hopman et al, 1988b, 1989, 1991) to single-cell suspensions of these tumors.
The results showed that in bladder tumors, which had a DNA index of approximately 1.0 as measured by FCM, aneuploidy could be frequently detected. Most strikingly, a monosomy for chromosome 9 was found in low-grade TCCs.

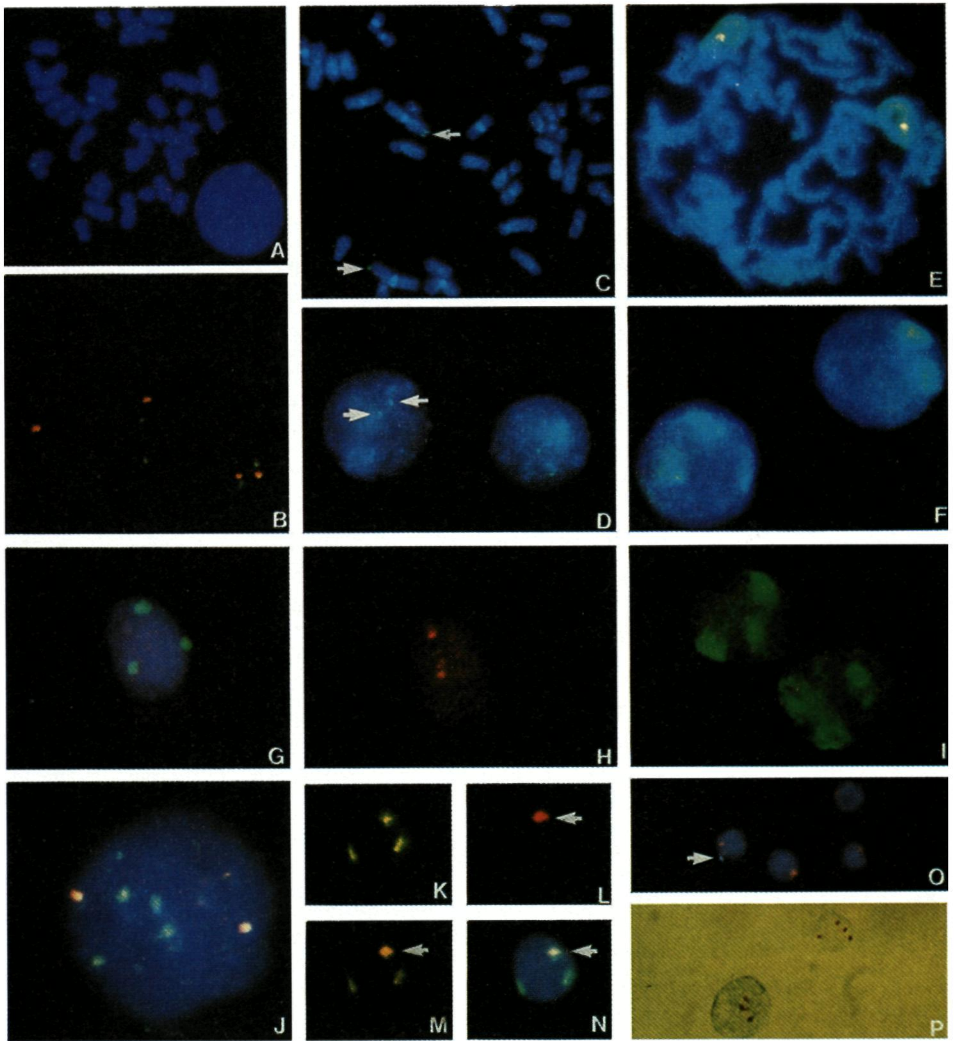
Tumors with a DNA index of 1.2 to 3.2, exhibited an even more profound heterogeneity when evaluating within one tumor or between different tumors with the same DNA-content. This phenomena is demonstrated by double-target ISH experiments in Figure 1J, showing an imbalance between chromosomes 1 and 18. Analysis of the total tumor cell populations by ISH has shown that minor cell fractions with extremely high chromosome copy numbers can be detected in these tumors. These cells may possibly originate from polyploidization. In high grade and/or invasive FCM aneuploid/tetraploid tumors chromosome 9 frequently appeared to be disomic, while the other chromosomes confirmed the tetraploidization.

One of the characteristic numerical aberrations which we detected in low-grade noninvasive TCCs included, next to the monosomy for chromosome 9, a trisomy for chromosome 1. Since the localization of many of the DNA targets will result in aberrant ISH spot numbers only when a complete loss or gain of the centromere or deletions including part of the centromere are involved, we hybridized low-grade noninvasive TCCs with a probe for the centromeric and telomeric region, as well as a library of DNA probes for chromosome 1 in single- and double-target hybridizations to detect selective loss or gain of the p- or q-arm. In this ISH study we determined frequent imbalance between the presence of centromere and telomere regions of chromosome 1. In some diploid cases three centromeric regions were found, while only two telomeric regions were detected. In those cases three domains for the library probe for chromosome 1 were seen (Figure 1G-I),

whereas in the tetraploid cases a selective loss of heterochromatin was observed (to be published).

We also detected numerical chromosome aberrations in paraffin sections of TCCs (Hopman et al, 1991). As an example, we show a TCC with a DNA index of 1.0, in which the main population contained a trisomy for chromosome 1 in single-cell suspensions. In the tumor areas, cell populations with spot numbers ranging from 1 to 4 were detected (Figure 2C). The inflammatory cells in the paraffin sections of this tumor mainly showed two spots for chromosome 1 (Figure 2D). Comparison of the ISH data from isolated tumor cells in suspension and paraffin sections show that aneuploidy can easily be detected. Figure 2E demonstrates a paraffin section of a TCC with a DNA index of 3.2. While in isolated cells, the average spot number for the chromosome 1 probe ranged from 6 to 9, in the paraffin sections this range was broader and no evident major peak could be detected. On basis of these comparative studies we conclude that a fast screening method for detection of numerical chromosome aberrations in routinely processed tumor material has become available. Hybridization with different probes on the same tumor areas in parallel sections or double-target ISH will enable to study chromosome ratios and therefore the specific loss of chromosomes.

Figure 1. *Single-target and double-target in situ hybridization on interphase nuclei and metaphase spreads In A and B, interphase nuclei and metaphase spreads of human lymphocytes are hybridized with probes for chromosomes 1 (TRITC fluorescence signals in B) and 18 (FITC fluorescence signals in B). The staining of the DNA in A is performed with DAPI. In C and D, interphase nuclei and metaphase spreads of human lymphocytes are hybridized with telomeric probe p1.79 for chromosome 1 (see arrows) In E and F, a phage probe library for chromosome 12 is hybridized on a metaphase spread (E) and interphase nuclei (F) of human lymphocytes. F shows the two domains of chromosome 12 in the interphase nuclei; this probe mixture has a preferential hybridization affinity to the centromere In G, H and I, interphase nuclei of flow cytometrically diploid transitional cell carcinoma of the urinary bladder are hybridized with a centromere probe pUC1 77 (trisomy, G), a telomere probe p1.79 (disomy, H), and a phage probe library (two and three domains, I) for chromosome 1. In J, double-target ISH using probes for chromosome 1 (FITC, green ISH spots) and chromosome 18 (TRITC, red spots) demonstrates the chromosome heterogeneity in the tumour cells of bladder. In K, L, M and N, double-target ISH was performed with the probe for chromosome 1 (FITC, green ISH spots) and the probe for chromosome 16 (TRITC, red ISH spots) on bone marrow cells of a patient suffering chronic myeloid leukaemia Colocalization of red and green spots, resulting in yellow/white signals in M and N, indicating the translocation product t(1;16). In O, the arrow shows a male cell in a female blood cell population, showing chimerism after bone marrow transplantation. In P, the combination of immunohistochemistry and the ISH technique is demonstrated and shown to discriminate between epithelial (keratin positive. Alkaline phosphatase/fast blue T24 cells, which are trisomic for the chromosome 1 probe) and non-epithelial (keratin negative Molt4 cells, which are tetrasomic for the chromosome 1 probe) cells*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Prostate cancer. The analysis of prostate tumors by ISH was reported by Van Dekken et al (1990). These authors found two typical chromosomal aberrations in adenocarcinomas of the prostate, i.e. a monosomy for chromosome 10 and a loss of the Y-chromosome. This Y-chromosome loss is possibly age-related, while the monosomy for chromosome 10 may correlate to the metastatic behavior of this carcinoma type, since in the literature a del(10q24) has been reported in such cases (Sandberg et al, 1988).

Renal cell carcinoma. Clinical evaluation of patients with renal cell carcinoma results in a TNM classification with a predictive value for prognosis after surgical treatment. This allows a rough subdivision of patients into groups with different survival rates. Additional techniques have been explored for their ability to obtain a more precise classification of patients Recently, Beck et al (manuscript in preparation) applied the ISH technique, using specific centromeric probes for chromosomes 1 and 7, to detect genetic events involved in the progression of this renal cell carcinoma. It is concluded that the group of renal cell carcinomas with a diploid DNA content as measured by flow cytometry can be subdivided into two subpopulations on basis of ISH. One group with an euploid spotnumber for chromosomes 1 and 7, and another group with an aberrant number of spots for these probes. Whether or not this refinement in diagnosis is of clinical importance remains to be evaluated.

Breast tumors

Devilee et al (1988) detected numerical aberrations for the target sites of chromosome 1 and/or chromosome 18 in seven studied primary breast tumors. Although all had a single peak in DNA flow measurements, six of the cases appeared to be heterogeneous with respect to the chromosome 1 and 18 content per nucleus, whereas no shift in DNA content was observed in the flow cytometer. Moreover, this finding is observed in more solid tumor types, since a minimal DNA difference of about 4% from DI=1.0 is required for obtaining a split peak in flow cytometry.

Testis tumors

One approach in the prevention of testicular germ cell cancer is to diagnose the neoplasia at an early stage, i.e. carcinoma in situ (CIS) or even earlier. So-called atypical germ cells morphologically resemble neoplastic cells in seminoma. The hyperdiploid DNA content, as measured by flow cytometry, is one of the markers of seminomas, next to numerical aberrations of chromosome 1. Giwercman et al (1990) developed a noninvasive procedure using the nonradioactive ISH assay with a probe for chromosome 1 on preparations of semen. In a blind study they identified samples from both patients with isolated CIS changes and patients in whom CIS was accompanied by a tumor, based on the percentage of the hyperdiploid cells present in such preparations. It was concluded that for detection of aneuploid cells in semen, ISH may be a more sensitive technique than flow cytometry, and may become a valuable and fast tool for diagnosis of testicular CIS and

thereby for prevention of testicular cancer

Walt et al (1989) used the ISH procedure with the probe for chromosome 1 to determine whether such aberrations can be detected in atypical germ cell nuclei in paraffin-embedded seminiferous tubules as well One-third of intratubular nuclei, containing atypical germ cells, consistently showed three hybridization signals in contrast to two signals regularly observed in normal intestine and spermatogonia Therefore, also cytogenetic studies of precancerous cells can be performed directly on paraffin embedded tissue which will now allow retrospective studies

Van Dekken et al (1990) applied the ISH to assess the presence of numerical chromosome aberrations in nuclei isolated from a fresh seminoma, using a panel of 12 centromeric probes Compared with the FCM analysis they found for almost all of these probes an aneuploid number of fluorescent spots

Neurological tumors

Cytogenetic studies of brain tumors have revealed numerical chromosome aberrations, such as a polysomy for chromosome 7 and losses for chromosomes 10, 22 and the sex chromosomes in glioma cells (both oligodendroglioma and glioblastoma cells), as well as monosomy for chromosome 22 in meningiomas Until now, these cytogenetic aberrations are only observed by karyotyping

Cremer et al (1988) have documented structural and quantitative changes in two glioma cell lines, including the loss and gain of entire individual chromosomes, and of chromosomal subregions They have been able to characterize both minor and predominant karyotypic features in each cell line The target sequences in chromosomes 1, 4, 7, 18 and 22, clearly highlighted numerical and/or structural aberrations, using library probes in the CISS hybridization technique They observed in both cell lines an under-representation of chromosome 22 and an over-representation of chromosome 7, in agreement with previous studies on gliomas In addition, these authors observed also an under-representation for chromosome 4

Recently, Arnoldus et al (1991) has screened for these chromosomal aberrations using 9 centromeric DNA probes on a procedure for interphase cytogenetics by ISH on brain tumors Apart from a discrepancy in the frequency of appearance of chromosomal aberrations as compared to the literature they found the similar cytogenetic aberrations

Hydatidiform moles and hydropic abortions

The differential diagnosis of complete (CM) and partial (PM) hydatidiform moles and hydropic abortions (HA) can be difficult when based on histology alone Therefore, van de Kaa et al (1991) performed the ISH technique on paraffin sections of hydatidiform moles (Figure 2F-H) and hydropic abortions using probes for chromosome 1, X and Y, to discriminate between maternal and trophoblast cells Using the ISH technique it was

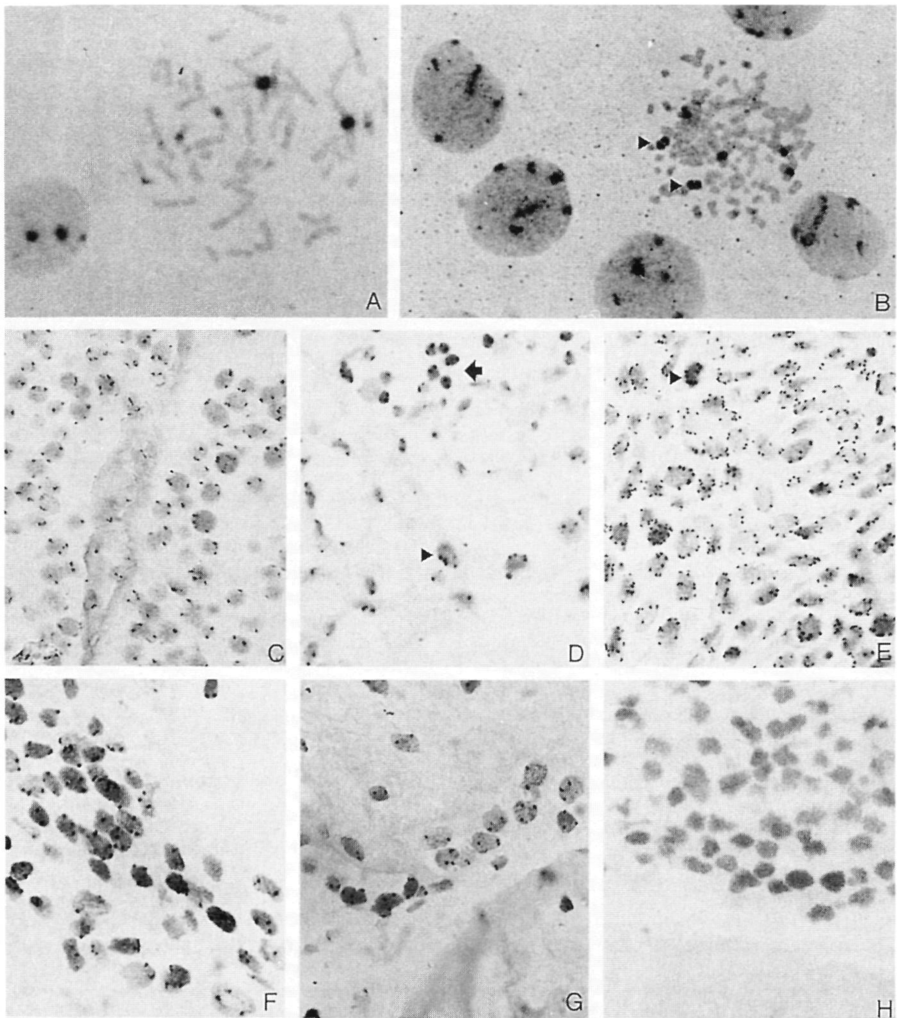

Figure 2. Results of the ISH procedures on routinely processed cytological and histological material. The ISH signals are immunologically detected with peroxidase/DAB for light microscopy. In A, metaphase spreads and interphase nuclei of human lymphocytes are hybridized with the repetitive probe for chromosome 1, demonstrating two ISH spots per nucleus. In B, a breast tumour in a cytological preparation from pleural fluid demonstrates an aneuploid ISH spotnumber for chromosome 1; the arrows indicate so-called iso-chromosomes. C and D show a flow cytometrically diploid transitional cell carcinoma of the human bladder, with two or three spots (see arrows) per nucleus for chromosome 1 (C). In D the tumour cells (three spots per nucleus) can be discriminated from the non-tumour cells, such as stromal cells (see arrow). In E a tetraploid tumour of the bladder is hybridized with a probe for chromosome 1, resulting in 3 to 9 ISH spots per nucleus. The arrow points to a metaphase nucleus, with 9 spots for chromosome 1. F, G and H show a case of hydropically degenerated abortion, with three copies of chromosomes 1 (F) and X (G), and none for chromosome Y (H), confirming triploidy as detected by cytometrical analysis.

demonstrated that it can be established whether a hydatidiform mole is diploid or triploid, which can be of great value in the differential diagnosis between complete and partial moles. Furthermore, as a result of the discrimination between maternal and trophoblast cells of complete moles, the cytogenetic heterogeneity as detected in the proliferating cytotrophoblasts, confirmed the relatively large hyperdiploid fraction observed by cytometric analyses.

Gastric tumors

DNA probes for chromosomes 1, 7, 17, X and Y were used for a cytogenetic study of ten histologically moderately or poorly differentiated gastric adenocarcinomas (Van Dekken et al, 1990). The chromosome aneuploidy as detected by ISH was shown to run parallel with the DNA-ploidy as detected by flow cytometry. Moreover, the ISH technique detected in a high percentage of cells the loss of the (small) Y chromosome, which could not be seen in the flow cytometric DNA histograms. This significant clonal absent of the Y chromosome possibly represents a chromosomal marker in this type of tumor.

Future Prospects

The ISH procedure gives rapid and statistically more reliable results about genomic alterations as compared to cytogenetic procedures. When compared to data obtained with (flow) cytometric procedures ISH gives more precise information about numerical and structural aberrations of the genome Because single cell suspensions of solid tumors often contain variable mixtures of non-malignant cells, such as stromal or inflammatory cells, ISH analysis of such tumor cell suspensions are often difficult to interpret and the real tumor cell fraction may be missed or grossly underestimated. Recently, Beck et al (1991) have described the application of keratin antibodies in the use of flow cytometry to sort the epithelial tumor cells, and to separate them from keratin negative stromal and inflammatory cells. In a multi-parameter flow cytometric analysis the tumor cells were sorted onto glass slides. After ISH analyses tumor cell populations of for example malignant body cavity fluids are enhanced and more convincingly shown to have aberrant numerical spot numbers for individual chromosomes. Therefore, a combination of immunocytochemistry and flow cytometry, followed by non-isotopic ISH methods, allows the detection of chromosome abnormalities in specific cell populations.

Immunohistochemistry for antigen detection is used successfully in the examination of histological and cytological preparations for many clinical and research purposes. While the ISH for the detection of nucleic acids is also being increasingly used, there are certain situations in which combined use of the two techniques on the same preparation can be advantageous. The simultaneous detection of proteins and (viral) DNA by immunohistochemistry and ISH, respectively (Mullink et al, 1989; Porter et al, 1990) permits the combined demonstration of antigen and nucleic acid on cytology preparations (Figure 1P),

but also on routinely processed tissue sections It is to be expected that the techniques for such combinations of immunohistochemistry and ISH will be optimized and find increasingly important applications in diagnostic and experimental procedure.

This review demonstrates that numerical chromosome aberrations can be detected in pathologic specimens using centromeric probes, detected with different markers Numerical as well as structural aberrations can be detected by the combined use of a centromeric and a telomeric probe (Van Dekken et al, 1989) In future, the application of chromosome specific probe libraries for interphase cytogenetics offers the opportunity to study structural chromosome aberrations in more detail, especially for solid tumors where the existing karyotyping procedures are not helpful Furthermore, the identification of marker chromosomes and analyses of complex karyotypes may be aided by using the combination of these types of DNA probes

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Interphase Cytogenetics of Hematological Cancer: Comparison of Classical Karyotyping and In Situ Hybridization using a Panel of Eleven Chromosome Specific DNA Probes

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Abstract

Numerical chromosome aberrations were detected in hematological cancers by non-radioactive in situ hybridization (ISH) procedures, using centromere specific probes for chromosomes 1, 7, 8, 9, 10, 11, 16, 17, 18, X, and Y. All 15 cases could be evaluated by ISH for these eleven probes. Our experiments show that in seven of these randomly selected leukemia bone marrow cell suspensions numerical aberrations for one or two chromosomes could be detected by this method. The results of ISH on interphase nuclei and in some cases on metaphase preparations were compared with karyotyping data. Seven cases of chromosomal aberrations observed with ISH (three for monosomy and four for trisomy) were confirmed by this classical cytogenetic technique, whereas in five instances an aberration was found only with ISH (twice for monosomy, twice for trisomy and one disomy for the Y-probe). One case of a trisomy for chromosome 1 observed by ISH on interphase nuclei could be explained by a marker chromosome, a finding that was further substantiated by ISH on metaphase spreads. In this case double-target ISH on interphase cells with the probes for chromosomes 1 and 16 strongly suggested a translocation between these chromosomes. Also, in one case a marker chromosome could be characterized as a translocation between chromosomes 7 and 17. In this latter case the cytogenetic examinations revealed only monosomy for chromosomes 7 and 17 in addition to non-characterized marker chromosomes.

Our results indicate that the nonradioactive ISH procedure in combination with chromosome specific repetitive centromeric probes is a powerful tool for studying both numerical and structural chromosomal aberrations in interphase nuclei of leukemias. It may therefore become a valuable and routine diagnostic tool in addition to the existing karyotyping procedures.

Introduction

Cytogenetic analysis, using karyotyping on basis of chromosome banding techniques, plays an important role in the detection of chromosomal abnormalities in human solid tumors (Teyssier, 1989). In some cases these aberrations can be correlated with diagnosis and/or prognosis of the malignant disease and may thus dictate therapeutic modalities. However, the interpretation of the chromosome banding patterns is often difficult due to the small number of recognizable metaphases, minimal chromosome spreading, poor banding quality, and condensed or fuzzy appearance of the chromosomes. A further limitation of this technique is that it requires dividing cells, and as a result the majority of the tumor cells are excluded from such analyses (Sandberg et al, 1988). Furthermore, in order to obtain analyzable metaphase chromosomes, culturing of tumor cells may be needed. This can introduce an additional unwanted parameter, i.e., the selection of a certain highly proliferative subpopulation of tumor cells.

Karyotyping of bone marrow cells from patients with hematological cancers has revealed many specific chromosomal abnormalities, which provide diagnostic as well as prognostic information (Croce, 1986; Fleischman et al, 1989; Rowley, 1990). A classical example of such a marker in chronic myeloid leukemia (CML) is a translocation, resulting in the Philadelphia chromosome (Rowley, 1973). However, in cases of terminally differentiated (tumor) cells, such as segmented neutrophils, or cells that have a low proliferative capacity, such as cells from patients with chronic lymphatic leukemia, karyotyping is often difficult or sometimes even impossible.

The cytogenetic analysis by ISH of non-mitotic cells was introduced by Cremer et al (1986) and is generally referred to as "interphase cytogenetics". The use of chromosome specific DNA probes in combination with the ISH technique enables the detection of numerical and structural chromosome aberrations in both metaphase spreads and interphase nuclei. As described previously by our group and others (Cremer et al, 1988; Devilee et al, 1988; Hopman et al, 1988, 1989; Van Dekken et al, 1990), this approach is of great importance in studies of solid tumors, where direct chromosome analysis is frequently hampered by the small number of recognizable metaphases or disturbed by the fact that the tested metaphases are not representative of the tumor because of in vitro selection.

Until now, only a few ISH studies have been performed in cases of leukemia and even fewer studies have correlated ISH results with classical karyotyping data (Nederlof et al, 1989; Van Dekken et al, 1989; Anastasi et al, 1990; Kibbelaar et al, 1991). In the underlying study we have analyzed bone marrow specimens from 15 randomly selected leukemia patients with the ISH procedure, using 11 DNA probes recognizing highly repetitive sequences in the (peri-)centromeric region of specific chromosomes. These results were correlated with cytogenetic analyses of G-banded metaphase chromosomes.

Materials and methods

Sample Preparation

Bone marrow aspirations of three cases of CML, eight cases of acute leukemia (AML/AUL/ALL)(Bennett et al, 1985) and four cases of myeloid dysplastic syndromes (MDS/CMMol)(Bennett et al, 1982) were randomly selected for our study. Cell preparations for ISH were made with freshly obtained bone marrow or peripheral blood. The samples were layered onto Ficoll Hypaque (Pharmacia/LKB, Sweden; specific density, 1077 g/ml) and centrifuged at 1200 rpm for 30 min. The low density cells were recovered, washed in PBS, fixed in 70% ethanol (-20°C), and stored at -30°C.

Metaphase spreads were obtained from bone marrow cells that had been cultured in RPMI-1640 for 1 or 24 h. Colcemid was present during the last hour of the culture. Before fixation in methanol:glacial acetic acid (3:1), the cells were exposed to a hypotonic solution (0.075 M KCl) for 15 min. Slides were made according to routine cytogenetic procedures. Karyotyping was performed using the GTG technique.

Tumor Cell Processing for In Situ Hybridization

For ISH, 5 μl of a cell suspension were dropped onto poly-L-lysine (Sigma Chemical Co , St Louis, MO, M_r 150,000 300,000) coated slides, air dried and heated at 80°C for 1 h To obtain an optimal recovery of cells and proper removal of cellular protein for improvement of DNA probe and antibody penetration, a proteolytic digestion step was applied as described before (Hopman et al, 1989) The digestion was performed with pepsin (Sigma) at a concentration of 100 μg/ml in 0 01 M HCl for 20 min at 37°C. After subsequent dip washes in H_2O and PBS, the nuclei were post-fixed in 4% formaldehyde in PBS for 20 min at 4°C Then the slides were subsequently washed in PBS and H_2O, respectively, and equilibrated in 60% formamide-2xSSC (0 3 M NaCl, and 30 mM Na-Citrate), pH 7 0, for 10 min at room temperature

DNA Probes

For the detection of the target sequences on chromosomes 1, 7, 8, 9, 10, 11, 16, 17, 18, X, and Y, the plasmid probes (as summarized in Table 1) were used The probes were labeled by nick translation with biotin-11-dUTP (Sigma)(Brigati et al, 1982), or modified with digoxygenin (Boehringer, Mannheim, Germany) according to the instructions of the supplier Several of these probes are commercially available from ONCOR (Gaithersburg, MD)

Table 1 *Chromosome specific centromeric probes used in this study*

Chromosome	Name	Vector	Insert (kilobases)	Sequence type	Reference
1	pUC1 77	pUC12	1 77	sat III	Cooke et al, 1979
7	p7t 1	pUC9	0 68	alphoid	Waye et al, 1987
8	D8Z2	pBS	2 55	alphoid	Donlon et al, 1986
9	pHUR98	pBR322	0 16	sat III	Moyzis et al, 1987
10	p10 1	pUC9	0 95	alphoid	Devilee et al, 1988
11	pLC11A	pSP65	0 85	alphoid	Waye et al, 1987
16	pHUR195	pBR322	1 20	sat II	Moyzis et al, 1987
17	p17H8	pSP65	2 70	alphoid	Waye et al, 1986
18	L1 84	pAT153	0 68	alphoid	Devilee et al, 1986
X	pBAMX5	pBR322	2 00	alphoid	Willard et al, 1983
Y	DYZ3	pSP65	2 45	sat III	Cooke et al, 1982

Table 2. Hybridization data of normal bone marrow and peripheral blood samples from male controls (n=6). Of each sample 200 nuclei were counted. Given are the mean percentages of the number of ISH signals per nucleus ± SD.

Probe for chromosome	Number of ISH signals per nucleus			
	0	1	2	3
1	0 5 ± 0 3	6 7 ± 2 8	92.8 ± 2.7	0.5 ± 0.8
7	0	5 5 ± 1 5	94.0 ± 1.7	0.5 ± 0 8
8	0	4 5 ± 1 5	95.0 ± 1 2	0.5 ± 0.5
9	0	6 2 ± 2.0	93 3 ± 1.6	0.5 ± 0.5
10	0 5 ± 0 6	7 1 ± 2.0	92.3 ± 2.1	0.3 ± 0.5
11	0	5 0 ± 1 1	94.5 ± 1.2	0.5 ± 0.8
16	0 5 ± 0 3	10.1 ± 1 1	89.3 ± 0.8	0.3 ± 0.5
17	0.5 ± 0.3	5.3 ± 2 1	94.0 ± 2 6	0.5 ± 0.6
18	0.5 ± 0.3	8 1 ± 2 7	92.9 ± 2.5	0.7 ± 0.8
X	0.5 ± 0.5	98.6 ± 0.5	1.0 ± 0.6	0
Y	1 1 ± 0.8	98 6 ± 1.8	0	0

In Situ Hybridization

The DNA probes were hybridized to the (tumor-) cell preparations (in single- and double-target ISH) as described before (Hopman et al, 1989, 1991) in 60% formamide, 2xSSC, and 10% dextran sulfate at a probe concentration of 1 ng/μl hybridization mixture. Under these stringency conditions, hybridization to minor binding sites was avoided Of the hybridization mixture, 8 μl were applied to the slides under a coverslip (18 x 18 mm) Denaturation of probe and target DNA was carried out by heating the slides in a moist chamber to 70°C for 2 5 min Hybridization was then performed overnight at 37°C. The coverslips were removed by immersing the slides in 60% formamide-2xSSC, pH 7.0. Next, the slides were washed three times for 5 min in the same buffer at 42°C and subsequently three times for 5 min in 2xSSC, pH 7 0 at 42°C

Detection of hybrids was accomplished with fluorescent reporter molecules as described previously (Hopman et al. 1991) The single-target ISH reactions were performed using biotinylated probes and immunocytochemically detected in 4xSSC-0.05% Tween 20-0 5% Boehringer milk, using FITC conjugated avidin (Vector Laboratories, Burlingame, CA) and if necessary immunologically amplified using biotin-labeled goat anti-avidin (Vector) followed by a second layer of FITC conjugated avidin (Pinkel et al, 1986) Double-target ISH using biotin-labeled and digoxygenin-labeled probes, which were hybridized simul-

taneously, were immunocytochemically detected using monoclonal anti-digoxin (Sigma) in PBS-0.05% Tween 20-0.5% Boehringer milk, followed by an incubation of FITC conjugated rabbit anti-mouse antibody (Dakopatts, Glostrup, Denmark) in the same buffer. After a brief wash in 4xSSC-0.05% Tween 20, the biotinylated probes were immunocytochemically detected using Texas Red conjugated avidin (Vector), and if necessary amplified with biotin-labeled goat anti-avidin and a second layer of Texas Red conjugated avidin. All the immunocytochemical steps were performed for 30 min at 37°C.

