Chromosome and flow cytometric studies of urinary bladder cancer

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Chromosome and flow cytometric studies of urinary bladder cancer.

Proefschrift

ter verkrijging van de graad van doctor in de geneeskunde aan de Rijksuniversiteit Limburg te Maastricht, op gezag van de Rector Magnificus, Prof. Dr. F.I.M. Bonke, volgens het besluit van het College van Dekanen, in het openbaar te verdedigen op 3 juli 1987 om 16.00 uur.

door

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geboren te Limbricht in 1941.

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Nichts ist geworden, alles ist stets im Werden; in dem ewigen Strom der Veränderung ist kein Stillstand.

Goethe

To: Winy
Caroline, Laura and Lidwien.

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Chapter I

Introduction

Frequency, diagnosis and prognosis of bladder cancer.

Urinary bladder tumors can be divided in epithelial (about 98%) and non-epithelial (about 2%) tumors. The epithelial tumors include three types: Transitional cell carcinoma (TCC), Squamous cell carcinoma and Adenocarcinoma. In the western world, the frequency of these three types is: 95-98%, 1-3% and 1-2%, respectively [26].

The mortality of bladder cancer patients in the Netherlands in 1984 was for males 10.4 out of 100,000 and for females 3.4 out of 100,000 [5].
This means that each year in the Netherlands about 1000 patients die due to bladder cancer. Death risk increases with age, while mortality increases strikingly after the age of 60 years.

For TCC classification the criteria of histological grade and the depth of infiltration into or through the bladder wall (= tumor stage) are used. The W.H.O. [55] has proposed a classification in three grades, i.e. G1, G2, G3, while the U.I.C.C. [21] introduced a staging system, indicating the depth of infiltration in the bladder wall, ranging from Ta to T4 (see addendum).
Superficial carcinomas (stage Ta and T1) generally are treated with transurethral-(electro-)resection (TURT), whether or not followed by intravesical chemotherapy. Patients with these tumors have a five year survival rate of about 80% [36]. Deeply invasive carcinomas (stage T2, T3 and T4) need radical therapy (radiation therapy and/or cystectomy). The five year survival rate of these patients is about 40% for T2 tumors and declines to about 6% for tumors growing through the bladder wall, regardless of the forms of treatment employed [24, 36].

The majority of patients presenting with bladder tumor for the first time are staged as Ta (40%) or T1 (27%) [9]. Between 10 and 20 percent of the Ta tumors develop into less differentiated and or invasive tumors (T1 or higher). About 30% of the T1 tumors will infiltrate to deeper layers. This behavior cannot be predicted with the current staging and grading systems. Tumors with identical histopathology are reported to vary significantly in their subsequent clinical course. Therefore, the clinical behavior of a certain tumor is hard to predict for each individual patient [9, 10].
A well known disturbing factor with respect to prediction is the discrepancy existing among pathologists in grading bladder tumors [16, 26, 35, 40].

From the foregoing it may be obvious that in addition to the current histopathological data more information about the biology of bladder cancer is needed for better management of the patients. In this respect it is of real importance to distinguish those Ta tumors which will infiltrate to stage T1 or deeper, from the non-infiltrating ones and also those T1 tumors which are able to invade into deeper layers, in spite of the therapy applied.
It is established that chromosomal studies and estimations of DNA content may contribute to the assessment of biological properties correlative with the prognosis of an individual patient with bladder cancer [12, 27].

Chromosomes and cancer.

In 1914 Boveri published the hypothesis that a change in chromosome constitution is a prerequisite for cancer development. To test this hypothesis it was necessary to know the exact chromosome number of normal somatic cells. Owing to technical progress in 1956 Tjio and Levan and shortly afterwards Ford and Hamerton described the definite chromosome number of normal cells [40].

In 1960 Nowell and Hungerford established in the leukemic cells of patients with Chronic Myelocytic Leukemia (CML) a striking abnormal small chromosome, the so-called Philadelphia chromosome [40]. This is the first onset of a cascade of chromosomal data of cancer patients.
The need for an accurate nomenclature for human chromosomes resulted in the first convention, to establish an international system for human cytogenetic nomenclature, held in Denver in 1960. This was followed by conferences in London in 1963, Chicago in 1966 and Paris in 1971.

Around 1970 chromosome banding techniques were introduced. These staining techniques visualise various intrachromosomal bands and/or regions of varying intensity, specific for the species. Since that time it is relatively easy to recognize the individual chromosomes and their structural abnormalities, if sufficient metaphases of adequate quality are present. The bulk of cytogenetic data presently available (more than 5000 cases with malignant disorders) is derived from various hematological malignancies. Solid tumors, quantitatively the largest group of human tumors, constitute less than 10% of the cases described [34]. This paucity of data in solid tumors attests to difficulties in obtaining adequate numbers of analyzable metaphases. Besides, the karyotypes of solid tumors generally are very complex in comparison with those of leukemia, and therefore very difficult to correlate with various biological, pathological and clinical aspects of these tumors [43, 50, 53 54].

In 1965 Shigematsu [44] was the first to study the chromosomes of transitional cell carcinoma of the bladder. He found a relation between the number of chromosomes and the histological type.
Comprehensive studies confirmed the positive correlation between invasion of the tumor and the increasing chromosome count [27, 47].
Next to numerical aberrations of the karyotype of bladder cancer, structural aberrations, on behalf of their unrecognizability called "marker chromosomes" were revealed [13, 14, 39]. The presence of marker chromosomes indicates a poor prognosis with respect to recurrence and progression of the tumor [14, 32, 48]. However, also in the absence of markers, recurrence was observed [11].

At the beginning of our study, in 1980, practically no new data regarding chromosome abnormalities in bladder had been presented during the last ten years. This was largely due to difficulties in disaggregation of the tumor specimens, low mitotic activity and the poor quality of the metaphases [6, 18, 19, 40, 52]. At that time Sandberg [40, 41] stipulated that it was imperative that the studies should be extended to a much larger series of patients at a variety of medical centers around the world, to obtain a body of data that would make it possible to establish the usefulness of the cytogenetic picture in the diagnosis, therapy and prognosis of cancer of the bladder. Direct preparations of the metaphases from tumor cells i.e. without previous cell culture, is preferred. Although

culturing of the cells provides more recognizable chromosomes, it also implies the risk of selecting some cell types and chromosomal changes during culture time [40, 52].

Non-random karyotypic changes in some solid tumors were emphasized, for example interstitial deletion of chromosome 13q14 in retinoblastoma and of 11p13 in Wilms' tumor [2, 46]. In an extensive study of bladder tumors published in 1984 [19] no specific chromosome abnormalities could be obtained after direct examination of the tumor specimens, due to insufficient quality of the preparations. Primary karyotypic changes are possibly related to the basic etiology of the disease. Secondary changes, that originate in a later stage of tumor growth, may play a crucial role in the biology of such tumors, and influence their invasiveness, metastatic spread and response to therapy [42, 57]. Establishing chromosomal aberrations is of great importance with respect to the study of the location of oncogenes. For example, the oncogene c-Ha-ras 1 on chromosome # 11 has drawn much attention [30, 31, 38, 45]. It is also of significance for obtaining insight in the process of oncogenesis [25, 42, 57].

Estimation of DNA content in bladder cancer.

Estimation of the relative DNA mass showed that as TCC becomes more aggressive, there is a progressive shift from the modal DNA content to higher values [8, 29, 49].
For measuring the DNA mass, the two major technical approaches are image cytometry and the flow cytometry.
In image cytometry the nuclear DNA-content of 100-200 cells spread on a glass slide and Feulgen stained is analyzed with an imaging system based on the microscope [22, 33].
In flow cytometry, cells are measured in a monodisperse suspension. With this technique, introduced in the mid seventies, thousands of cells can be analyzed per second [4, 33]. Cytochemical or immunocytochemical probes, such as fluorescent dyes and labeled antibodies, are used to examine the cell properties of interest [33]. For DNA estimation in FCM, the cells can be stained with a fluorochrome, for instance propidium iodide. When this fluorochrome is excited at a specific wave length, light of higher wave length is emitted, proportional to the amount of DNA. The signal per cell is registered and quantitatively analyzed with the DNA content of normal human lymphocytes as standard. As a result of the large number of cells that can be measured, different phases of the cell cycle can be analyzed and evaluated statistically. Therefore, flow cytometric DNA measurements can be used to assess the relative DNA mass and cell kinetic data of a certain tumor [1, 12, 20, 22, 28, 51, 56]. Normal DNA contents are characteristic for highly differentiated tumors [7, 29] which normally also show low proliferative activity. On the other hand, less differentiated tumors, normally show an abnormal DNA content and moderate or even high proliferative fractions [3, 51].

For DNA estimations of the cell type of interest, labeling with antibodies is possible. Tumors with near diploid and pseudo-diploid chromosome abnormalities cannot be detected by FCM [17, 40, 56]. This may be due to the fact that flow cytometric DNA analysis is often disturbed by the presence of stromal or inflammatory cells in the cell suspensions obtained from these neoplasms. Recent developments in cell biology have led to the identification and characterization of tissue specific proteins which make up part of the cell matrix. These so-called intermediate filament proteins (I.F.P.) have a diameter of 7-11 nm, and can be subdivided into five subgroups according to their protein type. Epithelial cells contain cytokeratins, while for instance mesenchymal cells contain

vimentin and muscle cells contain desmin [15, 37]. By application of antibodies to cytokeratin, the epithelial (tumor) cells of TCC can be stained specifically [15]. Immunolabeling of the cell suspension with these antibodies makes it possible to discriminate the epithelial cells from non-epithelial cells that are present in the cell suspension.

Aims of this study.

The aim of this study was to investigate bladder tumors in search for new diagnostic and prognostic factors. Especially the chromosomes and the DNA content of the tumor cells were examined in order to obtain information about the biological characteristics in addition to the routine histopathological data.
The techniques for obtaining chromosomes available at the beginning of this study did not result in quantitatively and qualitatively sufficient data. The development of a technique for obtaining representative chromosome pictures of the tumor cells was our first goal. In addition we wished a practicable, not-laborious technique. Ultimately, the chromosomes must be identified by means of banding techniques, in order to see whether or not non-random abnormalities in the chromosomes could be determined.

For estimation of nuclear DNA by means of flow cytometry, our second goal in this study, single cell suspensions were needed. Therefore, currently available methods for disaggregation of the solid tumor specimens were compared. The cytometrically obtained results were compared and related to the data of chromosomal analysis.

Addendum.

List of abbreviations used:

Grading system [55].

G1: Tumor with the least degree of cellular anaplasia, compatible with the diagnosis of malignancy.
G2: Intermediate cellular anaplasia.
G3: Tumor with the most severe degree of anaplasia.

Staging system [21].

Ta: The tumor is confined to the epithelial tissue, without infiltration.
T1: The tumor is infiltrated into the connective tissue. (Ta + T1 are superficial tumors).
T2: The tumor is invaded into the superficial muscular tissue.
T3: The tumor is invaded into the deep muscles or into the perivesical fat.
T4: The tumor has reached the adjacent organs.

TURT: Trans Urethral Resection of the Tumor.
FCM: Flow Cyto Metry.
TCC: Transitional Cell Carcinoma.

Some examples of cytogenetic nomenclature:

47,XY,+21 : 47 chromosomes, XY sex chromosomes and an additional chromosome # 21.
45,XX,-8 : 45 chromosomes, XX sex chromosomes and a missing chromosome # 8.
47,XY,+14p+ : Male karyotype with 47 chromosomes including an additional chromosome # 14 which has an increase in the length of its short arm.
47,XX,+1q- : Female karyotype with 47 chromosomes including an additional chromosome # 1 which has a loss of the length of its long arm.
deletion (abbreviated del): Loss of a chromosome part.
translocation (abbreviated t): Breakage followed by exchange of chromosome material between chromosomes.
triploid : All the chromosomes are present in triplicate i.e. 69 chromosomes (3n).
near diploid: The modal number cannot be given as a precise number (2n ±).
pseudo-diploid: The number of chromosomes is equal to the diploid number (for human beings 46) but numerical and/or structural aberrations are present.

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Chapter II

Chromosomal analysis of bladder cancer.

Technical aspects.

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Abstract

Of 77 patients with bladder carcinoma, 99 tissue specimens-including tissues of patients with recurrent tumors taken after radiotherapy or cytostatics - were subjected to chromosomal analysis. Of 42 specimens, recognizable metaphases could be obtained after conventional Giemsa staining and in a smaller number after C-and/or G-banding. All except one had abnormalities of the chromosomes. Short-term cultures for 24-48 hrs in RPMI-1640 plus 15% fetal calf serum plus penicillin-streptomycin gave better results than a direct technique (30 min in 0.075 M KCl + 0.1 µg colcemid/ml at 37°C, followed by fixation). In low stage/grade tumors the number of recognizable metaphases obtained after short-term cultures is lower than in higher stage/grade tissue specimens.

