REVIEW ARTICLE—CHROMOSOME PATHOLOGY

INTERPHASE CYTOGENETICS OF TUMOURS

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INTRODUCTION

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 tumour cell population, but it gives no information about 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 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.

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, such as proto-oncogenes and tumour 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 cytogenetic analysis by means of ISH applied to non-mitotic cells and was originally introduced by Cremer et al.1

About 20 years ago, the technique of in situ hybridization was developed using radioactive probes.2,3 Nowadays, radioactive ISH reaches a very high sensitivity. The detection of DNA sequences of single-copy genes, viral genomes,5,6 or mRNA molecules in individual cells7 or tissue slides8-11 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 hapten or other labels, which allow non-radioactive detection (e.g., by fluorochromes, enzymes or colloidal gold particles), have been developed.12 Moreover, 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.13,14,16,17 The combination of immunocytochemistry and in situ hybridization is also feasible.9

METHODOLOGICAL ASPECTS

To employ the ISH procedure in pathology practice and to apply it to single-cell suspensions,
Table I—Chromosome specific repetitive DNA probes*

<table>
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<tr>
<th>Chromosome recognized</th>
<th>Name of probe</th>
<th>Insert (kb)</th>
<th>Repetitive sequence</th>
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<tr>
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<td>1.77</td>
<td>Sat. III</td>
<td>49</td>
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<td>53</td>
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*Several of these probes are available from Oncor, Molecular Cyto- genetics, Gaithersburg, U.S.A.; American Type Culture Collection, Rockville, U.S.A.; and Amersham, Buckinghamshire, U.K.

paraffin sections, and frozen sections of solid tumours, the following methodological steps are required: (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) immunochemical detection of the hybridized probe.

**DNA probes**

Eukaryotic DNA contains many repeated basepair sequences, with highly repetitive DNA (satellite DNA) usually localized near centromeres. Some satellite DNAs have a heptanucleotide sequence repeated more than 10,000 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.

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 I), are now routinely applicable in pathology practice (Figs 1 and 2).

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, 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. This principle is also used to facilitate the selective hybridization of unique sequence subsets from cosmid DNA clones in ISH experiments. As a result, a more or less homogeneous hybridization signal on individual human chromosomes from pter to qter can be obtained (Fig. 1E), which can also be recognized in the interphase nuclei as chromosome domains (Figs 1F and 1I). Large single-copy genes 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 at a developmental stage, and the number of papers is limited.

**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, BrdU, and recently FITC-dUTP, or by chemical modification with, for example, an acety laminofluorene (AAF) group, mercury ions, or a sulphone group (for a review see ref. 12).

Recently, the chemical synthesis of oligonucleotide probes containing functional groups (i.e., primary aliphatic amines or sulphhydryl groups) has also been described, thus in principle allowing the application of nucleotide oligomers in non-radioactive ISH by coupling hapten, such as biotin, or reporter molecules like fluorochromes or enzymes. 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 hapten, or the modified probes, are detected by affinity cytochemical techniques, using monoclonal or polyclonal antibodies directed to the introduced hapten. Indirect immunocytochemical procedures use fluorochromes such as fluorescein (FITC), rhodamin (TRITC), Texas red, or AMCA (aminomethylcoumarin 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 the precipitation of enzyme substrates with different colours.

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

Processing of biological material for ISH in pathology

Fresh tumour material obtained after surgery or by fine-needle aspiration biopsies is divided for different diagnostic procedures, such as immuno-cytochemistry, histological or cytological diagnosis, flow cytometry, or in situ hybridization. Most of the fixatives used in pathology, such as 70 per cent ethanol in flow cytometry or formaldehyde in some immunohistological procedures, do not hamper the ISH reaction. To accomplish 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 by removing the cytoplasm and part of the nuclear proteins. 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 tumour material a higher concentration of pepsin has to be used. Sections which display nuclei will also remove part of them and hence the chromosome copy number per nucleus may be underestimated. We therefore correlated chromosome copy numbers as detected in single cell suspensions isolated from fresh tumour material with the number as detected in paraffin sections of the same tumour. Comparison of the ISH data shows that aneuploidy can easily be detected (Figs 2C–2H). The advantages of ISH on paraffin sections as compared to ISH on isolated tumour cells can be summarized as follows: (1) chromosome heterogeneity can be detected within a tumour; (2) focal tumour cell areas with chromosome aberrations can be recognized in the sections and can be correlated with the histological appearance; (3) discrimination between stromal, inflammatory cells, and tumour 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 a variety of reasons. Cells in which the chromosome copy number is underestimated result from inefficient hybridization and co-localization of signals. 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 sites, and spots in paired arrangement (split spots) can interfere with the estimation of chromosome copy number of a cell.
Fig. 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 these bladder tumour cells. 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, arrows) on bone marrow cells of a patient suffering from chronic myeloid leukaemia. Co-localization 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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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.