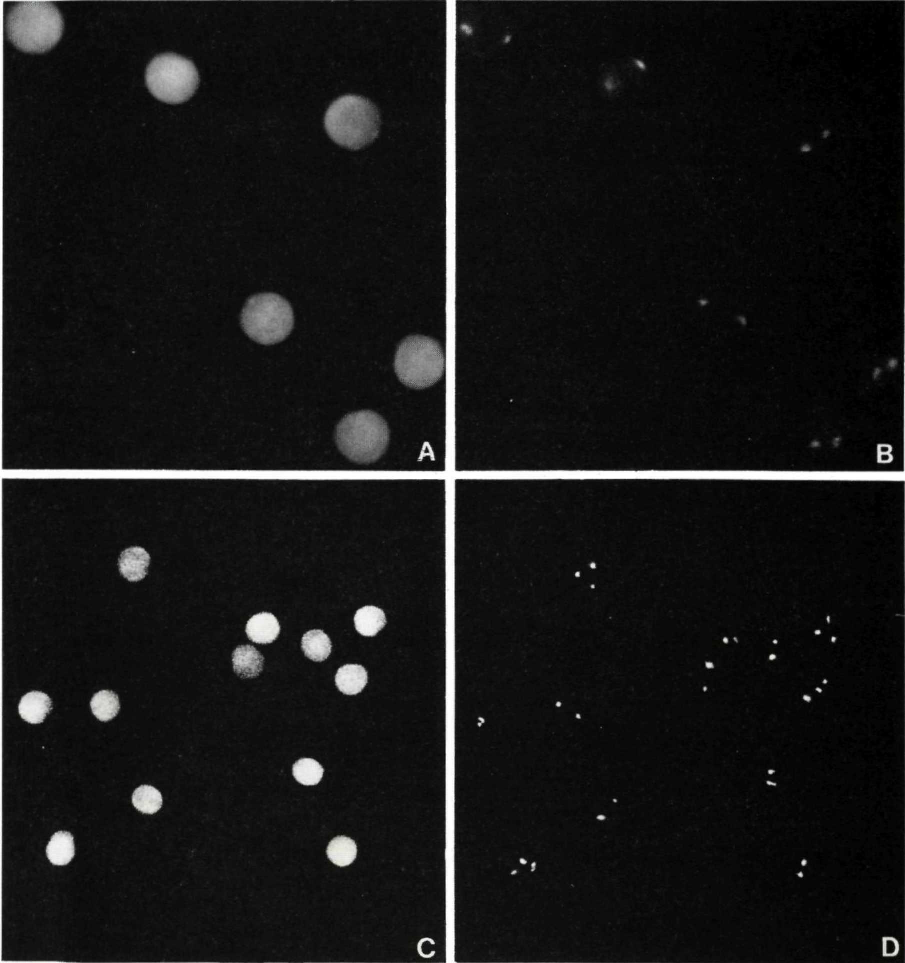


Figure 1. In situ hybridization results on interphase nuclei from normal lymphocytes with the probe for chromosome 1 (B) and to nuclei of bone marrow from case 3 with the probe for chromosome 10 (D). (A and C, DNA counterstaining with DAPI). In A and B more than 98% of the interphase nuclei exhibit two spots. In C and D, 31% of the nuclei show three spots, indicated as a trisomy.

Evaluation of ISH results

The ISH procedure was optimized with respect to sensitivity and specificity for the chromosome specific centromeric probes used in this study Evaluation and counting of fluorescent ISH signals were done by two trained observers according to criteria described before (Devilee et al, 1988, Hopman et al, 1988, 1989, Nederlof et al, 1989) Evaluation of the preparations was performed by counting 200 nuclei/ slide The analyses of the ISH preparations and karyotyping were done without previous knowledge of the results obtained by the complementary technique

Using this ISH procedure, at least 98% of the interphase nuclei of the human lymphocytes, the bone marrow cells and the leukemic cells showed one, two or three ISH signals in all preparations After the proteolytic digestion step with pepsin, which allowed a good penetration of the DNA probes and reporter molecules, the cells retained a good morphology, while discrete ISH signals with a high fluorescence intensity were obtained By counting the number of hybridization spots in 200 nuclei of individual control specimens from normal human lymphocytes and bone marrow of healthy donors, the percentages of cells containing the euploid number of spots for each of the eleven probes varied between approximately 90% and 98% (Table 2, Figure 1A and B) Intra- and interobserver studies revealed limited variability (< 2%) in the percentages of detected spotnumbers (see also Hopman et al, 1988, 1989) The percentage of cells with one ISH signal can be explained partly by close juxtaposition or overlapping of two ISH signals For the specimens used in our study we therefore decided upon a monosomy or trisomy for a certain chromosome only when the percentage of cells with one or three ISH signals, respectively, was greater than the mean ± 3xSD (standard deviation) of the controls The detection of three ISH signals in control cells was the case in less than 1% of the cells for all the probes A diagnosis of trisomy in leukemic specimens could theoretically be made if more than 2% of the nuclei showed three distinct hybridization signals Paired or split spots, as described previously (Hopman et al, 1989, Nederlof et al, 1989, Van Dekken et al, 1989), were normally counted as one ISH signal Finally, to avoid misinterpretations with regard to monosomy or trisomy, we performed double target hybridization experiments with the DNA probe showing the presumed aberration, in combination with a DNA probe, that revealed the euploid number of signals in more than 90% of the nuclei

Results

The clinical data cytogenetic analyses on GTG-banded metaphase spreads and ISH results of the 15 patients are summarized in Table 3

Twelve of 15 patients, which can be subdivided in three different groups, i e , those with chronic leukemia (patients 1 to 3) acute leukemia (patients 4 to 11) and myelodysplastic syndrome (patients 12 to 15), showed structural and/or numerical chromosome aberrations on basis of karyotyping assays For one patient with an AML (case 5), no

metaphases were found for karyotypıng, although a monosomy 16 was observed by ISH. Therefore, a comparıson of numerıcal chromosome aberratıons detected by cytogenetıc analyses of bone marrow cells wıth the results obtaıned by ın sıtu hybrıdızatıon to ınterphase nucleı, was possıble for 14 patıents screened wıth the 11 probes (154 of 165 probe hybrıdızatıons) In 144 of these 154 probe hybrıdızatıons a good correlatıon between results of both methods ıs found In 7 of the 144 probe hybrıdızatıons numerıcal chromosome aberratıons were observed wıth both procedures, ı e , three ınstances of monosomy and four ınstances of trısomy In 10 of the 154 probe hybrıdızatıons there was no correlatıon between the karyotypıng and ISH In 5 of these 10 tests an aberratıon was found only wıth ISH (two ınstances of trısomy, two ınstances of monosomy, and one ınstance of dısomy for the Y-probe) On the other hand ın the classıcal karyotypıng procedure 5 numerıcal aberratıons were observed (one trısomy, three monosomy, one nullısomy), whıch were not detected by ISH of ınterphase nucleı

Those cases ın whıch eıther the conventıonal cytogenetıc analysıs or the ISH procedure revealed aberratıons for the chromosomes, detectable by the probes used ın thıs study, wıll be dıscussed ın detaıl

Table 3 Clınıcal data, cytogenetıc analyses and ISH results on ınterphase nucleı of bone marrow from patıents wıth leukemıa

Case	Sex	Age (yr)	Dıagnosıs	Karyotype	Numerıcal and structural chromosome aberratıons detected by ISH usıng probes for chromosomes 1, 7, 8, 9, 10, 11, 16, 17, 18, X, and Y.
1	M	39	CML	46,XY,t(9,22)(q34,q11),ı(17q), ı(9q),+8, 9, 16, 16, 17,+mar1	trısomy 1(65%), trısomy 8(87%), t(1,16)(60%)
2	M	51	CML	46 XY,t(9,22)/44,Y,t(9,22), X	none
3	F	50	CML	46,XX,t(9,22)/47,XX,t(9,22),+Ph'	trısomy 10(31%), monosomy 16(16%)
4	M	40	AML,M1 M2	46,XY	none
5	F	43	AML Mo	no metaphases found	monosomy 16(18%)
6	M	43	AML-M4	46,XY	none
7	F	41	AML M5	46,XY,10p+,11p ,many addıtıonal non clonal aberratıons	none
8	M	44	AUL	46,XY/46,XY,t(9,22)/ 46,XY,t(9,22), 7,+der(7)t(7p,?)	none
9	F	44	AUL	46 XX,t(9,22)(q34,q11)	none
10	M	42	ALL	46,XY,t(9,22)/ 46,XY,t(9,22),dup(7q)/ 47,XY,t(9,22),9p+,+5,+8, 6, 7,+mar1	monosomy 7(65%), trısomy 8(67%)
11	M	61	AML M6 <RAEB t	46,XY/44,XY 1, 5, 7, 17,+mar1, +mar2, +2xmar3	monosomy 1(71%), t(7,17)(65%)
12	F	62	CMoL	46,XX	none
13	M	70	CMoL t	46,XY/45,XY, 16/46,XY,+11, 16	trısomy 11(72%); monosomy 16(97%)
14	M	47	RA	46,XY/47,XY,+8	none
15	M	58	RAEB,t	46,XY/44,XY,del(3q),+8, 5, 6	monosomy 17(96%), dısomy Y(20%), trısomy 8(94%)

In all three cells wıth 44 chromosomes one X chromosome was mıssıng next to other dıfferent aberratıons ın the dıfferent ındıvıdual cells

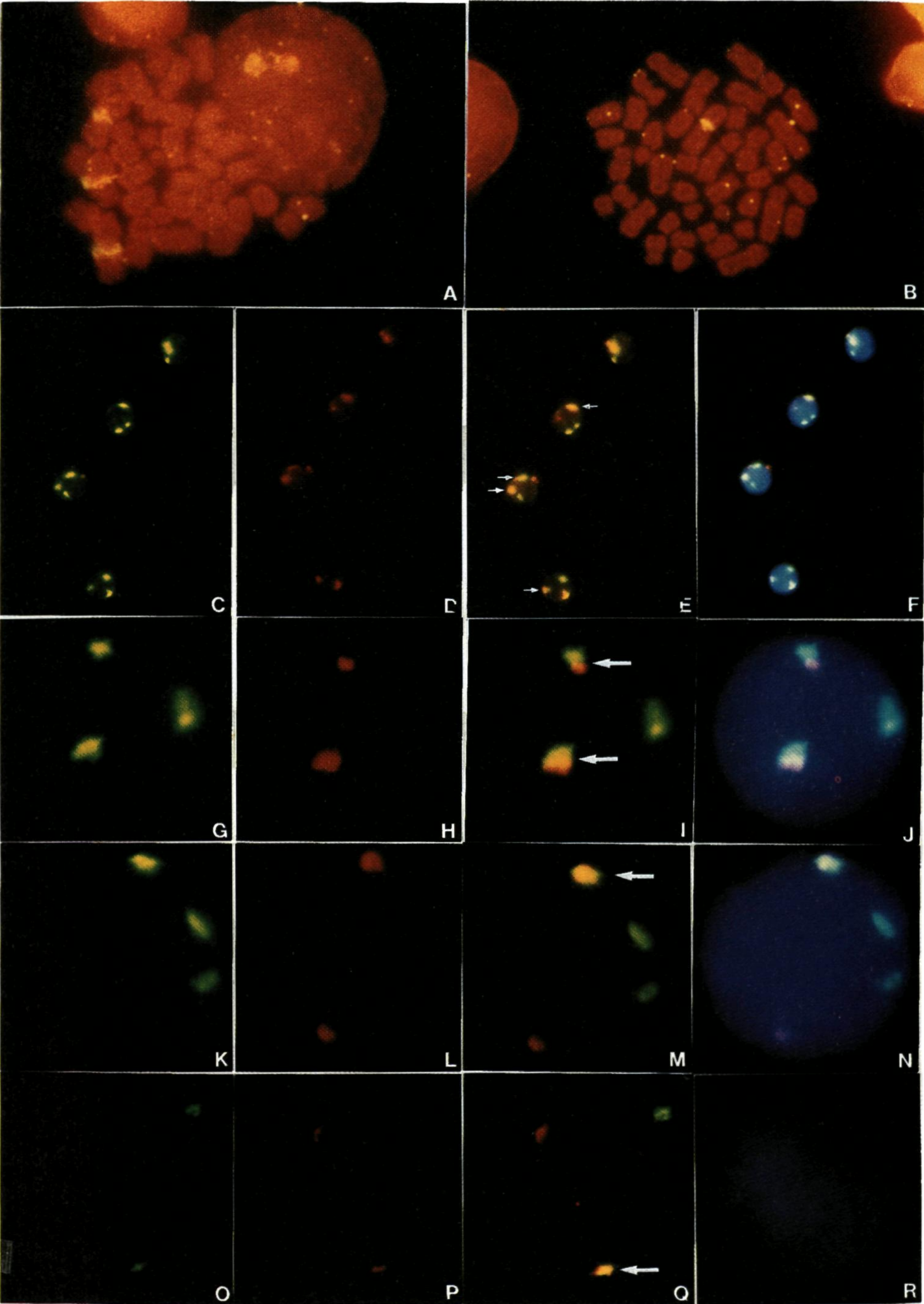
case 1

ISH on interphase nuclei with the panel of probes revealed significant aberrations for chromosome 1 (65% three spots)(Figure 2, C, G, and H) and chromosome 8 (87% three spots) Trisomy 8 could be confirmed by karyotyping

Hybridization on metaphase spreads with the probe for chromosome 1 (Figure 2A) showed a similar number and distribution of ISH spots, as shown in Figure 3A The karyotype observed in the bone marrow cells of this patient was extremely complex (Table 3) The alterations of chromosome 9, 16 and 17, detected by karyotyping, were not seen by our ISH assays However, the appearance of an i(9q) and i(17q) can explain the differences between karyotyping and ISH on chromosomes 9 and 17. Apparently these isochromosomes still contain the alphoid centromeric regions which are recognized by the probes used
The extra ISH spot with the probe for chromosome 1 as well as the normal number of spots with the probe for chromosome 16 could be explained after double-target ISH experiments, in which we combined the DNA probes for the chromosomes 1 and 16 The results showed a colocalization of ISH signals for chromosomes 1 and 16 in approximately 60% of the interphase nuclei (see Figure 2, C N) suggesting a translocation between (parts of) chromosomes 1 and 16 Reexamination of metaphases hybridized with the probe for chromosome 1 showed a marker chromosome (mar1) with two or perhaps even three centromeric regions as detected by DAPI counterstaining (Figure 4A) This marker could, however, not be characterized as being derived from chromosome 1 and/or chromosome 16 by karyotyping

case 2

Cytogenetic analysis of bone marrow from this patient with CML showed 2 cell populations, although only 8 mitoses were available for examination In 5 of the analyzed metaphases 46 chromosomes were counted, whereas in the other 3 metaphases only 44 chromosomes were counted In these three cells an X-chromosome was missing next to other aberrations in the different individual cells Virtually all the nuclei contained one fluorescent ISH spot after incubation with the X-probe The fact that karyotyping showed no marker chromosomes in the latter cell populations suggested that for this case a discrepancy in results of the two cytogenetic procedures exists Double-target ISH on interphase cells with the probes for the chromosomes X and Y showed that virtually all cells had one fluorescent signal per nucleus for each of these centromeric probes in both interphase nuclei as well as metaphase spreads The absence of the X chromosome was probably a preparation artifact, since in these metaphase spreads also another chromosome was missing (see Table 3)


A
B
C
D
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G
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K
L
M
N
O
P
Q
R

case 3

The nuclei of this patient with CML showed a significantly different distribution for chromosome 10 in ISH (31% three spots, 65% two spots and 4% one spot) as compared to karyotyping results. Furthermore, with the probe for chromosome 16 we detected a statistically significant percentage (16%) of cells with one fluorescent ISH spot. No numerical and/or structural chromosome aberrations for one of these two chromosomes was detected by cytogenetical analysis (Figures 1, C and D). The ISH results could not be examined on metaphase spreads because no samples where available.

case 7

Cytogenetic analysis of bone marrow cells from this patient with AML showed a normal number of chromosomes, but with many structural aberrations, including 10p+ and 11p-. ISH with the probes for chromosomes 10 and 11 showed that almost all cells contained two ISH fluorescent signals per nucleus (respectively, 96% and 93%).

case 8

ISH on interphase nuclei from this patient with AUL with the panel of probes revealed no aberrant distribution of spot numbers as compared to the control cells. The cytogenetic data showed a monosomy of chromosome 7, in combination with an additional marker chromosome, which contained parts of the lacking chromosome 7.

case 10

Hybridization to interphase nuclei from this patient with ALL, using the panel of probes, showed a significant number of cells that contain one ISH fluorescent signal (65%) with the probe for chromosome 7, and three ISH signals (67%) with the probe for chromosome 8. The cytogenetic analysis also observed a significant high number of metaphases with only one chromosome 7 and three chromosomes 8 (73%). As a result, in this case a good correlation between the two analytical procedures could be demonstrated.

Figure 2. Results of single- (A,B) and double-target (C-Q) in situ hybridization on interphase nuclei and metaphase spreads of specimens from case 1 (A, C-N) and case 11 (B, O-R) Blue (J,N,R) or red (A,B) counterstaining of DNA was done with DAPI or propidium iodide, respectively.
In A, three hybridization signals are demonstrated on a metaphase spread of a malignant cell from case 1 after incubation with the probe for chromosome 1. In B, one hybridisation signal with probe pUCl 77 is seen on a metaphase spread of case 11, indicating a monosomy for chromosome 1
In C-N, a double-target hybridization was performed with the probe for chromosome 1 (green spots) and the probe for chromosome 16 (red spots) Colocalization of red and green spots, resulting in yellow/white signals in E, F, I, J, M and N, suggest a translocation between a (part of) chromosome 1 and chromosome 16 (see arrow). In O-R, double-target hybridization was performed with the probe for chromosome 7 (green spots) and the probe for chromosome 17 (red spots) Again, colocalization of the red and green spots (resulting in the yellow signal in Q) indicates the translocation between chromosomes 7 and 17 The arrows in E, I, M and Q indicate a colocalization of red and green spots.

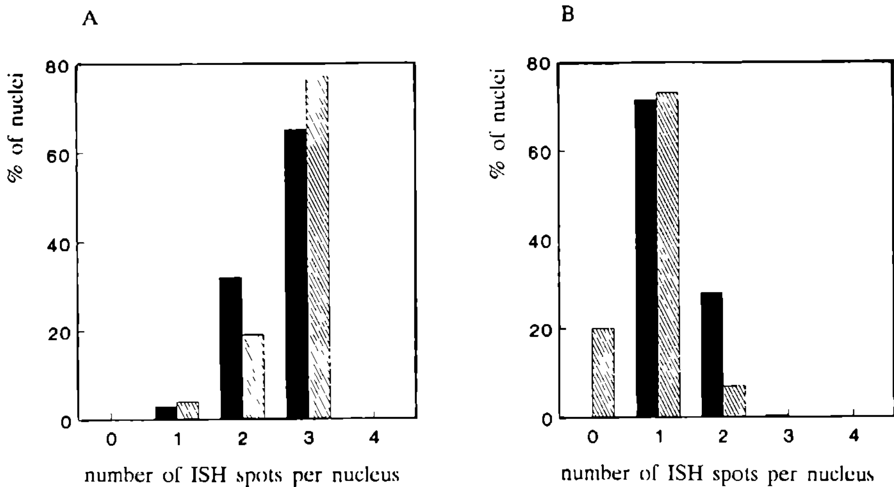

Figure 3. Evaluation of in situ hybridization reactions with the probe for chromosome 1 in case 1 (A) and case 11 (B). A comparison of the spot number distribution between interphase nuclei and metaphase spreads is shown The closed bars represent the percentages of interphase nuclei with the indicated spot number; the hatched bars represent the percentages of metaphase spreads with indicated spot number. In (A) 400 nuclei and 26 metaphase spreads ,and in (B) 400 nuclei and 15 metaphase spreads are evaluated

case 11

ISH on interphase nuclei with the panel of probes revealed that about 70% of the bone marrow nuclei from this patient with AML showed one fluorescent ISH signal with the probe for chromosome 1 (not shown). The same result was observed by ISH on metaphase spreads (Figure 2B). The distribution of spots in interphase nuclei and metaphase spreads are shown in Figure 3B. The cytogenetic analysis from bone marrow of this patient demonstrated two cell lines: one with a normal karyotype (46,XY, in two metaphases) and one with a complex karyotype (in 34 metaphases). A monosomy for chromosomes 1, 5, 7, 17, 20, and 21, and several marker chromosomes mar1, mar2 and 2xmar3 were found. Mar1 was cytogenetically characterized with a part of chromosome 1, mar2 was a translocation between a chromosome 17 and 7p. and mar3 was a translocation product of chromosome 20p and 21.

The single-target ISH results obtained with probes for chromosomes 7 and 17 showed two spots per nucleus in, respectively, 96% and 91% of the cells. Double-target ISH with the centromeric probes for chromosomes 7 and 17, however, demonstrates the translocation between chromosomes 7 and 17 in approximately 65% (Figure 2, O-R), as also characterized by the cytogeneticist (Figure 4B). Double-target ISH on metaphase spreads could not be performed because of lack of samples.

case 13

ISH on bone marrow cells from this patient with MDS (CMol.t) revealed significant aberrations for chromosome 11 (3% four spots; 72% three spots; 23% two spots; 2% one spot) and chromosome 16 (97% one spot; 3% two spots) (Figure 5, A-D). Both types of aberrations were confirmed by karyotyping. From the 19 analyzed metaphases, one had a normal chromosome number, 9 showed only a monosomy for chromosome 16, and 9 spreads revealed a monosomy for chromosome 16 and a trisomy for chromosome 11 (i.e., 93% monosomy 16; 47% trisomy 11). The lower number of cells with trisomy 11 found with the karyotyping could indicate that these cells show a lower proliferative capacity to the other cell population(s). In this case the interphase cytogenetic analysis gives a better estimation of the real number of cells than the conventional karyotyping procedure.

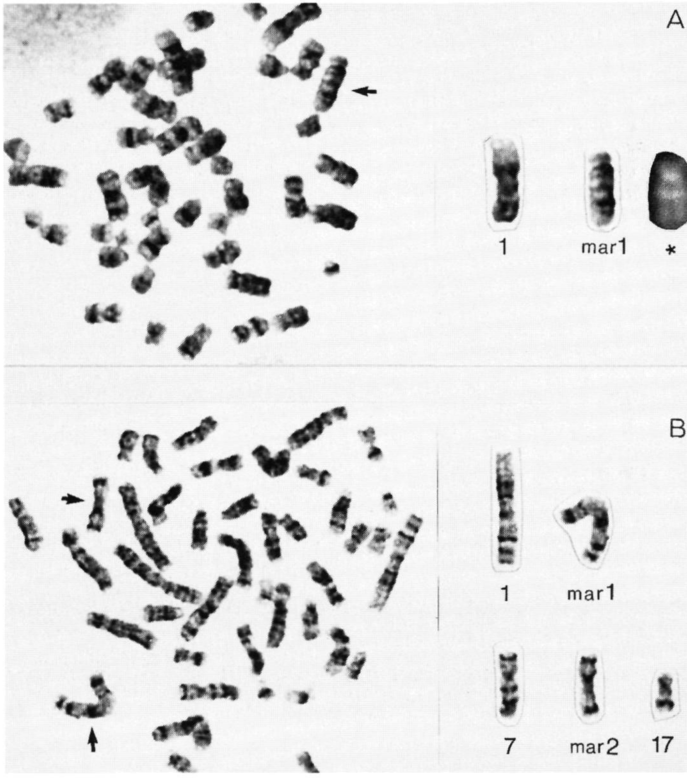

Figure 4. *Representative partial karyotypes from case 1 (A) and case 11 (B). Cytogenetic investigations were carried out from bone marrow as described in section Materials and Methods.*
In case 1 (A) a very complex karyotype was found (Table 3) with one marker chromosome (mar1), showing two centromeric regions in the DAPI counterstain (*). Double-target ISH proved that chromosomes 1 and 16 were involved (see Figures 2c-n). In case 11 (B) one chromosome 1 was absent in 34 out of 36 metaphases, while several marker chromosomes were present as a result of alterations, for chromosomes 1, 7, 17, 20 and 21. The arrows indicate several marker chromosomes that were also identified by ISH.

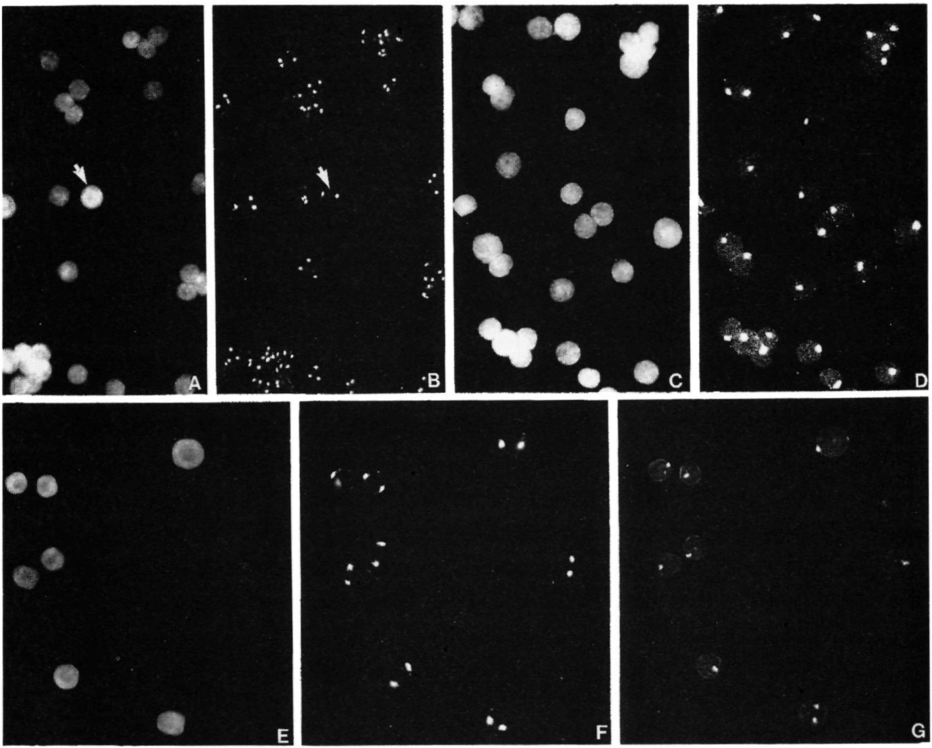

Figure 5. Results of single- (B,D) and double-target (F,G) in situ hybridization on bone marrow specimens from case 13 (A-D) and case 15 (E-G). In A, C and E, the DNA was counterstained with DAPI. In A and B, a significant number of nuclei showed trisomy for the chromosome 11 probe, while in C and D a significant number of interphase nuclei showed monosomy for chromosome 16. Both aberrations were also characterized by karyotyping (see Table 3). The arrow indicates a cell with two spots per nucleus. In E-G a double-target in situ hybridization procedure was performed with the chromosome 1 probe (F, two spots per nucleus) and the chromosome 17 probe (G, 95% of the nuclei show one spot), suggesting monosomy for chromosome 17.

case 14

ISH on interphase nuclei from this patient with MDS (refracting anemia) using the panel of probes revealed no aberrant distribution of spot numbers as compared to the control cells The cytogenetic analysis, however, showed two cell lines one with a normal karyotype (46,XY), and one with a trisomy 8 (in 4 of 38 metaphases) The discrepancy between the two cytogenetic analyses is most probably due to the fact that the sample used for karyotyping was analyzed 10 years before the ISH data were obtained To be able to perform these ISH reactions for our study, a new sample had to be used, which could not be karyotyped

case 15

Cytogenetic analysis of bone marrow cells from this patient with MDS (refracting anemia with excess of blast in transformation) showed two cell lines, one with a normal karyotype (46,XY, in five of the analyzed metaphases) and one with many chromosomal aberrations (44,XY, del(3q),+8,-5,-6, in five analyzed metaphases) ISH on interphase nuclei revealed a trisomy with the probe for chromosome 8 in 93% of the nuclei, and a monosomy for chromosome 17 in 95% of the tumor cells (Figure 5, E-G) The trisomy 8 could be confirmed by karyotyping, but the monosomy 17 was in contrast to the cytogenetic analysis, where two normal chromosomes 17 were observed Also, with the probe for chromosome Y there is a significant number of cells with two spots per nucleus, suggesting a disomy (in 20% of the tumor cells) Double-target ISH experiments on interphase nuclei with the X and Y probe supported these results (not shown)

Discussion

Several studies have demonstrated that DNA probes, recognizing specific repetitive sequences in the (peri-)centromeric region of a particular chromosome, can be used to detect and quantify their respective chromosomes or chromosome areas in the interphase nucleus (Cremer et al, 1988, Devilee et al, 1988, Hopman et al, 1988, 1989, Van Dekken et al, 1989, 1990, Nederlof et al, 1989, Anastasi et al, 1990, Kibbelaar et al, 1991) Recently, some studies were published, comparing the interphase cytogenetic procedure using nonradioactive ISH with conventional cytogenetic analyses of cell lines derived from solid tumors, in neoplastic cells from bone marrow and peripheral blood (Nederlof et al, 1989, Van Dekken et al, 1989, Anastasi et al, 1990, Kibbelaar et al, 1991) However, only a few DNA probes were used, furthermore, the number of specimens from patients with leukemia was rather small (Anastasi et al, 1990, Kibbelaar et al, 1991) In the present study we analyzed a group of 15 randomly selected patients suffering from leukemia with the ISH procedure, using 11 DNA probes, specific for repetitive target sequences on human chromosomes 1, 7, 8, 9, 10, 11, 16, 17, 18, X, and Y (see Table 1) and compared these results to data obtained through conventional karyotyping methods

Using the ISH procedure as described in Materials and Methods', at least 98% of the interphase nuclei of a human lymphocyte preparation, bone marrow cells and the leukemic cells showed one, two, or three ISH signals in all preparations After the proteolytic digestion step with pepsin, which guarantees a good penetration of the DNA probes and reporter molecules, the cells retained a good morphology, while discrete ISH signals with a high fluorescence intensity were obtained With ISH we detected 13 numerical chromosome aberrations in 165 analyses Seven were in good correlation with the classical karyotyping procedure (cases 1, 10, 11, 13, and 15) However, the trisomy for chromosome 10 (case 3, Figure 1, C and D) and monosomy for chromosome 16 (cases 3 and 5), and the monosomy for chromosome 17 and disomy for chromosome Y (case 15, male) could not be confirmed by the cytogenetic analysis, although the double-target ISH results further supported these aberrations (Figure 5, E G) This can be explained by the fact that the difficulties of the classical karyotyping techniques are often a consequence of the lack of mitoses and/or poor banding quality as a result of condensed or fuzzy appearance of the chromosomes (Sandberg et al, 1988, Teyssier, 1989) On the other hand, in addition to the 7 correlated aberrations, 14 abnormalities for the 11 examined chromosomes were found only with the classical karyotyping Some of these where structural aberrations, as in case 1 [i(9q) and i(17q), t(16q,16p)?], case 7 [10p+, 11p], case 8 [-7, +der(7)t(7p,?)] and case 10 [9p+] The nature of the probes used for ISH in this study was such that they did not allow detection of these aberrations Trisomy 8 as detected by karyotyping in case 14 and the discrepancy with the ISH results is due to the fact that the sample used for karyotyping was analyzed 10 years before the ISH data were obtained The new sample which had been used for our ISH studies could not be karyotyped The nullisomy for chromosome X as detected by the cytogeneticist in 3 of 8 analyzed metaphases in case 2 (male) was not in accord with the results obtained by single- and double-target ISH experiments, where one spot per nucleus for the X centromeric region was detected (94%) This finding can probably be explained by a preparation artifact, since other different chromosomes also were missing in these three individual cells This result illustrates an advantage of the ISH procedure, in which more cells can be analyzed, as compared to karyotyping

Although centromeric DNA probes are already able to detect numerical chromosome aberrations, our results suggest that ISH with centromeric probes is also capable of detecting structural aberrations The translocation in case 11 between chromosomes 7 and 17 as observed by karyotyping (Figure 4B), could also be demonstrated by ISH in interphase nuclei (Figure 2, O-R) The trisomy for chromosome 1 in case 1 (Figure 2A) could not be demonstrated with the classical karyotyping Double-target ISH experiments on interphase nuclei suggested that there might be a translocation between a chromosome 16 and a chromosome 1 (Figure 2, C N) Reexamination of the GTG-banded metaphase spreads of this case revealed a marker chromosome which contained two or three centromeric regions, probably derived from chromosome 1 and both chromosomes 16 (Figure 4A) Detection of chromosomal abnormalities in leukemia, using cytogenetic analyses on

the basis of chromosome banding techniques, plays an important role in determining diagnosis, prognosis, and treatment protocols of some specific types of cancer such as CML and AML

The DNA probes, specific for the (peri-)centromeric targets of chromosomes, enable the detection of part of a chromosome, only the copy number of the target sequence is obtained The development of chromosome specific library DNA probes (Cremer et al, 1988, Pinkel et al, 1988, Lichter et al, 1990) offers the opportunity to study structural aberrations in more detail Also the identification of marker chromosomes and analyses of complex karyotypes may in the future be solved by using the nonradioactive ISH with centromeric probes, single copy sequences, and total chromosomal libraries

It can be concluded that detection of numerical and structural chromosome aberrations as detected by ISH on interphase nuclei will in the future become an important additional technique to support and complement the classical karyotyping technique

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Interphase Cytogenetics on Agar Cultures: A Novel Approach to Determine Chromosomal Aberrations in Hematopoietic Progenitor Cells

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Abstract

We describe a novel approach to determine the presence of chromosomal aberrations in progenitor cells by in situ hybridization (ISH) on agar cultures Bone marrow cells of 3 patients suffering from acute myeloid leukemia (AML) were selected to develop the method In all 3 cases, numerical aberrations for chromosome 1 and/or 8 were detected by karyotyping and ISH using chromosome-specific centromeric-associated DNA probes. These aberrations were used as markers in this study After in vitro culture of the bone marrow samples in agar, the cells were pretreated in order to perform ISH This approach retains the cytological architecture of the agar assay, allowing discrimination between chromosomal aberrations detected in the clonogenic and non-clonogenic cells in culture. With this new technique, the presence of the cytogenetic aberration in clonogenic cells can be shown at the interphase level

Introduction

Hematopoietic neoplasms are assumed to be clonal diseases originating from a single cell (Messner et al, 1986, Fialkow et al, 1989); therefore, monoclonality can be used to further characterize these neoplasms In the majority of myeloproliferative disorders, progenitors can be cultured in vitro Information about the lineage involvement of these neoplasms may increase understanding of their pathogenesis and response to treatment Because the origin of clonogenic cells may be difficult to determine by morphology alone (Raskind et al, 1987), additional techniques have been developed to demonstrate the monoclonal origin of progenitors The X-chromosome-linked enzyme glucose-6-phosphate dehydrogenase (G6PD), or restriction fragment length polymorphisms (RFLP) of other X-chromosome-linked enzymes, can be used as a genetic marker in heterozygous women (Fialkow et al, 1989) Karyotyping and surface marker expression analysis can also be used (Dubé et al, 1981, Raskind et al, 1987)

Except for the latter technique, all methods analyze colonies that are individually harvested Apart from being time-consuming, harvesting may lead to sampling error because non-clonogenic cells may be removed in place of clonogenic cells due to colony overlap Since karyotyping analyzes dividing cells, cell populations with a selective growth advantage will be favored (Dubé et al, 1981) Furthermore, within a colony, sufficient proliferative cells must be available Only relatively large colonies (> 100 cells) could be analyzed, although with technical improvements smaller colonies (± 50 cells) are being investigated now (Dube et al, 1981)

Non-radioactive in situ hybridization (ISH) of chromosome-specific, centromeric-associated DNA probes to interphase cells has become available, determining the copy number of a target chromosome by counting the number of hybridization signals (Hopman et al, 1989, 1991, 1992, Poddighe et al, 1992) Although this interphase analysis provides

only limited information about the karyotype, it enables rapid screening of large numbers of cells.