Introduction

Since 1965 several studies have been published on chromosomal abnormalities in bladder tumors [3-8, 13, 14, 17-19, 21-23]. We have started a prospective study to see if information can be obtained at a very early stage about the future tumor behavior in superficial bladder carcinoma, based on the presence or absence of chromosomal abnormalities.

Chromosomes of bladder tumors usually were examined without prior cell culture. Cell suspensions were made using various protocols [4, 13].
Karyotyping bladder tumors has always been problematic, mainly because of the small amount of recognizable metaphases. Various techniques that ought to increase the number of good metaphases have been published. The results obtained with several of the published techniques are discussed for different stages and grades of tumor in this article.

Materials and methods

Patients

Between 1/1/81 and 6/30/83 material from 77 patients (67 male and 10 female) with a transitional cell carcinoma was subjected to chromosomal examination (Table 1). When the first material for cytogenetic examination was obtained, only one patient had been given cytostatic treatment and none of the patients had been subjected to radiotherapy before the first chromosomal analysis. In view of tumor recurrence, a number of patients had been examined two to four times, so that from these 77 patients a total of 99 biopsies of tumorous tissue were available for cytogenetic analysis. Specimens were taken by cold-cup biopsies or by transurethral diathermical resection (TURT). One part of the same biopsy went to the pathology laboratory, another to the cytogenetic laboratory.

Table 1
Patients examined.

	Tumor stage				
Grade	Ta	T1	T2	T3	Total
G1	3	–	–	–	3
G2	40	10	2	5	57
G3	–	5	4	8	17
Total	43	15	6	13	77

Staging was performed according to the TNM-classification for malignant tumors [26]. In the TNM-system, T stands for the degree of local growth of the tumor, N for the metastasis to lymphatic glands, and M for metastasis (at a wide range into other organs). Ta tumors are confined to the superficial epithelial tissue, without infiltration; T1 tumors infiltrate into the connective tissue below; T2 tumors invade the superficial muscular tissue; T3 tumors develop into the inner muscular tissue or into the perivesical fat; and T4 tumors grow into adjacent organs.

Ta and T1 tumors remain superficial, in the sense that they have a comparatively good prognosis, but have a high degree of recurrency. In general they can be treated by means of transurethral resection. T2 and T3 tumors have a lethal prognosis of approximately 50%, despite aggressive therapy (cystectomy and/or radiotherapy). T4 tumors are believed to be incurable.

The histologic grading was based on World Health Organization (W.H.O.) recommendations [27]. Grade 1 tumors show hyperplasia but are highly differentiated; grade 2 tumors show moderate polymorfia, nuclear atypia, and hyperplasia of the epithelium; in grade 3 tumors these phenomena are more serious, sometimes to the extent that the tissue is no longer recognizable as urothelial tissue.

Chromosome analysis

Technical variations tested in the beginning of this study

Collection and transportation of bladder tissue:
The biopsies were collected and transported in one of the following solutions: Ringer-glucose solution, Hanks' solution, or RPMI 1640 medium. All the solutions were at a temperature of ± 0° C or at roomtemperature, with or without addition of colcemid (between 0.01 μg and 10 μg/ml).

Preparation of cell suspension:
After mincing and scraping the tissues were mixed for 10-30 min in 0.9% NaCl in a magnetic stirrer. After mechanical pretreatment, the tissues were enzymatically treated with the enzymes protease and/or collagenase (Boehringer Mannheim B.V.).

Hypotonic treatment:
Tests were made with: 0.037 M KCl for 30-120 min at 37° C; 0.064 M KCl for 30-120 min at 37° C; 0.7% sodium citrate [16] and 0.6% glucose in 0.7% sodium chloride/0.44% sodium citrate (1 : 1) [17].

Cell fixation:
Cells were fixed with: 50% acetic acid [4]; 60% acetic acid; methanol/acetic acid 4:1 or methanol/acetic acid 3:1.

Preparation of slides:
The following techniques were used to prepare slides:
Application of cell suspension on a clean heated slide with 1 drop of 60% acetic acid already on the slide [2].
As a variation on Croizier's method, the cell suspension was mixed with the acetic acid as it fell onto the slide [11].
Slides were stored in 50% acetic acid of 4°C and the suspension was flame-dried [13].

A few drops were placed on clean wet slides, which were kept in distilled water at 4°C, and then flame or air-dried.

Tissue culture:
After dissociation of the tissue with collagenase, a short-term culture in Leibovitz L-15 medium with 20% fetal calf serum, 10-20 μg porcine insulin/ml, and 16 μg glutathione/ml was performed [12].

Current method

The tissue is put into a solution of 0.9% NaCl at approximately 0°C and within 30 min a cell suspension is made by mincing and scraping the material as finely as possible.

Treatment of the tumor:
Direct technique involves incubating the tumor material for 30 min in 0.075 M KCl plus 0.1 μg colcemid/ml at 37°C followed by fixation in methanol/acetic acid, 3:1. (Recently, we have also used an incubation period of 2 hrs in RPMI 1640 plus 0.1 μg colcemid at 37°C prior to hypotonic treatment).
Short-term culture, only used if a good cell suspension can be obtained, involves incubation for 24-48 hrs in RPMI 1640 plus 15% fetal calf serum and 4% penicillin-streptomycin (100 U/ml, respectively 100 μg/ml) at 37° C. Two hours before termination of the culture, 0.1 μg/ml colcemid is added, followed by hypotonic treatment, and fixation as above.
Long-term culture is performed only with cold-cup biopsy material, in the absence of a good cell suspension, in plastic bottles with Ham's F-10 plus 15, respectively 25% fetal calf serum 1-4 wk in 5% CO_2 atmosphere.

Treatment of the normal bladder tissue:
Normal bladder tissue is always subjected to a long-term culture, since it is very solid and cannot be easily worked into a cell suspension.

Preparation of slides:
Three or four drops of the cell suspension are placed on clean, wet object slides that have been stored in aqua dest of 4°C. The preparations are slowly flame-heated and then air-dried. The preparations are stained by conventional method with Giemsa.
In those cases where sufficient slides are available and the unbanded preparations show good metaphases, CBG-banding [24] and GTG-banding methods are used [20].

Results

Technical variations for chromosomal examination

All the solutions examined produced a worse, or in any case, no better metaphase result than the use of 0.9% NaCl at 0°C.
The use of a magnetic stirrer did not produce a better cell suspension than the conventional technique of mincing and scraping. The use of protease and/or collagenase damaged many cells or resulted in chromosomes with a very fuzzy appearance.
None of the solutions used gave a better spreading of chromosomes than 0.075 M KCl; the optimal time was 30 min.
Use of 60% acetic acid damaged the chromosomes and had negative effects on banding. The same effect on banding was noted with use of 50% acetic acid. This fixative did not increase the percentage of recognizable metaphases, as compared to methanol/acetic acid,

3 : 1. Methanol/acetic acid, 4 : 1 yielded no better results. Spreading of metaphases after 60% acetic acid was improved, as compared to methanol/acetic acid, but application of more than 50% acetic acid reduced the quality of the chromosomes.
Culture after treatment with collagenase only produced some metaphases, when a very large amount of material was available. The number of metaphases, spreading, and quality of chromosomes was worse with this method than after short-term culture with RPMI 1640, without enzymatic treatment.

Current method

In 42 of 99 tumors examined with the direct technique or with the short-time culture, recognizable metaphases were found (Table 2). Metaphases were considered recognizable if there were only a few overlaps, if the centromeres were visible, and if the A-G groups could be identified. The number of metaphases varied between three and a large number per tumor, or between 1 and 25 per slide.

Table 2
Number of patients and cases where metaphases were found in relation to T/G.

	ToGo	TaG1	TaG2	T1G2	T1G3	T2G2	T2G3	T3G2	T3T4G3	Total
n pat.	40	3	40	10	5	2	4	5	8	77
n cases	50	4	55	12	7	2	4	5	10	99
Inc. meta. I	11	2	39	7	6	2	4	4	8	72
Inc. meta. II	7	0	20	5	4	2	2	2	5	42

n pat. : number of patients.
n cases : number of cases.
Inc. meta. I : Incidence of metaphases with recognizable and/or unrecognizable chromosomes.
Inc. meta. II : Incidence of metaphases with recognizable chromosomes (more than three metaphases per case).
ToGo : Normal bladder tissue.

Of the 50 control experiments performed with normal bladder tissue metaphases were found in 11 cases, of which seven were recognizable: six tissue cultures with 46,XY and one with 46,XX. These metaphases were obtained after long-term culture.
Only one tumor (TaG2) showed a normal karyotype, in all other cases there were abnormalities that will be published in a separate report.
In total 78 superficial (Ta-T1) bladder tumors were examined from 58 patients. Recognizable metaphases were found in 29 tumors (37%). A group of 21 deeply infiltrating tumors (T2-T4) from 19 patients showed recognizable metaphases in 13 tumors (62%). These numbers include four tissue specimens from three patients with recurrent tumors who received radiotherapy after the first chromosomal analysis and which did not show recognizable metaphases after treatment.
The short-term culture of cold-cup biopsies yielded significantly better metaphases with the deep tumors than with the superficial tumors (Table 3). If we counted the recognizable and/or unrecognizable metaphases, there was no difference in this respect (Table 4).

Table 3

Recognizable metaphases in superficial and deep tumors in relation to the technique applied.

	direct techn. cold biopsy		direct techn. TURT		short-term cult. cold biopsy		short-term cult. TURT	
	+	–	+	–	+	–	+	–
Superficial tumors	7	52	8	39	5	7	18	12
Deep tumors	4	12	1	17	7	0	7	2
	$p>0.20$		$p>0.20$		$p=0.032$		$p>0.20$	

+ : number of cases with recognizable chromosomes.
– : number of cases without recognizable chromosomes.

Table 4

Recognizable and/or unrecognizable metaphases in superficial and deep tumors in relation to the technique applied.

	direct techn. cold biopsy		direct techn. TURT		short-term cult. cold biopsy		short-term cult. TURT	
	+	–	+	–	+	–	+	–
Superficial tumors	31	29	29	18	10	2	26	4
Deep tumors	12	4	13	5	7	0	8	1
	$p=0.16$		$p>0.20$		$p>0.20$		$p>0.20$	

+ : number of cases with recognizable and/or unrecognizable metaphases.
–: number of cases without metaphases.

If the percentage of metaphases, whether or not they were suitable for further cytogenetic analysis, is related to the technique applied (Table 5), it appears that with the direct technique metaphases are present in 52% of all cold-cup biopsies, (at least three per biopsy) and in 61% of the TURT material. When applied to those operations where both cold-cup biopsies and TURT tissues were removed, McNemar's test produces no significant difference in metaphase results with the direct technique ($p > 0.20$).

The use of short-term culture produced metaphases in 94% of the cold-cup biopsies and in 88% of the TURT-materials ($p > 0.20$, McNemar's test).
No difference was observed between either the metaphase production of the direct technique or short-term culture, when applied to cold-cup biopsies and TURT materials. In 12% of all cold-cup biopsies and in 13% of the TURT materials recognizable metaphases (≥ three per biopsy) were obtained with the direct technique ($p > 0.20$, McNemar's test). Recognizable metaphases with the use of short-term culture were found in 63% of the cold-cup biopsies and in 68% of the TURT tissues ($p > 0.20$, McNemar's test). McNemars'test, when applied to cold-cup biopsies where both the direct technique and short-term culture is applied, showed significantly more recognizable metaphases with the short-term culture ($p = 0.016$). The same test applied to TURT-materials, where both direct technique and short-term culture is applied, also showed significantly more recognizable metaphases with the short-term culture ($p < 0.001$).

Table 5
Recognizable and/or unrecognizable metaphases in relation to the technique applied.

	Direct method				Short-term culture			
	cold biopsy		TURT		cold biopsy		TURT	
	n cases	n meta.	n cases	n meta.	n cases	n meta.	n cases	n meta.
Rec. + unrec.	85	44 (52)	70	43 (61)	16	15 (94)	40	36 (88)
Rec.	85	10 (12)	70	9 (13)	11	10 (63)	41	28 (68)

n meta. : number of cases with metaphases.
In parentheses is the percentage of cases with metaphases.
Rec. + unrec. : metaphases with recognizable and/or unrecognizable chromosomes.
Rec. : metaphases with recognizable chromosomes.

Discussion

The overall result of recognizable metaphases was 37% with superficial tumors (Ta-T1) and 62% with deep infiltrating tumors (T2-T4). This may be related to a lower mitotic frequency in superficial tumors, as discussed by Sandberg [17]. Only by use of short-term culture with cold-cup biopsies did the deeply infiltrating tumors yield significantly more metaphases than superficial tumors (Table 3). However, taking together the unrecognizable and the recognizable metaphases in superficial and deep infiltrating tumors, there is no significant difference (Table 4).

