In situ hybridization as a tool
to study numerical chromosome aberrations
in solid bladder tumors

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Summary. Methods for single- and double-target in situ hybridization (ISH) to, cells isolated from solid transitional cell carcinomas (TCC's) of the urinary bladder are described. Single cell suspensions were prepared from solid tumors of the urinary bladder by mechanical disaggregation and fixed in 70% ethanol. Using two DNA probes specific for the centromeres of chromosomes #1 and #18, ISH procedures were optimized for these samples. Human lymphocytes and cells from the T24 bladder tumor cell line were used as controls. In lymphocyte nuclei and metaphase chromosome spreads, ISH showed two major spots for each of the probes. About 80% of the nuclei from T24 cells showed three spots for both the chromosome #1 and #18 specific probes. When nuclei from TCC's were analyzed, often the number of spots for chromosome #1, and to a lesser extent for chromosome #18, differed from the number expected on basis of flow cytometric ploidy measurements. The double target-ISH method in all cases allowed the correlation of numerical aberrations for chromosomes #1 and #18 in one and the same cell. By such analyses a profound heterogeneity in chromosome number was detected in most tumors. In order to optimize the reproducibility of the method and the interpretation of the ISH signals, criteria for their analysis have been determined. This procedure can now be applied on a routine basis to solid tumor specimens.

Introduction

The clinical course of bladder cancer is often unpredictable and as a result various efforts have been undertaken to develop prognostic parameters. In histopathology, next to the tumor stage, tumor grade is at present the most important indicator of the clinical behaviour of a bladder tumor (Cummings 1980; Cutler et al. 1982). At the cellular level the DNA content of the tumor cells may be another prognostic parameter (Levi et al. 1969; Tavares et al. 1980; De Vosee and Hijazi 1985) but chromosomal analysis is mentioned as one of the more objective criteria of the malignant potential of bladder cancer (Sandberg 1977; Summers et al. 1981; Tribukait et al. 1986; Wijkström et al. 1984). Cytogenetic studies showed a positive correlation between histo-

logical grade and modal chromosome numbers for low and high grade bladder tumors (Smeets et al. 1987a). Also non-random alterations in the karyotype of bladder cancer have been detected (Kovacs 1985; Atkin and Baker 1985; Smeets et al. 1987b).

Chromosome analysis of cancer cells by karyotyping is often only possible after tissue culturing. This method, however, 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 spreading, poor banding quality, and a condensed or fuzzy nature of the chromosomes (Granberg-Olman et al. 1980; Smeets et al. 1987c). In situ hybridization to interphase nuclei with chromosome specific DNA probes has the advantage that numerical chromosome aberrations can be detected without the necessity to culture tumor cells or to prepare chromosome spreads.

Here we describe the use of non-radioactive ISH methods for the detection of chromosome alterations in bladder cancer cells, and the development of criteria to distinguish between 'positive' and 'non-specific' signals. For this purpose we have applied several non-radioactive DNA labeling techniques (Langer et al. 1981; Brigati et al. 1982; Hopman et al. 1986a) and probes recognizing tandemly repeated DNA sequences in the centromere region of specific chromosomes (Devilee et al. 1986; Cooke and Hindley 1979). These probes show a high chromosome specificity and enable chromosome counting in non-mitotic single cells (Cremer et al. 1986; Hopman et al. 1987). Cremer et al. (1986) suggested the term 'interphase cytogenetics' for this technique. Here we report on our first experimental data obtained with cells isolated from solid bladder cancers which were analyzed for chromosome aberrations with non-radioactive ISH methods.

Materials and methods

Cell and tissue processing

Seven bladder tumor specimens as well as a bladder tumor cell line (T24) and human lymphocytes from healthy donors were used. Metaphase chromosome spreads and interphase nuclei were obtained from human peripheral blood lymphocyte cultures by standard procedures and were used as procedural controls. Cells of the bladder tumor cell line (T24) were grown as a monolayer,
trypsinized, and harvested by centrifugation at 400 × g for 7 min. Then, 70% ethanol (−20°C) was added rapidly to the cell pellet under constant shaking. The final concentration was about 3 million cells/ml. At this stage, the fixed cells could be stored at −30°C for flow cytometry and ISH analyses for up to several months.