Until now, few ISH studies have been published in which bone marrow and peripheral blood interphase nuclei from patients suffering from leukemia were analyzed at the total cell level (Nederlof et al, 1989; Anastasi et al, 1990; Poddighe et al, 1991; Jenkins et al, 1992; Price et al, 1992). Although the detection of structural aberrations is limited using centromeric DNA probes, double-target fluorescent ISH experiments on interphase nuclei have shown that the detection of structural chromosome aberrations is feasible (Lichter et al, 1988; Poddighe et al, 1991). A two-color ISH approach can be used to demonstrate the bcr-abl fusion, which is responsible for the appearance of the Philadelphia chromosome, in interphase cells of chronic myeloid leukemia (CML) patients (Arnoldus et al, 1990; Tkachuk et al, 1990). Also, marker chromosomes can be characterized by using these procedures.

In the present study, the application of interphase cytogenetics at the progenitor cell level is described. For this approach we developed a method to perform ISH on cells cultured in agar. Since the cytological architecture of the agar assay remains intact, discrimination between chromosomal aberrations detected in both the clonogenic and non-clonogenic cells can be detected.

Materials and Methods

Sample selection and preparation

Three karyotyped AML bone marrow samples with numerical chromosome aberrations were selected: monosomy for chromosome 1, trisomy for chromosome 8, and a polyploid karyotype. As a control, the samples were hybridized with a chromosome-specific probe for which no chromosomal aberration was detected. In addition, normal bone marrow cells of a healthy donor served as a control.

Preparations for ISH were made with freshly obtained bone marrow cells. The samples were layered onto Ficoll-Hypaque (specific density 1085 g/ml)(Pharmacia/LKB, Uppsala, Sweden) and centrifuged at 1200 rpm for 20 minutes.

Part of the collected low-density cells were washed in phosphate buffered saline (PBS), fixed in 70% ethanol (-20°C) and stored at -30°C. Shortly before ISH, 5 μl of a cell suspension were dropped onto slides coated with poly-L-lysine (molecular weight 150 to 300 kd)(Sigma, St.Louis, MO), air-dried and heated at 80°C for 1 hour.

The remaining low-density cells were cultured in Iscove's medium (Flow Laboratories, Irvine, Scotland), supplemented with 20% fetal calf serum (FCS), 50 IU/ml penicillin, 50 μg/ml streptomycin (both Flow Laboratories), and 0.3% bacto-agar (Difco, Detroit, MI). The cells were stimulated with the combination of IL-3 (40 ng/ml; Sandoz BV, Uden, The Netherlands), granulocyte-macrophage colony-stimulating factor (GM-CSF) (20 ng/ml, Sandoz BV) and granulocyte colony-stimulating factor (G-CSF) (5 ng/ml, Behring,

Marburg, Germany). Duplicates were cultured in 35x10 mm culture dishes (Costar, Cambridge, MA) at 37°C in a fully humidified atmosphere containing 5% CO_2 (Van Der Lely et al, 1991). The cells (2 x 10^5) were plated in a minimum volume of 0.85 ml Iscove's medium per dish in order to make them more accessible to the probes After 7 days, the total number of clusters (5 to 40 cells) and the total colony number (>40 cells) was counted The agar cultures were lifted out of the culture dishes and, after division into quarters, spun down on poly-L-lysine-coated slides (Sigma) by cytocentrifugation. Subsequently, the samples were fixed in 70% ethanol, air-dried and stored at 4°C until ISH was performed

Tumor Cell Processing for In Situ Hybridization

A proteolytic digestion step with pepsin at a concentration of 100 μg/ml in 0.01 M HCl for 20 minutes at 37°C was performed to obtain an optimal recovery of cells and proper removal of cellular protein for improvement of DNA probe and antibody penetration (Poddighe et al, 1991, Hopman et al, 1992) Thereafter, the nuclei were postfixed in 4% formaldehyde in PBS for 20 minutes at 4°C Subsequently, the slides were washed in PBS and H_2O and equilibrated in 2xSSC (0 3 M NaCl, and 30 Mm Na-Citrate), pH 7.0, for 5 minutes at room temperature

DNA Probes

The plasmid probes pUC1 77 (Cooke et al, 1979) and D8Z2 (Oncor, Gaithersburg, MD)(Donlon et al, 1986) were used for the detection of target sequences on chromosomes 1 and 8, respectively The probes were labeled by nick-translation with biotin-11-dUTP (Sigma), according to the supplier's instructions

In Situ Hybridization

The DNA probes were hybridized to the (tumor-)cell preparations as described before (Poddighe et al, 1991) in 60% formamide, 2xSSC and 10% dextran sulfate at a probe concentration of 1 ng/μl hybridization mixture Tem microliters of the hybridization mixture were applied to the slides under a coverslip (18x18 mm). Denaturation of probe and target DNA was performed simultaneously by heating the slides to 70°C in a moist chamber for 2 5 minutes Hybridization was then performed for 2 to 16 hours at 37°C. The coverslips were removed by immersing the slides in 60% formamide, 2xSSC, pH 7.0. Thereafter, the slides were washed 3 times for 5 minutes in the same buffer at 42°C and subsequently 3 times for 5 min in 2xSSC, pH 7 0 at 42°C

Immunocytochemistry

Detection of hybridized cells was accomplished using mouse anti-biotin (Dakopatts, Glostrup, Denmark) in PBS and 0 05% Tween 20 with 0 5% blocking milk (Boehringer, Mannheim, Germany), followed by an incubation with biotin-labeled horse anti-mouse

(Vector, Burlingame, CA) Next, a final incubation with the ABC complex (avidin-biotin-labeled peroxidase complex)(Vectastain Elite ABC Kit) was performed according to the supplier's instructions All immunocytochemical steps were performed for 30 minutes at 37°C Finally, the DNA probe was visualized with 0 5 mg/ml 3,3-diaminobenzidin tetrahydrochloride (DAB)(Sigma), 0 65% imidazole (Merck, Darmstadt, Germany) and 0 015% H_2O_2 (Merck), pH 7 8, in PBS for 5 minutes Slides were rinsed in distilled water and the signal was amplified with $CuSO_4$ (0 5% in 0 9% NaCl) for 5 minutes at room temperature, washed with distilled water, counterstained with hematoxylin and mounted in Permount (Fisher Scientific, Fair Lawn, NJ)

Evaluation of ISH results

Using the described ISH procedure, at least 90% of the interphase nuclei showed one, two or three distinct ISH signals in all preparations Evaluation of the preparations and counting of ISH signals were done by two trained observers according to criteria as described before (Poddighe et al, 1991, Hopman et al, 1992) Minor hybridization signals, which can be recognized by a lower intensity and spot area, and overlapping nuclei were not counted (Hopman et al, 1988) In the control cells, one ISH signal for a targeted chromosome was found in 5 to 10% of the cells, depending on the efficiency of the ISH procedure, or the colocalization of two ISH signals The detection of three ISH signals occured in less than 1% of the normal cells

Of the cells from suspension preparations, 200 nuclei per slide were counted. The agar preparations were evaluated by counting 200 single cells in agar (representing non-clonogenic cells), and approximately 50 aggregates (both colonies and clusters) Since the aggregates represent clonogenic cells, all the cells in an aggregate should be the same, and the distribution of ISH signals would be an indication for the clonality of the aggregates Therefore, a cell count of the distribution of ISH signals in an aggregate was not necessary Moreover, the same criteria for evaluation as described previously (Hopman et al, 1988; Poddighe et al, 1991) could be applied In an aggregate, about 90% showed ISH signals When more than 90% of the cells within an aggregate contained one ISH signal, it was classified monosomy Consequently, if it contained two or three ISH signals, it was classified as disomy and trisomy, respectively

Results

Development of the technique

The success rate of the ISH procedure using chromosome-specific DNA probes in interphase nuclei is mainly dictated by the accessibility of the target DNA Therefore, the effect of different conditions, such as the fixative, pretreatment with agarase and proteolytic digestion were studied

To avoid detachment of the agar preparations from the glass slides during pretreatment

and ISH denaturation, the slides were coated with poly-L-lysine. Fixation with 70% ethanol was sufficient to avoid loss of material from the slides and to preserve morphology during the permeabilization steps.

To improve the accessibility of the target DNAs and to achieve good penetration of DNA probes and immunological reagents through the agar, a proteolytic digestion with pepsin or agarase was performed. Pretreatment with pepsin, which removes a large part of the cytoplasmic and nuclear proteins, was shown to be more effective than agarase in improving sensitivity and reproducibility. The most crucial parameter for the success rate of ISH, however, was a reduction of the agar layer thickness. We observed that a reduced volume of agar medium at a technical minimum of 0.85 ml instead of the standard 2.0 ml per dish resulted in about 90% analyzable nuclei.

Table 1. *Results from karyotyping and in situ hybridization procedures in single-cell suspension preparations and in the semisolid assay of bone marrow cells of a healthy donor and acute myeloid leukemia patients.*

				cell suspension					semi solid assay					
									aggregates[a]	single cells in agar				
case no	diagnosis	karyotype	probe for chromosome	ISH signals/nucleus 0	1	2	3	4		ISH signals/nucleus 0	1	2	3	4
1	normal bone marrow	46 XY	#1	1	5	93	1	0	disomy (n = 50)[b]	2	4	94	0	0
			#8	0	4	95	1	0	disomy (n = 50)	3	3	92	2	0
2	AML	46 XY / 44,XY,-1,-5, -7,-17 +mar1,	#1	0	71	28	1	0	monosomy (n = 35)	0	88	12	0	0
		+mar2 +2xmar3	#8	0	9	91	0	0	disomy (n = 23)	0	8	90	2	0
3	AML	46,XY / 44,XY, 5, 6,	#1	0	6	94	0	0	disomy (n = 50)	0	6	93	1	0
		+8, +mar1	#8	0	1	5	94	0	trisomy (n = 64)	11	4	12	73	0
4	AML	polyploid 92-96 chromosomes XX (n = 6)	#1	0	5	33	18	44	disomy (n = 38)	0	2	32	16	50

[a] for evaluation see Materials and Methods
[b] number of aggregates (colonies >50 cells, clusters 20-50 cells) analyzed

For the immunological detection assay, fluorochromes or peroxidase-conjugated markers could be applied to detect the biotin-labeled probes The evaluation of ISH results by brightfield microscopy was preferred because of the permanency of the staining. Furthermore, fluorescent techniques are hampered by autofluorescence from the remaining agar.

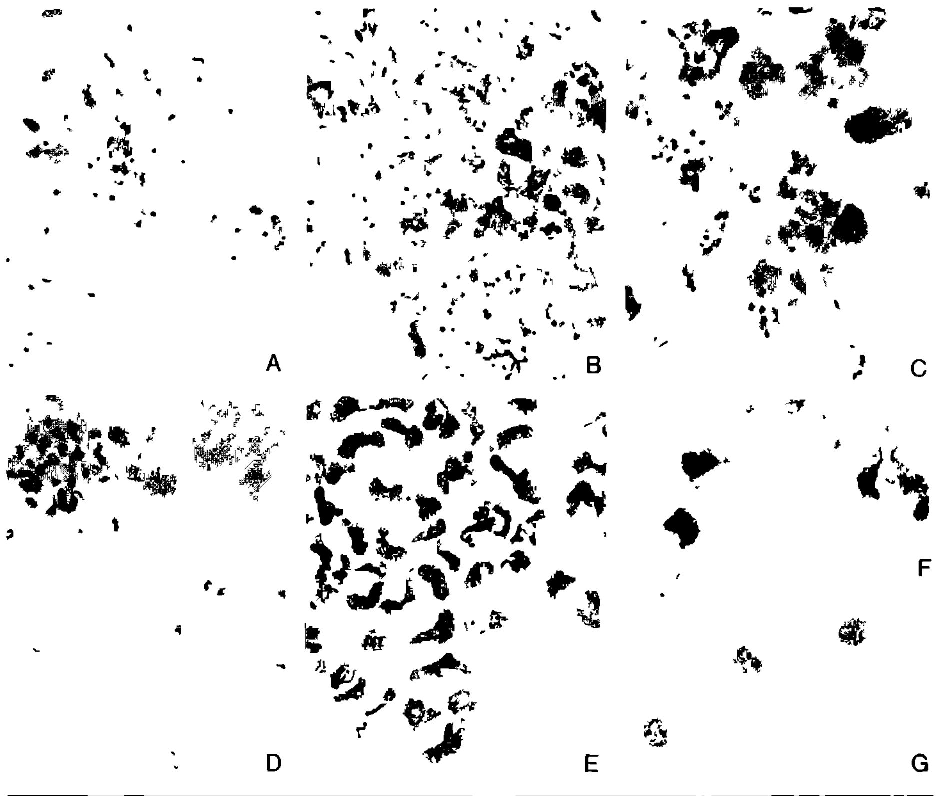

Figure 1. Application of the in situ hybridization procedure to in vitro cultured progenitor cells from patients with AML (A) Almost all clonogenic cells of case 2 demonstrate one ISH signal per cell for chromosome 1, as detected by ISH on single cells and by karyotyping (B) Part of an aggregate from normal bone marrow (case 1) showing two ISH signals per nucleus (C) Three ISH signals per cell for the chromosome 8 centromeric-associated DNA probe were detected in case 3, in which a trisomy 8 was karyotyped (D) The cytological architecture of the agar assay remains intact (E, F and G) Case 4 was karyotyped as polyploid The aggregates (E) demonstrated two ISH signals per nucleus, whereas single cells in the agar could be recognized showing three (G) or four (F) ISH signals with the chromosome 1 probe

Experimental results

The ISH results of the bone marrow samples with the probes for chromosome 1 and 8 are summarized in Table 1

Normal bone marrow cells from case 1 showed two ISH signals for both centromeric-associated probes (93% for chromosome 1 and 95% for chromosome 8) in the cells from the suspension preparation We also detected a low frequency of cells with one ISH signal for these two probes (5% and 4%) All the aggregates screened (n=50) in the semisolid assay contained a majority of cells with two ISH signals (Figure 1B) Within aggregates, about 10% of the nuclei showed one signal as a result of overlap of ISH signals No differences in this respect were seen between chromosome 1 and chromosome 8.

ISH on the interphase nuclei of the cells from the case 2 suspension preparation with a monosomy for chromosome 1, according to its karyotype, showed one ISH signal per nucleus in 71% and two ISH signals in 28% Analysis of the semisolid assay showed that the majority of cells (about 80%) in the aggregates (n=35) contained one ISH signal per nucleus for chromosome 1 (Figure 1A) A low percentage of cells (<10%) was found with two ISH signals The single cells in agar, however, showed 12% of the nuclei with two ISH signals and 88% with one ISH signal Karyotyping revealed no chromosomal aberration for chromosome 8 Hybridization of the cells from the suspension preparation and the semisolid assay with a chromosome 8 specific probe showed frequency distributions similar to the hybridization results for normal bone marrow cells

The nuclei of case 3 with a trisomy for chromosome 8 (46,XY/ 44,XY,-5,-6,+8, +mar1) showed three ISH signals in 94% of the nuclei in the cell suspension pre-paration. The majority of the nuclei in the aggregates (n=64) contained three ISH signals (Figure 1C), while the nuclei of the single cells in the agar demonstrated three ISH signals in 73% and two ISH signals in 12% Furthermore, in this case 11% of the single cells in the agar showed no signal at all Even after repeated hybridizations, a low frequency of nonreacting cells was observed The results of hybridization with the chromosome 1-specific probe were comparable to those of normal bone marrow cells

ISH on the interphase cells of case 4 with a polyploid karyotype (female, 92-96 chromosomes) showed for chromosome 1 two (33%), three (18%) and four (44%) ISH signals in the single cell suspension preparation The single cells in agar also demonstrated the same distribution of ISH signal counts (see Table 1), showing three and four ISH signals (Figures 1, F and G) The main population of cells within aggregates showed two ISH signals (Figure 1E) Within aggregates, however, a low percentage of cells with three or four ISH signals was observed (<20%)

Discussion

The approach described in this study demonstrates the application of the ISH technique for the determining chromosomal aberrations in hematopoietic progenitor cells cultured in a

semisolid assay

The distribution of the ISH signals in the interphase cells of the cell suspension preparations was shown to be similar to those of the single cells in agar in all four cases This means that these cells are representative of the total cell population In cases 2 and 4, a mixed cell population, according to the number of ISH signals for the probe for chromosome 1, was observed The cells in the aggregates in each of these cases, however, revealed one type of ISH signal In case 2, a monosomy and in case 4, a disomy could be detected, suggesting monoclonality of the progenitors

All the analyzed aggregates of cases 2 and 3 contained the clonal marker (monosomy 1 and trisomy 8, respectively) and no disomic aggregates were assessed Therefore, they all confirmed the leukemic origin In case 4, which had a polyploid karyotype, the aggregates were diploid The aggregates demonstrated a mixed population, however, predominantly diploid cells with a few polyploid cells A possible explanation is that the large colonies overlapped the aneuploid cells in this case This may be the result of the high plating concentration ($8x10^5$ cells per dish) that was required to obtain the colonies

The major advantage of the ISH approach over karyotyping is that not only metaphase spreads, but also interphase cells can be examined for chromosomal aberrations Therefore, at least 90% of the cells can be analyzed Another advantage of the ISH approach is that the cytological architecture of the agar assay remains intact (Figure 1D) This makes it possible to analyze small clusters containing about 5 to 40 cells, by previous techniques (Dubé et al, 1981), only large colonies could be analyzed Furthermore, the aggregates and single cells in agar can be analyzed individually This makes it possible to discriminate between the clonogenic and non-clonogenic cells in a mixed population The main advantage of ISH over the G6PD technique is that heterozygous females are not the only patients who can be monitored for the detection of progenitor cells

ISH, using a specific chromosomal aberration as a marker, can now be applied in addition to cytogenetic analyses to determine the leukemic origin of in vitro cultured cells Furthermore the ISH technique enables the origin of blood and bone marrow progenitor cells in patients with myeloproliferative disorders to be determined at different stages of the patients' disease Bone marrow can be maintained in culture for at least 10 days to serve as an autograft in patients with intensive chemo radiotherapy It is assumed that normal stem cells survive this period better than leukemic stem cells (Coulombel et al, 1985) The ISH technique facilitates the detection of numerical chromosome aberrations in progenitor cells to monitor the in vitro culture system

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Chapter four

Interphase Cytogenetic Analysis of Progenitor Cells in Patients with Acute Myeloid Leukemia and Myelodysplasia

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Abstract

Interphase cytogenetics was applied to investigate bone marrow (BM) cells, peripheral blood (PB) cells, and in vitro cultured progenitor cells of five patients with acute myeloid leukemia (AML) and myelodysplasia Recently, a new technique was developed to perform in situ hybridization (ISH) on agar cultures Two patients with respectively trisomy 8 and polyploidy as ISH marker were studied both at presentation and during remission. At presentation, the in vitro growth pattern of one leukemia consisted of small clusters, while the other demonstrated a hypoplastic growth pattern In both cases, the ISH marker was not present in aggregates Therefore, despite the abnormal growth patterns, the cultured progenitors could have been residual normal cells Alternatively, they could have originated from a preleukemic clone with a normal karyotype In both cases, a low percentage (maximally 6%) of abnormal BM and/or PB cells was detected with ISH during remission. Consequently, the remissions in these patients could have been partially or completely clonal

One patient with trisomy 10 as ISH marker was analyzed during myelodysplastic phase and after progression to AML On both occassions, abnormally appearing clusters were cultured However, only part of the clusters carried trisomy 10 The presence of a subclone characterized by trisomy 10 and an abnormally growing (pre)leukemic clone without trisomy 10 may explain this observation

Monosomy 1 and 17 were respectively used as ISH markers in two other AML patients. In one case, typed as M6, the percentage of BM cells with monosomy 1 was much higher (71%) than the amount of blasts (28%) This indicated that at least part of the normoblasts belonged to the abnormal clone, suggesting a multipotent stem cell origin In vitro, both leukemias exhibited a leukemic growth pattern of small clusters ISH confirmed the leukemic origin of these progenitors Long-term liquid cultures of these leukemias were performed for 10-20 days In both cases, no residual normal clonogenic cells could be detected Therefore, the selective growth advantage of normal progenitor cells in long-term marrow cultures cannot be demonstrated in all patients with leukemia

This paper illustrates the usefulness of the ISH technique to study the diverse biology of AML during preleukemic phase, active disease, remission, and under in vitro culture conditions

Introduction

Acute myeloid leukemia (AML) is considered to be the result of a clonal expansion of one single cell (Fialkow et al, 1981, Raskind et al, 1987) The origin of the leukemic clone may be either a totipotential hematopoietic stem cell (HSC) or a cell restricted to the granulocyte monocyte pathway (Fialkow et al, 1981, 1989, Messner et al, 1986, Raskind et al, 1987) AML is characterized by an accumulation of immature blasts that fail to

differentiate to functional granulocytes or monocytes Normal hematopoiesis is suppressed either by displacement of the stem cells by leukemic cells, by production of inhibitory factors, or directly cellular inhibition (Griffin et al, 1986, Messner et al, 1986) In myelodysplastic syndrome (MDS), the abnormal preleukemic clone is assumed to arise at stem cell level (Jacobs, 1990) MDS is characterized by dysmyelopoiesis and cytopenia in one to three cell lines (Raymakers et al, 1991) In time, the disease may progress to overt acute leukemia (Jacobs, 1990) Observations suggest that (pre)leukemic clones may be organized in a fashion similar to normal hematopoiesis, with a hierarchy of progenitor cells (CFU-L) generating large numbers of non-proliferating leukemic cells (Messner et al, 1986) The CFU-Ls, the putative leukemic stem cells, are held responsible for the maintenance of leukemia (Laitha, 1981) This property makes the leukemic progenitors an interesting population for further characterization In the majority of AML/MDS cases, progenitor cells can be cultured in vitro (Griffin et al, 1981, Laitha, 1981, Van Der Lely et al, 1991, Raymakers et al, 1991) The origin of these clonogenic cells may be difficult to assess, since colonies derived from (pre)leukemic progenitors are not always morphologically distinguishable from those derived from normal progenitors (Fialkow et al, 1987)

Several techniques are developed to determine the clonal origin of hematopoietic cells For example, in heterozygous females, the X chromosome linked enzyme glucose-6-phosphate dehydrogenase (G6PD) or restriction fragment length polymorphisms (RFLP) of other X-chromosomal enzymes can be used as a genetic marker (Fialkow et al, 1981, Pui et al, 1989, Raskind et al, 1987) Karyotyping and surface marker expression analysis offer another approach (Dubé et al, 1981, Raskind et al, 1987, Gerhartz et al, 1990) Progenitors of AML patients have been examined with these techniques both at presentation and clinical remission Mostly, at presentation the myeloid colonies derive from the malignant clone Occasionally, the myeloid colonies originate from normal stem cells (Fialkow et al, 1987) During remission, in some patients restoration of nonclonal hematopoiesis and repopulation of the marrow by normal stem cells has been observed These normal stem cells must have been present during the initial phase of the leukemia, but the suppression of their proliferation or differentiation along the granulocytic pathway in vivo prevented detection (Fialkow et al 1981) Clonal remissions have been observed the abnormal clone persists throughout complete clinical remission (Gerhartz et al, 1990, Fialkow et al 1989, 1991)

The results obtained with long-term bone marrow culture (LTBMC) of BM cells from AML patients additionally support the presence of residual normal stem cells in leukemic patients during active disease In most cases, the leukemic colonies and clusters are no longer detectable after one to four weeks of culture, whereas normal progenitors reached near normal numbers (Coulombel et al, 1985, Chang et al, 1986, 1989, Singer et al, 1988) The few reports about progenitors in MDS suggest the existence of a transformed stem cell either with or without an abnormal karyotype (Raskind et al, 1984, Jacobs, 1990)

Recently, we described a method to determine chromosomal aberrations by ISH in myeloid progenitor cells cultured in agar (Poddighe et al, 1993) The major advantage of

this technique is that numerical and structural chromosomal aberrations can be detected in interphase nuclei (Poddighe et al, 1991, 1992). Furthermore, the cytological architecture remains intact, allowing individual analysis of colonies and single cells in culture.

The present study describes the application of this ISH technique to determine the clonal origin of blood and bone marrow cells and myeloid progenitors in five patients with AML and MDS at different stages of their disease.

Patients and Methods

Patient selection

Patients with de-novo AML or AML after a myelodysplastic phase characterized by a numerical chromosomal aberration were selected for this study. AML and MDS were diagnosed according to the French-American-British (FAB) classification (Bennett et al, 1982, 1985)

Case 1

In February 1988, a 52-year-old man was diagnosed to have an AML, type M4. The relevant clinical and hematological data are shown in Table 1. The patient received combination induction chemotherapy He achieved complete remission (CR). Subsequently, he was treated with 2 intensive consolidation courses He relapsed in May 1992 and died of infectious complications during remission induction therapy.

Case 2

After a history of inflammatory bowel disease for 10 years, a 34-year-old woman suffered from a severe exacerbation of ulcerative colitis in March 1987. She was treated with prednison and sulfasalazine In the following weeks (April) she developed a leucocytosis with a pronounced shift to the left. The percentage of blasts in the bone marrow was 36% (see Table 1) One week later, a second bone marrow aspiration was performed, revealing only 2% blasts. The white blood cell count (WBC) had normalized as well, although myelopoiesis was still shifted to the left Despite specific therapy, the colitis deteriorated and a subtotal colectomy was performed. The condition of the patient improved and her blood counts normalized completely The reversible blood and bone marrow abnormalities were diagnosed retrospectively as a leukemoid reaction In September 1987, the patient presented with an increased bleeding tendency and she was found to have an AML, type M4 She received combination induction chemotherapy and achieved CR. In December 1988, just prior to a second consolidation course, she was still in CR and an autologous bone marrow transplant (ABMT) was planned. In February 1989, bone marrow was aspirated from the posterior iliac crests and cryopreserved in liquid nitrogen. Unfortunately, she relapsed one month later and died before ABMT could be performed.

Table 1. Clinical data

Case No	Date	Disease Status	Age (yr)	Sex	WBC (10^9/l)	Hb (mmol/l)	Platelets (10^9/l)	Blood % Blasts	BM % Blasts	FAB Type
1	2/88	presentation	52	M	10.5	6 4	26	3	85	M4
	5/91	CR			8 6	7 5	173	0	0	
	11/91	CR			5 4	6 9	155	1	NA	
2	4/87	colitis/ "leukemoid reaction"	34	F	43 6	7 7	236	2	36	M4?
	4/87 (1 week later)	colitis/ "leukemoid reaction"			4 6	7 1	266	0	2	
	9/87	presentation			46 9	7 4	23	42	80	M4
	12/87	CR			7 5	7.1	274	0	3	
	2/88	cryopreservation			6.7	7 8	140	0	2	
3	12/91	MDS	54	M	7 3	7 1	30	2	6	RAEB
	5/92	presentation			24.7	5 7	12		33	M2
4	5/89	presentation	60	M	21 6	5 7	16	27	28	M6
5	2/89	presentation	58	M	8 6	5 6	29	73	75	M2

Abbreviations. M, male; F, female; WBC, white blood cells; Hb, hemoglobin; BM, bone marrow; FAB, French-American-British classification; NA, not available.

Case 3

In November 1990, this 54-year-old man was diagnosed to have a MDS, type refracting anemia In October 1991, his disease had progressed to refracting anemia with excess of blasts (RAEB), and in May 1992 to AML, type M2 The patient was treated with poly-chemotherapy and died of bleeding during aplasia The clinical and hematological data of this patient are presented in Table 1

Case 4

In May 1989, a 60-year-old man developed an AML, type M6, three months after he was diagnosed to have a MDS, type RAEB Clinical and hematological data are shown in Table 1 The patient received polychemotherapy, but failed to achieve CR and died.