Haematological tumours

In haematological oncology, chromosome banding techniques play an important role in determining diagnosis and prognosis. It is to be expected that interphase cytogenetics will soon be implemented for diagnosis and monitoring of leukaemia and lymphomas.

Recently, studies were published comparing the interphase cytogenetic procedure using non-radioactive ISH with conventional cytogenetic analyses of cell lines derived from solid tumours, in neoplastic cells from bone marrow and peripheral blood. These studies demonstrated that repetitive DNA probes can be applied routinely in an ISH procedure and are useful in addition to conventional cytogenetic techniques for the detection of both numerical and 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. Figure 10 shows the 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 of the order of 0.01 per cent can be detected.

In addition to their role in detecting numerical chromosome aberrations, centromeric DNA probes can also be used to identify structural aberrations. We demonstrated that the use of double-target ISH experiments on interphase nuclei of bone marrow cells (Figs 1K–1N) can characterize marker chromosomes and translocations between chromosomes.

Interphase cytogenetics also provide an exquisitely sensitive means of monitoring patients for residual disease for which one or more cytogenetic hallmarks are known. For example, chronic myelogenous leukaemia (CML) is genetically characterized by fusion of the bcr and abl genes on chromosomes 22 and 9, respectively. This translocation can be detected by Southern blot analysis or in vitro amplification by the polymerase chain reaction (PCR). However, these techniques give no information about individual cells. A two-colour 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.

Urological tumours

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 chromosomes 1, 7, 9, 11, and 18 to single-cell suspensions of these tumours. The results showed that aneuploidy could be frequently detected in bladder tumours which had a DNA index of approximately 1.0 as measured by FCM. Most strikingly, a monosomy for chromosome 9 was found in low-grade TCCs.

Tumours with a DNA index of 1.2–3.2 exhibited significant heterogeneity, both within individual tumours and between different tumours with the same DNA content. This phenomenon is demonstrated by double-target ISH experiments in Fig. 1J, showing an imbalance between chromosomes 1 and 18. Analysis of the total tumour cell populations by ISH has shown that minor cell fractions with extremely high chromosome copy numbers can be detected in these tumours. These cells may possibly originate from polyploidization. In high-grade and invasive tumours with DNA aneuploidy or tetraploidy, 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 non-invasive 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 non-invasive TCCs with a probe for the centromeric and telomeric regions, 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 (Figs 1G–1N), whereas in the tetraploid cases selective loss of heterochromatin was observed (to be published).
Fig. 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 spot number for chromosome 1; the arrows indicate so-called isochromosomes. C and D show a flow cytometrically diploid transitional cell carcinoma of the human bladder, with an evident trisomy for chromosome 1 (C). In D, the tumour cells (four spots per nucleus, arrow-head) can be discriminated from the non-tumour cells, such as stromal cells (see arrow). In E, an aneuploid tumour of the bladder is hybridized with a probe for chromosome 1, resulting in 3–9 ISH spots per nucleus. The arrow-head 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 chromosome 1 (F) and X (G), and none for chromosome Y (H), confirming trisomy as detected by cytometrical analysis.
We also detected numerical chromosome aberrations in paraffin sections of TCCs.\textsuperscript{37} 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 tumour areas, cell populations with spot numbers ranging from 1 to 4 were detected (Figs 2C and 2D). The inflammatory cells in the paraffin sections of this tumour mainly showed two spots for chromosome 1 (Fig. 2D). Comparison with isolated tumour cells in suspension showed that aneuploidy can easily be detected in paraffin sections using ISH. Figure 2E shows a paraffin section of a TCC with a DNA index of 3.2. In isolated cells, the average spot number for the chromosome 1 probe ranged from 6 to 9, but in paraffin sections this range was broader and no major peak could be detected. On the basis of these comparative studies, we conclude that a fast screening method for detection of numerical chromosome aberrations in routinely processed tumour material has become available. Hybridization with different probes on the same tumour areas in parallel sections or double-target ISH will enable us to study chromosome ratios and therefore the specific loss of chromosomes.

Prostate cancer—The analysis of prostate tumours by ISH was reported by Van Dekken et al.\textsuperscript{37} These authors found two typical chromosomal aberrations in adenocarcinomas of the prostate—monosomy for chromosome 10 and loss of the Y chromosome. This Y chromosome loss is possibly age-related, while the monosomy for chromosome 10 may correlate to the metastatic behaviour of this type of carcinoma, since a del(10q24) has been reported in such cases.\textsuperscript{38}

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 renal cell carcinomas with a diploid DNA content (as measured by flow cytometry) can be subdivided on the basis of ISH into a subgroup with euploid spot number for chromosomes 1 and 7, and another with an aberrant number of spots for these probes. Whether or not this refinement diagnosis is of clinical importance remains to be evaluated.