Bladder tumor tissues were collected following transurethral resection. One part of each tumor was used for histopathological diagnosis, and another for flow cytometric studies and for in situ hybridization. The specimens were collected in 10 ml RPMI 1640 medium containing 17% fetal calf serum, 50 μg gentamycin/ml, 50 μg penicillin/ml and 50 μg streptomycin/ml. Single-cell suspensions of the fresh tissues were prepared as described before (Feit et al. 1985). 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, Bicester, Belgium). The filtered cell suspensions were fixed in 70% ethanol (−20°C) and stored at −30°C.

To improve cell recovery during isolation procedures the glassware (pipettes, etc.) was siliconized using dichloro-octamethyldisiloxane as described by the supplier (Surfasil, Pierce, USA). Clean microscopic glass slides were coated in 1 mg/ml poly-λ-lysine (MW > 150,000) for 10 min, washed with water, dehydrated via alcohol and air-dried.

Shortly before use, cells fixed in ethanol (T24 cells, tumors cells) were postfixed with freshly prepared methanol/glacial acid (3:1; four times, 5 min each at 0°C). Two or three drops of the cell suspension were placed on the poly-lysine coated slides and air-dried. Optionally, the cytoplasm was partly removed by dipping the slides in 70% acetic acid for 10–60 s, washed twice with distilled water, dehydrated in 100% ethanol and air-dried. The acid wash is expected to improve the penetration of both the probe during ISH and the antibodies during the immunocytochemical detection steps. We suggest to prepare slides shortly before use, since longer storage of the slides at 4°C may result in a significant enhancement of autofluorescence.

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 resuspended 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 fluorescence was measured using a 630 nm long pass filter for PI. The DNA content is expressed as the DNA index (DI). Chicken red blood cells were used as internal standard, while human lymphocytes were used as external standard.

DNA probes and labeling
The probe for chromosome #1 (pUC 1.77) is described by Cooke and Hindley (1979) and was kindly provided to us through Dr. Th. Cremer (Heidelberg). It recognizes the (pericentromeric region of which several ten thousand copies of a 1.77 kb sequence are present in a tandem repeat. The probe for chromosome #18 (L1.84) is described by Devißel et al. (1986).

Probe modification. Prior to mercuration of the probes, the DNA was sonicated to obtain fragments of about 400 bases. Mercuration was performed using mercury(II)acetate and the DNA isolated in the form of a cyanide complex (Hopman et al. 1986b, 1987). Biotinylation probes was performed using Bio-11-dUTP (BRL, USA) in a nick translation reaction as described by Brigati et al. (1982). The fragment length of the biotinylated probes was about 200–400 bases. This was achieved by variation of the DNase concentration during the polymerase reaction. After purification, the nucleic acids were dissolved to a concentration of 2–6 μg/ml in the hybridization mixture.

Hybridization. The probes used in this study do not only show binding to chromosomes #1 or #18 respectively, but also to chromosomes #9 and #13, #21, respectively when hybridization conditions of low stringency are applied (e.g. 50% formamide in 2 × SSC at 37°C). To avoid hybridization to such minor binding sites the hybridization conditions were optimized using peripheral blood metaphase chromosomes, resulting in a recipe with 60% (v/v) formamide, 2 × SSC of pH 5.0 (0.3 M NaCl, 30 mM Na-Citrate), 1 M KCl and salmon sperm DNA (1 μg/ml) as carrier DNA. Biotinylated probes could efficiently be hybridized in double-target ISH without interference of cyanide ions, which are essential for hybridization of mercured probes, and without interference of the mercured probes, as has been previously described (Hopman et al. 1986c). The hybridization mixture was added to dry slides (5 μl per 18 × 18 mm coverslip), sealed with rubber cement under a coverslip and denatured together with the target DNAs at 80°C on a heating plate. This procedure gave better results than denaturation of the targets with 0.07 N NaOH in 70% ethanol (Landegent et al. 1984; Raap et al. 1986) followed by proteinase K treatment (1–50 μg per 100 ml 20 mM Tris-HCl, pH 7.5, 2 mM CaCl2 at 37°C for 7.5 min).