In the majority of cases we could only perform chromosome analysis of superficial tumors with the direct technique, because the tissue specimen was too small ($< 2mm^3$) to perform short-term culture as well. Very solid material, which could not be dispersed easily into a cell suspension, was of no use for short-term culture.

If tumorous tissue is removed during a TURT, the amount of material available for analysis is usually much larger (sometimes more than $1\ cm^3$). For short-term culture it hardly matters if the tissue is a cold-cup biopsy or taken by TURT. A large amount of tissue is preferable because it is important to have many slides with many metaphases for banding techniques. After transurethral resection, the tissue becomes partially necrotic as a result of electroresection. This has a negatieve effect on the number of recognizable metaphases. Since the amount of resection material usually is larger, the final result is identical.

Culture of cells of a transitional cell carcinoma in vitro is difficult [15]. After long-term culture of tumor tissues, only 3 out of 10 tumors yielded recognizable metaphases. The use of the short-term agar culture in the cytogenetic analysis of human tumors, as mentioned by Hamburger and Salmon [10], has only rarely been reported. The preliminary results as reported by Trent and Salmon [25] demonstrated the potential application of this technique to cancer genetics. Because long-term culture is time consuming and provides poor metaphases (and it is uncertain which cells are growing), we prefer the use of the direct technique and short-term culture.

A comparison of our percentages of metaphases with the results of other investigators is difficult. Often, only recognizable metaphases are given and no mention is made of the number of non-recognizable metaphases or of their total absence. Although there are more metaphases observed in many tumors (Table 5), a great number is unrecognizable because of poor spreading or the fuzzy appearance of the chromosomes. To improve spreading,

fixation in acetic acid of higher concentration [2], was applied [11, 13]. Although use of 60% acetic acid afforded better spreading than with methanol/acetic acid 3:1, the chromosomes were thin, elongated, fuzzy, and even showed gaps in the chromatids. Even more important was the finding that after this procedure, banding was almost impossible. Harbott [11] had a similar experience. Flame-drying or air-drying slides provided no improvement in spreading. No improvement was seen when the slides were stored in 50% acetic acid at 4°C or room temperature, or in distilled water of differing temperature. Changing the concentration, the volume, or the kind of hypotonic solution did not give better metaphase spreads.

C-banding was attempted in 42 tumors with recognizable metaphases. Some banding pattern was obtained in 18 cases. In 13 of these the quality was good and in the remaining five cases the chromosomes were very contracted or had a very fuzzy appearance. G-banding was attempted in 15 tumors. Only three cases were successful, some chromosomes were recognizably banded in eight, and in the remaining four it did not work at all. The following factors may have contributed: the small number of metaphases; the very contracted or fuzzy chromosomes and the poor spreading. Tumor metaphases also might have shown an unexplainable resistance to banding.

It is the experience of many investigators dealing with bladder tumors that banding is a major problem. Atkin et al. [1] were successful in only 12 of 30 cases using C-banding, and a lower success rate using G-banding. The problem with banding might be attributable to the use of the direct technique [22]. Granberg-Öhman et al. [9] state that in spite of the numerous and well spread metaphases, banding was not of good quality. According to Sandberg [19], satisfactory banding analysis has been obtained in only 20%-30% of bladder tumors in the best hands.

Conclusions

Short-term cultures of the tumor tissues provided a larger percentage and better quality of metaphases than the use of the direct-technique. This applied to both cold-cup biopsies and TURT material. The short-term cultures of cold-cup biopsies yielded significantly more good metaphases in infiltrating tumors (T2-T4) than in the superficial tumors (Ta-T1).

Long-term cultures of tumor material as well as healthy bladder tissues was time consuming and the number of cultures with sufficient metaphases was limited. The successful cultures of normal tissue always revealed only normal karyotypes.

After the use of 60% acetic acid to obtain well-spread metaphases, the chromosomes were no longer suitable for G-banding. Banding was also difficult after fixation with methanol/acetic acid 3 : 1.

Technical improvements are necessary to obtain better spread metaphases without disturbing the chromosomes.

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Chapter III

Comparison of tissue disaggregation techniques of transitional cell bladder carcinomas for flow cytometry and chromosomal analysis.

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Abstract

DNA index (DI) measurements and chromosomal analysis of 42 transitional cell carcinomas were done after mechanical and after enzymatical disaggregation of the tumor specimens. The results obtained with these different disaggregation techniques were compared in the 33 cases (79%) which showed recognizable chromosomes. The enzymatically obtained cell suspensions could not be used for chromosomal analysis after short term culture of 24 hours. In four cases, the DI after enzymatical treatment could not be estimated. In most cases, the DI obtained from the tumor cells was similar for both aggregation techniques, with the exception of four cases of enzymatically treated cell suspensions in which the DI could not be estimated. The average DI of the aneuploid tumors was 13% higher than the corresponding chromosome count.
In 19% of the aneuploid tumors the proportion of aneuploid cells could not be measured after enzymatical treatment. In the remaining suspensions the proportion of diploid cells was higher after enzymatical disaggregation than after mechanical treatment. It is concluded that for flow cytometric and direct chromosomal analysis of bladder tumors, the mechanical disaggregation technique is most suitable.

Key terms: tissue disaggregation, bladder carcinoma.

Introduction

Flow cytometry and chromosomal analysis have been described as important methods for the examination of bladder carcinomas [10-12,23]. The degree of ploidy and the proliferative activity of the tumor can be used as additional parameters in patient treatment [3,20]. The same significance may be adjudged to the detection of chromosomal abnormalities in tumor specimens [6,15,17,18]. Essential for proper flow cytometric (FCM) investigations and chromosomal analyses are optimal single-cell suspensions of the (malignant) tissues under examination. Current methods for disaggregation of tumor specimens include mechanical and enzymatical treatment of the tissue. It has been reported that the proportions of aneuploid cells in cell suspensions obtained by mechanical disaggregation were higher than in those obtained after enzymatical disaggregation [5,8]. Differences may also be due to different enzymatical methods [4].
In this study the DNA content of tumor cells in the GO/G1 phase after mechanical and enzymatical disaggregation was measured. These data were correlated with histological as well as cytogenetical data of the same transitional cell carcinomas. The percentages of diploid cells in the GO/G1 phase present in the cell suspensions after the two disaggregation techniques were compared. The effects of disaggregation procedures and the usefulness of the different cell suspensions for FCM and chromosomal analysis are discussed.

Materials and methods

Forty-two tumor specimens, 38 primary and 4 recurrent ones, from 31 male and 7 female successive patients with carcinoma of the urinary bladder were investigated. They could be divided into tumor stages as folows: 14 pTa, 11 pT1, 17 pT2-pT4 tumors. The tissues were collected after transurethral resections.

One part of each biopsy was used for pathologic examination, while the remaining part was used for chromosomal and flow cytometric studies.
Clinical staging of the tumor was done according to the rules of Union International Contre le Cancer [13], and grading was done according to the WHO system [24]. Staging and grading were as follows: pTa tumors are noninvasive; pT1 tumors do not show invasion into the muscularis; pT2-pT4 tumors show invasion from superficial muscularis to neighbouring organs. Grade 1 tumors show hyperplasia but are highly differentiated. Grade 2 tumors show moderate polymorphia, nuclear atypia and hyperplasia of the epithelium. Grade 3 tumors show severe abnormalities, sometimes to the extent that the tissue is no longer recognizable as urothelial tissue.

Disaggregation procedures

The specimens were collected and transported in the following medium: 10 ml RPMI 1640 plus 17% fetal calf serum (FCS), 50 μg gentamicin/ml, 100 U penicillin/ml and 100 μg streptomicin/ml.

For mechanical disaggregation, the tissue was minced by scraping and cutting in a petri-dish and was filtered through a 100 μm nylon filter (Ortho Diagnostic Systems, Beerse, Belgium). The cell suspensions were divided in two parts, one for short-term or long-term chromosomal analysis and the other one for flow cytometry. For the latter purpose, the cells were centrifuged at 400g for 7 minutes. Then, 70% ethanol (-20°C) was added rapidly to the cell pellet under constant shaking. The final concentration was about 3 x 10 cells/ml ethanol. At this stage, the fixed cells could be stored several weeks at -20°C.

For enzymatical disaggregation, the specimen was cut into small fragments and incubated 1-2 hr at 37°C in the medium with 0.8 g/100 ml collagenase II (Worthington, Freehold, NJ). The cell pellet was washed with medium, filtered, and centrifuged. This washing step was repeated three times, and the cell suspension was divided into two parts, one for short-term or long-term chromosome culture and the other one for flow cytometry. For the latter purpose, the cells were fixed in 70% ethanol, at -20°C, as mentioned above.

For direct chromosomal analysis, the specimens were collected and transported in 10 ml sodium citrate + 0,5 μg colcemid/ml and disaggregated mechanically.

Chromosomal analysis

For chromosomal analysis one or more of the following techniques were applied:
Direct method (in preparation). Briefly: The tissue specimens were collected and disaggregated as mentioned above. After incubation in Hanks BSS again, a colcemid solution was added (2 μg/ml Hanks BSS). Hypotonic treatment was done with a mixture of 6 ml FCS and 24 ml 0.052 M KCl. Fixation was performed with acetic acid-methanol 3:7.
Short term culture [19]. Briefly: The cells obtained after the disaggregation procedures as described were incubated for 24 hrs in the medium. Metaphase arrest with colcemid 0.1 μg/ml was followed by hypotonic treatment with 0.075 M KCl and fixation in methanol-acetic acid 3:1.
Long term culture [9]. Briefly: The cells obtained after the disaggregation methods as described were incubated for 1-2 weeks in flasks containing the culture medium.

Methotrexate synchronization was done, followed by colcemid arrest, hypotonic treatment, and fixation.

All slides were stained with Giemsa. C-banding and, when enough appropriate metaphases were available, G-banding was performed.
Metaphases were photographed and analyzed according to the standard rules.
From every tumor, the chromosomal range, the modal number, the distribution according to ploidy ($n = 23 \pm 11$, $2n = 46 \pm 11$, $3n = 69 \pm 11$ etc.) and the presence of (double) minutes and marker chromosome(s) were recorded (see Table 1). In those cases in which metaphases were obtained with the direct method as well as with the short-term culture the results were pooled.
Our detailed chromosomal observations will be published elsewhere (in preparation).

Flow cytometry

For DNA measurements the sample was stained with the fluorochrome propidium iodide (PI) and thereafter the cells were treated with RNAse and inspected microscopically. Cell analysis was performed using a cytofluorograph 5OH (Ortho Instruments, Westwood, MA).
PI was excited at 488 nm with an argon ion laser (Spectra Physics, Mountain View, CA). Fluorescence was measured using a 630-nm long-pass filter. All data were stored on a PDP 11/34 computer (Digital Equipment Corporation, Marlboro, MA) for subsequent data analysis.
Chicken red blood cells (CRBC) were used as an internal standard, while human lymphocytes were used as an external standard. Ploidy measurements conformed to the method described by Jakobsen [14].
Samples with a DNA-index (DI) of more than 1.13 or lower than 0.89 (= mean ± 3 S.D. of measurements of normal bladder cells) after mechanical disaggregation and more than 1.12 or lower than 0.88 after enzymatical disaggregation were classified as abnormal.
Measurements of percentage of GO/G1 diploid cells conformed to the method described by Baisch et al. [1,2].

Results

Normal bladder tissue (7 cases)

The DI after mechanical disaggregation was 1.01 (range 0.95-1.07) with a s.d. = 0.04. After enzymatical disaggregation it was 1.00 (range 0.93-1.09) with a s.d. = 0.04.
The percentage of Go/G1 diploid cells after mechanical disaggregation was 82.5 (range 75.4 - 91.4) with a s.d. = 3.6. After enzymatical disaggregation it was 86.7 (range 71.1 - 94.5) with a s.d. = 4.7.

Bladder tumors

Of the 42 tumor specimens, analyzable metaphases were obtained in 33 cases (79%). The metaphases were obtained with the direct method and/or the short-term culture of 24 hrs and in two cases only after a long-term culture. Enzymatical disaggregation did not result in analyzable metaphases after 24 hrs culture, but in two out of five cases the long-term culture was successful. The DI could be obtained in all the specimens after mechanical

disaggregation and in 36 cases after enzymatical treatment. The mean coefficient of variation (c.v.) for the GO/G1 peak was 5.8 (range 3.5-9.3) after mechanical disaggregation and 6.2 (range 2.4-9.4) aftcr enzymatical disaggregation, respectively. As the study was restricted to the 33 cases with recognizable metaphases the DI of both pT1 and also of two pT2-pT4 tumor suspensions could not be examined after enzymatical treatment of the specimens. In five suspensions, the percentage of diploid cells in the GO/ G1 phase could not be estimated after enzymatical disaggregation of the specimens, owing to a large amount of debris.

Table 1

Flow cytometric and chromosome data of cell suspensions disaggregated by mechanical and enzymatical procedures in relation to stage and grade.