Case 5

In February 1989, a 58-year-old man was diagnosed to have AML, type M2. Five months earlier, he had presented with a MDS, type RAEB-t (RAEB in trans-formation). The clinical and hematological data concerning this patient are depicted in Table 1. For remission induction, he was treated with combination chemotherapy. Due to cardiac and infectious complications, the patient died during aplasia

Collecting of Cells, Cryo-Preservation, and Thawing

Bone marrow cells were collected in sterile buffered acid-citrate dextrose, pH=7.0. Peripheral blood cells were collected in preservative-free heparin After centrifugation on a Ficoll (1085 g/ml) density gradient, part of the collected cells were cryopreserved and another part prepared for ISH. Vials containing 5 to 20 x 10^6 cells in Iscove's medium (Flow Laboratories, Irvine, Scotland), supplemented with 10% fetal calf serum (FCS, heat inactivated, HyClone, Logan, Utah), 50 IU/ml penicillin, 50 μg/ml streptomycin (both Flow Laboratories) and 10% dimethylsulfoxide were frozen in liquid nitrogen using a temperature-controlled freezer (Kryo 10, Planer Biomed, Sunbury, Middlesex, UK). Just prior to the experiments, cells were thawed in a 37°C waterbath, resulting in a recovery of >90% Freezing and thawing procedures have been described in detail elsewhere (Van de Ouweland et al, 1982) For ISH, cells were fixed in 70% ethanol (-20°C), and stored at -30°C

Human Recombinant Growth Factors

Human recombinant interleukin 3 (IL-3) and human recombinant granulocyte-monocyte colony-stimulating factor (GM-CSF) were kindly donated by Sandoz BV (Uden, The Netherlands) Human recombinant granulocyte colony-stimulating factor (G-CSF) was a kind gift from Behring (Marburg, Germany) Final concentrations of 40 ng/ml, 20 ng/ml, and 5 ng/ml, respectively, were used and resulted in plateau stimulation

Clonogenic Assay (CFU C)

Cells were cultured in Iscove's medium, supplemented with 20% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 0 3% bacto-agar (Difco, Detroit, Michigan) The cells were stimulated with the combination of IL-3, GM-CSF, and G-CSF. Duplicates were cultured in 35- x 10-mm culture dishes (Costar, Cambridge, Massachusetts) at 37_C in a fully humidified atmosphere containing 5% CO_2 In order to make the cells better accessible for the DNA probes, a reduced volume of 0 85 ml per dish was plated (Poddighe et al, 1993) After 7 days, the total number of clusters (5-40 cells) and colonies (>40 cells) was counted The in vitro growth pattern was defined normal when the number of colonies exceeded 20 per 2 x 10^5 NC and the cluster to colony ratio did not exceed five When less colonies were cultured, but with a normal cluster to-colony ratio (less than five), the growth pattern was considered hypoplastic All other growth patterns were regarded leukemic Mostly, a pattern of small (maximum size, 20 cells) or large (maximum size, 40 cells) cluster formation was observed After counting, the agar cultures were dried by cytocentrifugation and processed for ISH or stained with hematoxylin solution (Merck, Darmstadt, Germany) to evaluate morphology (see below)

Liquid Culture

Cells were suspended in Iscove's medium supplemented with 20% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin and a combination of IL 3, GM-CSF, and G-CSF The liquid cultures were maintained in an incubator (37°C, 5% CO_2 and fully humidified atmosphere) for a period up to 20 days At regular intervals, part of the cells were collected, washed, and used for clonogenic assay and ISH

Drying Agar Cultures by Cytocentrifugation

Conventionally processed agar cultures have a relatively thick agar layer which prevents sufficient penetration and subsequent hybridization of DNA probes Therefore, a method based on a technique described by Baines (1989) was used The cultures were shaken into glucose-phosphate buffer and cut into quarters Each quarter was lifted onto a 76- x 26-mm poly-L lysine (Sigma Chemical Co St Louis Missouri, MW 150 000-300 000) coated glass slide and covered with a cellulose acetate strip (Sepraphore, Gelman Sciences, Ann Arbor, Michigan) Next, a piece of filter paper (Schleicher & Schuell, Dassel, Germany) was overlaid, followed by a filter card (Shandon, Pittsburgh, Pennsylvania) During 30 to 60 minutes the cultures were allowed to dry partially Thereafter, the slides were centrifuged in a cytospin centrifuge (Shandon) at 1,500 rpm for 10 minutes Subsequently, the filters were removed A thin agar layer, containing the cells, remained on the glass slide

Table 2. Bone Marrow and Blood, cases Nos. 1, 2, and 3

Case No	Date	Disease Status	Cell Sample	Suspension: Karyotype	Suspension: ISH	Semi-solid: ISH Colonies/Clusters	Semi-solid: ISH Single Cells	Semi-solid: CFU-C* Colonies/Clusters
1	2/88	presen-tation	BM	46,XY/47,XY,+8 (n=1/12)#	trisomy 8 (71%)	disomy 8 (n=17)@	trisomy 8 (14%)	0/89
	5/91	CR	BM	46,XY (n=32)	trisomy 8 (6%)	disomy 8 (n=21)	trisomy 8 (19%) tetrasomy 8 (4%)	99/34
	5/91	CR	PB	no metaphases found	NA	disomy 8 (n=18)	trisomy 8 (12%) tetrasomy 8 (2%)	33/20
	11/91	CR	PB	no metaphases found	NA	disomy 8 (n=18)	trisomy 8 (7%)	42/20
2	4/87	colitis/ leukemoid reaction	BM	46,XX (n=4)	trisomy 1 (1%) tetrasomy 1 (3%)	disomy 1 (n=17)	trisomy 1 (2%) tetrasomy 1 (6%)	12/21
	4/87 (1 week later)	colitis/ leukemoid reaction	BM	46,XX (n=5)	NA	NA	NA	68/76
	9/87	presen-tation	BM	polyploid (about 96 chromosomes, n=6)	trisomy 1 (18%) tetrasomy 1 (44%)	disomy 1 (n=38)	trisomy 1 (16%) tetrasomy 1 (50%)	4/5
	12/87	CR	BM	46,XX (n=9)	NA	NA	NA	114/112
	2/88	cryopre-servation	BM	46,XX/polyploid (n=14/2)	trisomy 1 (2%) tetrasomy 1 (3%)	disomy 1 (n=39)	trisomy1 (1%) tetrasomy 1 (1%)	140/14
3	12/91	MDS	BM	46,XY/47,XY,+10 (n= 24/8)	trisomy 10 (26%)	disomy 10 (n=6) trisomy10 (n=14)	trisomy10 (35%)	1/116
			PB	NA	trisomy 10 (14%)	disomy 10 (n=18) trisomy10 (n=17)	trisomy10 (37%)	1/43
	5/92	presen tation	BM	no metaphases found	trisomy 10 (16%)	disomy 10 (n=23) trisomy10 (n=7)	trisomy 10 (8%)	18/2160

Karyotyping

Metaphase spreads were obtained from BM or PB cells after culturing in RPMI 1640 (Flow Laboratories) for 1 or 24 hrs Colcemid was present during the last hour of the culture Before fixation in methanol glacial acetic acid (3 1), the cells were exposed to a hypotonic solution (0 075 M KCl) for 15 minutes Slides were prepared according to routine cytogenetic procedures Karyotyping was performed using the GTG technique.

In Situ Hybridization

For the detection of the target sequences on chromosomes 1, 8, 10, and 17, the plasmid DNA probes pUC1 77 (Cooke et al, 1979), D8Z2 (Donlon et al, 1986), p10 1 (Devilee et al, 1988), and p17H8 (Waye et al, 1986) were used, respectively ISH was performed on single cells and dried agar cultures, fixed in 70% ethanol as described elsewhere (Poddighe et al, 1993) Five μl of a cell suspension were dropped onto a poly-L lysine coated glass-slide, air dried, and heated at 80°C for 1 hour For a good penetration of the DNA probes and antibodies, a proteolytic digestion step with pepsin (P7000 Sigma) was performed at a concentration of 100 μg/ml in 0 01 M HCl for 20 min at 37°C After several dip washes in H_2O and phosphate-buffered saline (PBS), the nuclei were postfixed in 4% formaldehyde in PBS for 20 min at 4°C Of the hybridization mixture (60% formamide-2xSSC-10% dextran sulphate, pH 7 0, probe concentration 1 ng/μl), 10 μl were applied under a coverslip. Denaturation of target DNA and probe was carried out simultaneously at 70°C for 2 5 min in a moist chamber Hybridization was performed for 2 to 16 hours at 37°C After three stringent washes in 60% formamide-2xSSC, pH 7 0, at 42°C, and subsequent washes in 2xSSC, pH 7 0 at 42°C for five minutes each, the hybridized probes were detected by horseradish peroxidase (HRP) conjugated avidin (Dakopatts, Glostrup, Denmark) Finally, the DNA probe was visualized with 0 5 mg/ml 3,3-diaminobenzidin tetrahydrochloride (DAB, Sigma), 0 65% imidazole (Merck), 0 015% H_2O_2 (Merck), at pH 7 8 in PBS As a control, the samples were hybridized with a chromosome-specific DNA probe for which no chromosomal aberration was detected In these controls, the cells from suspension preparations demonstrated one ISH signal for a target chromosome in 5 to 10% of the cells, and three ISH signals in less than 1% of the cells Of these suspension preparations, 200 nuclei per slide were counted

The agar preparations were evaluated by counting 100 single cells in agar, and approximately 50 aggregates In these controls, at least 90% of the cells contained ISH signals The percentages of cells containing the euploid number of ISH signals for the control DNA probe-targets varied between 85-90% A detailed description of the evaluation of the ISH results has been presented elsewhere (Poddighe et al, 1991, 1993)

Results

Case 1

Presentation

At presentation, 12 of the 13 analyzed BM cell metaphases showed trisomy 8 (see Table 2). With ISH, trisomy 8 was found in 71% of the BM cells. A leukemic growth pattern was observed when BM cells were cultured in agar. The clusters consisted of 5 to 25 immature myeloid cells. After hybridization with the probe for chromosome 8, disomy was found in all analyzed clusters, whereas 14% of the single cells in the semi-solid culture had trisomy 8.

Complete remission

In May 1991, karyotyping revealed only normal BM cells, whereas no metaphases could be detected in the PB. However, by ISH, 6% of the BM cells showed trisomy 8. Both BM and PB exhibited a normal in vitro growth pattern. The colonies appeared morphologically normal and were diploid when analyzed with the probe for chromosome 8. However, part of the single cells in the semi-solid cultures contained three ISH signals. In the BM culture respectively 19% and 4% of the single cells had trisomy and tetrasomy for chromosome 8, whereas the single cells of the PB cultures showed trisomy and tetrasomy for chromosome 8 in 12% and 2%, respectively.

In November 1991, the patient was still in clinical CR although his Hb had decreased to 6.9 mmol/l. Only PB was analyzed. No metaphases were obtained. ISH was not performed by lack of a sample. A normal growth pattern of the cells was observed, demonstrating a disomy 8 by ISH in all aggregates, while part of the single cells in culture had trisomy 8 (7%).

Case 2

At leukemic presentation, all investigated BM metaphases appeared to be polyploid, containing about 96 chromosomes (see Table 2). For ISH analysis, we applied a centromere associated DNA probe for chromosome 1.

Colitis/"leukemoid reaction"

During "leukemoid reaction", on two occasions a normal karyotype was found in a limited number of BM metaphases (see Table 2). In contrast, ISH revealed that 4% of the BM cells contained three or four ISH signals with the chromosome 1 probe. The number of colonies in the semi-solid culture was depressed on one occasion and normal one week later. Morphology and size of the colonies appeared normal. All analyzed colonies were disomic, whereas 8% of the single cells in agar contained three or four ISH signals.

Presentation

During overt leukemia, when all analyzed BM metaphases were polyploid, tri- or tetrasomy 1 was found by ISH in 62% of the BM cells. Only very few colonies and

clusters could be cultured in agar. They appeared to have normal morphology and size and were disomic for chromosome 1 after ISH. Sixty-six percent of the single cells in those cultures were tri- or tetrasomic.

Complete Remission

During CR in December 1987, only BM cells with a normal karyotype were found. Normal numbers of colonies and clusters could be cultured in vitro. Since no material was cryopreserved, ISH could not be performed. In February 1988, when bone marrow was aspirated for cryopreservation and the patient was still in CR, both normal and polyploid cells were detected by chromosomal analysis. ISH revealed tri- or tetrasomy 1 in 5% of the BM cells. Normal colony numbers were observed. Their morphology and size appeared also normal. All analyzed colonies were disomic, whereas 2% of the single cells in the semi-solid culture showed tri- or tetrasomy.

Case 3

MDS

Karyotyping of the BM revealed that eight of 32 analyzed metaphases had trisomy 10 (see Table 2). With ISH 26% of the BM cells had trisomy 10. In the PB cells, on which no karyotyping was performed, 14% showed trisomy 10 by ISH. Both BM and PB cells showed a leukemic growth pattern characterized by clusters consisting of 10 to 35 immature

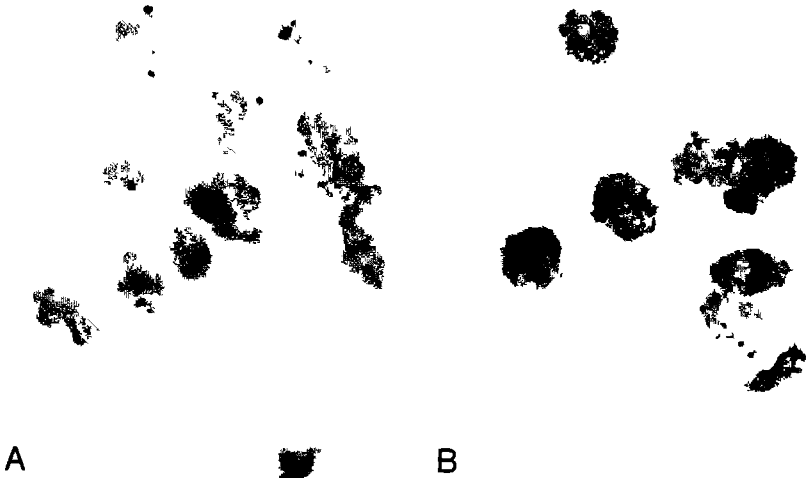

Figure 1. Examples of ISH results to in vitro cultured progenitor cells from patients with AML. (A) represents a small cluster of clonogenic cells of case 5 (after 20 days of liquid culture), demonstrating one ISH signal per cell for chromosome 17 (B)represents a small cluster of clonogenic cells of case 3 at presentation, demonstrating three ISH signals per cell for chromosome 10.

Table 3. Bone marrow cases Nos. 4 and 5

		Suspension		Semi-solid		
Case No	Days of Liquid Culture	Karyotype	ISH	ISH Colonies/Clusters	ISH Single Cells	CFU-C* Colonies/Clusters
4	presention	46,XY/44,XY,-1,-5,-7, -17,-20,-21,+mar1, +mar2,+2xmar3 (n=2/34)#	monosomy 1 (71%)	monosomy 1 (n=35)@	monosomy 1 (88%)	0/380
	day 10	NA	monosomy 1 (91%)	monosomy 1(n=43)	monosomy 1 (91%)	0/180
5	presention	46,XY/43,X,3q,+8,+10, -5,-6,-12,-13,-17,-22,-Y +mar1-3 (n=1/9)#	monosomy 17 (96%)	monosomy 17 (n=42)	monosomy 17 (82%)	0/5400
	day 5	NA	monosomy 17 (99%)	monosomy 17 (n=38)	NA	0/5100
	day 10	NA	monosomy 17 (96%)	monosomy 17 (n=55)	NA	0/11000
	day 14	NA	monosomy 17 (95%)	monosomy 17 (n=27)	NA	0/4500
	day 20	NA	monosomy 17 (96%)	monosomy 17 (n=26)	monosomy 17 (84%)	0/900

Abbreviations ISH, in situ hybridization, NA, not analyzed
Number of mitosis, @ Number of analysed aggregates, * Number of CFU-C per 2×10^5 NC

cells. Although morphologically not distinguishable, both clusters with and without trisomy 10 were observed by ISH in the BM and PB cell cultures. The single cells in the BM and PB cultures demonstrated a trisomy for chromosome 10 by ISH in 35% and 37%, respectively.

Presentation

At leukemic presentation, no BM metaphases were detected. By ISH, 16% of the BM cells showed a trisomy 10. The clusters appeared morphologically identical to the clusters cultured during MDS phase. Trisomy 10 was found in seven of 30 clusters (Figure 1A), whereas 8% of the single cells in culture showed trisomy 10.

In the following two cases, BM was cultured in liquid in the presence of IL-3, GM-CSF, and G-CSF for up to 20 days to investigate whether residual normal progenitors had a growth advantage compared to the leukemic clone.

Case 4

Presentation

At leukemic presentation, karyotyping of BM cells revealed multiple abnormalities in almost all metaphases (see Table 3). The probe for chromosome 1 was selected as a marker for ISH. Monosomy 1 was found in 71% of the BM cells. In vitro, a leukemic growth pattern was observed. The small clusters (5 to 20 cells) appeared immature. All analyzed clusters and 88% of the single cells showed monosomy 1.

Liquid culture

Liquid culture for 10 days did not significantly influence the number of abnormal cells and clusters. Ninety-one percent of the cells in suspension demonstrated monosomy 1. Again, only immature appearing small clusters could be cultured, which were all monosomic for chromosome 1. The single cells in the semi-solid assay showed in 91% monosomy 1.

Case 5

Presentation

During active disease, one of 10 BM metaphases was normal. The other nine metaphases showed multiple chromosomal aberrations (Table 3). In this case, monosomy 17 was chosen as ISH marker. Monosomy 17 was detected by ISH in 96% of the BM cells. In the clonogenic assay, a leukemic growth pattern was observed. The clusters consisted of five to 25 immature cells and showed all a monosomy for chromosome 17. Moreover, in 82% of the single cells in the semi-solid assay a monosomy 17 was detected.

Liquid culture

The high plating efficiency allowed in liquid culturing of the leukemic cells up to 20 days. Liquid culture cells and agar cultures (both single cells and clusters) were analyzed at regular intervals. During the whole period, the proportion of abnormal cells remained

essentially the same (95% to 99%) At any time, only aggregates with monosomy 17 were detected by ISH, as is demonstrated in Figure 1A

Discussion

Five patients with de novo AML or AML after myelodysplastic phase and abnormal clones characterized by numerical chromosomal abnormalities were subject of this study In all cases, the chromosomal aberrations found with cytogenetics could also be detected by ISH However, the incidence of abnormal cells was not always the same for both methods Conventional cytogenetic analyses examines fewer cells than ISH and may result in a less accurate estimation of the real number of abnormal cells Furthermore, it can be performed solely to dividing cells, which can lead to a selective growth advantage for the abnormal subpopulation (Dube et al, 1981) Non dividing interphase lymphocytes are included in the ISH analysis and cannot be analyzed by karyotyping The lymphoid lineage is usually not a part of the abnormal clone and thus may account for the lower incidence of abnormal cells found with ISH (Raskind et al, 1987)

A leukemic growth pattern was observed in case no 1 at presentation, but the ISH marker for the abnormal cells (trisomy 8) was not detected in the cells of the clusters These clusters could be normal progenitor cells which exhibited an abnormal growth pattern probably due to influences of the leukemic clone In the majority of reported cases, myeloid progenitors are part of the leukemic clone at presentation However, in a few patients normal CFU-GM has been detected (Fialkow et al, 1987) If the clusters in case no 1 did originate from normal cells, then the in vitro growth pattern and morphology by itself are not sufficient to determine the origin of progenitor cells The inaccuracy of morphology has been described before by Singer et al (1988), where LTBMC of leukemic BM resulted in morphologically normal colonies which nevertheless belonged to the leukemic clone An alternative explanation for our results could be that these progenitor cells represent a part of the leukemic clone without trisomy 8 This concept can be confirmed by a multistep leukemogenesis with a preleukemic stage an early step causes clonal proliferation, and a later step results in a chromosomal abnormality in descendants of these progenitors (Pui et al, 1989, Fialkow et al 1991)

During CR, abnormal cells were detected by ISH in both BM and PB cells of case no 1 A growth advantage for the clonogenic cells with a normal karyotype and the lower number of analyzed cells may explain why karyotyping detected only normal BM cells The absence of metaphases in the PB demonstrated one of the limitations of cytogenetic analysis In vitro normal appearing, disomic colonies could be cultured, but part of the single cells were abnormal, containing three or four ISH signals The number of ISH signals is constant during the whole cell cycle, and in normal diploid cells the number of cells with four ISH signals is maximally 1% (Poddighe et al, 1991) Therefore, the detected tetrasomy 8 should be considered significant, suggesting an additional chromosomal

aberration Since the differential counts of both BM and PB did not contain blasts, and blood consisted only of segmented neutrophils (81%), monocytes (11%), and lymphocytes (8%), apparently differentiated cells must have had trisomy 8. This suggests that morphologically normal differentiated blood cells developed from the abnormal leukemic clone. Similar observations have been made in G6PD and RFLP studies (Fialkow et al, 1987). At least two and possibly three populations were present in this patient during remission. One population of trisomy 8 cells capable of maturation and another colony-forming population without trisomy 8. This latter population could either have originated from residual normal stem cells, a preleukemic clone, or a combination of both Conse-quently, the remission might have been partially or completely clonal.

In case no 2, 4% abnormal BM cells, containing three and four ISH signals, were observed at the time of "leukemoid reaction". This suggests that the leukemic clone was already present at that time The diagnosis leukemoid reaction has to be considered incorrect. The activation of the BM as a physiological response to the colitis apparently lifted the number of abnormal cells above the detection level of ISH By cytogenetic analysis at presentation, only cells with an abnormal karyotype were found, whereas ISH revealed 62% polyploid cells The same explanations as given in case no 1 can be applied to this observation One month before relapse, at the time of cryopreservation, abnormal cells were detected with both ISH and cytogenetic analysis, indicating the presence of the leukemic clone. In contrast to the colitis period, a higher number of metaphases could be karyotyped This probably explains why in this occasion cytogenetic analysis revealed abnormal cells as well

Since in case no 2 the ISH marker was not detected in the cells of colonies and clusters cultured at the different stages of the disease, these cells may have originated from residual normal progenitors. The depressed colony number observed during leukemoid reaction and full-blown situation could indicate that in vivo the number of normal clonogenic cells was reduced It also may have been the result of inhibition by the leukemic cells or the concomitant prednison/sulfasalazine medication As in case no. 1, it can not be excluded that these progenitors originated from a preleukemic, abnormal clone without polyploidy. As a result, the remission in this patient may be partially or completely clonal.

In case no. 3 the progression from MDS to AML could be observed. During MDS, the percentage of BM cells with trisomy 10 found by karyotyping and ISH were the same, probably since a considerable number of cells could be analyzed by both techniques. The abnormal but similar appearing clusters could be distinguished in two subpopulations, consisting of progenitors with and without trisomy 10 However, no increase in either BM cells or progenitor cells with trisomy 10 was observed when the patient developed AML. Only the plating efficiency of cultured progenitors increased This observation matches the theory that MDS arises from a transformed progenitor cell and progresses from a preleukemic state to overt leukemia by successive genetic changes (Raskind et al, 1984; Jacobs, 1990) In that case, all cultured clusters belong to a (pre)leukemic clone with abnormal

growth characteristics, but only part of this clone obtains a chromo-somal aberration (trisomy 10)

In case no 4, the percentage of abnormal BM cells as detected by ISH was much higher than the percentage of BM blasts, but still lower than the karyotyped incidence Due to overlap of ISH signals, an overestimation of monosomy of maximally 10% can occur (Poddighe et al, 1991) This can never explain completely the observed difference between the number of cells with one ISH signal for chromosome 1 and the percentage of blasts. In this BM, 28% blasts and 50% normoblasts were present Considering the high percentage of monosomy 1 (71%), this implicates that at least part of the normoblasts belonged to the abnormal clone This suggests that the origin of this clone was a multipotent progenitor cell The 60-year-old patient developed AML after a period of MDS, which is assumed to originate at stem cell level (Griffin et al, 1981) Furthermore, it has been reported that especially in elderly patients leukemias may arise from stem cells with multipotent differentiative expression (Fialkow et al, 1987)

The cultured clusters from case nos 4 and 5 contained the same ISH marker as found in the total blast population This was in concordance with the observed leukemic growth pattern and undifferentiated appearance of the clusters, and confirmed the leukemic origin of these myeloid progenitors Although in both cases part of the BM cell metaphase spreads and interphase nuclei appeared normal, no residual normal clonogenic cells could be detected after long-term culture in liquid In the majority of reported cases, long-term marrow cultures provide a selective growth advantage for normal progenitor cells But in some AML cases, like in cases nos 4 and 5, persistence of the leukemia has been observed (Coulombel et al, 1985, Chang et al, 1986, 1989, Singer et al, 1988)

Forty to 70% of the AMLs and 25 to 50% of the MDSs have numerical chromosomal abnormalities (Yunis, 1984, Jacobs et al, 1986), indicating that a substantial number of patients with AML and MDS can be analyzed by ISH using centromere associated DNA probes Moreover, probes against fusion genes, like the bcr-abl gene, have become available (Tkachuk et al, 1990) This may further increase the number of patients which can be studied by ISH techniques By performing ISH on agar cultures, the cytological architecture remains alo intact This enables individual analysis of aggregates and single cells in the culture Sample errors are avoided since removal of colonies is not necessary In contrast to the conventional cytogenetic techniques, not only large colonies but also small clusters can be analyzed Furthermore, it is less time consuming to investigate large numbers of aggragates Therefore, ISH may help to clarify the complex biology of MDS and AML both during full blown situation and clinical remission In addition, it can be applied to monitor in vitro culture systems and in vitro purging of autologous bone marrow grafts

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Detection of Numerical Chromosome Aberrations in Bladder Cancer by In Situ Hybridization

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Abstract

The nuclear DNA content of 53 transitional cell carcinomas (TCCs) of the urinary bladder, as determined by flow cytometry (FCM), was compared with chromosome ploidy as detected by nonradioactive in situ hybridization (ISH). For this purpose, probes for repetitive DNA targets in the (peri)centromeric region of chromosomes 1 and 18 were used. Hybridization results with both probes of 35 TCCs, which had a DNA index of approximately 1.0 as concluded from FCM, showed evident chromosome 1 aberrations in approximately 25% of the tumors, and in a few cases an aberration for chromosome 18 was detected. Comparison of the ISH spot numbers for both chromosomes showed in most cases a higher number for chromosome 1 than for chromosome 18. ISH on 18 cases of TCCs, which showed a single peak in FCM with a DNA index of 1.2 to 3.2, exhibited a profound heterogeneity. In these TCCs the ratio between chromosomes 1 and 18 varied over a wide range, resulting in cases showing more hybridization signals for chromosome 1 than for chromosome 18 or the opposite. Furthermore, using ISH minor cell populations showing polyploidization and giant cells containing numerous ISH signals could occasionally be detected. Results showed that interphase cytogenetics by ISH enable a fast screening of numerical chromosome aberrations and detection of different cell populations within one tumor, which was apparently homogeneous according to FCM.

Introduction

Because the DNA index is a prognostic parameter, flow cytometry (FCM) has become a method for rapid and objective screening of malignant tumors (Barlogie et al, 1983; Tribukait et al, 1986, Merkel et al, 1987, Smeets et al, 1987). However, by FCM the total DNA content of a cell nucleus is estimated and no information about the specific chromosome aberrations is obtained. Chromosomal analysis is described as a more objective criterion of the malignant potential of, for example, bladder cancer (Sandberg, 1977; Summers et al, 1981, Wijkstrom et al, 1984; Kovacs, 1985; Atkin et al, 1985, 1986; Tribukait et al, 1986, Babu et al, 1987, Smeets et al, 1987, Vanni et al, 1988), although it should be mentioned that cytogenetic studies of transitional cell carcinomas (TCCs) showed a positive correlation between the modal chromosome number and the DNA index as measured by FCM (Tribukait et al, 1986; Smeets et al, 1987).

Chromosome analysis of solid tumors is possible with or without previous culturing of the tumor cells. Culturing may result in a selective growth of cells and changes in chromosomal material. Direct analysis is frequently hampered by the small number of recognizable metaphases and cannot be performed routinely.

Recently it was demonstrated that in situ hybridization (ISH) using chromosome-specific probes allows the detection of chromosome aberrations at the cellular level (Cremer et al, 1986, 1988a, 1988b, Hopman et al, 1986a, 1988, Devilee et al, 1988; Nederlof et al,

1989) Because such ISH analyses can be performed on nonmitotic cells, it is generally referred to as interphase cytogenetics (Cremer et al, 1986) and has become a powerful tool in the study of numerical and structural chromosome aberrations in tumor cells (Cremer et al, 1988a, 1988b, Hopman et al, 1988, Nederlof et al, 1989) In this study ISH DNA probes recognizing highly repetitive sequences in the centromeric region of chromosomes were applied to a series of TCCs, which had been analyzed previously by FCM We applied a probe specific for chromosome 1 (Cooke et al, 1979) because for this chromosome nonrandom numerical aberrations were described in several tumor types (Atkin, 1985) Furthermore, a probe for chromosome 18 (Devilee et al, 1986) was used, because this chromosome is not specifically or nonrandomly affected in bladder cancer (Teyssier, 1989)

Materials and Methods

Patient Material

Sixty TCC specimens were analyzed, of which ten were collected after chemotherapy or radiotherapy Tissues were obtained after transurethral resection One part of each tumor was used for histopathologic diagnosis, whereas the other part was used for FCM and ISH studies The tumors were collected in 10 ml RPMI 1640 (Flow Laboratories, Ltd, Irvine, CA) medium containing 17% fetal calf serum (Flow Laboratories), 50 μg gentamycin, 50 μg penicillin, and 50 μg streptomycin/ml Single-cell suspensions of fresh tissues were prepared as previously described (Smeets et al, 1987) In brief, the tissues were mechanically disaggregated by scraping and cutting in a Petri dish and filtered through a 100 μm nylon filter (Ortho Diagnostic Systems, Beerse, Belgium) The filtered cell suspensions were fixed in 70% ethanol (-20 °C) and stored at 30 °C The cell suspensions can be stored for more than 3 years without noticeable differences in the ISH results

Flow Cytometry

DNA analysis was performed using a Cytofluorograph 50H (Ortho Instruments, Westwood, MA) The pellet obtained after centrifugation of the ethanol-fixed cells was suspended in propidium iodide (PI, final concentration 20 mg/l in 150 mM sodium phosphate buffer, pH 7 4) The fluorochrome PI was excited at 488 nm with an argon laser (Spectra Physics, Mountain View, CA), and PI fluorescence was measured using a 630 nm pass filter The DNA content is expressed as the DNA index The TCCs were qualified 2C, 3C, 4C, and >4C, 2C DNA index = 0 9 to 1 1, 3C DNA index = 1 2 to 1 7, 4C DNA index = 1 8 to 2 2, >4C DNA index = >2 2 Chicken red blood cells were used as an internal standard whereas human lymphocytes were used as the external standard

Tumor Cell Processing for In Situ Hybridization

The suspensions that were not immediately used had been stored from a few months to

several years Cells fixed in ethanol were postfixed in methanol/acetic acid (3/1; four times, 5 minutes at 0°C), and spotted onto poly-L-lysine-coated slides Optionally, the cytoplasm was partly removed by a 70% acetic acid wash (Hopman et al, 1988), for improvement of DNA probe and antibody penetration A second procedure that was applied and that gave an optimal recovery of cells and proper removal of cellular protein was proteolytic digestion of the cells before ISH For this purpose, 5 μl of a suspension was dropped onto the slides, air-dried, and then digested with pepsin (Burns et al, 1986) from porcine stomach mucose (2500 to 3500 units per mg protein, Sigma Chemical Co , St. Louis, MO) at a concentration of 40 to 200 μg/ml in 0 01 M HCl for 10 min at 37°C. After subsequent dip washes in H_2O and phosphate-buffered saline (PBS) and fixation in 4% paraformaldehyde in PBS for 5 minutes at 0°C, the slides were dehydrated, air dried, and heated at 80 °C for 30 minutes

Metaphase spreads of human lymphocytes, ethanol fixed T24 Bubeník et al, 1973) and Molt-4 (Minowada et al, 1972) cells were used as controls for the detection of disomy, trisomy, and tetrasomy for the chromosome 1 and chromosome 18 probe

DNA Probes and Nonradioactive Labeling

The DNA probe for chromosome 1 (pUC 1 77, indicated as probe 1c) has been described by Cooke and Hindley (1979) and recognizes a tandem repeat of 1 77 kb in the (peri)centromeric region (1q12) The probe for chromosome 18 (L1 84, probe 18c) has been described by Devilee et al (1986) and recognizes a tandem repeat in the centromeric region of chromosome 18 Biotinylation of the probes (Langer et al, 1982) was performed using Bio-11-dUTP (Enzo, New York, NY) in a nick translation reaction as described by Brigati et al (1982) Mercuration of the probes was performed by using the mercury(II) acetate method described by Hopman et al (1986b, 1988)

In general, when double target ISH was performed the chromosome 1 probe was biotinylated, whereas the chromosome 18 probe was mercurated

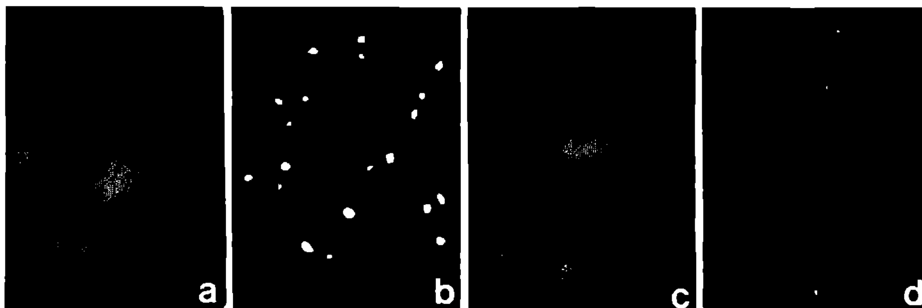

Figure 1. In situ hybridization on the bladder tumor cell line T24 with probe 1c (b) and probe 18c (d) (a,c DNA counterstaining with DAPI)

In Situ Hybridization (ISH)

The probes were hybridized (in single- and double-target ISH) in 60% formamide, 2 x SSC of pH 5.0 (0.3 M NaCl, 30 mM Na-citrate), 1 mM KCN and 1 μg/μl salmon sperm DNA as carrier DNA, at a probe concentration of 2 to 6 ng/μl hybridization mixture. Under these stringency conditions, hybridization to minor binding sites was avoided. Of the hybridization mixture, 5 μl was added to the slides and sealed with rubber cement under a coverslip (18x18 mm). Denaturation was performed at 70 to 80°C on a heating plate during 2.5 minutes. Hybridization was performed overnight at 37°C. Posthybridization washings and fluorescent detection of hybrids were performed as previously described (Hopman et al 1986c, 1988). Evaluation and counting of fluorescent ISH signals were done according to criteria as described before (Hopman et al, 1988). Microphotographs were taken with a Leitz Dialux 20 EB microscope (Leitz Wetzlar, Germany), equiped for FITC, TRITC, and DAPI fluorescence using Tmax Kodak 400 ASA film (Kodak, Rochester, NY). Exposure times were approximately 2 minutes for FITC fluorescence, approximately 40 seconds for TRITC fluorescence, and 1 second for DAPI fluorescence. Evaluation of the preparations was performed by counting approximately 200 nuclei per slide. With respect to the interpretation and statistical analysis of the ISH preparations, it must be stated that in all samples nuclei not reacting with the DNA probes were seen. These negative nuclei were not counted and therefore did not contribute to the data mentioned in the text and figures. When the cell population with negative nuclei exceeded 15%, the procedure was regarded as suboptimal and, therefore, the data from seven of 60 cases could not be used.