Hybridizations were performed overnight at 37°C. Post-hybridization washings were done in 60% formamide, 2 × SSC of pH 5.0 and 1 M KCl for 15 min, once in 2 × SSC of pH 5.0 for 15 min and twice in 3 × SSC of pH 5.0 containing 1 mM EDTA, 0.05% Tween 20 (peroxide free, Pierce, USA) for 5 min. Immuno-cytochemical detection of the mercured probe was performed by subsequent incubation with a mercury-binding ligand Trinitrophenyl-Lys-Lys-NH2-CH2-CH2-SH (Hopman et al. 1986a) (available from Euro-Diagnostic BV, The Netherlands) dissolved in 1 M EDTA, 3 × SSC of pH 5.0 at a concentration of about 1 μM. Then sheep anti-trinitrophenyl serum, diluted 1:200 in phosphate buffered saline (PBS: 0.15 M NaCl, 10 mM Na-phosphate pH 7.2) containing 2% normal rabbit serum and 0.05% Tween 20 was added (buffer A), followed by incubation with FITC-conjugated rabbit anti-sheep serum, diluted 1:80 in buffer A (Nordic Immunology, The Netherlands). Biotin labeled DNA was detected by TRITC-conjugated avidin (Sigma, USA) at a dilution 1:1000 in buffer A. After each incubation step the slides were washed 3 times for 10 min in PBS. For the fluorescent approach, the slides were dehydrated and mounted in PBS/glycerol (1:9, v/v) containing 2.3% 1,4-di-azobicyclo(2,2,2)-octane (Sigma, USA) (Bock et al. 1985). In the double-target ISH method the FITC- and TRITC-conjugated reagents were incubated simultaneously.

Microphotographs were taken with a Leitz Dialux 20 EB microscope, equipped for FITC, TRITC, and DAPI fluorescence using a 3 M colour slide film (640-T, 3200 K). Exposure times were 2–3 min for FITC fluorescence, approximately 40 s for TRITC fluorescence, and 1 s for DAPI fluorescence.

Results

In situ hybridization procedures
Interphase nuclei and metaphase spreads of human lymphocytes and nuclei of the human bladder tumor cell line T24 were used to optimize the single- and double-target hybridization protocols using probes for chromosomes #1 and #18. Figure 1a–c shows examples of interphase nuclei and a metaphase spread of such cells, while Fig. 2a–c shows the schematic representation of cell and chromosomes with their respective ISH signals and counted spot number. The hybridization conditions as described in materials and methods were in general of sufficient stringency to detect only the targets on chromosomes #1 and #18 since each gives rise to intense doublets of fluorescent spots, both on the chromosome spreads as well as in the interphase nuclei. Occasionally, however, minor binding sites on other chromosomes were observed. If present, such signals could be distinguished from the signals on chromosome #1 and #18 respectively, because they were far less intense.
The bladder tumor cell line (T24) was originally derived from a primary transitional-cell carcinoma and its karyotype was characterized by an average chromosome number of 86, ranging from 44 to 108 chromosomes (Bubenik et al. 1973). Single-target ISH experiments showed mainly three spots for each probe (Fig. 3a) which corresponds well with the ploidy of the cell line as estimated by flow cytometry (DNA index = 1.6, Fig. 3b). About 15% to 20% of the T24 nuclei showed different spot numbers. In order to test the reproducibility of the ISH methods, and to examine whether single- and double-target ISH methods give comparable results for a specific probe the number of hybridization spots for probe #1c and probe #18c when applied to the bladder tumor cell line were compared for both methods. Results of the double-target ISH are given in Fig. 3c. Again, the main population of cells exhibited a triplet for probes #1c and #18c. Minor fractions of the cell culture showed different combinations of spot numbers, with for example two spots for probe #1c and three spots for probe #18c and vice versa. Figure 1d shows an example of the double-target ISH on this cell line. In Table 1 the percentages of T24 cells containing different spot numbers for probes #1c and #18c as counted in the single- and double-target ISH-method are compared. From these data it has become clear that both methods give similar results, although it appears that for probe #18c a slightly higher percentage of cells with four and five hybridization spots are seen in the single-target method.