						Chromosomal results							
Tumor	Age (years)	Sex	pT/G	DI^1	DI^2	mn	range	2n	3n	4n	5n	dm	M
1	70	M	TaG2	1.00	0.99	46	45-47	10					
2	72	M	TaG2	1.03	1.03	46	34-48[b]	30					
3	53	M	TaG2	0.99	0.98	43	40-45	40					+
4	70	M	TaG2	1.02	0.99	46	38-47	20					+
5	64	M	TaG2	1.11	1.14	43	40-90	19	1	5			+
6	54	M	TaG2	1.05	0.97	46	46[b]	6					
7	70	M	TaG2	1.02	0.97	46	46	10					
8	78	M	TaG2	0.97	1.05	46	39-87	32	1	2			+
9	66	M	TaG2	1.01	0.97	45	36-47	15					+
10	61	M	TaG2	1.06	1.05	46	46	10					
11	44	M	TaG2	1.03	0.98	46	36-46	27					+
12	48	M	TaG2	1.00	1.00	45	37-46	15					
13	86	M	T1G2	1.45	1.61	69	40-90	11	43	2		+	
14	50	M	T1G3	2.37	2.19	87	65-90		3	4			
15	55	M	T1G2	1.09	1.03	44	37-47	40					+
16	84	F	T1G2	1.11	1.05	46	42-50	10					+
17	53	M	T1G2	0.98	1.00	43	35-60	14	1				+
18	62	F	T1G2	2.11	2.14	87	44-87	2	1	15			+
19	86	F	T1G2	1.94	–[a]	80	75-86		16	4			+
20	75	M	T1G3	1.96	–[a]	73	44-73	3	4				+
21	73	M	T2G2	1.76	1.80	46 (70)	43-75	27	6				
22	71	M	T2G3	1.69	1.87	46 (70)	42-70	5	3				
23	74	F	T2G3	2.37	2.50	98	46-110	3	1	15	1		
24	54	M	T2G3	1.68	1.69	73	65-73		14				+
25	75	M	T2G2	1.95	1.87	85	59-93		22	28			+
26	53	M	T2G3	1.74 (3.28)	1.70	68	54-117	2	8		3		+
27	64	M	T3G2	2.00	–[a]	74	45-78	4	6				+
28	67	F	T3G3	1.83	1.76	77	46-87	4	24	4			+
29	67	F	T3G3	1.74	1.79	77	53-89	1	28	2			+
30	70	M	T3G3	1.85	1.70	73	53-76	2	23				+
31	74	M	T3G3	1.84	–[a]	73	62-140		38	2			+
32	73	F	T4G3	1.61	1.57	69	46-120	14	18	3		+	+
33	66	M	T4G3	1.67	1.65	69	65-125		13	1	5		+

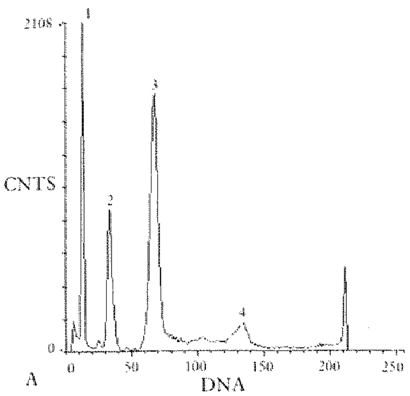
DI^1, DNA index after mechanical disaggregation; DI^2, DNA index after enzymatical disaggregation; mn, modal number; dm, double minutes; M, marker chromosome(s); a, not measured; b, long term-culture.

pTa tumors (12 cases)

The DI of all the cases was within the normal range (= 2c) after mechanical disaggregation (table 1). After enzymatical treatment the DI of one case (No. 5) was slightly higher than normal.
The modal chromosome number was diploid in eight cases, including the two in which only long-term cultures were applied, and hypodiploid in four cases. These 12 samples were obtained from ten patients. Two specimens had a wide range in chromosome number (Nos. 5 and 8).
Marker chromosomes of different types could be recognized in six cases. In three out of 12 cases, neither numerical nor structural abnormalities could be observed.
By comparison of the percentages of diploid cells present in the single cell suspensions after the two disaggregation methods were applied, no significant difference was seen ($p = 0.41$; Wilcoxon test, paired case). The mean percentage of diploid cells after mechanical disaggregation was 85.9 (s.d. = 4.5), and after enzymatical disaggregation it was 84.8 (s.d. = 3.8).

pT1 tumors (8 cases)

The DI after mechanical disaggregation was normal in three cases and varied from about 3c to nearly 5c in the other ones. After enzymatical treatment the DI was normal in the same three cases as above. In three other cases the DI varied from about 3c to about 4c. In two cases the DI could not be measured, owing to marked reduction of the fraction of GO/G1 cells. Comparison of the DI's obtained with the two disaggregation methods showed that the results were identical within a 10% deviation, with the restriction that after enzymatical treatment in two cases the DI could not be measured.
The modal chromosome number varied from hypodiploid to near-tetraploid. All the specimens had an abnormal chromosome constitution. Especially in the aneuploid tumors, a wide range was seen, and cells with different levels of ploidy were present. Marker chromosomes of different types were present in six out of eight cases. In one specimen a lot of double minutes could be observed.
The DI in four out of five cases with an abnormal DNA value was higher than the corresponding chromosome counts. This difference varied from 12% (No. 18) to about 23% (No. 20).
Comparison of the percentages of diploid cells present in the single cell suspension after mechanical (mean 42.1 with a s.d. = 30.3) or enzymatical (mean 65.8 with a s.d. = 21.3) treatment showed that after enzymatical treatment significantly more diploid cells were present in the cell suspension (Fig. 1) ($p= 0.04$). From two suspensions these percentages could not be estimated owing to bad quality of the cell suspensions.



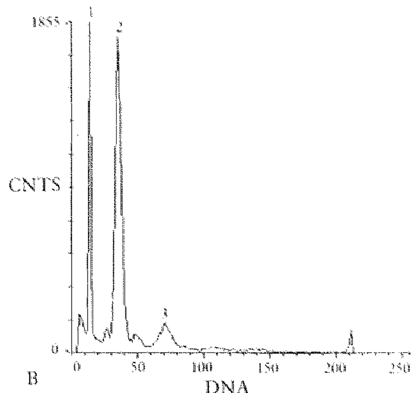


Fig 1.
DNA histogram of a T1G3 bladder carcinoma (No. 20) after mechanical (A) and after enzymatical (B) disaggregation of the tissue specimen.

A) 1, CRBC-peak; 2, normal GO/G1 phase; 3, abnormal GO/G1 Phase (DI 1.96); 4,G_2M phase of the cells indicated under peak 3.

B) 1, CRBC-peak; 2, normal GO/G1 phase; 3, the abnormal cells in B can not be indicated, since they seem to be masked by the G_2M-phase cells of the normal cells.

pT2-pT4 tumors (13 cases)

The DI after mechanical disaggregation varied from about 3c to nearly 5c. In one case (No. 26) two different populations were found. After enzymatical treatment the DI could not be estimated in two cases, owing to strong reduction of the GO/G1 cells. In the remaining cases the DI varied from about 3c to 5c. Also in this group of tumors, the DI's obtained with the two disaggregation methods were identical.
The modal chromosome number varied from diploid to about tetraploid. All the specimens showed an abnormal chromosome constitution. It is conspicuous that most infiltrating tumors (pT3-pT4) showed a modal number in the triploid range.
Marker chromosomes of different types could be observed in ten out of 13 cases. In one specimen many double minutes were seen.
The DI as estimated by flow cytometric analysis was in all cases higher than the corresponding chromosome counts. This difference ranged from 4% (No. 29) to about 24% (No. 30), with an average of 12%. Comparison of the percentages of diploid cells obtained after mechanical treatment (mean 26.9 with a s.d. = 13.0) and after enzymatical treatment (mean 62.7 with a s.d. = 17.2) showed that after enzymatical treatment significantly more diploid cells were present in the suspension (p= 0.004). In two suspensions these percentages could not be obtained, owing to bad quality of the cell suspensions.

Cell morphology

Four cell suspensions, one from a non-infiltrating tumor and three from deeply infiltrating tumors, obtained after mechanical or enzymatical disaggregation were examined for cell morphology. The percentage of epithelial cells was reduced after enzymatical treatment. The enzymatical treatment also had a degenerative effect on tumor cells.

Discussion

Flow cytometric investigations and chromosomal analyses need optimal single-cell suspensions. Bladder tumor tissue is often very adherent, and a single-cell suspension is often difficult to prepare by mechanical disaggregation. Therefore, enzymatical disaggregation is frequently used. In this study, the usefulness of mechanically and enzymatically obtained cell suspensions for flow cytometry and chromosomal analysis were compared.

Flow cytometry gave data in all cases examined after mechanical disaggregation. In four of the 33 cases compared, the DI could not be examined after enzymatical disaggregation. The DI of the GO/G1 phase of the tumor cells after both disaggregation methods was, with a marginal exception, identical.

The DI of the pTa tumors was 2c. In superficially infiltrating tumors (pT1), normal as well as abnormal DNA values were found, whereas in deeply infiltrating tumors only abnormal DNA values were seen. This agreed with others [23].

For chromosomal analysis, analyzable data were obtained in 33 of the 42 cases (79%). In our hands, it appeared that enzymatically obtained cells were not suitable for the 24-hour short-term culture. For long-term culture we agree with Wake et al, [22] who found that, after enzymatical disaggregation, a large number of cells attached themselves to the bottom of the culture flasks. We do not prefer this long-term culture, since it is time consuming and it is uncertain whether all the cells present in the initial cell suspensions are able to grow, and if so, it is uncertain whether they grow at the same speed. For this reason we remain in doubt about the representativity of the chromosome constitution obtained after long-term culture. Owing to technical improvements at this moment, it is possible to obtain chromosomes in more than 90% of the tumor specimens, with a direct technique, after mechanical disaggregation.

In cell suspensions, epithelial as well as non-epithelial cells are present. For this reason, in order to reduce this risk of false-negative karyotypes, a great number of cells must be analyzed chromosomally. However, when a few chromosomally abnormal cells among a large number of normal ones are found, it may be assumed that these cells represent the malignant part of the tissue, as soon as two or more cells show the same abnormalities.

The mean value of the DNA content of abnormal pT1-pT4 tumor cells, as estimated flow cytometrically, was 13% above the value expected from the corresponding chromosome count, assuming that the average chromosomal DNA is 1/46 of the mean DNA content of the normal diploid genome. In normal bladder tissue a 12% deviation is accepted (DI= 2c ± 3 s.d.). When this percentage also is used in aneuploid tumors, the DI measured is still above or in the upper range of the DI expected on the basis of the chromosome count. This difference has been described earlier [11,23]. Contribution of double stranded RNA is excluded, since a RNAse treatment was applied before flow cytometric analysis of the cell suspensions. A disproportionate increase of large chromosomes, as present in many tumors, can not explain this difference, as the ratio of large to small chromosomes did not deviate from normal diploid cells [21]. Premature chromosome condensation also focused attention on the need to explain this difference [3]. However, no integral explanation for the difference between the DNA values and cytogenetic data could be obtained and therefore this phenomenon needs further investigation.

Another difference between both methods of analysing the cells was noted with respect to the chromosomal range. By means of FCM analysis, tumors with two or more abnormal

cell lines were rarely observed. It is possible that cells with an abnormal DNA value were masked by the S-phase in flow cytometric examination. Chromosomal analysis especially of infiltrating tumors, showed a great range in the chromosome counts. Possible this represents stem-line(s) with side-line(s), however, without banding analysis it is impossible to discriminate among such lines. Nevertheless, it remains worthwhile to examine the clinical significance of this wide distribution.
When the percentages of diploid cells obtained with the two disaggregation techniques were compared, it appeared that in the aneuploid tumors a higher percentage of diploid cells was present in the cell suspension after enzymatical treatment. In other words, the percentage of aneuploid cells was higher after mechanical disaggregation than after enzymatical disaggregation. This difference is probably due to an increase in the release of stromal cells into the cell suspension as a result of collagenase treatment. The possibility can not be excluded that other enzymes would yield other results. Also, many tumor cells were degenerated. Frankfurt et al. [8] found that in 14 of the 16 aneuploid tumors the proportion of aneuploid cells was higher in the suspensions that were obtained mechanically. In the remaining two cases, enzymatically obtained suspensions contained a slightly higher percentage of aneuploid cells. In three of their cases, the aneuploid cells, which were observed in the DNA histogram after mechanical disaggregation, were not evident in the histogram of enzymatically obtained cell suspensions from the same tumors. In our study, in all of the 15 tumors with an abnormal DI that could be compared, the percentage of aneuploid cells after mechanical treatment was higher than after enzymatical disaggregation. Moreover, in four (= 19%) cases of infiltrating tumors, the percentage of aneuploid cells could not be obtained after enzymatical treatment.
Small numbers of aneuploid cells in enzymatically obtained cell suspensions are easily masked by the released non-epithelial cells. Also, in multiparameter analyses, for instance in the determination of percentages of S and G_2M phase, stromal and inflammatory cells disturb these estimations. To avoid this problem, mechanical disaggregation can be applied, although a better procedure for discriminating the epithelial from non-epithelial cells is to apply cytokeratin antibodies in a twodimensional FCM analysis [7,16].
From our results we conclude that disaggregation of bladder tumor tissue with the enzyme collagenase II is not suitable for short-term chromosome culture. For flow cytometric analysis it does not influence the DI, as compared to mechanical disaggregation, but in four cases the DI could not be estimated after enzymatical treatment. The percentage of diploid cells is higher after enzymatical treatment of aneuploid tumor specimens. Mechanical disaggregation must therefore be preferred to enzymatical methods in the preparation of cell suspensions to be used for FCM and chromosomal analysis without previous culture.