Results

Technical aspects

The ISH procedure was tested on human lymphocytes, the bladder carcinoma cell line T24, and the leukemic tumor cell line Molt-4, which showed a maximum of two, three, and four ISH signals per nucleus, respectively. These cells were used to develop proper cell preparation techniques and to optimize the specificity and sensitivity of the ISH procedure. In Figure 1a through d, examples of ISH on T24 with probes 1c and 18c are shown, indicating that both probes detect the three DNA targets per nucleus. When the ISH procedure was performed as described in Materials and Methods, at least 80% of the interphase nuclei of the lymphocytes, the T24 cells, and the Molt-4 cells reacted as expected. In the remaining 20% of the cells, mainly lower copy numbers for both chromosomes were found. This percentage varied between the two different procedures used. The least reproducible results were obtained when the methanol/acetic acid postfixation step was used. The reproducibility was highest when a proteolytic digestion step was included in the procedure to remove cytoplasmic and nuclear proteins. For this purpose, an optimal pepsin concentration had to be established to obtain a maximal penetration of DNA probes and antibodies and, thus, a maximal ratio between the nuclei showing the real copy number and

nuclei with lower copy numbers For a statistical analysis of human lymphocytes and T24 cells hybridized with several DNA probes, see Hopman et al (1988, 1989) The optimalization of the proteolytic digestion step for Molt-4 cells has also been described before (Hopman et al, 1989) The cells from the solid TCCs were isolated by mechanical disaggregation technique and fixed in 70% ethanol As a result of this technique, some cytoplasm was left around the nucleus This remainder can give rise to a high autofluorescent background, which is higher when the storage time is increased A low fluorescent ISH reaction was noticed in these cases, and this was most pronounced in those cells having the nucleus partly covered by cytoplasm To remove the cytoplasm and part of the nuclear proteins two procedures were followed

1 Postfixation of the samples with a methanol/acetic acid mixture and preparation of the microscopic slides by dropping the cell suspension onto glass slides, which was sometimes followed by dipping in acetic acid

2 Dropping of the cell suspension onto glass slides and proteolytic digestion with pepsin Coating of the slides with poly L-lysine, fixation with paraformaldehyde and heating as described in Materials and Methods were found to be essential for retention of the cells on the glass slides, as well as for optimal preservation of nuclear morphology and DNA staining

The latter approach was the most reproducible one with respect to removal of protein and, thus, for penetration of the probe or for exposing the DNA target for reaction Furthermore, in general it gave the best morphology and the most discrete ISH signals, with a high fluorescence intensity Cells fixed with methanol/acetic acid were often flat compared to the pepsin-treated cells Microscopic examination of pepsin-treated cells was, however, difficult, and counting of ISH signals needed focusing at different levels of the nucleus With respect to the pepsin treatment of the cells, it can be stated that cells with a higher DNA content and larger nuclei generally needed lower enzyme concentrations to obtain optimal hybridization signals than cells with smaller nuclei For practical reasons we normally used the highest concentration of pepsin (50 to 200 μg/ml) still having an acceptable nuclear morphology With respect to the appearance of the ISH signal, we noted split-spots and blurred spots next to the more discrete signals Examples of such a deviating spot morphology are shown in Figure 2b, Figure 3b, f, and m, and Figure 4d We earlier listed the criteria for the evaluation of such signals (Hopman et al, 1988) To analyze the variations that have been introduced into the final result by different observers and between different experiments, we performed comparative studies with three observers that analyzed two different ISH incubations of 21 TCCs Figures 5A and B show examples of a diploid and an aneuploid TCC analyzed by the three observers The results obtained by two observers in two independently repeated experiments on the same 21 TCCs are compared in Figure 5C This plot illustrates the limited variability in the detection of aneuploid cells that occurs among different experiments, even when only 200 (randomly chosen) nuclei per tumor are examined

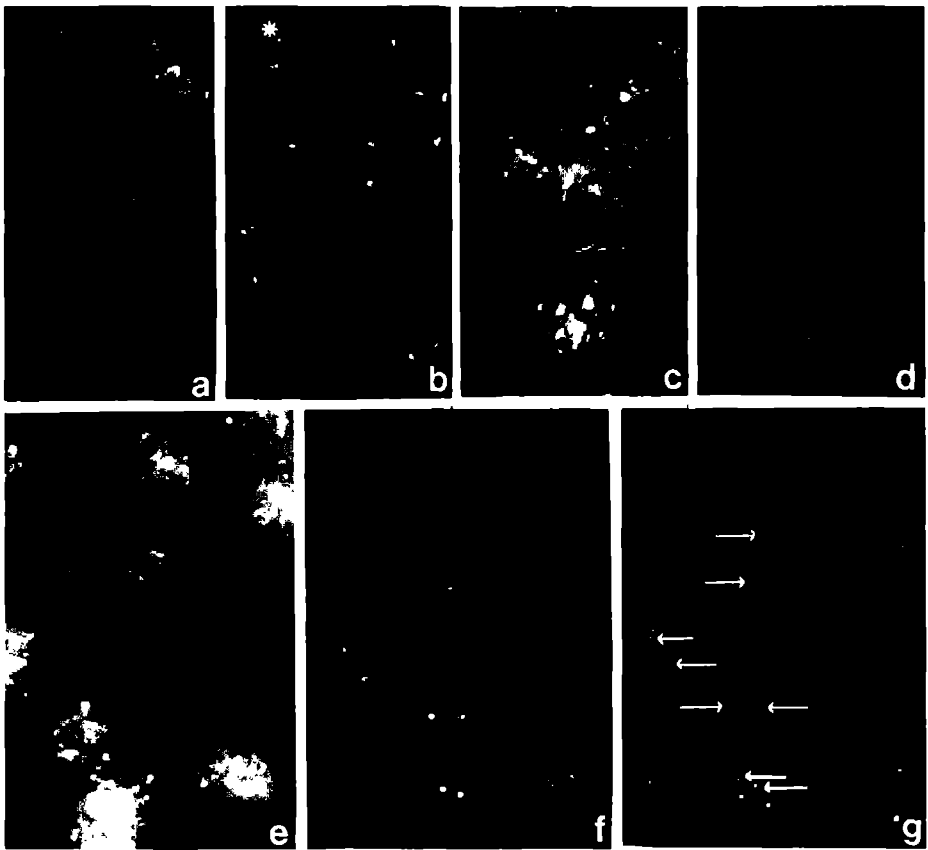

Figure 2. Single- and double-target ISH on a 2C diploid bladder tumor. ***a-d****: Single-target ISH on isolated bladder tumor cells with probe 1c (****b****) and probe 18c (****d****). Asterisk in b indicates a split or paired spot for probe 1c.* ***e-g****: Double-target ISH of the same tumor showing DNA staining with DAPI (****e****), TRITC-fluorescence signals of probe 1c (****f****), and FITC-fluorescence signals of probe 18c (****g****). Overlap of the TRITC-fluorescence signals in the FITC channel is indicated by the arrows in g. (****a****,* ***c*** *and* ***e****, DNA counterstaining with DAPI.*

In Situ Hybridization on Cell Suspensions of TCCs

2C TCCs (35 Cases)

These tumors had a single peak in FCM and a DNA index of 0.9 to 1.1. The expected number of ISH signals for both probes in the cells with a normal DNA content (DNA index = 1.0) was two, as is the case with normal human lymphocytes. Our ISH results in these

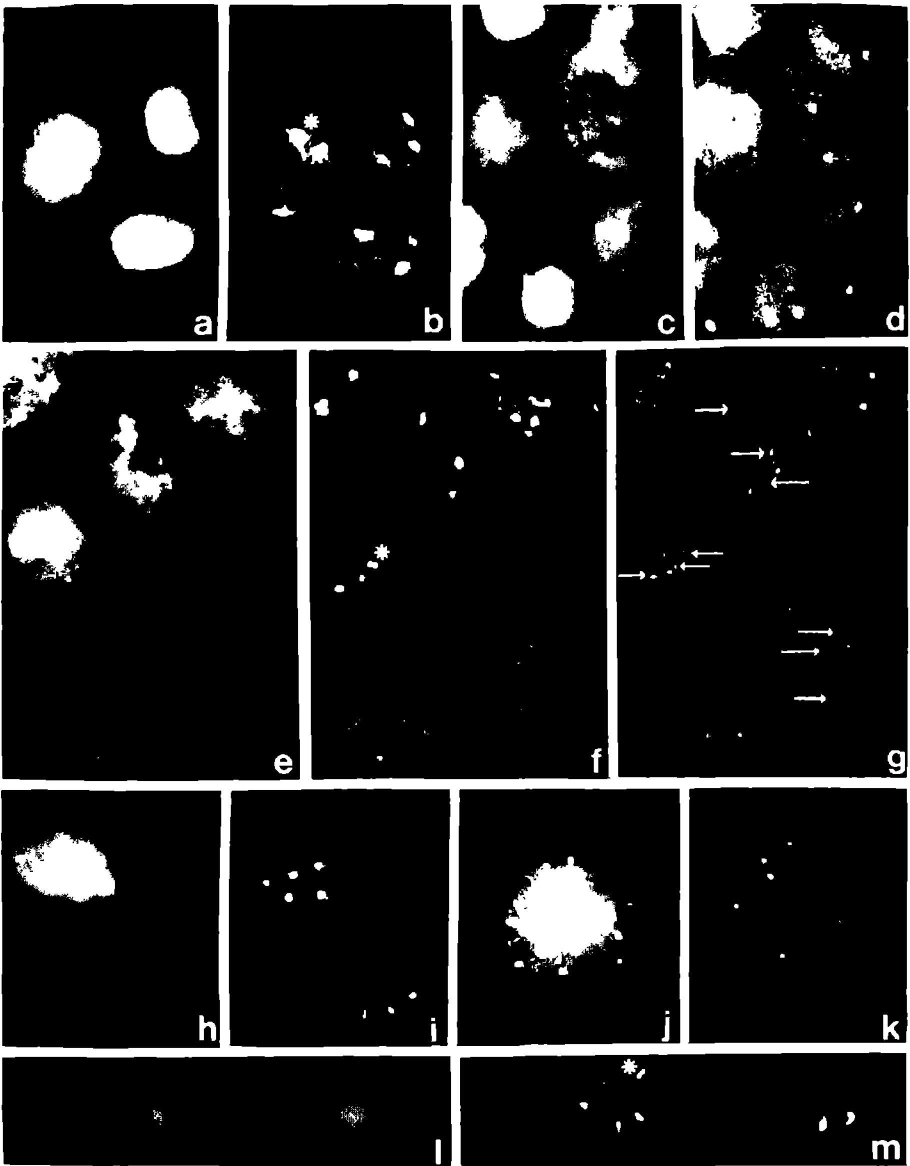

Figure 3. Single- and double-target ISH on 2C bladder tumors showing numerical chromosome aberrations. ***a-d:*** *Single-target ISH on a bladder tumor (DNA index, 1.0) with probe 1c* ***(b)*** *and probe 18c* ***(d)***. ***e-g:*** *Double-target ISH on the same tumor showing DNA staining with DAPI* ***(e)***, *probe 1c ISH signals* ***(f)***, *and probe 18c hybridization* ***(g)***. *Overlap of the TRITC-fluorescence signals in the FITC channel is indicated by the arrows in g.* ***h*** *and* ***i:*** *Cell nucleus showing five ISH signals for probe 1c next to a cell nucleus with three ISH signals within the same tumor cell suspension for probe 1c.* ***j*** *and* ***k:*** *Giant cell within this suspension showing multiple spots for probe 1c.* ***l*** *and* ***m:*** *Example of a 2C bladder tumor with a tetrasomy for probe 1c. Asterisk in b indicates blurred spots and asterisks in f and m indicate examples of split spots. (****a, c, e, h, j,*** *and* ***l****, DNA counterstaining with DAPI.*

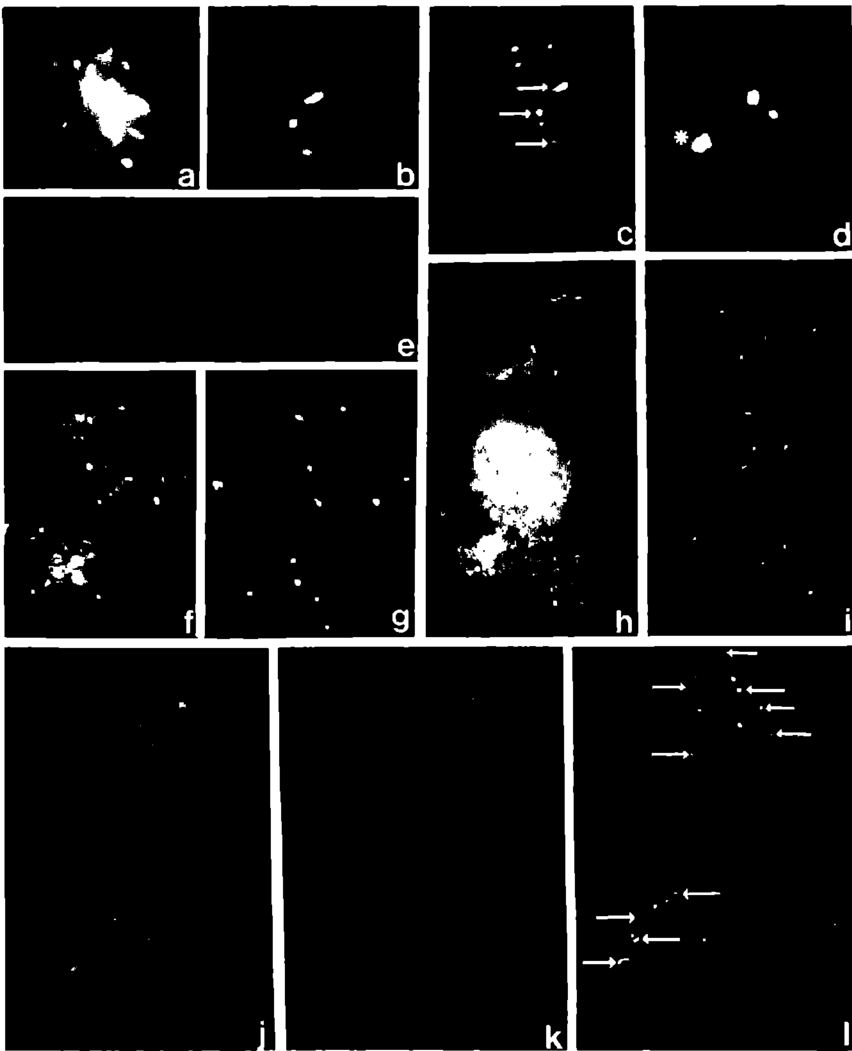

Figure 4. Single- and double-target ISH on 3C and 4C tumors. a-c: Double-target ISH on an aneuploid tumor (DNA index, 1.5) with probe 1c (b) and probe 18c (c). d-i: Single-target ISH on 4C bladder tumors with probe 1c (d, e, and g) and probe 18c (i). j-l: Double-target ISH on the same tumor shown in f to i with probe 1c (k) and probe 18c (l). Overlap of the TRITC fluorescence signals in the FITC channel is indicated by the arrows in c and l. Asterisk in d indicates a large ISH spot as compared to the other two hybridization signals in the same nucleus. (a, f, h, and j, DNA counterstaining with DAPI).

TCCs using probe 1c showed that all tumors contain cell populations with two copies for chromosome 1. However, in nine of these 35 cases, evident additional cell populations (i.e.

comprising more than 35% of the total cell population) were detected showing three or four ISH signals for probe 1c Examples of those cell populations with a disomy, trisomy, and tetrasomy for chromosome 1 are shown in Figures 2 and 3 The 35 analyzable diploid tumors showed different patterns Twenty TCCs showed a major cell fraction (approximately 80% of the cells) with two ISH signals for chromosome 1, and minor cell populations with one (an average of 8% of the cells, range, 2% to 24%), three and four ISH signals (an average of 8% of the cells, range, 2% to 12%) Fifteen TCCs showed two major cell populations, one showing two ISH signals for chromosome 1 (approximately 60% of the cells) and another showing three ISH signals (an average of 28% of the cells; range, 11% to 83%) and a minor population with four ISH signals In four of these tumors the population of cells with four ISH signals exceeded 10% By FCM none of these aneuploid cell populations could be detected in different types of TCCs

Evaluation of ISH signals using probe 18c was possible in 19 of 35 cases of the TCCs analyzed with probe 1c Comparison with the ISH data for probe 18c showed that in two of the cases with a monosomy for chromosome 1 a monosomy for chromosome 18 was also detected, whereas in one of these cases the copy number for 18c was higher In the tumors with a disomy for chromosome 1, the copy number for probe 18c corresponded to that of probe 1c (compare Figure 2b to 2d) By means of the double target ISH procedure we also demonstrated that most of these cells contained two copies of both chromosome targets (Figure 2e-g) Hybridization of probe 18c on tumors with three and four copies for chromosome 1 showed in most cases a higher copy number for probe 1c than for probe 18c Figure 3d illustrates an example of disomy for 18c, whereas the same fraction of cells within this TCC showed a trisomy for probe 1c (Figure 3b) Figure 6a to c depicts the FCM analysis and the frequency distribution of ISH signals for probe 1c and 18c, in both the single- and double-target ISH method, for this tumor The latter procedure proves that in one and the same cell different copy numbers for probe 1c and 18c can be detected This is illustrated in Figures 2g and 3f where an example of this double-target ISH method clearly shows different ploidies Within the same tumor cells with five and six ISH signals were also seen (Figure 3i), and in the double-target ISH we even found one cluster of cells containing six copies for probe 1c and four copies for probe 18c (Figure 3f, g: the cell in the upper right corner) In four TCCs, a fraction of cells was found with four ISH signals for probe 1c (Figure 3m) In these tumors, however, larger fractions with a trisomy for chromosome 1 were also detected In a number of the tumors with a DNA index of 1 0, cells with a very high copy number for probe 1c were found (Figure 3k)

3C, 4C, >4C TCCs (18 Cases)

These tumors had a DNA index between 1 2 and 3 2 as measured by FCM. ISH of probe 1c on these TCCs showed an extensive heterogeneity in the number of hybridization signals, on the one hand between TCCs with a similar DNA index, and on the other hand, within one tumor In eight tumors with a DNA index between 1 2 and 1 7, the copy

number for chromosome 1 ranged between three (Figure 4b) and five, but most of these TCCs contained cell populations (accounting for more than 10% of the total cell population) with both three and four ISH signals per cell (Figure 6d-f) Two of eight TCCs had a major fraction showing chromosome 1 trisomy, whereas one tumor showed mainly tetrasomy for probe 1c.

In the eight TCCs with a DNA index between 1.8 and 2 2, most tumors contained fractions with trisomy, tetrasomy, and pentasomy for chromosome 1. Four of these cases had a major fraction with tetrasomy (Figure 5B). However, within these TCCs cell populations with ISH signals for chromosome 1 ranging from one to six were observed. In one case a monosomy was frequently detected next to the disomic fraction (Figure 4e). In some cells of this case one large ISH signal next to some smaller ISH signals was also observed (Figure 4d).

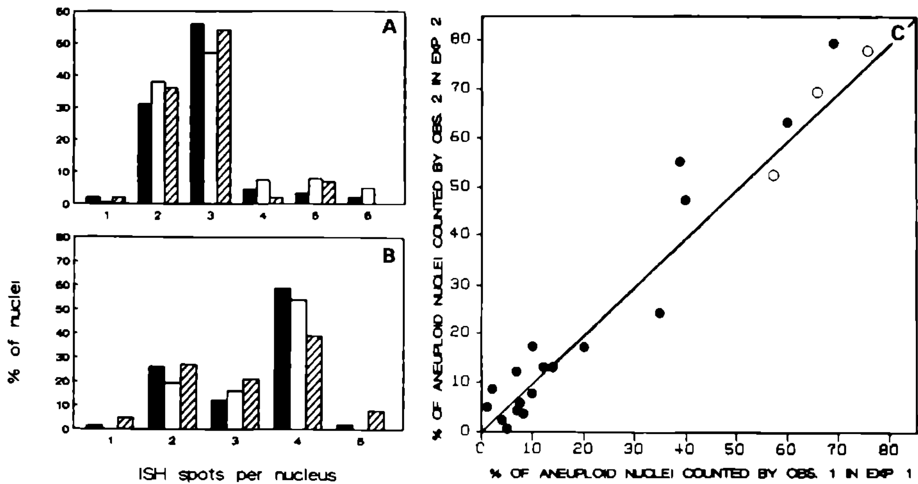

Figure 5. A and B Interobserver variability for the evaluation of in situ hybridization reactions in a diploid (A) and tetraploid (B) transitional cell carcinoma Three different observers analyzed at least 200 nuclei in two experiments The results found by the three individual observers are presented as closed, open and hatched bars C Comparison of the results of two different ISH experiments of 21 tumors analyzed by two independent observers plotted in a scatter diagram. The closed circles indicate the percentage of aneuploid cells in 18 diploid TCCs, as detected by observer 1 in experiment 1 (on the X-axis) and by observer 2 in experiment 2 (on the Y-axis) Similarly, the open circles represent these values for three aneuploid TCCs, indicating a statistically highly significant correlation (correlation coefficient Pearson's r=0 98 with a P < 0 0001) between both experiments

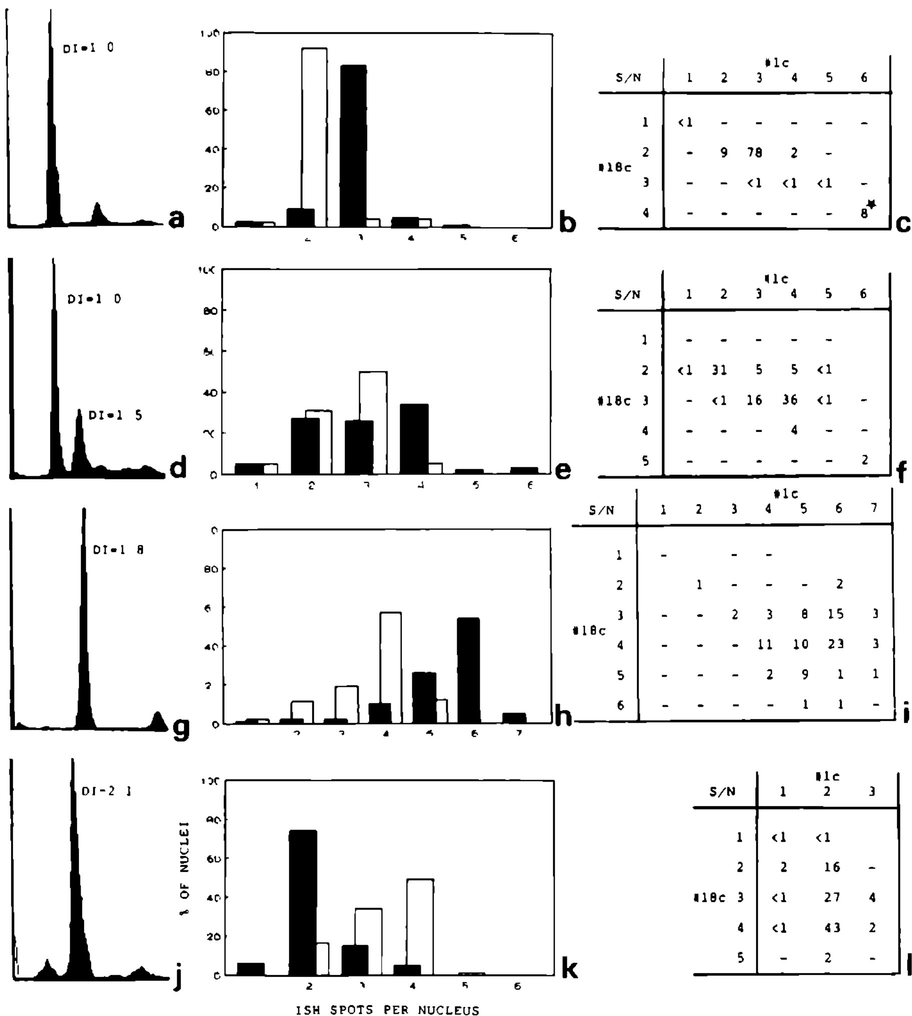

Figure 6. Typical examples of flow cytometric analyses of a 2C (a-c), 3C (d-f), and two 4C (g-l) TCCs and ISH analyses of chromosome 1 and chromosome 18 **a, d, g,** *and* **j** *DNA histogram of TCC cell suspensions stained with propidium iodide and analyzed by flow cytometry* **b, e, h,** *and* **k** *Frequency distribution of the number of ISH signals per nucleus (S/N) after the single-target procedure with probe 1c (closed bars) and probe 18c (open bars) Usually, 100 to 200 nuclei were analyzed* **c, f, i,** *and* **l** *Percentage of cells with the indicated number of ISH signals per nucleus after a double-target ISH with probe 1c (vertical rows) and probe 18c (horizontal rows) Asterisk in c indicates that six spots for probe 1c and four spots for probe 18c were found in a cluster of cells*

Figures 4g and k depict results obtained with the tetraploid TCC also shown in Figure 6g to i, which contained four, five, and six ISH signals, respectively, for chromosome 1. The ISH results with probe 1c of the TCC evaluated in Figure 6j to l (DNA index, 2.1) are

depicted in Figure 7b and f It can be seen that most of these tumor cells show two strong hybridization signals for probe 1c, whereas in this case several minor, less intense ISH signals were also detected It could not be concluded whether these signals represented a specific hybridization with chromosome 1 or minor binding sites on other chromosomes The two tumors with the highest DNA index (2 6 and 3 2) also showed high copy numbers for chromosomes 1 and 18 Figure 7i shows an example of the case with a DNA index of 3 2, illustrating cells with eight and nine ISH signals for probe 1c

The profound heterogeneity observed for chromosome 1 became even more pronounced after application of probe 18c in the double-target ISH Evaluation with probe 18c was possible in 12 of the 18 cases analyzed with probe 1c In five cases studied with probe 18c a heterogeneity similar to that observed with probe 1c was seen, whereas in four cases the number of ISH signals counted for probe 1c was greater than for probe 18c (Figure 6b, d, e, f, h, and i) Examples of such preparations are shown in Figure 7f to i, in which the chromosome 1 number varied between 4 and 6 whereas the copy number for chromosome 18 varied between 3 and 5 Figure 7h to k shows the hybridization results with probe 18c on the TCC with a DNA index of 3 2 and also reveals that in this case the copy number for chromosome 18 is smaller than for chromosome 1 Analyses showed ratios of 8 to 9 and 5 to 6 for probes 1c and 18c respectively Examples of cases with higher spot numbers for probe 18c than for probe 1c are given in Figure 4b and c and Figure 7a to g This latter case is evaluated in Figure 6k, which shows that the spot numbers for probe 1c varied between 2 and 3 whereas the spot numbers for probe 18c varied between 2 and 4

Discussion

Earlier studies showed that probes to repetitive DNA sequences located predominantly in the (peri)centromeric region of one particular chromosome will, under proper hybridization conditions, exclusively reveal their targets as distinct spots in the interphase nucleus (Cremer et al, 1986, Devilee et al, 1988, Hopman et al, 1988, Nederlof et al, 1989) In this study we showed that nonradioactive ISH in combination with two of these repetitive DNA probes (for chromosomes 1 and 18) can detect numerical chromosome aberrations in cases of solid bladder tumors, i e , transitional cell carcinomas (TCCs) This approach provided a simple means for the rapid detection and statistical analysis of numerical chromosome aberrations in interphase tumor cells and allowed us to evaluate and detect individual tumor cells and tumor cell populations within TCCs

A number of technical items needed for the technique need discussion Single cell suspensions prepared by a mechanical disaggregation method from fresh tumor material followed by fixation in ethanol were stored for up to several years and still could be used for ISH Treatment of the cells by postfixation in methanol/acetic acid or by pepsin digestion enables the evaluation of approximately 90% and 70% of the tumors with probes 1c and 18c, respectively Evaluation of the ISH signals by counting the number of hybridi-

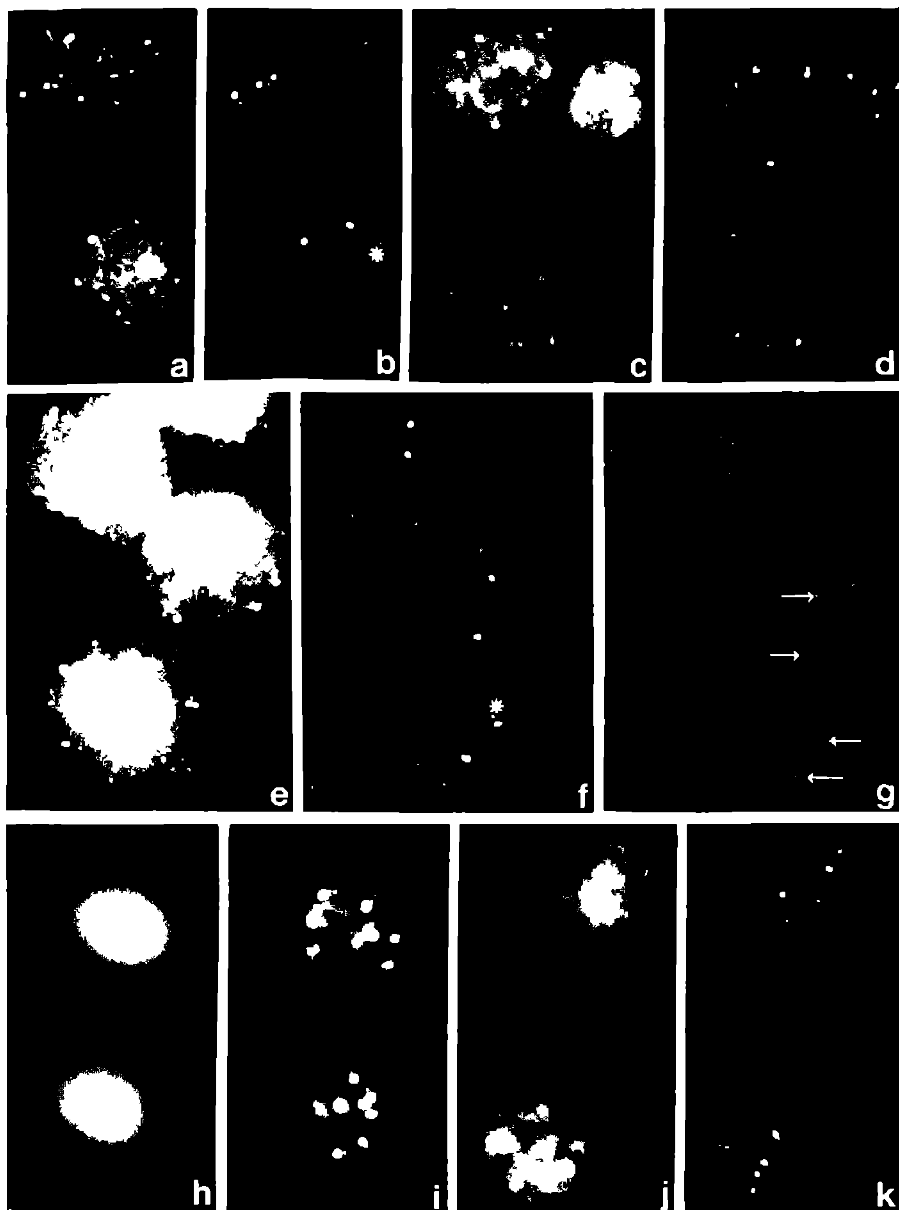

Figure 7. *Single- and double-target ISH on a 4C and a >4C bladder tumor* ***a-d*** *Single-target ISH on 4C tumor with probe 1c* ***(b)*** *and probe 18c* ***(d)*** ***e-g*** *Double-target ISH on the same tumor with probe 1c* ***(f)*** *and probe 18c* ***(g)*** *Overlap of the TRITC fluorescence signals in the FITC channel is indicated by the arrows in g* ***h-k*** *Single-target ISH on a >4C tumor (DNA index, 3 2) with probe 1c* ***(i)*** *and probe 18c* ***(k)*** *Asterisks in b and f probably indicate minor hybridization signals* ***(a, c, e, h, and j,*** *DNA counterstaining with DAPI)*

zation spots is, however, sometimes difficult because of the way the hybridization signals are manifested Paired (or split) spots and a blurred appearance of the spots have been described previously (Devilee et al, 1988, Hopman et al, 1988, Cremer et al, 1988). Part of these patterns may be artifacts resulting from the methanol/acetic acid fixation, the fragile structure of the nucleus of some large tumor cells, or both, which may lead to a disaggregation of the heterochromatin. Mild pepsin digestion or heat denaturation resulted in more distinct signals similar to those seen in ISH studies on paraffin sections (Hopman et al, 1989) We also noticed that in most TCCs a population of cells showed no spots at all or cells showing only one ISH spot for chromosome 1 and 18. It can furthermore be expected that inefficient protein removal may block penetration of probe and antibodies, that paired localization of target DNAs may occur, and that DNA may be lost as a result of overdigestion and the denaturation Therefore, these analyses may underestimate the numerical chromosome aberrations or number of targets Thus it is easier to detect cell populations with an increased numbers of the tested chromosomes than cell populations with chromosome losses On the contrary, clumping of cells during the isolation procedure or the presence of split-spots may result in the counting of apparently higher copy numbers The validity of the ISH method for detecting of numerical chromosome aberrations has been tested in cell lines with known chromosome number In these preparations the published karyotyping results for chromosomes 1 and 18 correspond with the number of detected ISH signals for probes 1c and 18c (Cremer et al, 1986, 1988b). In general, ISH on cell lines resulted in one major peak representing the cells with the real copy number for the individual chromosomes In all cases minor fractions of cells with a lower spot number were counted. Similarly, in diploid TCCs with mainly two ISH signals for chromosome 1 a relative small fraction (<10%) of nuclei with none or one ISH signal for individual chromosomes was always detected next to the major fraction with two ISH signals. However, in several of these malignancies a fraction with higher spot numbers (three and four) was also found From comparisons of the results in the cell lines and normal lymphocytes with the TCCs we are convinced that even these minor fractions in these TCCs with higher spot numbers represent aneuploid cells (see also Figure 5C). Preliminary results of karyotyping and ISH on paraffin sections of the same tumors as described above support this assumption (Hopman et al, to be published).