Preparation of single cell suspensions from solid tumors for ISH

The reproducible results obtained with the fixation procedures for the single cell suspension as described above prompted us to see whether for solid tumor samples a similar preparation technique could be designed. Two disaggregation techniques were investigated. One involves mechanical and the other enzymatic treatment. The mechanical disaggregation method proved to be most favorable both in terms of cell recovery and ease of performance. The cell suspensions were subjected to fixation in 70% ethanol. As a result such cell suspensions could be stored for several months before being analyzed, at least when kept at -30°C. Postfixation of these samples with methanol/acetic acid and preparation of the microscopic slides by dropping of the cell suspension on to glass slides was performed in the same way as done for lymphocyte preparations. The approach gave good ISH results and good morphology even after storage of the suspensions in ethanol during several months. However, mechanical disaggregation occasionally leaves some cytoplasm around the nucleus. This may then give rise to a high autofluorescent background and/or a strongly reduced ISH hybridization reaction. The latter phenomenon was noticed most pronounced in cells in which the nucleus was partly masked by cytoplasm. Here the ISH fluorescence intensity in uncovered areas was higher than in covered parts of the nucleus. Washing of the slides with 70% acetic acid prior to ISH diminished these problems, because it removed the residual cytoplasm, resulting in a reduction of autofluorescence. It also strongly improved the final ISH signal intensities. In all seven cases the heat denaturation with an optional 70% acetic acid wash prior to ISH gave the best results. Proteinase K treatment followed by an alkaline ethanol denaturation proved to be less effective.

ISH on bladder cancer preparations

The bladder tumors are grouped according to their DNA content.

a) Near-diploid tumors (four cases). The expected number of ISH spots for cells with a near-diploid character (DNA index = 2c + /-0.15 c) is two, similar to the number in human lymphocytes. Two of the near-diploid tumors did indeed show two spots for probes #1c and #18c. In Fig. 1e an example of such a tumor hybridized with probe #1c is shown. It can be seen that the signal for probe #1c in some cells clearly occurs as split spots lying close to each other (see also Fig. 2e). We decided to count these split spots as one ISH signal, that is one chromosome. Of 91 analyzed nuclei of this tumor, 47% contains two strong ISH fluorescent spots, 31% one strong fluorescent spot and two weaker ones and 22% of the nuclei contained two split spots (Fig. 1e). In the double-target ISH of this case, it was clear that the nuclei which contain two spots for probe #1c also contain two spots for probe #18c (Figs. 1f and 2f). Statistical evaluation was only performed when cells did not overlap so that the ISH spots could be designated to individual nuclei. However, in these two cases, occasionally some cells with three spots for probe #1c were seen which contained only two spots for probe #18c.

With two other near-diploid tumors, which showed similar DNA profiles, different results were obtained in ISH. In these two cases a large population of cells showed three spots for probe #1c (Figs. 1g and 2g) and two spots for probe #18c. In double-target hybridization experiments, illustrated in Figs. 1h and 2h, the different copy numbers for chromosomes #1 and #18 were clearly seen in one and the same cell. Occasionally cells with six spots for probe #1c and four spots for probe #18c were seen, probably representing, tetraploid cells. In one of these two cases, also a large fraction of the cells showed, however, two spots for probes #1c and #18c, thus indicating the presence of different cell lines within the tumor cell population.