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Chapter IV

Tissue specific markers in flow cytometry of urological cancers.

III. Comparing chromosomal and flow cytometric DNA analysis of bladder tumors.

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Abstract

Thirty-seven transitional-cell carcinomas (TCC) of the urinary bladder were analyzed by DNA flow cytometry (FCM). After labeling of the cell suspensions with antibodies to cytokeratin, the cytokeratin positive cells and the non-epithelial cytokeratin negative cells could be analyzed separately.
After estimation of S- and G_2M phase, 3 of the 17 cases (18%) with a normal DNA index showed elevated proliferative levels, among cytokeratin labeled suspensions only. Of these 17 cases, 14 showed chromosomal abnormalities. The remaining 20 cases were abnormal irrespective of the technique used.
Although immuno-labeling of tumor cells for cytokeratin in FCM increases the sensitivity of this method in detecting aneuploid tumors or tumors with high proliferation fractions, the discriminating power of chromosomal analysis of TCC is greater than FCM.

Introduction

Chromosomal analysis as well as DNA flow cytometry have been described as important methods for studying the biology of urinary bladder cancers (Granberg-Öhman et al., 1980, 1982, 1984; Wijkström et al., 1984). An increase in chromosome numbers has been reported to correlate with invasiveness and loss of differentiation (Granberg-Öhman et al., 1980; Lamb, 1967; Spooner and Cooper, 1972). Furthermore, marker chromosomes are regarded as characteristics of clinically more aggressive bladder tumors (Falor and Ward, 1978; Sandberg, 1980, 1982; Summers et al., 1981).
DNA measurements of transitional cell carcinomas (TCC) showed that an abnormal DNA value is a sign of malignancy (Levi et al., 1969; Collste et al., 1980; Tribukait et al., 1982; Barlogie et al., 1983; Frankfurt et al., 1984). DNA values obtained through FCM and proliferative data are useful tools for diagnosis, management and prognosis of patients with bladder tumors (Devonec et al., 1981; Gustafson et al., 1982; Klein et al., 1982; Chin et al., 1985). Normal modal chromosome number and DNA index obtained by FCM are in good agreement with each other in TCC. However, tumors with near diploid chromosome counts and pseudo-diploid abnormalities can apparently not be detected by FCM (Granberg-Öhman et al., 1980; Wijkström et al., 1984). This may be due to the fact that the DNA flow cytometric analysis of carcinomas is often disturbed by the presence of stromal or inflammatory cells in the cell suspensions obtained from these neoplasms.
Recently the use of antibodies to cytokeratin and FCM has made it possible to distinguish between the different tissue types present in such suspensions (Ramaekers et al., 1983, 1984). Virtually all carcinomas, including TCC, contain cytokeratins, contrary to diploid stromal and inflammatory cells. Therefore, immuno-labeling of tumor cell suspensions with cytokeratin antibodies makes possible the analysis of epithelial cells by FCM (Feitz et al., 1985). In this way, the proliferative fraction among tumor cells can be estimated apart from stromal and inflammatory cells. This method also allows cytokeratin-negative diploid cells to be used as internal standards for calculation of the DI values of the tumor cells. This cannot be done without this labeling procedure, since in that case diploid carcinoma cells cannot be distinguished from non-epithelial cells.
In this study, data on chromosomal analysis and on FCM DNA measurements in tissue specimens from patients with TCC of the urinary bladder, with and without application of antisera to cytokeratin, were compared. The proportion of tumor cells in S-phase, estimated after application of these antisera was correlated with tumor stage.

Materials and methods

Thirty-seven tumor specimens, 33 primary and 4 recurrent cases, from 26 male and 7 female patients with TCC of the urinary bladder were succesfully analyzed. According to tumor stage, they could be divided as follows: 14 cases of pTa, 11 of pT1 and 12 of pT2-pT4.
The tissues were collected following transurethral resection. One part of each biopsy specimen was used for pathologic examination, while the rest was used for chromosomal and flow cytometric studies with and without application of antisera against cytokeratin. Clinical staging of the tumor was done according to the UICC TNM system (Harmer, 1978) and grading according to the WHO system (WHO, 1973).

Chromosomal analysis.

One or both of the following techniques were used:
Direct method.
The tissue specimens were collected and transported in 10 ml 0.5% sodium citrate + 0.5 μg colcemid/ml (1 hr). They were disaggregated by scraping and cutting in a Petri dish and filtered through a 100 μ nylon filter (Ortho Diagnostic Systems, Beerse, Belgium). After incubation in Hanks' balanced salt solution (BSS obtained from Gibco, Paisley, U.K.) colcemid was added (2 μg/ml Hanks' BSS 0.5 hr). Hypotonic treatment was done with a mixture of 6 ml foetal calf serum (FCS, Gibco) and 24 ml 0.052 M KCl. Fixation was performed with acetic acid-methanol 3 : 7.

Short-term culture (Smeets et al., 1985).
The tissue specimens were collected and transported in 10 ml RPMI 1640 medium plus 17% FCS, 50 μg gentamycin/ml, 50 U penicillin/ml and 50 U streptomycin/ml. They were disaggregated mechanically, then incubated for 24 hrs in the medium mentioned above. Metaphase arrest with colcemid (0.1 μg/ml) was followed by hypotonic treatment with 0.075 M KCl and fixation in methanol-acetic acid 3 : 1. All slides were stained with Giemsa.
C-banding and G-banding were performed (if sufficient appropriate metaphases were available) and metaphases photographed and analyzed according to the standard rules (ICSN, 1978).

Chromosomal range, modal number, distribution according to ploidy ($n= 23\pm 11$, $2n=46\pm11$, $3n=69\pm11$ etc.) and the presence of marker chromosome(s) were registered. Results were pooled in those cases where metaphases were obtained with the direct method as well as with the short-term culture method.

Flow cytometry.

The specimens were collected in 10 ml RPMI 1640 medium plus 17% FCS, 50 μg gentamycin/ml, 50 U penicillin/ml and 50 U streptomycin/ml. The cell suspensions were prepared mechanically as described above for the suspensions used for chromosomal analysis. The filtered cell suspensions were centrifuged at 400 g for 7 min. Then, 70% ethanol (-20°C) was added rapidly to the cell pellet with constant shaking. The final concentration was about 3 x 10^6 cells/ml ethanol. At this stage, the fixed cells could be stored for several weeks at -20°C.

Immunohistochemical cell staining procedures:
Cells were incubated with a polyclonal antibody to cytokeratin (pKer; Eurodiagnostics, Apeldoorn, The Netherlands) for an indirect immunofluorescence technique (Ramaekers et al., 1983). About 1 x 10^6 cells in 70% ethanol were centrifuged (400 g; 1.5 min) and the pellet was resuspended with 1 ml of 5% FCS in buffer A (8.01 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.2 g KH_2PO_4 in 1000 ml water. pH 7.4) and pelleted again. The cell pellet was resuspended in 0.2 ml of the pKer antiserum, diluted 1 : 5 in 5% FCS in buffer A and incubated for 30 min at room temperature with regular shaking. Then the cells were washed 3 times in 1 ml buffer A containing 5% FCS. After the last washing step the cell pellet was resuspended in 0.5 ml of FITC conjugated goat-anti-rabbit IgG, diluted 1 : 25 (Nordic, Tilburg, The Netherlands). After incubation for 30 minutes with this second antibody, the cells were washed again.
The cell pellet was then resuspended in 1 ml of a propidium iodide (PI) solution (20 mg/l A-grade in 150 m*M* sodium phosphate buffer, pH 7.4; Calbiochem-Boehringer, La Jolla, CA). To 1 ml of this cell suspension 0.1 ml of a stock-solution of RNAse (1% type A in the sodium phosphate buffer; Sigma, St. Louis, MO) was added, then the cell suspension was incubated for 10 min at 37°C. Finally, the cell suspension was filtered through a 100 μ filter and the cells were kept in the dark at room temperature prior to FCM analysis (Ramaekers et al., 1984; Feitz et al., 1985).

DNA measurements:.
Cell analysis was performed using a Cytofluorograph 5OH (Ortho Instruments, Westwood, MA). The fluorochromes PI and FITC were excited at 488 nm with an argon ion laser (Spectra Physics, Mountain View, CA). Fluorescence was measured simultaneously using a 515-530 nm band pass filter and a 630 nm long pass filter for FITC and PI, respectively. A correction was made for leakage of FITC fluorescence into the PI channel. All data were stored in list mode in a PDP 11/34 computer (Digital Equipment Corporation, Marlboro, MA) for subsequent data analysis.
Ploidy measurements of the unlabeled tumor cells were done (Jakobsen, 1983). The DNA content was expressed as DNA index (DI) (Hiddemann et al., 1984).
The DI of the tumor cells labeled with pKer was estimated using the non-pKer-labeled cells as internal standards. The DI of normal bladder cells was 1.01 (range 0.95-1.07) with a s.d. = 0.04.
Samples with a DI of more than 1.13 or less than 0.89 (= mean ± 3 s.d. of measurements of normal bladder cells) were classified as abnormal (Smeets et al., 1987).
Percentages of cells in S- and G_2M phases were measured (Baisch et al., 1975, 1982).
The mean coefficient of variation (c.v.) for the Go/G1 peak of the tumor cells was 5.8 (range 3.5 - 9.3).

Results

Chromosomal abnormalities were observed in 34 out of 37 cases that were analyzed successfully (92%). Both unlabeled and labeled in 20 cases the DI was abnormal and 17 specimens showed a normal DI. In 31/37 tumors which did not contain excessive debris, the percentage of cytokeratin positive cells in S- and G_2M phase could be calculated (Fig. 1) which was not possible without pKer labeling. Among the 17 tumors with a normal DI, in one case the S-phase and in 2 cases the G_2M-phase were conspicuously high. Thus in 3

of the 17 cases, formerly thought to have a normal DNA content, application of cytokeratin antibodies gave indications of abnormalities which were not seen without such labeling.

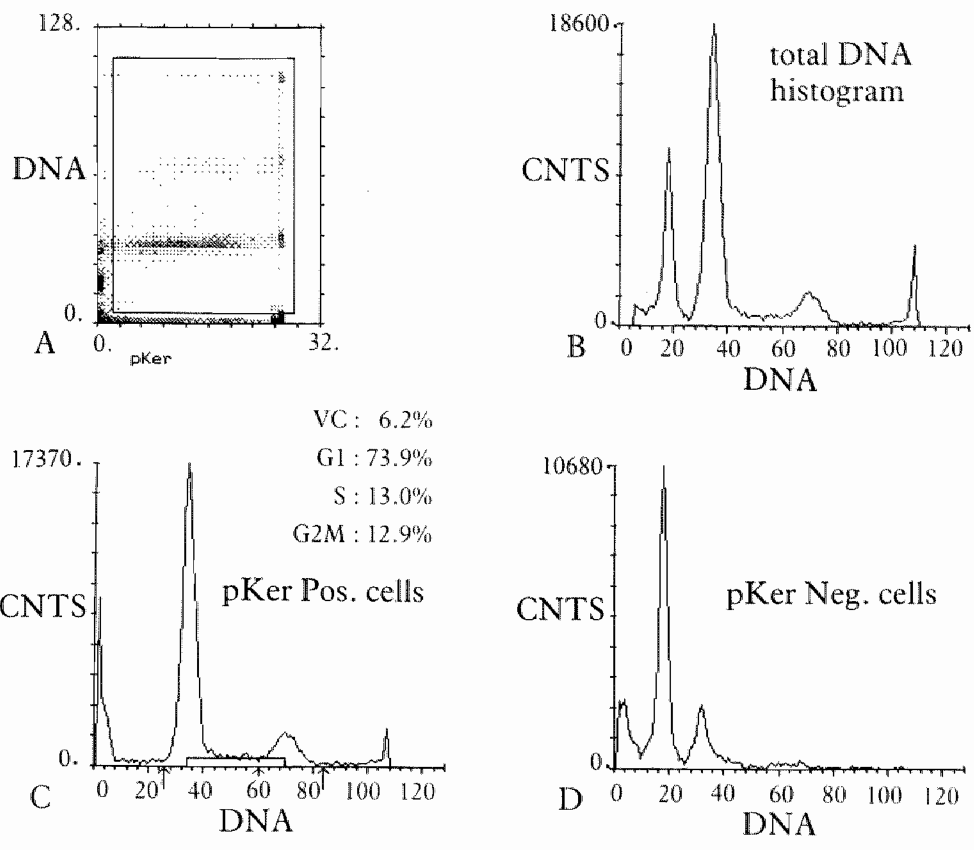

Fig. 1.
FCM analysis of a transitional bladder cell carcinoma after labeling for cytokeratin (pKer) and staining of DNA with P.I.