Thus far no comparative data are available that draw parallels between classic karyotyping and interphase cytogenetics on the same solid tumors In five cases karyotyping data could be obtained concerning chromosomes 1 and 18 In one case the aberration detected by karyotyping was a +1p- This aberration was detected by the ISH as an extra spot with probe 1c (Figure 3b and f) In another case an isochromosome 1q and two normal copies of chromosome 1 were seen by the cytogeneticist ISH occasionally showed one large spot, in some cases consisting of two signals, and two smaller spots (Figure 4d), confirming the cytogenetic data A trisomy for chromosome 18 was detected by ISH in a TCC with a DNA index of 1 0, in which karyotyping showed a dicentric chromosome 18, thus

explaining the extra spot with probe 18c Although these data already demonstrate the validity of the ISH-method, proper evaluation and comparison of these two methods must be performed on a larger scale in the future

TCCs with a DNA index of 0 9 to 1 1, as measured by FCM, all contained a population of cells with two ISH signals for probes 1c and 18c, most probably representing or containing the normal epithelial and stromal cells next to diploid tumor cells In these tumors a monosomy, trisomy, or tetrasomy for probe 1c could frequently be detected, thus demonstrating the profound ploidy aberrations in tumors with an apparently normal DNA content In approximately 25% of these TCCs evident chromosome aberrations were detected The number of 2C tumors with a numerical chromosome 18 aberration was much lower than for chromosome 1 By means of the double-target ISH-method, we nicely demonstrated these numerical differences of chromosomes 1 and 18 in one and the same cell From the foregoing it may be obvious that apparently homogeneous tumor cell populations within one TCC, as concluded from DNA FCM, may show a heterogeneous composition when analyzed by ISH (Figure 5C) Furthermore, cells with a tetrasomy for 1c were seen in these 2C tumors by ISH That these cells are no normal G_2M cells, as measured by FCM, can be detected because the number of the ISH signals for the DNA targets were not doubled during proliferation due to their (peri)centromeric localization Finally, cells with a very high spot number for probe 1c were occasionally found These cells, which probably represent the giant cells as described for TCCs, are most probably formed by polyploidization (Fossa et al, 1976) These cells are normally not detected by FCM because of their low frequency, or cannot be distinguished from cell clusters by this method

In most aneuploid and tetraploid tumors a cell population was also present that showed two ISH signals for probes 1c and 18c, most probably representing the normal epithelial and stromal cells However, in all these TCCs a cell population with an ISH spot number deviating from two was detected for probes 1c and 18c In most tumors this cell population exceeded 30% of the total cell number Comparison of the FCM data with the chromosome copy number detected by ISH showed that single, sharp DNA peaks in FCM contained several populations with different chromosome numbers In FCM, furthermore, proliferating cells in the G_2M phase, which have twice the DNA content of the G_0G_1 cells, cannot be discriminated from the tetrasomic tumor cells ISH can distinguish between these populations, because mitotic cells show the same spot numbers as nonmitotic cells, at least with probes used in this study

In the TCCs with a DNA index greater than 1 2, a large heterogeneity was detected for both probes 1c and 18c This means that within a certain TCC two or more evident cell populations were detected that had different spot numbers for probe 1c and probe 18c. Also, when comparing different TCCs with a similar DNA index, profound differences in copy numbers for both probes were observed Tumors with higher copy numbers for chromosome 1 than for 18 were detected and, on the other hand, tumors with higher copy

numbers for chromosome 18 than for chromosome 1 were seen This was best demonstrated in the double-target ISH It should be stated, however, that the number of probe 18c spots normally correlated better with the DNA index as estimated by FCM than did the probe 1c spot numbers This was perhaps to be expected on the basis of karyotyping results, because chromosome 1 aberrations contrary to chromosome 18 aberrations were reported by several authors in various types of human malignancies This chromosome has frequently been reported to undergo structural along with numerical changes (Sandberg, 1977, Atkin et al, 1985, 1986, Smeets et al, 1987, Devilee et al, 1988, Teyssier, 1989) Breakpoints within this chromosome are mostly situated in or near the centromere heterochromatin region and duplications of a part of the long arm are common (Atkin, 1986) The latter aberrations could result in a doubling of the target for probe 1c (located on the 1q arm) and thus give an extra ISH signal An example of a structural aberration that we observed by our ISH approach is shown in Figure 4d and dealt with an isochromosome 1q In general, chromosome 1 aberrations have been described as late events Our results with the diploid TCCs indicate that such aberrations may also occur in an early stage of malignant transformation Future studies comparing conventional karyotyping data with ISH data on an extended series of TCCs, as well as in other malignancies, should reveal the usefulness of interphase cytogenetics Furthermore, clinical follow-up data must be correlated with the ISH data, to investigate the prognostic value of this technique

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Structural Chromosome 1 Aberrations in Transitional Cell Carcinoma of the Bladder: Interphase Cytogenetics Combining a Centromeric, Telomeric, and Library DNA Probe.

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Abstract

Fluorescence in situ hybridization (FISH) was used to study numerical and structural chromosome 1 aberrations in interphase nuclei of transitional cell carcinomas (TCCs) of the urinary bladder One of the characteristic numerical aberrations, as detected previous-ly in low-grade noninvasive TCCs, included trisomy for chromosome 1 (AHN Hopman et al, Cancer Res 51 644, 1991) We examined in more detail 22 cases with a centromeric (1q12) and a telomeric associated (1p36) DNA probe, and with a library DNA probe from sorted human chromosome 1 in single- and double-target FISH procedures All flow cytometrically determined DNA diploid TCCs (13 cases), which showed three spots for 1q12 (6 cases), had two spots for 1p36 Since the library DNA probe showed three separate domains in the nuclei of these cases, the additional copy for 1q12 could be explained as an extra chromosome 1p-, containing the 1q12 target

In the flow cytometrically DNA tetraploid/aneuploid tumors, the results were more complex In 6 of 9 cases, we observed an overrepresentation of 1q12 as compared to 1p36, also suggesting the presence of extra copies of 1p- chromosomes

The results of the present study demonstrate the utility of the FISH method to assess structural chromosome aberrations in interphase nuclei of solid tumors

Introduction

The malignant potential of transitional cell carcinoma (TCC) of the bladder can be assessed on basis of its extent of invasion (stage) and histological appearance (grade) (Cummings, 1980) Furthermore, quantitative DNA analysis by FCM has provided complementary prognostic information However, the detection of specific chromosome aberrations and minor genomic changes is limited by means of FCM (Smeets et al, 1987a; Koss et al, 1989) Although genetic aberrations can be detected more precisely by karyotyping, for solid tumors it is hampered by difficulties such as a low mitotic index, poor banding quality, and a condensed or fuzzy appearance of the chromosomes Furthermore, the number of cells that can be analysed is extremely limited (Atkin et al, 1985b, Smeets et al, 1987b, Sandberg et al, 1988, Teyssier, 1989)

FISH using repetitive DNA probes that hybridize to the centromeric associated region of a specific chromosome is a powerful technique to study numerical, and in a limited number of cases, structural chromosome aberrations within the interphase nuclei of tumor cells (Cremer et al, 1988a, Hopman et al, 1988a, Nederlof et al, 1989, Raap et al, 1990, Perez Losada et al, 1991) It has been shown that this method of interphase cytogenetics enables routine screening of a large number of tumor cells (Hopman et al, 1989, 1991; Poddighe et al, 1991) and allows the detection of minor cell populations or imbalance in chromosome copy number within one tumor (Hopman et al, 1988b, Devilee et al, 1988, Van Dekken et al, 1990, Poddighe et al, 1992) The application of chromosome in situ

suppression-hybridization using library DNA probes can specifically stain individual human chromosomes to identify chromosomal aberrations. This approach has been used for the rapid detection of numerical aberrations, such as trisomy for chromosome 21 (Lichter et al, 1988b; Pinkel et al, 1988, Fuscoe et al, 1989), and analysis of chromo-some changes in metaphase and interphase cells (Cremer et al, 1988b, Lichter et al, 1988a; Jauch et al, 1990)

Numerical and structural aberrations involving several chromosomes, such as chromosomes 1, 7, 9, 11, and 17, have been detected for bladder cancer using metaphase and interphase cytogenetics (Atkin et al, 1985b, Hopman et al, 1989, 1991; Waldman et al, 1991). In particular, allelic loss on 9q, 11p, and 17p has been found recently in high-stage, high-grade TCCs using restriction fragment length polymorphism analysis (Tsai et al, 1990; Olumni et al, 1990).

In the present study, we have focussed on chromosome 1, since aberrations in chromosome 1 copy number have been previously detected in TCCs of the bladder by FISH with centromeric associated probes (Hopman et al, 1989, 1991) In general, numerical and structural chromosome 1 aberrations have been found in many solid tumors (Atkin, 1985a; Croce, 1986, Sandberg et al, 1988, Teyssier, 1989) Therefore, we combined two probes that recognize repetitive sequences in the centromeric (1q12) and the telomeric (1p36) region and a library DNA probe from sorted human chromosome 1 to study structural chromosome 1 aberrations and the imbalance in FISH copy number for 1q12 and 1p36 in interphase nuclei of TCCs

Materials and Methods

Tumor Samples

For FISH analysis, seven cases with a disomy for 1q12 and six cases with a trisomy for 1q12 were selected from a series of 28 FCM-determined diploid TCCs (Hopman et al, 1991) Nine TCCs were DNA tetraploid/aneuploid. The TCC specimens were obtained immediately after transurethral resection FCM quantitation of DNA content was performed as described previously (Smeets et al, 1987a) The DNA content is expressed as the DNA index (DI) The TCCs were qualified as DNA (near-)diploid (DI = 0.9-1 1) and DNA tetraploid/aneuploid (DI > 1 2-3 2)

Tumor Cell Processing

Preparation of slides from the TCC cell suspensions, fixed in 70% ethanol, and steps necessary to remove cellular proteins for an improvement of DNA probe and antibody penetration were performed as described before (Hopman et al, 1989b, 1992; Poddighe et al, 1991). Briefly, 5 μl of a TCC cell suspension were dropped onto glass slides coated with poly L-lysine (Sigma Chemical Co , St Louis, MO; M_r 150,000-300,000), air dried, and heated at 80°C for 1 h Thereafter, a proteolytic digestion was performed with pepsin

(P 7000, Sigma) at an optimized concentration of 100 μg/ml in 0 01 N HCl for 20 min at 37°C After dipwashes in H_2O and PBS, the nuclei were postfixed in 4% formaldehyde in PBS for 20 min at 4°C Then, the slides were subsequently washed in PBS and H_2O and, finally, dehydrated in an ethanol series of 70, 90 and 100%

DNA Probes and Nonradioactive Labeling

For the detection of the target sequences on chromosome 1, we used the repetitive DNA probe pUC1 77 (Cooke et al, 1979) for the detection of the pericentromeric region 1q12, the repetitive DNA probe 1p79 (Buroker et al, 1987) for the telomeric region 1p36, and a library DNA probe from sorted human chromosome 1 The probes were labeled by nick-translation with biotin 11-dUTP (Sigma) (Pinket et al, 1986) or digoxygenin-11-dUTP (Boehringer, Mannheim, Germany) according to the instructions of the supplier

FISH

The single-target FISH with the repetitive DNA probes for 1q12 and 1p36 was performed as described previously (Hopman et al, 1989, 1992, Poddighe et al, 1991) in 60% formamide 2xSSC, pH 7 0-10% dextran sulfate-1 μg/μl salmon sperm DNA as carrier DNA, at a probe concentration of 0 4 and 1 0 ng/μl hybridization mixture, respectively Hybridization mixture (10 μl) was applied to the slides under a coverslip (18 x 18 mm) and sealed with photo-paper glue Denaturation of probe and target DNA was carried out simultaneously by heating the slides in a moist chamber at 70°C for 2 5 min

For the chromosome in situ suppression hybridization procedure, the library DNA probe from sorted human chromosome 1 was hybridized to the cells at a concentration of 12,5 ng/μl hybridization mixture containing 50% formamide-2x SSC, pH 7 0-10% dextran sulfate 1 μg/μl salmon sperm DNA as carrier Furthermore, for the suppression of hybridization signals from repeated sequences, such as Alu and KpnI elements, human cot1 DNA at a concentration of 600 ng/μl was used in a reannealing procedure The probe mixture was heated at 95°C for 5 min to denaturate the DNA and then incubated at 37°C for 20 min to promote partial reannealing The preparations on the glass slides were separately denaturated in 70% formamide-2x SSC, pH 7 0 at 70°C for 2 5 min and then dehydrated in a series of ethanol (70, 90 and 100%) at -20°C For the hybridization, 10 μl of the preannealed probe mixture were applied to the prewarmed slides (37°C) under a coverslip and sealed with photo-paper glue

In the double-target FISH procedure with the repetitive probes for the 1q12 and 1p36 target, the hybridizations were performed in 60% formamide hybridization mixture When the library DNA probe was involved, in combination with the probe for either 1q12 or 1p36, the reaction was performed in 50% formamide hybridization mixture In these experiments, the denaturated repetitive probe was added to the preannealed library probe mixture just before applying to the slides

All hybridization steps were performed overnight at 37°C

Table 1 Summary of results of interphase cytogenetics in 22 TCCs

		in situ hybridization copy number				
Case	DNA index	1q12 target[b]		1p36 target[b]		Chromosomal Domains[a]
1	1 0	disomy	(90%)[c]	disomy	(95%)	two
2	1 0	disomy	(87%)	disomy	(98%)	two
3	1 0	disomy	(93%)	disomy	(97%)	two
4	1 0	disomy	(95%)	disomy	(97%)	two
5	1 0	disomy	(96%)	disomy	(92%)	two
6	1 0	disomy	(91%)	disomy	(92%)	two
7	1 0	disomy	(85%)	disomy	(91%)	two
8	1 0	trisomy	(74%)	disomy	(95%)	three
9	1 0	trisomy	(71%)	disomy	(87%)	three
10	1 0	trisomy	(76%)	disomy	(94%)	three
11	1 0	trisomy	(70%)	disomy	(91%)	three
12	1 0	trisomy	(62%)	disomy	(93%)	three
13	1 0	trisomy	(63%)	disomy	(70%)	three
14	1 3	trisomy	(79%)	disomy	(70%)	three
15	1 6	tetrasomy	(38%)	trisomy	(81%)	three
		trisomy	(41%)			
16	1 6	pentasomy	(35%)	trisomy	(68%)	n d[d]
		tetrasomy	(37%)			
17	1 7	tetrasomy	(81%)	trisomy	(85%)	four
18	1 7	tetrasomy	(81%)	tetrasomy	(71%)	four
19	1 8	tetrasomy	(73%)	tetrasomy	(66%)	four
20	1 8	hexasomy	(50%)	tetrasomy	(59%)	n d
		pentasomy	(24%)	trisomy	(30%)	
21	2 0	disomy	(51%)	pentasomy	(74%)	five
		monosomy	(41%)			
22	3 2	nonasomy	(20%)	tetrasomy	(70%)	n d
		octasomy	(36%)			
		septasomy	(17%)			
		disomy	(12%)			

[a] The maximum number of FISH domains is mentioned In all diploid cases, one and two domains were observed Also, in the tetraploid/aneuploid cases the number of domains varied from one to the maximum number

[b] In all cases, the major population is mentioned If more than one subpopulation was present, with < 15% in discrepancy, then both subpopulations are mentioned

[c] Percentage of cells

[d] n d , not determined

Immunocytochemical Detection

After FISH, the slides were washed three times for 5 min under stringent conditions (60% formamide-2xSSS, pH 7 0 for the repetitive DNA probes, and 50% formamide-2xSSC, pH 7 0 when the library DNA probe was involved) at 42°C and, subsequently, three times for 5 min in 2xSSC, pH 7 0 at 42°C

Detection of hybrids was accomplished with fluorescent reporter molecules as described previously (Hopman et al, 1991, 1992) The single-target FISH reactions were performed using biotinylated probes, detected with FITC-conjugated avidin (Vector Laboratories, Burlingame, CA), and, if necessary, amplified using biotin-labeled goat anti-avidin (Vector) followed by a second layer of FITC-conjugated avidin (Pinkel et al, 1986) After double-target FISH, the digoxygenin-labeled probe was detected using monoclonal anti-digoxin (Sigma), followed by incubation with FITC-conjugated rabbit anti-mouse antibody (Dakopatts, Glostrup, Denmark) The biotinylated probe was detected using Texas Red-conjugated avidin (Vector), and, if necessary, the FISH signals were amplified as described above All the immunocytochemical steps were performed for 30 min at 37°C

Results

Evaluation and Interpretation of In Situ Hybridization Signals

The validity and specificity of the detection of the FISH signals were determined on metaphase spreads and interphase nuclei of lymphocytes from normal healthy donors The evaluation of the single-target FISH results with the centromeric associated probe for 1q12 and telomeric associated probe for 1p36 was done according to criteria as described previously (Nederlof et al, 1989, Poddighe et al, 1991, Hopman et al, 1991) For these probes, the number of signals per nucleus was determined by counting 200 nuclei per slide We performed double-target FISH reactions to confirm the results with the probes for 1q12 and for 1p36 and to avoid misinterpretations with regard to the imbalance of FISH spot numbers for these regions in the TCCs Evaluation of these results was done by counting 100 nuclei/slide

In contrast to the repetitive DNA probes, interpretation of the FISH results in interphase nuclei with the chromosome 1 library probe was more difficult The domains in these preparations are not always clearly separated, because of the 3-dimensional structure of the nuclei, the close juxtaposition of two individual domains in some cells, or the inability to resolve domains that actually occupy different areas within the nucleus According to results observed by others (Lichter et al, 1988a, Cremer et al, 1988b), the number of interphase nuclei with (artificially) one single domain was 10-20%, whereas in the metaphase spreads of the control human lymphocytes both chromosomes were decorated. Also, about 5-10% of the nuclei showed no signals Therefore, the evaluation of these domains in the interphase nuclei of the TCCs was mostly done qualitatively Furthermore,

double-target FISH reactions of the library DNA probe in combination with the probe for either 1q12 or 1p36 were performed to investigate whether small or substantial portions of chromosome 1 were duplicated in these tumor cells

DNA Diploid TCCs (n=13)

Hybridization results of the three probes in the FCM-established DNA diploid TCCs in single target FISH are summarized in Table 1 Using the centromeric associated probe, we detected three copies for the 1q12 target in six of 13 cases The percentage of trisomy detected by FISH in the individual cases ranged from 62-76% of the cells For the telomeric associated probe, in all cases, two copies for the 1p36 target were detected (range 70-98% of the cells) Hybridization with the library DNA probe in the diploid interphase nuclei resulted in the detection of three domains in all cases in which three FISH signals for the centromeric probe were detected To prove imbalance between the centromere and telomere targets, double-target FISH was performed In Figure 1, A and B, we illustrate the trisomy for 1q12 and disomy for 1p36 in a double-target procedure In Figure 1, C and D, the imbalance between the chromosomal domains and telomere sequences is illustrated Figure 1D shows the three separate domains for chromosome 1 with the library DNA probe In Figure 2, two typical examples, namely, case 3 (A and B) and case 11 (C and D), are schematically represented The histograms demonstrate the frequency distribution in the single-target FISH, using the probes for 1q12, 1p36 and the library probe (Figure 2, A and C) The double-target FISH results, combining the centromere and telomere probes, are shown in Figure 2, B and D For case 3, the copy number for 1q12 (disomy in 93% of the cells) was equal to the copy number for 1p36 (disomy in 97% of the cells) Counting of the fluorescent domains after FISH with the library DNA probe in the interphase nuclei revealed also 95% of the nuclei with two domains (Figure 2A), in agreement with the number of FISH signals for the 1q12 and 1p36 regions For case 11 (Figure 2, C and D), however, the imbalance between 1q12 (trisomy in 70% of the cells) and 1p36 (disomy in 91% of the cells) can be clearly illustrated Counting the chromosomal domains after FISH with the library DNA probe revealed three domains in about 50% of the nuclei of this case (Figure 2C)

DNA Tetraploid/Aneuploid TCCs (n=9)

Single-target FISH in the flow cytometrically determined DNA tetraploid/aneuploid TCCs (DI=1 3-3 2) revealed a more complex distribution of FISH spot numbers as detected in the DNA diploid TCCs (Table 1) The cases were selected on the basis of their chromosome 1 centromere copy number varying from one to nine

For example, case 14 showed a trisomy for 1q12 (in 79% of the cells), whereas two FISH signals for 1p36 (in 70% of the cells) and three domains for the library DNA probe were detected In case 15, a heterogeneous population was detected for 1q12, showing a tetrasomy in 38% of the cells and a trisomy in 41% of the cells, while an evident trisomy

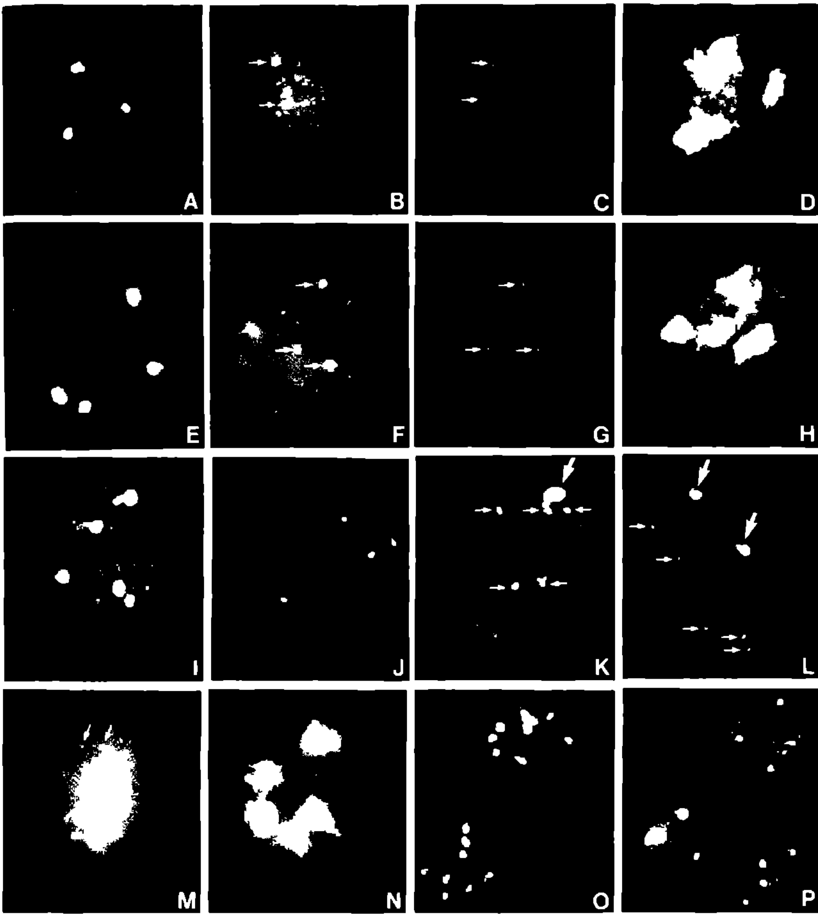


Figure 1. Results of double-target (A-H and K-N) and single-target (I, J, O, and P) FISH procedures in interphase nuclei of case 11 (A-D), case 17 (E-H), case 20 (I,J), case 21 (K-N), and case 22 (O and P) using probes for chromosome 1. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). In A and B, and in C and D, two double-target FISH reactions demonstrate the overrepresentation of the centromere (A) as compared to the telomere region (B and C, arrows) in a diploid TCC. Furthermore, nuclei showing two FISH signals for the telomere (C, arrows) displayed three discrete and separate chromosome 1 domains with the library DNA probe (D). In E and F, and in G and H, two double-target FISH reactions in an aneuploid TCC (case 17, DI=1.7) illustrate that four copies of the centromere (E) are combined with three copies of the telomere (F, arrows), whereas four chromosomal domains (H) contain only three telomere regions (G, arrows), demonstrating the imbalance between these three chromosome 1 targets. In I and J, two nuclei of case 20 show five FISH spots for the centromere (I) and four FISH spots for the telomere region (J) in single-target FISH reactions. In K and L, two nuclei of case 21 illustrate the imbalance between the number of centromeres (large arrows), which are one (K) and two (L) and the number of telomeres (small arrows), showing five signals per nucleus. These two photographs are double-exposures of the double-target FISH reactions. In M and N, a double-target FISH reaction demonstrates that the nuclei having five spots for the telomere probe (M, arrows), also show five chromosomal domains (N). In O and P, the nuclei of case 22 demonstrate the imbalance between centromere (O, eight and nine spots/nucleus), and telomere (P, three and four spots/nucleus).

for 1p36 in 81% of the cells was detected, as well as three domains for chromosome 1. Also, in case 16, an overrepresentation of 1q12 was observed (35% pentasomy and 37% tetrasomy), as compared to the trisomy for the 1p36 target (in 68% of the cells).

In two of three cases with a tetrasomy for 1q12 (cases 18 and 19), we could not detect an imbalance between the three chromosome 1 targets However, in the other case (case 17), an imbalance between 1p36 as compared to both 1q12 and the chromosomal domain probe was detected Figure 2, E and F, schematically illustrates the frequency distribu-tions of the single target FISH with the three probes (E) and the double-target FISH, using 1p36 and 1q12 (F) These data show the tetrasomy for 1q12 in 81% of the cells, and the trisomy for 1p36 in 85% of the cells The maximum number of chromosomal domains was four (in 24% of the cells), while the largest cell population (43%) showed three domains Figure 1, E and F, shows the tetrasomy for 1q12 and the trisomy for 1p36 in a double-target FISH experiment, whereas in Figure 1, G and H, the imbalance between the telomere sites and the chromosomal domains of case 17 is illustrated

In case 20, containing a heterogeneous population of cells, the main numbers of FISH signals for 1q12 were six (in 50% of the cells) and five (in 24% of the cells), while for 1p36 the main copy numbers were four (in 59% of the cells) and three (in 30% of the cells) (Figure 1, I and J)

In case 21, evident monosomy (in 41% of the cells) and disomy (in 51% of the cells) for the chromosome 1 centromere were detected, whereas the copy number for 1p36 targets was five (in 74% of the cells) (Figure 2, G and H) Counting the number of chromosome 1 domains revealed a broad distribution, varying from two to five fluo-rescent regions per nucleus, with no evident peak fraction The imbalance between centromere and telomere for this case is illustrated in Figures 1, K and L In Figure 1K, an interphase nucleus shows a monosomy for 1q12 and a pentasomy for the 1p36 target Also, in the nuclei with a disomy for 1q12, a pentasomy for 1p36 was detected (Figure 1L) Evaluation of these nuclei in double-target FISH, using the library DNA probe and the telomere probe, demonstrated that the copy number of these targets were in balance, as is illustrated in Figures 1, M and N

Case 22 demonstrated an evident heterogeneous population of cells, in which the maximum number of FISH signals for 1q12 was nine (in 20% of the cells), but also cells with eight (in 36% of the cells), seven (in 17% of the cells), and two (in 12% of the cells) FISH signals were observed (Figure 1O) In contrast, the maximum number of FISH signals for the telomeric target 1p36 was four (in 70% of the cells) (Figure 1P)

Discussion

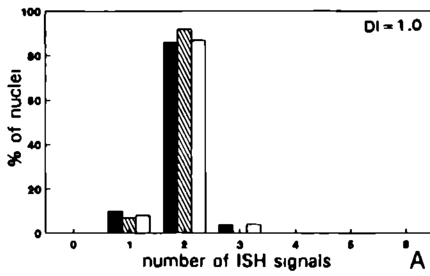
FISH using centromeric associated repetitive DNA probes, is a powerful technique for the detection of numerical chromosome aberrations in the interphase nuclei of tumors (Poddighe et al, 1992) Recently, studies were published that compared the results of

interphase cytogenetics with conventional karyotyping analyses of cell lines derived from solid tumors and of neoplastic cells from bone marrow and peripheral blood (Smit et al, 1990, Poddighe et al, 1991) This combined approach has become a rapid procedure for the detection of numerical and structural chromosome aberrations, such as translocations, and for identification and subsequent characterization of marker chromosomes; statistical evaluations are also possible (Rappold et al, 1984; Cremer et al, 1988b, Nederlof et al, 1989, Smit et al, 1990, Poddighe et al, 1991). A combination of a centromeric and a telomeric associated DNA probe, specific for chromosome 1, was described (Van Dekken et al, 1989) and was used to investigate structural chromosome aberrations in more detail.