In a large fraction of these cells (about 30%), ISH signals occur as a diffusely spotted area, different from the commonly seen discrete distribution in the human lymphocytes, in T24 cells and in the nuclei of the cases described above. The difference in distribution is clearly seen in Fig. 1g and h (see also Fig. 1i; for a schematic representation see Fig. 2g, h and i). Note, however, that within one preparation nuclei exhibiting discrete spots are seen next to nuclei showing such diffuse fluorescent areas. These patches were more frequently seen for probe #1c than for probe #18c. It is noteworthy that the ISH signal and such nuclei either have a discrete or a fuzzy appearance and that generally few nuclei are seen containing both types of spot distribution.

To exclude that this phenomenon is a result of the acetic acid treatment prior to hybridization we compared the distribution for probe #1c in acid and non-acid treated tumor cells and lymphocytes. Both treatments resulted in tumor cell nuclei in which patches were observed, while for lymphocytes always discrete ISH spots were seen.

b) Aneuploid bladder tumors. One of the tumors examined flow cytometrically, showed two major peaks (Fig. 4b), one with a diploid DNA content and the other with a tripliod
Fig. 1a–o. Single- and double-target in situ hybridization results on human lymphocytes, the bladder tumor cell line T24, and human bladder tumor cells. For explanation see legend to Fig. 2.
DNA content. On basis of these flow cytometric data one would expect two cell populations with different ISH spot numbers, i.e. two and three respectively to occur in this suspension. The single ISH showed indeed major populations of cells containing two and three ISH signals. However, also four copies for probe #1c were seen, while for probe #18c two and three copies were detected (Fig. 4a). The double-target ISH showed that cells with two spots for probe #1c also contained two spots for probe #18c, probably indicating cells with a diploid DNA content. The
Table 1. Comparison of the percentages of T24 cells showing different spot numbers for probes #1c and #18c in single- and double-target in situ hybridization. In the single-target hybridization procedure 100 nuclei were counted, while in the double-target hybridization method 200 nuclei were evaluated.

<table>
<thead>
<tr>
<th>ISH spot number</th>
<th>#1c</th>
<th>#18c</th>
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<tbody>
<tr>
<td></td>
<td>Double</td>
<td>Single</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
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<td>3</td>
<td>85</td>
<td>83</td>
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<tr>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
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cells with and four spots for probe #1c had in both cases three spots for probe #18c (Fig. 4b). Occasionally nuclei with four to six spots for both probes were seen, probably representing mitotic cells of the diploid and triploid tumor cell population, respectively. In Fig. 1j an example of a cell containing four spots for probe #1c and three spots for probe #18c is shown. A nucleus from this tumor with an even higher copy number for #1c is depicted in Fig. 1k.

To check the reproducibility of these observations the spot numbers for chromosomes #1 and #18 were counted for both the single- and double-targetISH and compared to each other. For this purpose the following criteria for counting of the ISH signals were applied: a) count only non-overlapping spots; b) count split spots as one; c) count patches only if they are well separated. In Table 2a the percentages of nuclei which were counted by two observers in a single-targetISH for both probes are compared. It is evident that the data are in good agreement. An even more important result is the observation that for the double-targetISH experiment both observers recognized similar sub-populations (Table 2b). For this experiment the spot numbers for probe #18c in nuclei which showed two, three and four copies for probe #1c were counted. It is obvious that the population with two spots for probe #1c was recognized by both observers to contain two spots for probe #18c. In case of three spots for #1c the largest fraction of nuclei contained three spots, and a smaller fraction two spots for probe #18c. In cells containing four spots for probe #1c both observers found three spots for probe #18c in about 80% of the nuclei. We therefore conclude that this tumor contains three main sub-populations of cells each representing more than 15%.