A. Two parameter analysis, showing position of the window containing epithelial cytokeratin positive tumor cells.
B. DNA histogram of the total cell suspension.
C. DNA histogram of the cytokeratin positive cells, selected by placing a window as illustrated in A.
D. DNA histogram of cytokeratin negative cells.

pTa tumors

The DI of the Go/G1 fraction in 13/14 cases of superficially growing bladder tumors was normal (2c), irrespective of the labeling for cytokeratin (Table 1). In one case (number 13) the DI was highly abnormal and after labeling a high percentage of cells was found in S-phase (mean 4.7 with a s.d. = 4.1). In one diploid tumor (number 5) the S-phase percentage and in another case (number 9) the G_2M-phase percentage were very high. Without labeling the S-fractions of the pTa tumors were not abnormal. In 11/14 cases

Table 1.

Results of DNA flow cytometric and chromosomal analyses of bladder transitional cell carcinomas in relation to tumor stage.

number	T/G	DI^1	DI^2	%S	$\%G_2M$	modal chrom. number	range	2n	3n	4n	≥5n	M	banding
1.	TaG2	1.00	0.98	3.7	17.9	46	45-47	10					
2.	TaG2	1.03	1.02	1.5	16.6	46	34-48	30					
3.	TaG2	0.99	1.00	2.3	12.3	43	40-45	40				+	+
4.	TaG2	1.02	1.05	2.5	15.9	46	38-47	20				+	+
5.	TaG2	1.11	1.09	12.6	19.8	43	40-90	19	1	5		+	
6.	TaG2	1.05	1.13	4.3	21.4	46	46	6					
7.	TaG2	1.02	1.04	2.4	9.7	46	46	10					
8.	TaG2	0.97	1.02	3.5	14.5	46	39-87	32	1	2		+	+
9.	TaG2	1.01	1.02	3.8	32.5	45	36-47	15				+	+
10.	TaG2	1.06	1.05	5.5	14.7	46	46	10					
11.	TaG2	1.03	1.04	1.7	15.5	46	36-46	27				+	+
12.	TaG2	1.00	1.10	1.5	6.2	45	37-46	15					+
13.	TaG2	1.93	1.91	15.7	15.5	78	40-83	11	13	1			
14.	TaG2	1.00	1.04	4.3	20.2	45	36-47	15				+	+
15.	T1G2	1.45	1.64	12.4	26.0	69	40-90	11	2				+
16.	T1G2	2.37	2.23	8.3	8.7	87	65-90		3	4			
17.	T1G2	1.09	1.06	7.4	21.9	44	37-47	40				+	+
18.	T1G2	1.11	1.02	2.6	37.9	46	42-50	10				+	+
19.	T1G2	0.98	1.07	10.2	16.5	43	35-60	14	1			+	+
20.	T1G2	2.11	2.24	9.6	14.5	87	44-87	2	1	15		+	+
21.	T1G2	1.94	1.85	-	-	80	75-86		16	4		+	+
22.	T1G2	1.73	1.91	11.0	18.4	72	62-77		25			+	+
23.	T1G3	1.96	2.00	16.6	15.1	73	44-73	3	4			+	+
24.	T1G2	1.12	1.08	10.3	23.0	44(68)	43-70	28	6			+	+
25.	T1G2	1.86	1.82	16.4	16.7	83	75-88		1	17			+
26.	T2G2	1.76	2.02	9.8	26.4	46(73)	43-75	27	6				
27.	T2G3	1.69	1.85	-	-	46(70)	42-70	5	3				
28.	T2G3	2.37	2.53	-	-	98	46-110	3	1	15	1		+
29.	T2G3	1.68	1.80	13.3	18.4	73	65-73		14			+	+
30.	T2G2	1.95	1.91	14.2	13.5	85	59-93		22	28		+	+
31.	T2G3	1.74	1.87	11.0	11.8	68	54-117	2	8		3	+	+
		(3.28)	(3.60)										
32.	T3G2	2.00	2.35	-	-	74	45-78	4	6			+	+
33.	T3G3	1.83	1.79	16.2	12.1	77	46-87	4	24	4		+	+
34.	T3G3	1.74	1.75	16.4	14.6	77	53-89	1	28	2		+	+
35.	T3G3	1.84	2.04	11.0	18.4	73	62-140		38		2	+	+
36.	T3G3	2.83	2.61	-	-	115	73-121		3	2	35	+	+
37.	T4G3	1.67	1.77	-	-	69	65-125		13	1	5	+	+

DI^1 : DNA index of the Go/G1 fraction of the tumor cells, estimated without labeling with pKer.
DI^2 : DNA index of the Go/G1 fraction of the tumor cells after labeling with pKer.
M : Marker chromosome(s).
%S : % cells in S-phase after labeling with pKer.
$\%G_2M$: % cells in G_2M phase after labeling with pKer.

chromosomal abnormalities were observed. With one hyperdiploid exception the modal chromosome number of these cytogenetically abnormal tumors was (hypo-)diploid, while 3 cases (numbers 5, 8 and 13) showed a wide range in their chromosome number. Marker chromosomes of different types could be obtained in 7 cases. A G-banded karyotype with some abnormal chromosomes is shown in Figure 2.

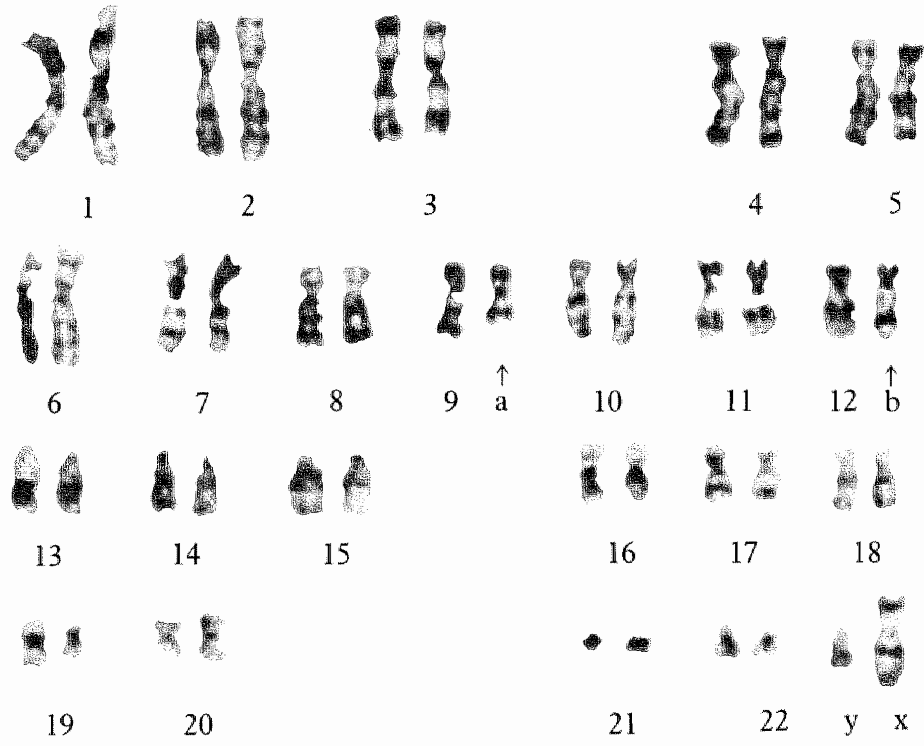

Fig. 2.
A G-banded karyotype of a Ta tumor (x 4000). Note the presence of abnormal chromosomes indicated by arrows. This tumor was not found to be abnormal by FCM.

pT1 tumors

The DI of the Go/G1 fraction of 7/11 cases was abnormal, either with or without labeling. The mean percentage of cells in S-phase was 10.5 (s.d. = 4.1). In one case with a normal DI (number 18) the percentage of cells in G_2M phase was extremely high.
All cases showed chromosomal abnormalities. The modal chromosome number was (hypo-)diploid in 4 cases and hyperdiploid in 7 specimens. Marker chromosomes of different types were found in 8/11 cases.

pT2-pT4 tumors

The DI of the Go/G1 fraction of all these cases of deeply infiltrating tumors was abnormal either with or without labeling. In one case (number 31) 2 different cell populations with an abnormal DI were found.

The mean percentage of cells in S-phase was 13.1 (s.d. = 2.6). All 12 cases showed chromosomal abnormalities. The modal chromosome number was diploid in 2 cases and hyperdiploid in 12 specimens. Marker chromosomes of different types were found in 9/12 cases.

Correlation between flow cytometric and chromosomal data.

The mean value of the DNA index, as determined after labeling of pTa tumors, was 6% above the value expected from the corresponding chromosome count. For pT1 tumors this was 12% and for pT2-pT4 tumors 18% (Fig. 3).

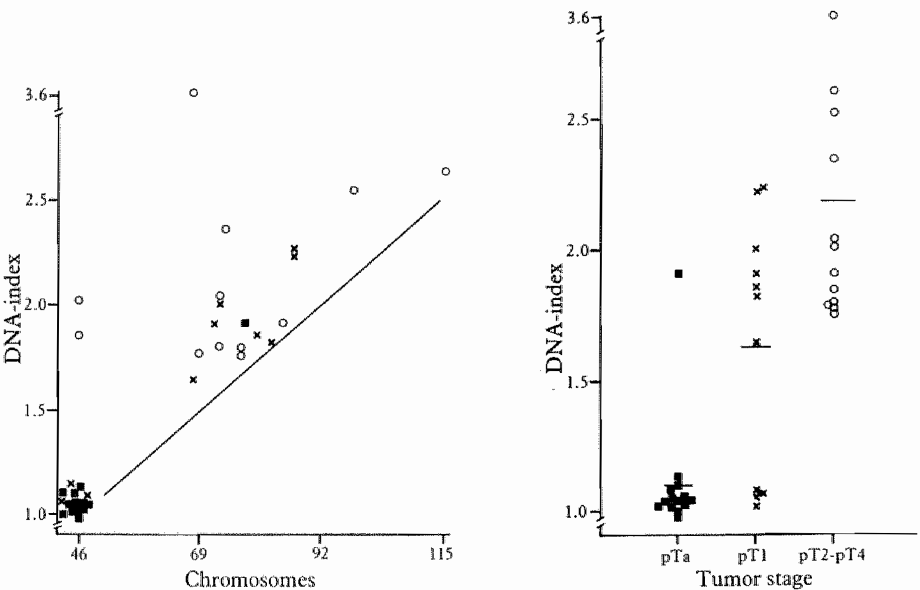

Fig. 3.
Correlation between the DNA index of cytokeratin positive cells and modal chromosome number.
□ = pTa tumor, x = pT1 tumor, o = pT2-pT4 tumors.

Fig. 4.
Correlation between the DNA index of cytokeratin positive cells and tumor stage.
Mean pTa: 1.18 (s.d. = 0.23). pT1: 1.63 (s.d. = 0.45).
pT2-pT4: 2.02 (s.d. = 0.29). The horizontal bars indicate the mean values. Wilcoxon test for degree of significance: pTa-pT1: p = 0.005. pTa-p(T2-T4): p = 0.0001. pT1-p(T2-T4): p = 0.069.

Correlation between DNA index, proliferative fraction and chromosomal number with tumor stage.

The different parameters estimated from the flow cytometric studies and obtained from chromosomal analysis (Table 1) were correlated with tumor stage. It appeared that DNA

index, S-phase and modal chromosome number showed a strong correlation with tumor invasiveness (Fig. 4-6). Strikingly, however, 2 cases of pTa tumors (cases 5 and 13) could clearly be distinguished in these comparative studies, since they showed high values for one or more parameters. Similarly, some cases of pT1 tumors had values lower than average.