Breast tumours

Devilee et al.\textsuperscript{39} detected numerical aberrations for the target sites of chromosome 1 and/or chromosome 18 in seven primary breast tumours. 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 tumour types, since a minimal DNA difference of about 4 per cent from DI = 1.0 is required to obtain a split peak in flow cytometry.

Testis tumours

One approach in the prevention of testicular germ cell cancer is to diagnose the neoplasm 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.\textsuperscript{40} developed a procedure using non-radioactive ISH with a probe for chromosome 1 on preparations of semen. In a blind study based on the percentage of hyperdiploid cells, they identified samples both from patients with isolated CIS changes and from patients in whom CIS was accompanied by a tumour. 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 the prevention of testicular cancer. By similar methods precancerous cells can be identified directly in paraffin-embedded tissue\textsuperscript{41} which will now allow retrospective studies. Van Dekken et al.\textsuperscript{37} also demonstrated numerical chromosome aberrations in nuclei isolated from a fresh seminoma, using a panel of 12 centromeric ISH probes. Compared with FCM analysis, they found for almost all of these probes an aneuploid number of fluorescent spots.

Neurological tumours

Cytogenetic studies of brain tumours have revealed numerical chromosome aberrations, such as polysomy for chromosome 7 and losses of chromosomes 10, 22, and the sex chromosomes in
glioma cells (both oligodendrogioma and glioblastoma cells), as well as monosomy for chromosome 22 in meningiomas. Until recently, these cytogenetic aberrations were only observed by karyotyping, but Cremer et al. have no documented numerical and structural aberrations using library probes in the CISS hybridization technique. In two glioma cell lines, they showed underrepresentation of chromosome 22 and overrepresentation of chromosome 7, in agreement with previous studies on gliomas. In addition, these authors also observed underrepresentation for chromosome 4. Recently, Arnoldus et al. applied similar methods to brain tumours.

Hydatidiform moles and hydropic abortions

Van de Kaa et al. performed ISH on paraffin sections of hydatidiform moles and hydropic abortions (Figs 2F–2H) using probes for chromosome 1, X and Y, to discriminate between maternal and trophoblast cells. Diploid and triploid cells were clearly distinguished, which can be of great value in the differential diagnosis of complete and partial moles. Cytogenetic heterogeneity was detected in the proliferating cytotrophoblasts, confirming the relatively large hyperdiploid fraction observed by cytometric analyses.

Gastric tumours

DNA probes for chromosomes 1, 7, 17, X and Y were used in a cytogenetic study of ten histologically moderately or poorly differentiated gastric adenocarcinomas. The chromosome aneuploidy detected by ISH was shown to run parallel with the DNA ploidy detected by flow cytometry. Moreover, the ISH technique, detected in a high percentage of cells the loss of the Y chromosome, which is too small to be seen in flow cytometric DNA histograms. This significant clonal absence of the Y chromosome possibly represents a chromosomal marker in this type of tumour.

FUTURE PROSPECTS

As compared with cytogenetic procedures, ISH gives rapid and statistically more reliable information about genomic alterations. It gives more precise information about numerical and structural aberrations of the genome than flow cytometry. ISH analysis of tumour cell suspensions is often difficult to interpret, however, since the real tumour cell fraction may be missed or grossly underestimated because of admixture of non-malignant stromal or inflammatory cells. Recently Beck et al. have described the application of keratin antibodies in the use of flow cytometry to sort the epithelial tumour cells and to separate them from keratin-negative stromal and inflammatory cells. In a multiparameter flow cytometric analysis, the tumour cells were sorted onto glass slides. After ISH analyses, tumour cell populations (for example, in malignant effusions) 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 ISH is also being increasingly used for the detection of nucleic acids, there are certain situations in which combination of the two techniques on the same preparation can be advantageous. For example, viral antigens and DNA can be detected simultaneously by immunohistochemistry and ISH, respectively, in both cytology preparations (Fig. 1P) and routinely processed tissue sections. It is to be expected that such combined techniques will be improved and find increasing applications in diagnostic and experimental pathology.

This review demonstrates that numerical chromosome aberrations can be detected in pathological 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 telomeric probe. 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 tumours where the existing karyotyping procedures are not helpful. Furthermore, the identification of marker chromosomes and analyses of complex karyotypes may be aided by using a combination of these types of DNA probes.

ACKNOWLEDGEMENTS

We wish to acknowledge Olof Moesker, Eric van Hoorn for technical assistance, and Dr C. van de Kaa for microphotographs from ISH on
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hydatidiform moles. This work was supported by The Netherlands Cancer Foundation, IKL 88-7.

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