Two TCC's with a tetraploid DNA content as determined by flow cytometry did not show the expected number of four copies for both probes in the largest population of nuclei. The copy number for probes #1c and #18c varied within a wide range. In one case the major fraction of cells showed six copies for probe #1c, but also nuclei with five, four and three spots of #1c were seen. In Fig. 11 examples of these tumor nuclei are shown, with one nucleus showing the typical patches of #1c fluorescence. The copy number for probe #18c appeared to be lower, in this case showing four copies in the major fraction. The discrete localization for probe #18c in this tumor is shown in Fig. 1m. In the double-target hybridization procedure it was found that this tumor was very heterogeneous with respect to the copy number ratio for probes #1c and #18c. Although a limited number of nuclei was evaluated, six discrete cell populations were detected, all representing 10% or more of the total cell population. An example of such a double-target hybridization result is given in Fig. 1n. Occasionally giant nuclei with ten or more spots were seen.

In the other tetraploid tumor case a completely different pattern for probes #1c and #18c was seen. For probe #18c a higher ISH spot number was counted than for probe #1c. The largest cell population contained four spots for #18c and two spots for #1c. In Fig. 1o an example of such a nucleus is shown.
Table 2a and b. Interobserver variation in scoring of ISH spots after the single- and double-target hybridization procedures.

<table>
<thead>
<tr>
<th>Observer</th>
<th>DNA probe</th>
<th>Percentage of nuclei with spot number</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>A</td>
<td>t1c</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td>t1c</td>
<td>4</td>
</tr>
<tr>
<td>A</td>
<td>t18c</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td>t18c</td>
<td>8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Observer</th>
<th>Spots for t18c</th>
<th>Percentage of nuclei with fixed spot number for t1c, n=145</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
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<td>82</td>
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<td>0</td>
</tr>
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<tr>
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<td>4</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
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Discussion

We show that non-radioactive in situ hybridization can be used to determine numerical chromosome aberrations in cases of solid bladder tumors. The bladder tumors analyzed in this study were selected on basis of their DNA index as estimated by flow cytometric analyses and covered the range of (near) diploid, aneuploid, and tetraploid tumors. Single cell suspensions were prepared from fresh material following mechanical disaggregation. During our initial ex-
periments it became clear that for optimal ISH results single cell suspensions could be prepared in a similar fashion as described for flow cytometric (FCM) procedures. Also fixation in 70% ethanol is similar to the fixation procedure applied for FCM. As a result, FCM and ISH of one and the same sample can easily be combined, especially since only very low amounts of cells (a few thousands) are needed to make the ISH procedure statistically evaluable. Furthermore, in future this will allow the analysis of flow sorted fractions by ISH.

For this study the chromosome specific probes of the satellite family were used to detect chromosomes \#1 and \#18. The targets of these probes are localized in the centromere regions of chromosomes \#1 and \#18. The ISH can be performed under conditions which are stringent enough to guarantee specific hybridization only to these chromosomes, at least in nuclei from normal human lymphocytes. In tumor cell nuclei, however, occasionally minor binding sites were observed (see Figs. 1 and 2). It has been described before that probes recognizing targets on chromosome \#1 may show some cross hybridization with chromosome \#9 (Cremer et al. 1988). The probe for chromosome \#18 has been shown to have minor binding sites on chromosomes \#13 and \#21 (Devilee et al. 1986; Cremer et al. 1986). The fact that such minor binding sites are sometimes seen in tumor cell nuclei, but not in normal human lymphocytes may be a result of a better penetration of the probe and/or antibodies. For evaluation of the hybridization results minor spots were not counted as stated below.

Before discussing the results obtained with these chromosome specific probes \#1c and \#18c we want to stress that it is important to realize that the ISH with these probes detects DNA-target-sequences localized on the q arm of the respective chromosomes. For this reason we refer to DNA targets, ISH-signals, or spots rather than to chromosomes or fragments of chromosomes, since certain deletions in the respective chromosomes may or may not result in a disappearance of such signals.