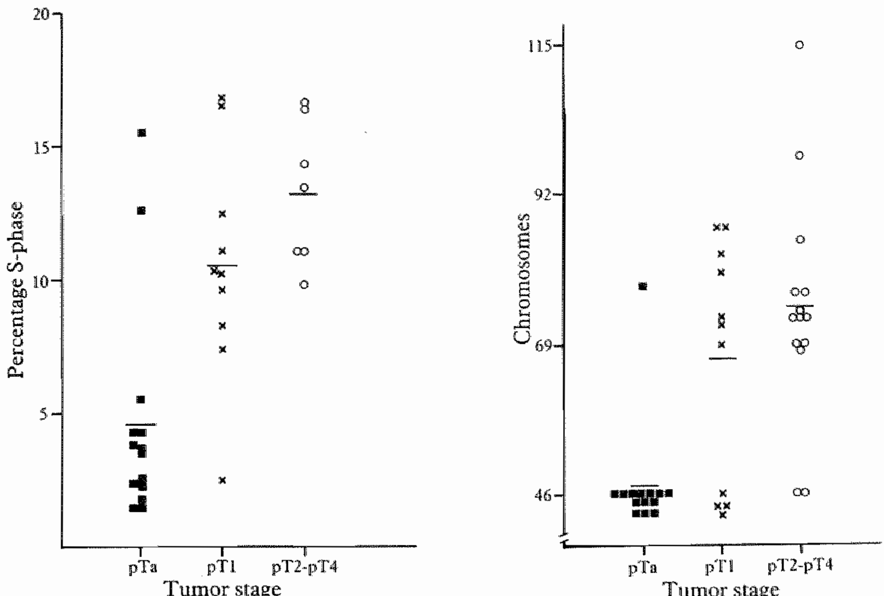

Fig. 5.
Correlation between the percentage of cytokeratin positive cells in S-phase and tumor stage. Mean pTa: 4.7 (s.d. = 4.1), pT1: 10.5 (s.d. = 4.1), pT2-pT4: 13.1 (s.d. = 2.6). The horizontal bars indicate the mean values. Wilcoxon test for degree of significance: pTa-pT1: p = 0.005, pTa-p(T2-T4): p = 0.002, pT1-p(T2-T4): p = 0.170.

Fig. 6.
Correlation between the modal chromosome number and the tumor stage. The horizontal bars indicate the mean values.
Wilcoxon test for degree of significane: pTa-pT1: p = 0.052. pTa-p(T2-T4): p = 0.0001. pT1-p(T2-T4): p = 0.441.

Discussion

DNA flow cytometric (FCM) analysis of carcinoma cells has been described as a useful and reproducible method in diagnosis and management of patients with bladder tumors (Devonec et al., 1981; Wijkström et al., 1984). In order to analyse the carcinoma cells separately from contamination of non-epithelial cells, antibodies to cytokeratin can be used in a 2 parameter analysis (Ramaekers et al., 1983, 1984; Feitz et al., 1985, 1986). Although chromosomal analysis of bladder cancer is hampered by practical difficulties, essential information such as range in chromosome counts, marker chromosomes and the association of some chromosomal changes and bladder cancer can be obtained (Atkin and Baker, 1977, 1985; Gibas et al., 1984; Sandberg, 1984; Smeets et al., 1985; Vanni et al., 1985; Pauwels et al., 1987).

In this study, the data obtained by 2 methods of DNA analysis of cells recognized with antibodies to cytokeratin and chromosomal analysis have been compared.
The advantages of using cytokeratin antiserum have been documented (Feitz et al., 1985). This study revealed 17 tumors with an apparent diploid DI. But, after labeling with the cytokeratin antiserum 3 of these cases showed strikingly high percentages of cells in S- and G_2M phase. This is of importance, since high S-phase may be interpreted as an adverse prognostic factor (Barlogie et al., 1983). The high percentage of cells in G_2M phase may possibly indicate the presence of a tetraploid cell population in the tumor. Chromosomal analysis did not show any tetraploid cells in the cases mentioned. However, there is always a risk of missing some cells by cytogenetic examination especially in specimens with low mitotic activity.
In bladder cancer cases it is of extreme prognostic and therapeutic importance to be able to distinguish between pTa and pT1 tumors and furthermore to recognize potentially invasive pTa tumors. Of the 17 cases of pTa with a normal DI, 3 showed indications of abnormalities when S-and G_2M fractions were estimated after labeling, which thus improved the discriminatory power of FCM by 18%.
The two cases of pTa tumors with a high DI and/or S-phase are particularly interesting in this respect. These tumors did also show significant chromosome abnormalities and, therefore, should be considered potentially more aggressive cases.
Chromosomal abnormalities were present in 14/17 cases with a normal DI, while 11/17 cases showed marker chromosomes. The 20 tumors with an abnormal DI showed, within a 10% variation the same DNA value with or without labeling for cytokeratin. All these cases showed chromosomal abnormalities. The fact that the DI is above the values expected from the corresponding chromosome count (in this study 6-18%) has been described earlier (Granberg-Öhman et al., 1982; Wijkström et al., 1984).
Neither premature chromosome condensation (Barlogie et al., 1983) nor an increase in the number of large chromosomes, as present in many tumors (Tribukait et al., 1986), can explain this difference. Contribution of double stranded RNA is excluded since an RNAse treatment was applied before flow cytometric analysis of the cell suspensions. It is possible that a random loss of chromosomes especially in the strongly hyperdiploid tumors, may contribute to this difference.

The wide range of chromosome numbers, seen in non-infiltrating as well as in infiltrating tumors, is the result of a great heterogeneity and indicates a more aggressive behavior of the tumor (Pauwels et al., 1987). Banding of chromosomes is still problematic. C-banding could be done in most cases, but G-banding, which is necessary for karyotyping and identification of structurally abnormal chromosomes, could only be achieved in a minority of cases. Nevertheless, the presence of chromosomal abnormalities was unequivocal in most instances.
Although labeling for cytokeratin improved the value of FCM analysis, the discriminatory power of chromosomal analysis appeared greater than that of FCM. Near diploidy, marker chromosomes and wide range in chromosome count which is present in many superficial tumors could be not recognized by FCM in most cases.
We thus suggest a combination of chromosomal analysis and flow cytometric estimation of DI, S- and G_2M phase following prior labeling with antibodies to cytokeratin, when trying to recognize the potentially invasive non-infiltrating tumors.

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Chapter V

Grading in superficial bladder cancer.

Cytogenetic classification.

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Summary

Cytogenetic analysis was performed in 92 newly diagnosed transitional cell bladder carcinomas. The results of this analysis were correlated with the clinical course in the superficial tumors. Low grade tumors appeared to have hypo- or peri-diploid chromosomal numbers. Intermediate grade tumors were characterized by chromosomal counts up to the hyperdiploid range, but could have a peri-diploid modal number. With respect to the clinical course of superficial tumors the range in chromosomal counts appeared to be more reliable than the modal number. The significant difference in chromosome numbers between low, intermediate and high grade tumors may be considered as a biological basis for grading.

Introduction

The natural history of a papillary tumor of the bladder is unpredictable. Next to tumor stage, grade is at present the most important indicator of the clinical behavior of a bladder tumor (Kern, 1984). However, reports regarding grade are not always completely comparable because different grading systems are used, or different interpretations are given to one grading system (Hofstädter et al., 1984). Chromosomal analysis is mentioned as one of the more objective criteria of the malignant potential of bladder cancer (Lamb, 1967; Spooner and Cooper, 1972).

Cytogenetic studies showed a positive correlation between histological grade and modal chromosome numbers for low and high grade bladder tumors (Spooner and Cooper, 1972; Wijkström et al., 1984). In intermediate grade tumors normal as well as abnormal modal numbers were found. In another study (Pauwels et al., 1987) an interpretation of the W.H.O. grading criteria was established which appeared to correlate with rate of recurrences and tumor progression.

The aim of this study was to investigate whether this interpretation of the W.H.O. grading classification could be confirmed by a cytogenetic study.

Patients and methods

Between 1980 and 1986, from 92 patients with a primary transitional cell carcinoma of the bladder only treated by TUR,chromosomal data from tumor specimens were achieved (see table 1).

Table 1.
Number of patients in relation to tumor stage and grade.

Grade	Tumor stage			Totals
	Ta	T1	T2-4	
G1	31	1	–	32
G2	9	15	11	35
G3	–	5	20	25
Total	40	21	31	92

Staging was done according to the T.N.M.-system (Harmer, 1978). As grade 1 were classified tumors showing increased cellularity with or without slight nuclear and cellular variations but with normal polarity of individual cells. Tumors showing clear cytological

deviations with a tendency to loose normal polarity were classified grade 2. Tumors were called grade 3 if considerable cellular deviation was seen, with strong variations in size and shape of cells and nuclei and a loss of normal cellular architecture.
Patients with a superficial (Ta-T1) low or intermediate grade tumor were followed by cystoscopy for 3 to 62 - mean 28 - months. If chemotherapy, immunotherapy or radical therapy was applied, or tumor progression occurred, follow-up ended.

Cytogenetic analysis.

For microscopic analysis of chromosomes, cells in metaphase were obtained from a suspension of single cells. After colcemid arrest, hypotonic treatment and fixation, the chromosomes were routinely stained with Giemsa. For further identification of chromosomes, specific banding techniques are available. The techniques used for cytogenetic analysis -direct method or short term culture - have been described previously (Smeets et al., 1985).
Analysable metaphases, at least 5 (average 28) per case were photographed and karyotyped according to the Paris nomenclature (I.S.C.N., 1978). The tumors were classified according to the modal chromosome number and chromosome range. The latter classification distinguished the tumors with cells only in the hypo-diploid or peri-diploid range (49 or less) and cells with more than 49 chromosomes (hyperdiploid).
Marker chromosomes, structurally abnormal chromosomes, as mentioned in this study were shown by routine techniques, mostly in non-banded preparations.

Results

Normal bladder tissue.

In 15 patients with non-infiltrating tumors, random biopsies from cystoscopically normal bladder tissue showed neither histopathological nor cytogenetic abnormalities.

Tumor stage and grade.

The majority of non-invasive tumors (Ta) had peri-diploid chromosomal numbers (see table 2).

Table 2.
Tumor stage in relation to chromosomal numbers.

chromosomal number	Number and stage of tumors		
	Ta	T1	T2-4
modal number ≤ 49	35	9	6
modal number > 49	5	12	25
range ≤ 49	31	–	–
range > 49	9	21	31

In all infiltrating tumors, cells with more than 49 chromosomes were present, although the modal number often was peri-diploid. The difference in the range of chromosomal numbers between non-invasive and invasive tumors is significant ($p < 0.001$).
As is shown in table 3 all except one grade 1 tumors had a modal number of 49 or less. In nearly half of the 35 grade 2 tumors the modal number was peri-diploid. Nearly always, however, cells in the hyperdiploid range were seen like in all grade 3 tumors. The

differences in chromosomal numbers (mode and range) between grade 1 and grade 2 tumors are significant ($p < 0.001$). Between grade 2 and grade 3 tumors there was a significant difference in modal number ($p < 0.002$).

Table 3.
Tumor grade in relation to chromosomal numbers.

chromosomal number	Number and grade of tumors		
	G1	G2	G3
modal number ≤ 49	31	17	2
modal number > 49	1	18	23
range ≤ 49	28	3	-
range > 49	4	32	25

Marker chromosomes were found in 17 of the 40 non-invasive tumors (43%) and in 31 of the 52 invasive cases (60%) ($p = 0.22$). Nine out of 32 grade 1 tumors showed markers (28%) compared with 39 of the 60 grade 2 and grade 3 cases (65%). So, in higher grade tumors more often marker chromosomes were found ($p = 0.003$).

Follow up of superficial low and intermediate grade tumors.

Fourty-two patients with a superficial low or intermediate grade tumor were evaluable for follow up (see table 4).

Table 4.
Correlation of results of chromosomal analysis with clinical course for superficial (Ta - T1) low and intermediate grade bladder tumors.

	Results of chromosomal analysis.					
patients	modal number		chromosomal range		marker chromosomes	
	≤ 49	> 49	≤ 49	> 49	no	yes
evaluable	37	5	28	14	24	18
disease free	19 (51%)	0^a	16 (57%)	3 (21%)^b	13 (54%)	6 (33%)^c
with tumor progression	7 (19%)	2 (40%)^d	3 (11%)	6 (43%)^e	4 (17%)	5 (28%)^f
recurrence rate	3.0	2.7	2.9	3.0	2.2	3.9

Follow up 3 to 62 - mean 28 - months.
Recurrence rate: n recurrences/n months patient follow up x 100 $^ap = 0.07$; $^bp = 0.16$; $^cp = 0.50$; $^dp = 0.62$; $^ep = 0.05$; $^fp = 0.63$

Fifty-one percent of the tumors with a peri-diploid modal number did not recur. In the group with hyperdiploid modal numbers all tumors recurred ($p = 0.07$). With respect to tumor progression no significant difference was seen between these two groups ($p = 0.62$). If, however, in a tumor chromosomal numbers in the hyperdiploid range were shown, tumor progression was seen significantly more often than in the group with lower chromosomal numbers ($p = 0.05$).
The tumor recurrence rates showed no significant differences between the groups. However, estimations using the Kaplan-Meier method show that in the group with a hypo- or peri-diploid chromosomal range the first 18 months only 10% had a recurrence,

which figure rose to 69% after 42 months. In the group with a hyperdiploid chromosomal range, 54% had at least one recurrence after 18 months. So, tumors with a hypo- or peri-diploid chromosomal range clearly recurred much later.
In four cases progression was seen in tumors with near normal modal numbers but a hyperdiploid chromosomal range. With respect to the clinical course, no sifnificant difference between the groups with or without marker chromosomes was observed.
An addendum shows the histopathological and cytogenetic data, and follow up of the clinical course for each patient.