The FISH method using library DNA probes visualizes whole individual human chromosomes as discrete territories in interphase nuclei and can thus be of value in the analysis of structural chromosome changes of solid tumors (Rappold et al, 1984, Burns et al, 1985, Lichter et al, 1988a, 1988b, Cremer et al, 1988b). In the present study, we applied a combination of a centromeric associated, a telomeric associated and a library DNA probe for chromosome 1 to examine the feasibility of detecting numerical and structural aberrations in solid tumors by means of such probes in combination with FISH. Analyzing flow cytometrically determined DNA diploid TCCs, we have found that in all cases in which three FISH signals for the 1q12 target were detected (six of 13 cases), two signals for the 1p36 target were observed Furthermore, all cases with an over-representation of the centromeric region, as compared to the telomeric region, showed three separate domains for chromosome 1, suggesting the presence of an additional large part of chromosome 1 in these TCCs

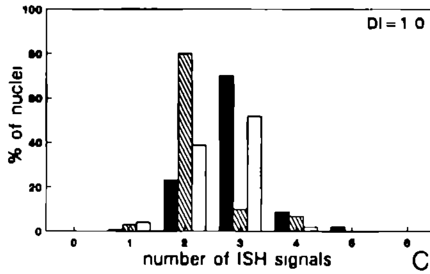
Structural changes in chromosome 1 were recognized in bladder cancers by means of karyotyping These aberrations were characterized by breakpoints in the proximal regions of the chromosome arms In case of FCM near-diploid TCCs, +1p- and 1q+ chromosomes have been reported frequently (Kovacs, 1985, Smeets et al, 1987a) The 1q+ chromosomes, with one or two duplications of part of the long arm, are rather common in such tumors and, if the break has occurred in the heterochromatin, one or two interstitial C-bands may be present on the long arm (Atkin et al, 1986) If we compare our data with the conventional cytogenetic analyses, the structural chromosome aberration in the 6 diploid cases with an imbalance between centromere and telomere most probably represents +1p-.

Analysis of the (im)balance between 1q12 and 1p36 in the tetraploid/aneuploid TCCs results is more complex because of the heterogeneous nature of these tumors. Also, the interpretation of the results obtained by FISH with the library DNA probe proved to be difficult because of overlap of the individual domains in the nuclei However, comparison of the number of 1p36 and 1q12 signals showed in six of nine cases an evident overrepresentation of 1q12 whereas in two cases a balance between both targets was detected In the cases analyzed with the library DNA probe, the number of FISH domains was equal to the number of 1q12 targets In this respect the balance between 1q12 and chromosomal domains, as well as overrepresentation of 1q12 as compared to 1p36, are comparable to



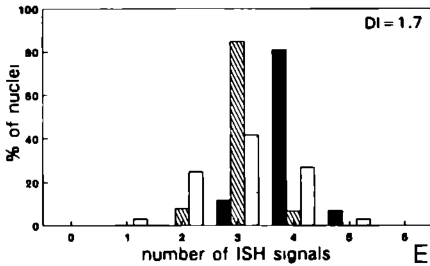
B

#1p36 \ #1q12 (ISH spots)	0	1	2	3	4
4	-	-	-	-	-
3	-	-	-	2	-
2	-	10	86	2	-
1	-	-	-	-	-
0	-	-	-	-	-



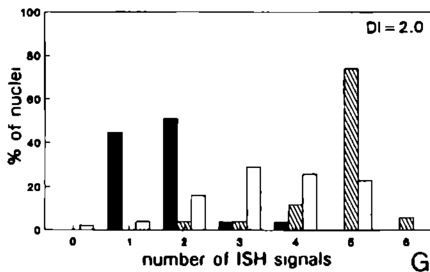
D

#1p36 \ #1q12 (ISH spots)	0	1	2	3	4
4	-	-	-	-	6
3	-	-	-	8	2
2	-	-	6	72	2
1	-	2	2	-	-
0	-	-	-	-	-



F

#1p36 \ #1q12 (ISH spots)	1	2	3	4	5
5	-	-	-	-	-
4	-	-	-	8	3
3	-	-	12	69	-
2	-	-	2	6	-
1	-	-	-	-	-



H

#1p36 \ #1q12 (ISH spots)	1	2	3	4	5
6	1	1	-	-	-
5	33	46	3	-	-
4	7	7	-	-	-
3	1	1	-	-	-
2	1	-	-	-	-
1	-	-	-	-	-

Figure 2. Evaluation of FISH reactions with the three different probes for chromosome 1 in case 3 (A and B), case 11 (C and D), case 17 (E and F), and case 21 (G and H). A, C, E, and G, demonstrate the frequency distributions of the number of hybridization spots obtained after a single-target FISH procedure. Two hundred nuclei were counted. ■ probe 1q12; ▨ probe 1p36; ▭ chromosome 1 library DNA probe. In B, D, F, and H, percentages of FISH spot numbers detected in interphase nuclei after the double-target procedure with the probes for the telomere (1p36) and the centromere (1q12) are shown. One hundred nuclei were counted.

results in the DNA diploid TCCs Only in one tetraploid/aneuploid TCC (case 21, DI=2 0) was a strong underrepresentation of 1q12 as compared to 1p36 observed This case was selected from a group of 21 tetraploid/aneuploid TCCs, of which it appeared to be the only tetraploid/aneuploid tumor sample with a high percentage of cells containing one FISH signal for 1q12 per nucleus (41%)

It is generally accepted that tetraploidization is a crucial step in the progression of many solid tumors (Kovacs, 1985, Shackney et al, 1989), in which random as well as nonrandom loss of chromosomes could lead to selection and growth of aneuploid or tetraploid tumors. In DNA diploid TCCs, we observed gain of 1q12 We have to realize that the cases with a trisomy for 1q12 were selected from a series of 28 cases and occur at a frequency of about 20% (Hopman et al, 1991) In the tetraploid/aneuploid TCCs, gain of 1q12 is difficult to determine since the DNA index ranged from 1 3-3 2 However, in four cases (cases 15, 16, 20, and 22), the copy number was higher, as was expected on basis of the DI In the latter cases, the copy number for 1p36 is in concordance with the DI However, on the basis of the limited number of the tetraploid /aneuploid cases, we could speculate about selective gain of 1q12 or loss of 1p36 on the basis of tetraploidization Only in case 20 (DI=1 8) did we detect a ratio of FISH signals between 1q12 and 1p36 of 6/4, which could be the result of tetraploidization

Abnormalities involving both the long arm (q) and the short arm (p) of chromosome 1 have been observed in many types of human tumors (Atkin, 1985, Olah et al, 1989), including hematological disorders, lymphomas, childhood tumors (including Wilms' tumor and neuroblastoma) colorectal, breast and ovarian cancers, and carcinoma of the cervix uteri (Sandberg et al, 1988, Olah et al, 1989) Furthermore, abnormalities involving chromosome 1 are implicated in tumor progression and in in vitro immortalization of human colorectal adenoma cell lines These abnormalities can either involve deletions of the short arm of chromosome 1 or formation of an isochromosome 1(q) resulting in 3 or 4 copies of the long arm of chromosome 1 in each cell (Paraskeva, 1989) On the other hand, loss of genes on chromosome 1(q) allows cells to escape cellular senescence, which appears to be an important step in carcinogenesis (Sugarawa et al, 1990)

The present study demonstrates the routine utility of the FISH methods to assess structural aberrations in interphase nuclei of solid tumors It is to be expected that in future with the use of regional-specific, single copy DNA probes, a more precise definition of breakpoints and of the chromosomal regions involved in deletion, translocation, duplication, or amplification can be achieved

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Comparison of Interphase Cytogenetics and RFLP Analysis of Transitional Cell Carcinoma of the Urinary Bladder

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Abstract

The purpose of this study was to compare the outcome of RFLP analysis and interphase cytogenetics on transitional cell carcinomas (TCCs) of the urinary bladder, to obtain more insight into the mechanism and genetic background of progression of TCC

TCC DNA samples, obtained from tumors of different stage and grade, were screened for loss of heterozygosity for chromosome loci 9q34, 11p15, 16q22-24, 17p13, and 18q21

In situ hybridization (ISH) was performed on serial frozen tissue sections of the same tumors Centromere associated DNA probes for the chromosomes 1, 7, 9, 10, 11, 16, 17 and 18 were used to determine chromosome ploidy, numerical chromosome aberrations, the imbalance between chromosomes, and heterogeneity of aberrations within one tumor

The results demonstrate a good correlation between RFLP and ISH approaches It shows that loss of heterozygosity (LOH) for chromosome locus 9q34 in early phases of TCC development is most probably due to loss of the complete chromosome 9, since ISH using the centromeric probe for this chromosome showed in all but one of these cases an underrepresentation No correlations were found for the loci on chromosomes 11p, 16q, 17p and 18q, suggesting either an interstitial deletion or LOH in combination with a duplication of (a part of) that chromosome in the aneuploid cases

Introduction

Transitional cell carcinoma (TCC) is the most common form of urinary bladder cancer, comprising a heterogeneous group with markedly different neoplastic features (Cummings, 1980) Approximately two thirds of the patients with TCC finally present recurrences, of which ten to twenty five percent will be of higher grade or stage (Tribukait et al, 1982) Currently, the most important predictors of the behaviour of TCCs are histological stage and grade The DNA ploidy, as determined by flow cytometry (FCM), may have additional value to predict the biological behaviour of the tumor (Pauwels et al, 1987) For example, FCM has revealed that noninvasive (pTa) TCCs are predominantly DNA diploid, while invasive behaviour of these malignancies is highly correlated with DNA aneuploidy or tetraploidy (Tribukait et al, 1986, Blomjous et al, 1988, Koss et al, 1989)

Although conventional karyotyping allows the identification of chromosomal aberrations, characteristic for bladder cancer, involving chromosomes 1, 7, 9, and 11, the technique is generally hampered by difficulties such as low mitotic index, poor banding quality, or limited number of analyzable cells (Gibas et al, 1984, 1986, Atkin et al, 1985, Kovacs, 1985, Sandberg et al, 1986, 1988, Smeets et al, 1987a, 1987b, Vanni et al, 1988, Teyssier, 1989)

Recently, interphase cytogenetics using centromere-associated DNA probes has been shown to enable a routine screening of tumor cells in both single cell suspensions (Cremer et al, 1988, Hopman et al 1988a, Devilee et al, 1988b, Nederlof et al, 1989, Raap et al,

1990; Lichter et al, 1991, Poddighe et al, 1991, 1992b) and sections of frozen tissues or formalin-fixed, paraffin-embedded tissues (Naoumov et al, 1988; Hopman et al, 1990, 1992, Arnoldus et al, 1991, Poddighe et al, 1992a). For TCCs the most striking findings seem a primary loss of chromosome 9 in diploid tumors, and gain of chromosomes 1 and 7 in aneuploid tumors (Hopman et al. 1988b, 1989, 1991).

Comparison of tumor DNA and constitutional DNA for polymorphic markers by restriction fragment length polymorphism (RFLP) analysis allows the detection of loss of heterozygosity (LOH) at specific chromosomal loci LOH at chromosomal regions in tumor tissue may indicate a deletion of putative tumor suppressor genes (Fearon et al, 1990b). RFLP analyses have shown allelic loss on 9q, 11p, 16q, 17p, and 18q being involved in various human cancers, including urinary bladder cancers (Tsai et al, 1990, Olumni et al, 1990; Presti et al, 1991, Proctor et al, 1991; Sidranski et al, 1991, 1992).

In earlier studies we used centromere associated DNA probes for chromosomes 1, 7, 9, and 11 in ISH, because of the apparant correlation of changes in these chromosomes and TCC (Gibas et al. 1984, 1986, Sandberg, 1986; Sidranski et al, 1991, 1992; Presti et al, 1991) Chromosomes 10, 16 and 18 were examined as controls and for the determination of chromosome ploidy

Screening of 24 TCCs by RFLP analysis and ISH with the probes described above was done in view of the following questions a) is there a correlation between imbalance of different chromosome copy numbers and selective LOH? ; b) is underrepresentation of certain chromosomes, as detected by ISH, correlated with LOH of markers on that same chromosome? , c) although the DNA probes for ISH detect only centromeric regions, can the ISH approach be used instead of or as an adjunct to the LOH approach in a clinical and routine setting to detect (numerical) chromosome aberrations? , d) how is the underrepresentation of chromosome 9 followed during the process of tetraploidization by ISH and RFLP? , e) to what extent do both techniques overlap, and thus reveal the same information? ; f) can heterogeneity of chromosome aberrations be detected in TCC by means of ISH and how does such heterogeneity influence the RFLP results? Answers to these questions may result in a better definition of the indication and application of each of these techniques

Materials and Methods

Sample Selection and Preparation for ISH

The tissue samples studied were selected for their tumor cell content, which was at least 60-70%. Classification of histological stage and grade (I-III) was performed in paraffin sections.

For this study 24 frozen tissue samples of TCC were selected on which both ISH and RFLP techniques could be performed For ISH, 5 μm sections were mounted on organosilane coated slides, air dried and stored at room temperature. For RFLP analysis, 20 μm

sections were collected and stored at -20°C Also, peripheral blood lymphocytes (PBLs) were collected of the same patients for RFLP analysis

Pretreatment of Frozen Sections

Prior to the ISH procedure, the sections were fixed in 70% ethanol/1% formaldehyde for 20 min at -20°C After subsequent washings in phosphate buffered saline (PBS)/0 05% Tween-20, and water for 5 min at room temperature, the slides are incubated for 15 min at 37°C in 100 μg pepsin per ml 0 01 N HCl Then the slides were rinsed in five dip washes of water and five dip washes of PBS, and dehydrated in an ascending alcohol series After air-drying, the sections were post-fixed in 1% formaldehyde/PBS for 10 min at room temperature

DNA Probes for ISH

The centromere associated DNA probes pUC1 77 (Cooke et al, 1979), p7t 1 (Waye et al, 1987b), pHUR98 (Moyzis et al, 1987), pHUR195 (Moyzis et al, 1987), p10 1 (Devilee et al, 1988a), pLC11A (Waye et al, 1987a), p17H8 (Waye et al, 1986) and L1 84 (Devilee et al, 1986) were used for the detection of target sequences on chromosomes 1, 7, 9, 16, 10, 11, 17, and 18, respectively The probes were labeled by nick-translation with biotin-11-dATP, according to the supplier's instructions (Boehringer, Mannheim, Germany)

In Situ Hybridization

The DNA probes described above were hybridized to the (tumor-)cell preparations at a probe concentration of 1 ng/μl hybridization mixture, containing 60% formamide-2x standard sodium citrate (SSC) 10% dextransulfate, pH 7 0 Under these stringent conditions, 15 μl of the hybridization mixture was applied to the slides under a coverslip (18 x 18 mm) Denaturation of probe and target DNA was performed simultaneously by heating the slides in a moist chamber to 70°C for 3 min After hybridization for 2 to 16 hours at 37°C, the coverslips were removed by immersing the slides in 2xSSC, pH 7 0 at 42°C Posthybridization washing steps were performed twice in 60% formamide-2xSSC, pH 7 0 for 5 min and twice in 2xSSC, pH 7 0 for 5 min at 42°C

Immunocytochemical detection of the hybridized DNA probes was performed as previously described (Hopman et al 1992) with mouse anti-biotin (Dakopatts, Glostrup, Denmark), biotin-labeled horse anti mouse IgG (Vector, Burlingame, CA), and a final incubation with the avidin-biotin-labelled peroxidase complex (Vectastain Elite ABC Kit, Vector) All immuno-cytochemical steps were performed for 30 min at 37°C The DNA probe was visualized with 0 5 mg/ml 3,3 diaminobenzidin tetrahydrochloride (DAB, Sigma), 0 65% imidazole (Merck, Darmstadt, Germany), 0 015% H_2O_2 (Merck), in PBS for 3 min at pH 7 8, and the signal was amplified with $CuSO_4$ (0 5% in 0 9% NaCl) for 3 min The slides were counterstained with hematoxylin and mounted in Permount (Fisher Scientific, New Jersey, NJ)

Evaluation of ISH Results

The reproducibility and validity of a protocol to detect chromosome copy numbers by ISH in 5 μm routinely processed tissue sections have been described elsewere (Poddighe et al, 1992a).

Evaluation of the preparations was done according to criteria as described before (Hopman et al, 1990; Poddighe et al, 1992a). A disadvantage of the use of tissue sections is, that, due to truncation of a significant number of nuclei, the number of ISH signals per nucleus will be underestimated as compared to the actual copy number of the target chromosome (Naoumov et al, 1988; Hopman et al, 1990; Arnoldus et al, 1991). Therefore, we evaluated separately within each section the tumor area, normal tissue and stromal cells, since no simple correction factors for truncation of nuclei are sofar available. In diploid tumors the true chromosome copy number is detectable in about 50% of the nuclei, so that monosomy or trisomy can be determined conclusively. However, with increasing nuclear size the true chromosome copy number will be increasingly underestimated.

Hybridization with different probes on the same tumor areas in parallel sections enables the study of imbalance in chromosome copy number and, as a result, the specific loss of chromosomes.

The TCCs were classified as diploid or aneuploid on basis of the mean number of ISH signals of at least six of the eight DNA probes.

DNA Isolation and Southern Blot Analysis

High molecular weight DNA was isolated from frozen tissue samples of the 24 TCCs, and from whole blood samples of the same patients as described (Miller et al, 1988). Ten μg of DNA digested with the appropriate restriction endonuclease was separated on a 0.8% agarose gel and transferred to Hybond N+ (Amersham). Blots were hybridized to the following radioactive labelled probes: EFD126 (Nakamura et al, 1987) for 9q34, c-H-ras (Pulciano et al, 1982) for 11p15, PV962 (Mansouri et al, 1988) and 79.2.23 (Bufton et al, 1986) for 16q22, 144D6 (Schwartz et al, 1988) for 17p13, and 15.65 (Fearon et al, 1990a) for 18q21.

Results

Table 1 summarizes the RFLP and ISH results for each of the 24 TCCs. These were ranged according to their chromosome ploidy as determined on basis of ISH. Case nos. 1 to 13 were classified as chromosome diploid, case nos. 14 to 21 as aneuploid, and case nos. 22 to 24 as heterogeneous, i.e., mixed diploid and aneuploid.

RFLP analysis.

DNA isolated from these 24 TCCs was examined by Southern blotting for LOH for regions on chromosomes 9q, 10p, 11p, 16q, 17p, and 18q. In thirteen patients we found 24

Table 1. A summary of the RFLP and ISH results for the 24 Transitional Cell Carcinomas. For ISH we used the centromere-associated DNA probes for chromosomes 1, 7, 9, 10, 11, 16, 17, and 18. Abbreviations: NI=probe is not informative, nd=not done, + means no LOH; - means LOH.

		RFLP analysis					ISH analysis	
case No	grade/stage	chromosomal locus detected on					chrom. ploidy	numerical aberration(s)
		9q	11p	16q	17p	18q		
1	G1 / pTa	+	+	+	+	+	diploid	None
2	G1 / pTa	+	+	+	+	NI	diploid	-9, -17
3	G1 / pTa	-	+	-	+	+	diploid	-9
4	G1 / pTa	+	+	+	+	NI	diploid	-9, -17
5	G1-2 / pTa	+	+	+	+	+	diploid	-10
6	G1-2 / pTa	-	-	+	+	+	diploid	-9
7	G2 / pTa	+	NI	+	-	-	diploid	-9, -18
8	G2 / pT1	-	+	+	+	NI	diploid	-9
9	G2 / pT1	-	-	+	+	NI	diploid	+7, -9
10	G2 / pT1	+	+	+	+	NI	diploid	-18
11	G2 / pT1	+	NI	+	NI	+	diploid	None
12	G3 / pT3	-	+	+	NI	nd	diploid	-9
13	G3 / pT3	NI	+	+	+	nd	diploid	None
14	G2-3 / pTa	NI	+	+	+	NI	triploid	-9
15	G2 / pT1	+	+	+	+	+	triploid	+1, -9
16	G2 / pT1	-	+	+		-	tetraploid	+1, -9
17	G2 / pT1	NI	-	+	+	-	hexaploid	-9, -9
18	G1-2 / pT1-2	+	NI	+	+	+	tetraploid	None
19	G3 / pT2	+	+	-	+	NI	tetraploid	-18
20	G3 / pT>2	NI	NI	+	+	NI	tetraploid	-9, -17
21	G3 / pT3	NI	NI	-	-	nd	triploid	None
22	G1-2 / pTa		+	+	-	nd	tetraploid / diploid	+1, -9, -9 / +1, -9
23	G1-2 / pTa	-	-	+	+	+	tetraploid / diploid	None
24	G3 / pT>2	-	+	+	-	+	tetraploid / diploid	-9,-9,-17,-17 / -9, -17

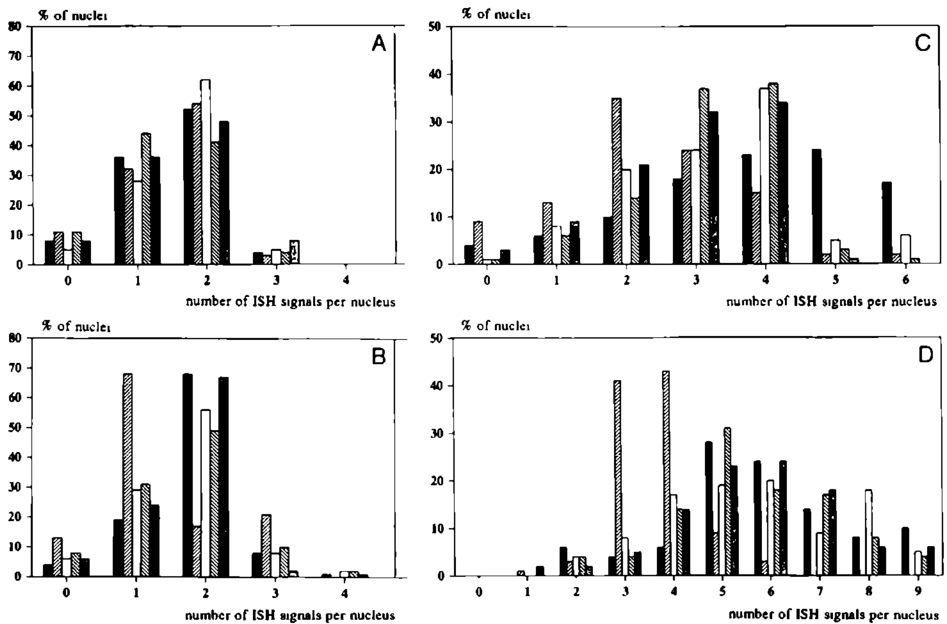
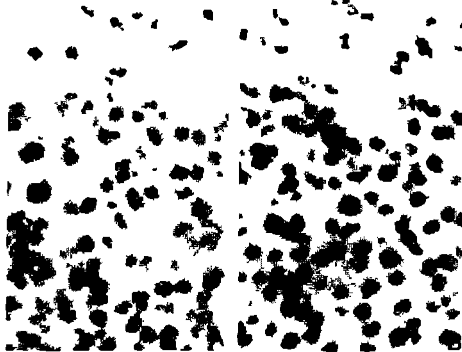
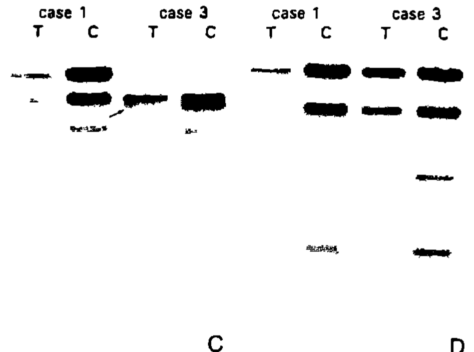


Figure 1. Frequency distribution patterns of in situ hybridization signals obtained with different centromere-associated DNA probes for chromosome 1 (▬), chromosome 9 (▨), chromosome 11 (☐), chromosome 17 (▧), and chromosome 18 (▩) on frozen tissue sections of TCCs A) case no. 1, representing a diploid tumor with no numerical aberrations. B) represents case no 3, showing an underrepresentation for chromosome 9. C) Aneuploid tumor, case no. 16, demonstrating an overrepresentation for chromosome centromere 1, and loss for the centromeric target of chromosome 9. D) case no. 17, representing a highly aneuploid tumor with a mean chromosome copy number of six. Note that in the distribution patterns for the analyzed chromosomes an underrepresentation of the real copy number occurs due to truncation of nuclei in the five micrometer thick sections

cases of LOH for one or more markers. The most frequent allelic loss was seen for the marker on chromosome 9q. Of nineteen informative cases (79%) we found LOH of the 9q34 locus in nine tumors (47%). For the chromosome 11p marker we found that 21% of the nineteen informative cases (79%) had allelic loss. Although the 16q marker was informative in all 24 tumors, only three cases (12,5%) showed LOH for this locus. For the marker on 17p twenty-two cases (92%) were informative, of which five cases (23%) had LOH. Finally, the marker on 18q was less informative (60%), and demonstrated allelic loss in three cases (25%).




case 1
T C
case 3
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case 1
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case 3
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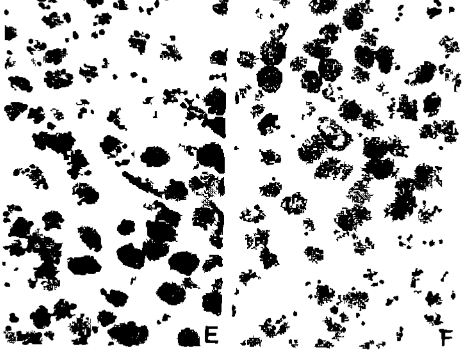
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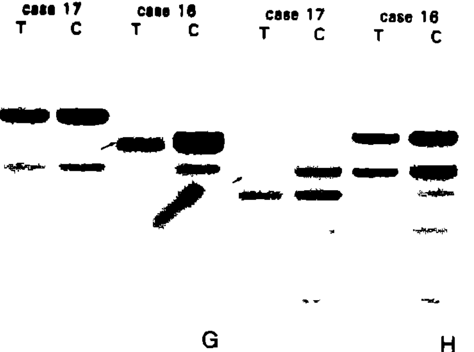
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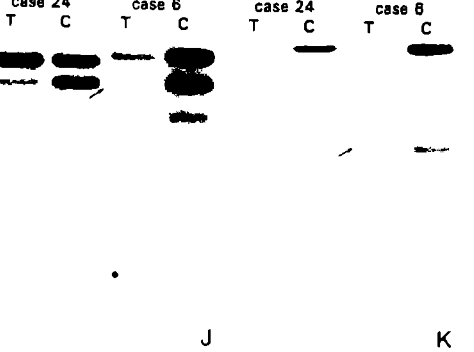
case 24
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case 6
T C
case 24
T C
case 6
T C
J
K

ISH analysis

Evaluation of the ISH reactions on frozen tissue sections of the 24 TCCs with the eight centromeric DNA probes revealed that 18 out of 24 cases showed 27 times loss or gain of ISH signals as compared to the mean chromosome ploidy (determined according the criteria described in M & M sections) A gain of ISH signals was observed for chromosome 1 in three cases (12,5%), and for chromosome 7 in one case (4%). Loss of ISH signals as compared to the modal ISH number was most frequently found for chromosome 9, showing 15 cases (62,5%) with a numerical loss The centromeric probe for chromosome 10 demonstrated in one case (4%) an loss, while for the chromosomes 17 and 18 in three cases (respectively 12,5% and 13%) loss of ISH signals was observed For chromosome 16 no numerical aberrations were detected

Chromosome diploid tumors

Numerical chromosome aberrations were detected in ten out of 13 diploid TCCs (Table 1, case nos 1-13) In eight cases a loss for chromosome 9 was observed. In two cases an additional loss of chromosome 17 was detected One case (no. 4) demonstrated a loss for chromosome 10, whereas case no 6 showed loss of chromosome 18, next to a loss for chromosome 9. Loss of chromosome 18 was also observed in case no. 9 Figure 1A shows

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Figure 2. *In situ hybridization on frozen tumor tissue sections with centromere-associated DNA probes for chromosome 11 (A), chromosome 9 (B and F), and chromosome 1 (E and I) Southern blot hybridizations of tumor (T) and constitutional (C) DNA, digested with TaqI, and hybridized to polymorphic probes for 9q34 (C, G, and J), and 11p15 (D, H, and K) A-D represent a chromosome diploid TCC (case no 3) A and B demonstrate a tumor area in serial sections, showing two ISH signals for the probe for chromosome 11 (A), while the centromere probe for chromosome 9 demonstrates one ISH signal in the same tumor area (B) C and D demonstrate Southern blot analyses for case no 1 and 3 For the 9q34 probe case no 1 retained this locus in the tumor (C lane 1), while case no 3 demonstrated LOH (C lane 3) Both case nos 1 and 3 were heterozygous for the 11p probe (D lane 2 and 4, respectively) and demonstrated no LOH (D lanes 1 and 3) E-H represent a chromosome aneuploid TCC (case 17) A representative tumor area, in which most nuclei contain five to six ISH signals with the probe for chromosome 1 (E) The arrow indicates a nucleus with eight ISH signals In F the same tumor area in a serial section is hybridized with the probe for chromosome 9, showing a mean ISH copy number of four Southern blot analyses of case nos 16 and 17 for the 9q34 probe revealed that tumor DNA from case 16 showed LOH (G lane 3), whereas case no 17 retained the 9q34 locus (G lane 2) For the 11p probe both tumors were informative (H lanes 2 and 4) Case no 17 demonstrated LOH (H lane 1), and case 16 no LOH for this locus (H lane 3) I-K represent a heterogeneous tumor (case 24) In I, the tumor tissue section was hybridized with the centromere DNA probe for chromosome 1 The heterogeneity of the tumor is demonstrated In the left area the nuclei contain mainly two ISH signals while in the right area the nuclei contain three and four ISH signals In J, TaqI digested DNA from case nos 24 and 6 was hybridized with the 9q34 probe, demonstrating in both cases LOH (J lanes 1 and 3) Case no 24 showed no LOH with the 11p probe (K lane 1), whereas case no 6 demonstrated LOH for this locus (K lane 3)*

the quantitative evaluation of the hybridization signals of case no 1 for the centromere-associated DNA probes for chromosomes 1 9, 11, 17, and 18 In this particular case the mean number of ISH signals for the chromosomal probes was two in about 45-50% of the nuclei, and one in 28 42% of the nuclei Therefore, this case was classified as a chromosome diploid tumor Figure 1B represents case no 3, in which a significant loss of chromosome 9 was detected, since 68% of the nuclei contain only one ISH signal for the chromosome 9 probe In about 55% of the nuclei of this case two ISH signals for the other chromosomes were detected Figure 2, A- and B, shows tumor areas of this diploid case (no 3), hybridized with the probe for chromosome 11 (Figure 2A), showing two ISH signals, and the probe for chromosome 9 (Figure 2B), showing one ISH signal in a high frequency of the cells

The RFLP results for the case nos 1 and 3 are shown in Figure 2C and D, on which TaqI digested DNA is hybridized to a probe for 9q and 11p, respectively Tumor DNA from case 3 showed LOH for the 9q probe (Figure 2C), whereas for the 11p probe no LOH was observed (Figure 2D)

Chromosome aneuploid tumors

Screening of the eight chromosome aneuploid tumors (cases no 14-21) with the eight centromere-associated DNA probes revealed a profound heterogeneity with respect to the chromosome copy numbers In five cases an underrepresentation for chromosome 9, as compared to the other chromosomes, was detected In two cases (case nos 15 and 16) an overrepresentation for chromosome 1 was observed An additional loss for chromosome 17, next to loss of chromosome 9, was detected in case no 20 Case no 19 showed a loss for chromosome 18 as the only numerical aberration

Figure 1C shows the frequency distribution of case no 16 by evaluating the ISH signals in 200 nuclei in serial sections, showing a mean between 3 0 and 3 3 for the copy number of chromosomes 7, 11, 16, 17 and 18, and a modal copy number of four ISH signals per nucleus For chromosome 1 the mean copy number was 3 9, and modal copy number was five to six ISH signals per nucleus, representing a relative overrepresentation or gain of this chromosome The mean and modal copy number for chromosome 9 were 2 4 and 2, respectively, demonstrating an underrepresentation or loss of this chromosome

The frequency distribution of ISH signals for the chromosomes 1, 9, 11, 17, and 18 of case no 17 is shown in Figure 1D The mean copy number for the chromosomes 1, 11, 17, and 18 was between 5 6 and 5 9, and a modal copy number of six ISH signals per nucleus For chromosome 9 the mean copy number was 3 6, with a modal copy number of four, representing a loss of two copies of this chromosome Figure 2E-F represent a part of the tumor of case 17 where the nuclei contain 5/6 ISH signals (with a maximum ISH copy number of 9) for chromosome 1 (Figure 2E), whereas for chromosome 9 the nuclei contain 3/4 ISH signals (with a maximum ISH copy number of 6)(Figure 2F)

Southern blots of tumor and constitutional DNA from case nos 16 and 17 are shown in

Figure 2G and H, hybridized to the 9q and 11p probe, respectively. For the 9q probe, case no 16 demonstrates LOH, while case no 17 is not informative (Figure 2G). For the 11p probe, case no 16 retains the 11p allele, whereas case no 17 revealed LOH (Figure 2H).