For application on isolated bladder cancer cells heat denaturation of the target DNA in the presence of the chromosome specific probes was shown to be the most reproducible procedure for these single cell suspensions. Washing of the preparations with acetic acid can lead to stronger signals as result of protein removal. In our hands the immunofluorescent approach as compared to immunoperoxidase in combination with brightfield light microscopy and reflection contrast microscopy (Landegent et al. 1985) was most informative, and gave optimal results with respect to spot localization. Furthermore, differences in signal intensities allow a discrimination between major and minor binding sites, as well as the background.

Our results with the ISH on bladder cancer cell suspensions led to the following conclusions:

1. The following criteria have to be applied to allow a proper evaluation of in situ hybridization signals on isolated tumor cells: a) nuclei should not overlap; b) cells should not be asymmetrically covered by cytoplasm; c) ISH signals should have more or less the same homogeneous fluorescence intensity; d) minor hybridization spots should not be counted. Since these spots have a lower intensity they can be recognized by using filters to diminish the fluorescence intensity of the spots; e) fluorescent spots or patches of fluorescence may only be included when the signals are completely separated from each other; f) spots in a paired arrangement (split spots), close to each other are counted as one chromosome complement.

When the criteria, formulated above were applied, interobserver variations were minimal. In all seven tumor cases no significant differences in percentages of cells positive for probes \#1c and \#18c, both in single- and double-target ISH were observed. This high degree of reproducibility is probably a result of the high sensitivity of non-radioactive ISH methods using probes \#1c and \#18c.

2. In several tumors a profound heterogeneity was observed with respect to the number of ISH-signals that could be detected for the two individual DNA probes. Detection of sub-populations of tumor cells was found to be relatively easy, especially in the double-target hybridization procedure where a good agreement was found between two observers. The results strongly indicate that an apparently homogeneous tumor cell population, at least with respect to DNA content, can contain (minor) fractions of cells containing chromosome numbers which deviate from the expected number as calculated on basis of flow cytometry. The tumors with the highest ploidy seem to have the greatest number of sub-populations.

3. Large variations in ISH signal intensities were observed between the different nuclei. However, within one nucleus the signals have a rather similar intensity. This phenomenon may be explained by differences in penetration of the probes or antibodies into the different nuclei. This may be a result of differences in the compactness of the nucleus or may be caused by residual cytoplasm covering the nucleus. Furthermore ISH signals could be detected either as discrete condensed spots or as fuzzy patches. Especially for probe \#1c patchy signals were frequently seen in the TCC nuclei. No clear correlation was found between nuclear size and shape, DAPI fluorescence intensity, ploidy of the tumor, ISH signal intensity or loss of morphology. No explanation so far can be given for this observation, although important parameters could be the procedure used for the isolation of the single cell suspensions, the stage of the cell in the cell cycle, rearrangements which occur during carcinogenesis, the presence of fragile sites at the centromere, polymorphism of the site recognized by the probes, or compactness of the chromatin at the site of the target sequences.

4. Large numbers of nuclei can be screened easily for copy numbers of chromosomes \#1 and \#18.

So far no typical marker chromosomes have been found by karyotyping bladder tumors. However, analysis of the TCC of the urinary bladder has revealed that a number of non-random chromosome aberrations may be involved. Numerical aberrations for chromosome \#1 have been reported to be correlated to various types of human neoplasia (see Atkin 1986). The chromosome \#1 specific probe, isolated by Cooke and Hindley (1979), will therefore become an important marker for various types of human malignancies. Recently Smeets et al. (1987b) found a structural rearrangement for this chromosome in 6 out of 13 TCC upon karyotyping. In another series of bladder cancers structural changes in chromosome \#1 were recognized, which were characterized by breakpoints in the proximal regions of the chromosome arms. In cases of near-diploid tumors, frequently +1p− and 1q+ chromosomes have been reported.
of the bladder: Involvement of chromosomes 1 and 11. Cancer Genet Cytogenet 15: 253–268
Landegent JE, Jansen in de Wal N, Baan RA, Hooijmakers JH,