Heterogeneous tumors

Evaluation of ISH signals for the cases no. 22-24 revealed extensive chromosome heterogeneity, both between ISH signals for the eight different DNA probes in the individual tumor cells, as well as between different tumor areas in the same case of TCC. Therefore, we have classified these three cases as being heterogeneous. In two cases (case nos. 22 and 24) loss for the centromere of chromosome 9 was detected after screening of serial sections with the eight centromere-associated DNA probes. In the tetraploid tumor areas, we observed an ISH copy number for chromosome 9 of maximum two, indicated as an apparent loss of two copies of chromosome 9 (Table 1) Figure 2I demonstrates the tumor heterogeneity of ISH signals within case no. 24, in which one part of the TCC contains 3 or 4 ISH signals for the chromosome 1 probe, while in another part of the TCC mainly 1 or 2 ISH signals for the same probe are found

Figure 2J and K demonstrates the RFLP results of DNA from this tumor hybridized to the 9q and 11p probe Although LOH for the 9q locus was concluded, the heterogeneity of the tumor DNA is reflected by the faint band in the blot (arrow in Figure 2J)

Discussion

Several investigations have demonstrated that centromere associated DNA probes can be applied in ISH techniques to determine numerical chromosome aberrations in interphase nuclei of tumors (for reviews, see Raap et al, 1990, Poddighe et al, 1992a). Other studies have compared the results of interphase cytogenetics with those of conventional karyotyping of neoplastic cells from leukemia or cell lines derived from solid tumors (Nederlof et al, 89, Poddighe et al, 1991) Such a combined approach is particularly suited to determine or confirm structural chromosome aberrations, such as translocations, and to identify and characterize marker chromosomes

In this study we have applied ISH to frozen tissue sections of TCCs and compared these results with those of RFLP analyses of the same tumor cases. In Table 2 we summarized these results per chromosome, in order to see whether a correlation exists between an underrepresentation, as detected by ISH, and LOH of a marker on that same chromosome.

The most characteristic numerical aberration as detected by ISH in the 24 TCCs was a loss for chromosome 9 in 15 out of 24 cases, as compared to the mean chromosome ploidy as determined with the panel of 8 centromeric probes These observations strongly confirm other published data (Hopman et al, 1988b, 1989, 1991), where the cytogenetic observations concerning loss of chromosome 9 in low-grade, low-stage TCCs were confirmed by the ISH approach Also, the frequent occurrence of loss of chromosome 9 in early stages of

CHROMOSOME 9 (n = 19)

	RFLP		
	LOH	no LOH	N
ISH loss	8	4	12
no ISH loss	1	6	7
N	9	10	19

CHROMOSOME 11 (n = 19)

	RFLP		
	LOH	no LOH	N
ISH loss	0	0	0
no ISH loss	4	15	19
N	4	15	19

CHROMOSOME 16 (n = 21)

	RFLP		
	LOH	no LOH	N
ISH loss	0	0	0
no ISH loss	3	18	21
N	3	18	21

CHROMOSOME 17 (n - 22)

	RFLP		
	LOH	no LOH	N
ISH loss	1	3	4
no ISH loss	4	14	18
N	5	17	22

CHROMOSOME 18 (n = 12)

	RFLP		
	LOH	no LOH	N
ISH loss	1	2	3
no ISH loss	2	7	9
N	3	9	12

Table 2. *Two-way tables demonstrating the comparison of both RFLP and ISH analyses per chromosome In brackets are given the number of cases, on which both techniques revealed results This means informative for the RFLP analysis, and evaluable for ISH Loss means underrepresentation for that particular chromosome as detected by ISH, no loss means within the ploidy range as detected by ISH with the other centromere associated DNA probes*

TCC is in agreement with the observation of Tsai et al (1990), who showed that LOH of markers on chromosome 9q could be detected in 67% of the informative cases Comparison of the ISH results with the RFLP analyses of the same tumors in this study, revealed a high concordance for LOH and chromosomal loss as detected by ISH (Table 2). For the 13 chromosome diploid cases studied, the ISH approach showed monosomy for chromosome 9 in 8 cases The process of tetraploidization is a generally accepted concept in tumor progression (Shackney et al, 1989, Fearon et al, 1990b) In the aneuploid TCCs the apparent loss of two copies of chromosome 9 strongly indicates that the loss of one copy of this chromosome occurred before tetraploidization took place This confirms our previous

suggestion that loss of chromosome 9 is a primary or early event in carcinogenesis of the urinary bladder (Hopman et al, 1991), and that it is conserved during the process of tumor progression and invasion

The loss of chromosome 9 as detected by ISH is most probably not limited to a small part of 9q Because this DNA target is situated in the heterochromatin on 9q, close to the centromere, only a complete loss of this heterochromatin region would result in a complete disappearance of the chromosome 9 signal Deletion of either 9q or 9p would preserve a part of this target sequence, resulting in positive, but less intense or less extended ISH signals Therefore, candidate genes that may play a relevant role in the oncogenesis of the urinary bladder can be located on both arms of chromosome 9. Recently, Cairns et al (1993) published a study, using 13 polymorphic markers for chromosome 9 in order to map the region of deletion in human bladder carcinomas Their results suggest that the target locus of a putative tumor suppressor gene involved in bladder carcinoma is located at the region between 9p12-13 and 9q22 In our RFLP analysis we used a polymorphic marker located at 9q34. This may explain our observation that in four cases (Nos. 2, 4, 7, and 15) an ISH loss for chromosome 9, but no LOH with RFLP analysis was observed.

For the other chromosomes studied, we found no correlation between LOH and centromeric loss The biological meaning of these observations could be that in those cases where LOH was found, but no loss was observed by ISH for that particular chromosome, an interstitial deletion, chromosomal rearrangement, or duplication of a part of that chromosome was involved As far as heterogeneity is concerned, ISH analysis on tissue sections is a more specific approach as compared to RFLP or karyotyping, since it is possible to evaluate results at the individual cell level, while Southern blot analysis provides a quantitative but integral overview of changes that dominate within the tumor This means that by RFLP analysis the genomic heterogeneity that might exist between subclones of neoplastic cells remains undetected. Furthermore, by ISH a numerical chromosome aberration can be observed, which is not possible by the RFLP approach Therefore, ISH can be applied in a clinical setting on a large scale basis for the analysis of tumors. The use of centromere-associated probes enables the detection of numerical chromosome aberrations. The development and application of region specific cosmid probes should increase the clinical applicability of ISH

Recently, Matsumura et al (1992) showed that LOH of 17p in breast tumors correlated with 17 centromere and 17p loss, as detected by ISH, indicating that for breast tumors ISH can be used as a relevant clinical parameter. Therefore, we suggest that the ISH and RFLP techniques can be applied as complementary approaches for the study of clinical tumor material

The approach as described in this paper, combining both Southern blotting and ISH, has the advantage that more insight into the two mechanisms of LOH and chromosomal evolution of tumors can be obtained A further step, however, could be the use of both centromeric and cosmid probes of the same chromosome

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Summary and Future Directions

Interphase cytogenetics by in situ hybridization (ISH) techniques, allows the detection of specific nucleic acid sequences in morphologically preserved interphase cells or tissue sections The copy number of a specific chromosome in the nucleus can be determined by counting the number of signals after ISH, using specific DNA probes In this study chromosome specific DNA probes were applied in order to detect chromosome aberrations in tumor cells in a routine setting

In hematological malignancies, chromosome banding techniques play an important role in the detection of genetic aberrations Since the chromosome aberrations are mostly well-characterized, these banding techniques are used in diagnosis and determining prognosis Although a growing number of chromosomal abnormalities in solid tumors are being reported, cytogenetical analysis of solid tumors is still limited due to technical difficulties, such as low mitotic index, neccessity of culturing the tumor cells, leading to induced changes in DNA content, low banding quality and fuzzy chromosomes

Chapter 1 reviews the clinical applications and results obtained by single and multicolor ISH techniques in tumors Information on the different DNA probe types that are available for the detection of chromosomal abnormalities in interphase cells is presented A summary is given of the results obtained with these probes in different types of tumors, reveiling both numerical and structural chromosome aberrations in metaphase spreads and interphase nuclei

In the three subsequent chapters the ISH technique was applied to interphase nuclei of leukemias These studies were performed in order to investigate whether interphase cytogenetics can be applied as a routine diagnostic tool in addition to the existing karyotyping procedures

Chapter 2 describes numerical chromosome aberrations in hematological cancers as detected by non radioactive ISH procedures, using centromere-specific DNA probes for chromosomes 1 7, 8, 9, 10, 11, 16, 17, 18, X and Y In seven out of fifteen randomly selected cases with leukemia numerical aberrations for one or two chromosomes were demonstrated Comparison of the ISH results with classical karyotyping revealed that seven numerical chromosome aberrations were detected by both techniques, whereas in five instances an aberration was only found with ISH Furthermore, double target ISH using centromeric associated DNA probes demonstrated translocations between two chromosomes in two cases Using the ISH approach on interphase nuclei allowed the identification of non-characterized marker chromosomes, found by classical cytogenetic analysis Therefore, interphase cytogenetics using chromosome specific repetitive centromeric probes may be an additional tool for studying both numerical and structural chromosomal aberrations of leukemias

Chapter 3 descibes a novel approach to determine the presence of chromosomal aberrations in progenitor cells of bone marrow By using this method, a numerical chromosome aberration, as detected by both karyotyping and interphase cytogenetics, can be used as a marker for the aberrant cells After in vitro culturing of the bone marrow samples in agar, the ISH technique was applied Since our approach retains the cytological architecture of the agar assay, it allows discrimination between chromosomal aberrations detected in the clonogenic and non clonogenic cells With this new technique, the presence of the cytogenetic aberration in clonogenic cells can be detected at the interphase level.

The application of this new ISH approach is demonstrated in a study of the diversity of acute myeloid leukemia (AML) during preleukemic phase, active disease, remission, and under in vitro culture conditions (Chapter 4) Firstly, in long-term liquid cultures of these leukemias upto 10 20 days, the numerical ISH marker was present in all clusters, which were morphologically typical for a leukemic growth pattern Since no residual normal clonogenic cells could be detected, it showed that not all leukemias appear to have the reported selective growth advantage for normal progenitor cells in long term liquid cultures Secondly, two AML patients with a numerical ISH marker were studied both at presentation and during remission In both cases the marker was not present in the colonies nor in the clusters Therefore despite the abnormal growth patterns, the cultured progenitors may well be residual normal cells Alternatively, they could have originated from a preleukemic clone without abnormal karyotype In both cases a low percentage (maximally 6%) of abnormal bone marrow or peripheral blood cells were detected by ISH during remission Consequently, remissions in these patients could have been partially or completely of clonal origin Finally, one patient with trisomy for chromosome 10 as marker was analyzed during myelodysplastic phase and after transformation to AML During both stadia uniform but abnormal appearing cell clusters were cultered However, these clusters were either disomic or trisomic for chromosome 10, indicating that a subclone within an abnormal growing preleukemic cell line might have been present

The second part of this thesis deals with interphase cytogenetic analyses of a solid type of malignancy, i e the transitional cell carcinoma (TCC) of the urinary bladder As already mentioned before, karyotyping analyses of solid tumors are hampered by different technical aspects, while also the flow cytometric (FCM) analysis of such lesions poses several problems

Chapter 5 compares the nuclear DNA content of 53 TCCs of the urinary bladder as determined by FCM to chromosome ploidy as detected by ISH Therefore, DNA probes specific for the centromeric region of chromosomes 1 and 18 were used In about 25% of the FCM diploid tumors evident chiomosome 1 aberrations were found by ISH, while in a few cases an aberration for chromosome 18 was detected FCM aneuploid cases with a DNA-index of 1 2 to 3 2 exhibited a profound heterogeneity In these TCCs the ratio between chromosomes 1 and 18 varied in a wide range, resulting in cases that showed more

ISH signals for chromosome 1 as compared to chromosome 18 or the opposite. Also minor cell populations showing polyploidization and giant cells containing numerous ISH signals could be detected It is concluded that interphase cytogenetics by ISH enables a fast screening of numerical chromosome aberrations and detection of different cell populations within one tumor that is apparently homogeneous after FCM analyses.

Structural chromosome aberrations, such as +1p- and duplication of 1q, are frequently encountered chromosomal anomalies in bladder cancer Similarly, extra chromosome 1 copy numbers were detected by ISH in FCM diploid and aneuploid cases. Chapter 6 therefore concentrates on structural chromosome 1 aberrations in 22 TCCs. For this purpose, a centromeric (1q12) and a telomeric (1p36) DNA probe, as well as a library DNA probe from sorted human chromosome 1 were applied in single- and double-target ISH procedures. All FCM diploid TCCs, which showed three ISH signals for 1q12, had two ISH signals for 1p36. Since the library DNA probe showed three separate domains in the nuclei of these cases, the additional copy for 1q12 could be explained as an extra chromosome 1p-, containing the 1q12 target In the FCM tetraploid and aneuploid tumors the results were more complex In several cases we observed an overrepresentation of 1q12 as compared to 1p36, also suggesting the presence of extra copies of 1p- chromosomes.

Chapter 7 compares RFLP analyses and interphase cytogenetics in 24 TCCs in order to obtain an impression of the degree of overlap of both techniques The tumor DNA samples were screened for loss of heterozygosity (LOH) for chromosome loci 9q34, 11p15, 16q22-24, 17p13, and 18q21 ISH was performed on serial frozen tissue sections with centromeric-associated DNA probes for chromosomes 1, 7, 9, 10, 11, 16, 17 and 18 to determine chromosome aneuploidy, numerical chromosome aberrations, imbalance between chromosomes, and heterogeneity of those aberrations within the tumor The results demonstrate a good correlation between both approaches, showing that LOH for chromosome locus 9q34 is mainly due to loss of the complete chromosome 9, since ISH with the centromeric probe for this chromosome showed in almost the same cases an underrepresentation No correlations were found for the loci on chromosomes 11p, 16q, 17p and 18q, suggesting either an interstitial deletion or LOH in combination with a duplication of (a part of) that particular chromosome in the aneuploid cases

Future Directions

The approach of interphase cytogenetics using ISH techniques has not yet reached the end of its possibilities with regard to its technical developments and applications in the tumor analysis The approaches, as described in this thesis, allow mainly the identification of numerical changes of specific chromosomes Most of the repetitive DNA sequences applied so far recognize targets located in the centromeric or heterochromatin regions of the respective chromosomes

Interphase cytogenetics can be applied to monitor patients for minimal residual disease.

Especially in hematological malignancies, where a vast knowledge of chromosomal aberrations exists, interphase cytogenetics will be carried out in the near future for diagnosis and monitoring

In solid tumor diagnosis, where classical cytogenetic analyses are hampered by technical difficulties, interphase cytogenetics may reveal information of subclonal populations with chromosomal aberrations For example, in FCM near-diploid bladder tumors, we found that about 25% of these tumors contain numerical chromosome aberrations.

Counting of hybridization signals is a crucial part of the evaluation of chromosomal changes in interphase nuclei. Heterogeneity of tumor cell populations, and variety of spot morphology may often lead to interpretation problems Furthermore, the number of ISH signals for a specific chromosome probe is not neccessaraly in agreement with the copy number of that particular chromosome, since most solid tumors consist of complex karyotypes with complex marker chromosomes The use of chromosome specific DNA libraries will facilitate the detection of numerical and structural chromosomal aberrations. This technique, known as chromosome in situ suppression (CISS or chromosome painting) hybridization, has already general applications for the identification of complex chromosome translocations in metaphase spreads The application of this technique for interphase cytigenetics is limited, since the staining patterns are difficult to interpret

Another development in ISH is based on the combination of ratio-labeled DNA probes with digital imaging devices, like CCD cameras This enables the simultaneous detection of up to twelve distinctly painted chromosomes, both in metaphase spreads and interphase nuclei However, for the analysis of a tumor cell sample, previous knowledge of the expected chromosomal aberrations is required in such analyses

A new approach to analyse tumor samples for unknown chromosomal changes has been described, termed as reverse chromosome painting. The principle of this technique is that isolated tumor DNA serves as probe in a CISS hybridisation to normal metaphase chromosome spreads Evaluation of signal intensities may reveal amplified sequences, but also loss of chromosomal material A further improvement of this technique for the detection of small differences of DNA sequences, is the so-called comparative genomic hybridization (CGH) technique In this approach, the labeled tumor DNA competes with differently labeled control genomic DNA for the same targets Hybridized tumor and control DNA sequences are detected by different fluorochromes. The resulting fluorescence ratios for each chromosome should reflect the relative copy number of the homologous sequences containing the two genomic DNAs

The detection of chromosome target sites in both frozen or paraffin-embedded tissue sections allows the study of chromosome aneuploidy within the context of a variety of histological and morphological parameters The question whether progression of the tumor has given rise to cytogenetically distinct foci of cells becomes amenable for analysis, although further developments of the ISH approaches are necessary before applying in a routine setting.

To conclude, detailed genetic characterization of tumor cell populations by means of ISH techniques has already found its application in tumor pathology, especcially by the use of repetitive DNA probes Double (or multiple) target ISH may be applied in order to detect chromosome imbalances and tumor heterogeneity. Furthermore, it is to be expected that in combination with other molecular techniques, such as immunophenotyping, Southern blotting and PCR, the identification of chromosomal abnormalities and genetic imbalances in the tumor genome will help in the search for oncogenes and tumor suppressor genes specifically involved in certain tumor types Such improved and combined approaches could then increase our knowledge of tumor progression, and subsequently improve tumor diagnosis.

Samenvatting

De interfase cytogenetica, gebruik makend van de in situ hybridisatie (ISH) technieken, biedt de mogelijkheid om specifieke nucleinezuur-sequenties te detecteren in interfase cellen of weefselcoupes Het kopie-aantal van een specifiek chromosoom in de kern kan worden bepaald door het aantal signalen te tellen na ISH met behulp van specifieke DNA probes In deze studie zijn chromosoom-specifieke DNA probes toegepast om routinematig chromosoom-afwijkingen in tumorcellen te detecteren

Bij kwaadaardige hematologische ziekten spelen chromosoom banderingstechnieken een belangrijke rol in de detectie van genetische afwijkingen Aangezien de meeste chromosoom afwijkingen thans goed gekarakteriseerd zijn worden deze banderingstechnieken gebruikt bij het vaststellen van de diagnose en prognose Hoewel steeds meer chromosomale afwijkingen in solide tumoren bekend worden, zijn cytogenetische analyses van solide tumoren beperkt als gevolg van technische problemen, zoals een lage delingscapaciteit van de tumorcellen, slechte banderingskwaliteit en vage chromosoom-contouren Ook kan de noodzaak van het kweken van de cellen leiden tot geinduceerde veranderingen in DNA samenstelling

Hoofdstuk 1 geeft een overzicht van de klinische toepassingen en resultaten, zoals verkregen met behulp van enkel- en meerkleurige ISH technieken in verscheidene tumor typen Informatie omtrent de verschillende typen DNA probes die beschikbaar zijn voor de detectie van chromosomale afwijkingen in interfase cellen wordt gevolgd door een samenvatting van de resultaten zoals verkregen met deze probes Deze betreffen zowel numerieke als structurele chromosoom-veranderingen in metafase preparaten en in interfase kernen Tevens wordt aangegeven hoe deze gegevens ertoe bijdragen de tumordiagnose te vergemakkelijken

In de drie daarop volgende hoofdstukken wordt de toepassing beschreven van de ISH techniek toegepast op interfase kernen van leukemieen Dit onderzoek werd gestart om uit te zoeken in hoeverre de interfase cytogenetica kan worden toegepast als een routinematig diagnostisch hulpmiddel naast de bestaande karyotyperingsprocedures

Hoofdstuk 2 beschrijft de detectie van numerieke chromosoom veranderingen in hematologische maligniteiten met behulp van ISH procedures, gebruik makend van centromeer specifieke DNA probes voor de chromosomen 1, 7, 8, 9, 10, 11, 16, 17, 18, X en Y In zeven van de vijftien willekeurig gekozen gevallen van leukemie zijn numerieke veranderingen voor een of twee chromosomen aangetoond Vergelijking van de ISH resultaten met de klassieke karyotypering liet zeven maal een numerieke chromosoom afwijking zien, die door beide technieken werd aangetoond, terwijl vijf maal een afwijking alleen met ISH werd gevonden Verder toonde de dubbel ISH procedure met twee verschillende centromeer geassocieerde DNA probes translocaties tussen twee chromosomen

aan in twee gevallen. Met behulp van de ISH benadering op interfase kernen bleek het verder mogelijk niet-gekarakteriseerde markerchromosomen, zoals verkregen met de klassieke cytogenetische analyse, te karakteriseren. Daardoor kan de interfase cytogenetica met behulp van chromosoom-specifieke, repetitieve centromeer-probes additionele informatie opleveren bij de bestudering van numerieke en structurele chromosomale veranderingen in leukemieën.

In Hoofdstuk 3 is een nieuwe benadering beschreven voor de bestudering van chromosomale veranderingen in progenitor cellen in beenmerg. Daarvoor kan een numerieke chromosoom verandering, zoals aangetoond met zowel karyotypering als interfase cytogenetica, als merker voor de afwijkende cellen worden gebruikt. Na in vitro kweek van beenmerg cellen in agar kan de ISH techniek worden toegepast met behoud van de cytologische informatie van het agar assay. Hierdoor is het mogelijk een onderscheid te maken tussen chromosomale veranderingen in de kolonievormende en niet-kolonievormende cellen in kweek. Met deze nieuwe benadering kan de aanwezigheid van de cytogenetische afwijking in de klonogene cel worden aangetoond op interfase niveau.

De betekenis van deze nieuwe ISH benadering is aangetoond door de bestudering van de biologische verscheidenheid van acute myeloïde leukemie (AML) gedurende de preleukemische fase, de aktieve ziekte, tijdens remissie, en onder in vitro kweek condities (Hoofdstuk 4). In lange termijn cultures van deze leukemieën (10-20 dagen) is de numerieke chromosomale verandering aanwezig in alle celklusters, die morfologisch kenmerkend zijn voor het leukemische groeipatroon. Aangezien er geen residuale normale klonogene cellen konden worden aangetoond, blijkt dat niet voor alle leukemieën het beschreven selectieve groeivoordeel van normale progenitor cellen optreedt. Twee AML patiënten met een numerieke ISH merker werden bestudeerd zowel ten tijde van eerste presentatie als ook na remissie van de ziekte. In beide gevallen was de merker niet aanwezig in de kolonies of in de klusters, hetgeen wijst op een normaal karakter van de gekweekte progenitor cellen. Ook is het mogelijk dat ze ontstaan zijn uit een preleukemische celkloon met een normaal karyotype. In beide gevallen is een laag percentage (maximaal 6%) van abnormale beenmerg- en perifere bloedcellen gedetecteerd met ISH tijdens remissie. Dit betekent dat de remissies in deze patienten partieel of compleet klonaal kunnen zijn geweest. Tenslotte is één patiënt met een trisomie voor chromosoom 10 als merker geanalyseerd tijdens de myelodysplastische fase en na transformatie tot AML. Tijdens beide stadia zijn gelijke, maar abnormaal ogende celklusters gekweekt. Echter, de celclusters vertoonden hetzij een disomie hetzij een trisomie voor chromosoom 10. Dit duidt erop dat een subkloon in een abnormaal groeiende preleukemische kloon aanwezig kan zijn geweest.

Het tweede deel van dit proefschrift beschrijft de toepassing van de interfase cytogenetica op een solide tumor, namelijk het overgangscel-carcinoom (TCC) van de urine blaas. Zoals al eerder genoemd, worden karyotypering-analyses van solide tumoren belemmerd door verschillende technische aspecten, terwijl ook de flowcytometrische (FCM)

analyses van dergelijke afwijkingen verscheidene problemen met zich meebrengen

In Hoofdstuk 5 is de DNA inhoud van 53 TCCs, zoals bepaald met behulp van FCM, vergeleken met de chromosoom-ploidie, zoals die bepaald wordt met behulp van de ISH procedure Daarvoor zijn DNA probes specifiek voor het centromeer gebied van de chromosomen 1 en 18 gebruikt In ongeveer 25% van de FCM diploide tumoren zijn chromosoom 1 afwijkingen gevonden met ISH, terwijl in enkele gevallen een afwijking voor chromosoom 18 gedetecteerd is De FCM aneuploide gevallen met een DNA index van 1 2 tot 3 2, toonden een hoge mate van heterogeniteit In deze TCCs varieerde de ratio tussen de chromosomen 1 en 18 aanzienlijk, resulterend in gevallen die meer ISH signalen voor chromosoom 1 vertoonden in vergelijking tot signalen voor chromosoom 18, en omgekeerd Ook werden kleine celpopulaties waargenomen waarbij polyploidisering was opgetreden, en reuzecellen met zeer vele ISH signalen De interfase cytogenetica kan een snelle screening van numerieke chromosoom afwijkingen mogelijk maken en verschillende celpopulaties detecteren binnen eén tumor die aanvankelijk homogeen leek zoals bepaald met behulp van FCM

Structurele chromosoom afwijkingen, zoals +1p- en duplicatie van 1q, zijn vaak waargenomen chromosomale veranderingen bij blaaskanker Ook extra kopie-aantallen voor chromosoom 1 zijn met ISH gedetecteerd in zowel FCM diploide als aneuploide tumoren Hoofdstuk 6 beschrijft een studie naar structurele chromosoom 1 veranderingen in 22 TCCs Daarvoor zijn centromeer (1q12) en telomeer (1p36) DNA probes, evenals een DNA chromosoom 1 bank gebruikt in enkel en dubbel-target ISH procedures Alle FCM diploide TCCs, die drie ISH signalen per kern voor 1q12 bevatten, toonden twee ISH signalen voor 1p36 Aangezien de chromosoom 1 bank drie aparte domeinen in de kernen van deze gevallen toonde, kon het additionele signaal voor 1q12 worden verklaard als een extra chromosoom 1p-, dat het 1q12 target bevat In de FCM tetraploide en aneuploide tumoren waren de resultaten complexer In enkele gevallen werd een overrepresentatie van 1q12 ten opzichte van 1p36 waargenomen, hetgeen eveneens de aanwezigheid van extra kopie-aantallen van 1p- suggereert

In Hoofdstuk 7 zijn de resultaten van RFLP analyses en die van de interfase cytogenetica op 24 TCCs met elkaar vergeleken De tumor DNA samples, verkregen uit tumoren van verschillende gradatie en stadia, werden gescreend op verlies van heterozygotie voor de chromosoom gebieden 9q34, 11p15, 16q22-24, 17p13 en 18q21 ISH werd op seriecoupes van vriesmateriaal van dezelfde tumoren uitgevoerd Centromeer-geassocieerde DNA probes voor de chromosomen 1, 7 9 10, 11, 16, 17 en 18 werden gebruikt om chromosoom ploidy, numerieke chromosoom afwijkingen, imbalans tussen chromosomen en heterogeniteit van afwijkingen binnen eenzelfde tumor te bepalen Er bleek een goede correlatie te bestaan tussen de RFLP en ISH benadering, waarbij het verlies van heterozygotie voor het chromosoom locus 9q34 in de vroege fase van de TCC-ontwikkeling voornamelijk het gevolg is van verlies van het complete chromosoom 9, aangezien ISH met de centromeer probe voor dit chromosoom in bijna alle gevallen een ondervertegenwoordi-

ging liet zien. Voor de loci op chromosomen 11p, 16q, 17p, en 18q werd geen correlatie tussen beide benaderingen gevonden. Het gevonden verlies van heterozygotie kan duiden op een interstitiele deletie, maar ook verlies van heterozygotie in combinatie met een duplicatie van (een deel van) het betreffende chromosoom in de aneuploide gevallen.

List of Publications

Hopman A H N , *Poddighe P J* , Smeets A W G B , Moesker O , Beck J L M , Vooijs G P , Ramaekers F C S (1989) Detection of numerical chromosome aberrations in bladder cancer by in situ hybridization American Journal of Pathology 135 1105-1117

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Poddighe P J , Bringuier P P , Schalken J A , Ramaekers F C S , Hopman A H N (1993) Comparison of interphase cytogenetics on frozen tissue sections and Southern Blot analysis of transitional cell carcinoma of the urinary bladder Submitted

Vet J A M , Bringuier P P , *Poddighe P J* , Karthaus H F M , Debruyne F M J , Schalken J A (1993) Prognostic significance of p53 mutations in bladder cancer Manuscript in preparation

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de afdeling Anthropogenetica
de afdeling Medische Fotografie (zwart-wit en kleur)
de afdeling Moleculaire Biologie en Genetica te Maastricht
mijn familie
mijn vrienden, vriendinnen en kennissen
Hannie
Olof

Bedankt!

Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 18 januari 1963 te Sittard. Aldaar bezocht hij het Bisschoppelijk College St. Jozef, en behaalde in het voorjaar 1981 zijn diploma Atheneum-b In het najaar begon hij aan de studie biologie aan de Katholieke Universiteit te Nijmegen Tijdens de doctoraalfase liep hij een eerste bijvakstage op de afdeling Chemische Cytologie (Dr F Wanka), waarbij hij onderzoek verrichtte aan de origins van replicatie en hun associatie met de kernmatrix Zijn hoofdvakstage doorliep hij op de afdeling Anthropogenetica (Prof. Dr. H.H Ropers / Dr. B. Wieringa). Aldaar werkte hij mee aan de typering van humane apolipoproteine E varianten met behulp van in vitro DNA amplificatie (PCR). Tevens werd een studie gedaan naar de koppeling van dit gen met myotone dystrofie (DM) in 16 families. Tussendoor behaalde hij zijn kandidaats-examen biochemie (B4) in 1986. De doctoraalfase sloot hij af met een tweede bijvak op het Urologisch Research Laboratorium (Dr J Schalken), alwaar hij meewerkte aan de keratine-expressie van het Dunning R-3327 rat prostaatkanker modelsysteem. Op 30 augustus 1988 studeerde hij af

Van september 1988 tot september 1992 werd het in dit proefschrift beschreven onderzoek verricht op het Instituut voor Pathologie (Prof Dr. G.P. Vooijs, na 1-2-1992 Prof. Dr. D J Ruiter) van het St Radboudziekenhuis te Nijmegen. Aansluitend kreeg hij een aanstelling als wetenschappelijk medewerker, op basis van een NKB project, binnen de Moleculaire Pathologie-groep van hetzelfde Instituut.

STELLINGEN

behorende bij het proefschrift.

Interphase Cytogenetics in Tumor Pathology

door Pino J Poddighe
19 januari 1994

1 Het verlies van chromosoom 9q is een belangrijke vroege gebeurtenis bij het ontstaan van het overgangscelcarcinoom van de urineblaas
(Dit proefschrift)

2 Bij het in combinatie gebruiken van glucose-oxidase- en peroxidase-geconjugeerde antilichamen in de immunocytochemie wordt onvoldoende rekening gehouden met het ontstaan van peroxides door auto-oxidatie van DAB.
(Kuhlmann WD et al , Histochemistry (1986) 85 13-17)

3. Cellen met een abnormaal chromosoomaantal zijn focaal al aanwezig in intraepitheliale afwijkingen graad 1 van de cervix uteri
(NKB KWF-project no 92-34)

4 De ontwikkeling van geautomatiseerde systemen voor ISH signaal analyse resulteert in een schijnbaar objectieve benadering van de werkelijkheid door het niet geobjectiveerd zijn van de selectie-criteria

5. Bij de interpretatie van in situ hybridisatie resultaten op weefselcoupes is het aansnij-effect ondergeschikt aan het behoud van de weefsel-architektuur.

6. De in situ detectie van DNA of RNA sequenties dient, evenals immunocytochemie, een vaste plaats in de diagnostiek te krijgen.

7. Prenatale diagnostiek van de menselijke geaardheid is irrelevant en maatschappelijk ongewenst.

8. De toegankelijkheid van klassieke muziek wordt verminderd doordat de cd-booklets zelden in het Nederlands verschijnen.

9. Het lot van het stedelijk knooppunt Arnhem-Nijmegen wordt tijdens de slag om Elst beslist.

10. Voor de wetenschapper geldt: Onbegrepen maakt onbemind.

11. Een Sardijn in Nederland is als een peper in hutspot.

12. Voorzitters zijn vaak ook lijders.

13. Schrijven is zilver, schrappen is goud.

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