Discussion

In a previous study about grading in superficial bladder cancer, a clear correlation between morphological criteria and the clinical course of bladder tumors was found (Pauwels et al., 1987). The present study was done to see whether a correlation between chromosomal abnormalities and the grading criteria used could be demonstrated.
Grade 1 tumors appeared to have hypo- or peri-diploid chromosomal numbers. Grade 2 tumors show in half of the cases a peri-diploid modal number but nearly always they contain a number of cells with more than 49 chromosomes. In grade 3 tumors the chromosomal mode is strongly hyperdiploid. Other authors also reported that grade 2 tumors are heterogeneous with respect to modal number (Spooner and Cooper, 1972; Wijkström et al., 1984). The significant difference in chromosomal range between grade 1 and grade 2 tumors is not reported before.
Tumors graded by us as intermediate recur significantly earlier, and progress more often than low grade tumors (Pauwels et al., 1987). These grade 2 tumors appear to have chromosomal numbers up to the hyperdiploid range. This means that nuclear pleomorphism, enlargement and hyperchromasia probably reflect the increase in chromosome number and the increased aggressiveness of a tumor.
Tumors having a peri-diploid modal number but a range till in the hyperdiploid region, tended to behave worse than tumors with only near normal chromosomal numbers. The finding of hyperdiploid modal numbers in intermediate grade superficial bladder tumors, like by FCM analysis higher DNA values, is a bad prognostic sign (Wijkström et al., 1984). However, a near normal modal chromosomal number does not always indicate a benign behavior of the tumor.
Marker chromosomes in superficial bladder cancers were regarded as a very bad prognostic factor (Falor and Ward, 1978; Summers et al., 1981). In our study this could not be confirmed. For establishing structural abnormalities so called chromosome banding techniques are necessary.
By the use of different banding techniques non-random chromosomal changes have been demonstrated in bladder tumors (Gibas et al., 1984; Atkin and Baker, 1985). The prognostic significance of the different changes are not yet known. The increasing variation in chromosomal number during the progression of the neoplasia is believed to confer proliferative advantage to the tumor cells (Yunis, 1983).
Our grading criteria in superficial bladder cancer were proved to have a clinical basis with respect to tumor stage, rate of recurrences and tumor progression (Pauwels et al., 1987). The conclusion of this study may be that with chromosomal analysis we were able to give a biological meaning to our grading system.

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Addendum:

Histopathologic and cytogenetic data for the initial biopsy from individual bladder carcinoma patients with follow up.

Patient			Tumor		Chromosomes								Follow up	
							Ploidy[a]							
Case	Age	Sex	T	G	Range	Mode	n	2n	3n	4n	5n	Mar	n Recurr.[b] n Months	Progression
1	66	M	a(m)	1	19-46	46	5	21				–	1/16	+ (T2G3)
2	51	M	a(m)	1	40-45	43		40				+	3/41	–
3	27	M	a	1	45-46	46		5				–	–/26	–
4	63	M	a	1	34-48	46		7				–	–/38	–
5	64	M	a	1	40-90	43		19	1	5		+	–/30	–
6	65	M	a(m)	1	35-71	43/70		8	8			–	1/29	–
7	66	M	a	1	30-46	43		10				–	–/12	–
8	72	F	a	1	19-39	30	2	3				–	1/51	–
9	70	M	a	1	40-47	46		20				–	1/ 4	–
10	58	F	a(m)	1	40-45	43		16				–	2/16	+ (T1G3)
11	57	M	a(m)	1	38-78	43		19	3			+	3/53	+ (T1G1)
12	41	M	a(m)	1	45-46	45		5				+	4/24	–
13	59	M	a(m)	1	40-44	43		5				–	–/30	–
14	63	M	a	1	41-47	43		16				–	–/12	–
15	73	M	a	1	46-48	48		8				–	–/ 3	–
16	55	M	a	1	43-46	44		7				–	1/30	–
17	17	M	a	1	45-46	46		5				–	1/48	–
18	77	M	a	1	42-47	46		11				–	1/21	–
19	56	M	a	1	35-45	42		10				+	chemotherapy	
20	60	F	a	1	32-49	46	10	74				+	–/42	–
21	58	M	a	1	34-46	46		10				–	–/52	–
22	91	M	a(m)	1	38-46	46		46				–	–/12	–
23	65	M	a	1	46	46		5				–	–/54	–
24	56	M	a	1	36-46	44		13				–	3/48	–
25	71	M	a	1	35-48	46		5				+	–/42	–
26	61	M	a(m)	1	30-46	40		5				–	2/66	–
27	63	M	a	1	42-44	43		5				–	1/36	–
28	60	M	a	1	44-46	46		10				–	–/12	–
29	76	M	a	1	40-45	45		9				–	chemotherapy	
30	48	M	a	1	37-46	45		15				–	–/12	–

Patient			Tumor		Chromosomes								Follow up	
								Ploidy[a]						
Case	Age	Sex	T	G	Range	Mode	n	2n	3n	4n	5n	Mar	n Recurr.[b] n Months	Progression
31	66	M	a	1	36-47	45		16				+	–/15	–
32	65	M	a	2	19-45	42	11	16				+	4/24	+ (T1G2)
33	73	M	a	2	60-89	70			7			+	1/28	+ (T2G3)
34	82	M	a	2	50-90	67		2	7	4		+	not fit for treatment	
35	61	M	a	2	50-75	64			20			+	1/60	–
36	72	F	a	2	38-57	45		11				+	–/18	–
37	66	M	a	2	23-44	40	4	31				+	1/26	–
38	78	M	a	2	39-87	45		31	1	2		+	1/25	–
39	44	M	a	2	36-49	46		27				+	chemotherapy	
40	83	M	a	2	40-83	78		13	13	1		+	chemothearpy	
41	55	M	1	1	37-72	44		47	1			+	1/20	–
42	78	F	1(m)	2	46-92	46		38	6	4		–	1/ 5	+ (T2(m)G2)
43	66	M	1(m)	2	35-72	65		4	7			+	1/ 4	+ (T2(m)G3)
44	77	M	1	2	28-73	53	2	9	5			+	1/62	–
45	86	M	1	2	46-120	70		14	53	2		+	death unrelated to cancer	
46	59	M	1	2	35-72	42		38	2			+	–/40	–
47	72	M	1	2	62-77	72			25			+	chemotherapy	
48	53	M	1	2	35-57	43		13				+	radiotherapy	
49	54	M	1	2	19-69	44	11	38	6			+	death unrelated to cancer	
50	62	F	1	2	44-89	87		2	1	16		+	chemotherapy	
51	86	F	1	2	75-86	80			16	4		+	chemotherapy	
52	70	F	1	2	43-70	44		18	6			+	chemotherapy	
53	74	M	1	2	44-86	47		21		1		–	chemotherapy	
54	81	M	1	2	75-88	84				18		–	chemotherapy	
55	69	F	1(m)	2	23-80	45	4	7	4			–	1/ 4	+ (T3G3)
56	69	M	1	2	30-87	46		32	1	2		+	1/ 8	+ (T2G3M1)
57	73	F	1	3	70-89	73			3	3		+	radiotherapy	
58	68	M	1	3	86-100	90			5			–	radiotherapy	
59	45	M	1	3	65-90	70			5	2		–	–/ 9	–
60	60	M	1	3	43-100	88		1	1	13		–	1/ 3	–
61	65	M	1	3	43-147	146		1	1	1	4	+	1/ 5	–
62	73	M	2	2	43-75	46		27	6			–	–/24	–
63	75	M	2(m)	2	59-93	85			22	28		+	cystectomy	
64	84	M	2	2	30-89	72	1	2	15	2		+	radiotherapy	+ cystectomy
65	54	M	2	3	65-73	72			6	8		+	radiotherapy	+ cystectomy
66	70	M	2	3	53-76	73		2	23			+	radiotherapy	+ cystectomy
67	74	F	2	3	46-110	98		3	1	16	1	–	death unrelated to cancer	
68	82	M	2	3	19-93	58	3	3	3			+	radiotherapy	+ cystectomy
69	53	M	2	3	54-117	60		2	8		3	+	cystectomy	
70	71	F	2	3	42-70	44		5	1			–	radiotherapy	+ cystectomy
71	87	F	2	3	50-220	110				15	10	+	radiotherapy	
72	56	M	2	3	46-90	46/90		3	1	4		–	radiotherapy	+ cystectomy
73	70	F	2(m)	3	36-90	69		17	2	10		+	radiotherapy	
74	76	M	2	3	30-69	45/90		20	–	24		–	death unrelated to cancer	
75	67	F	3	2	53-81	77		2	28	1		+	cystectomy	
76	66	M	3	2	46-100	69		1	3	1		–	radiotherapy	
77	72	M	3(m)	2	40-69	46		4	2			+	cystectomy	
78	78	M	3	2	65-80	73			13			+	radiotherapy	
79	73	M	3	2	42-62	46		9	1			–	radiotherapy	
80	80	M	3	2	40-90	46/90		10		10		–	death unrelated to cancer	

Patient			Tumor		Chromosomes								Follow up	
							Ploidy[a]							
Case	Age	Sex	T	G	Range	Mode	n	2n	3n	4n	5n	Mar	n Recurr.[b] / n Months	Progression
81	69	M	3	2	46-90	46		6		2		−	cystectomy	
82	64	M	3	3	45-75	75		4	6			+	cystectomy	
83	74	M	3(m)	3	62-140	73			37		2	+	radiotherapy	
84	75	M	3	3	90-95	92				5		+	radiotherapy	
85	73	M	3	3	42-90	45		3	2			−	cystectomy	
86	56	M	3(m)	3	64-90	75			5	5		+	radiotherapy	
87	74	F	3	3	50-90	70			4	1		+	radiotherapy	
88	64	M	3	3	19-69	50	1	4	4	13		−	death of cancer	
89	70	M	3(m)	3	73-121	115			3	2	35	+	radiotherapy	
90	60	M	4(m)	2	40-140	43		19	4	1	5	−	radiotherapy	
91	66	M	4	3	65-135	69			13	1	6	−	death of cancer	
92	44	F	4	3	46-120	56		8	3	1		−	radiotherapy	

[a] Ploidy: n = 23± 11; 2n = 46 ± 11; 3n = 69 ± 11 etc.

[b] n Recurr. / n Months : number of recurrences per total number months of follow up.

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Chapter VI

Chromosomal analysis of bladder cancer. II.

A practical method.

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Abstract

Twenty-seven successive bladder cancer tissue specimens varying from non-infiltrating to deeply infiltrating ones, were chromosomally analyzed using a new direct method. Recognizable metaphases were obtained in twenty-five specimens (93%). In nearly all cases suitable C-banding, and in nine cases G-banding was achieved.
Essential steps in this method are colcemid application in two steps, interrupted by washing with Hanks BSS and two separated hypotonic treatments.
This method is easy to use and not time expensive.

Introduction

Chromosome analysis of bladder cancer is often hampered by several of the following factors: small number of recognizable metaphases, lack of spreading and condensed or fuzzy nature of the chromosomes. Successful chromosome analysis, using a direct technique combined with conventional staining appeared possible in about 50% of the tumor specimens [1-3].
This percentage decreased when banding was applied [4,5]. As pointed out in the past, most of the tumor specimens contained metaphases but they could not be analyzed due to the poor morphology. Hence, technical improvements are necessary to obtain recognizable metaphases [2]. Several laboratories introduced methodologic improvements, especially with regard to tumor disaggregation and in vitro culture [6]. In this study a simple direct technique is described which can give recognizable metaphases in nearly all stages and grades of bladder tumors.

Materials and methods

Twenty-seven tissue specimens were obtained by transurethral resection (TURT) or by cold-cup biopsy from 26 successive patients, 25 with primary and one with recurrent tumors. None of these patients had previously been treated with radiation or intravesical chemotherapy. The specimens came from the urological department of one clinic and were taken by two urologists with many years of experience. The tumors varied from non-infiltrating to deeply infiltrating ones (Ta-T3).
The specimens were cut in two parts in the operating room. One part was put in 10 ml 0.5% sodium citrate + 0.5 μg/ml colcemid (Gibco) and the other in 10 ml of Earl's solution (room temperature). The specimens arrived at the cytogenetic laboratory within one hour.

Method I
- The tissue, collected in 0,5% sodium citrate + 0,5% μg/ml colcemid, was cut in some pieces and incubated for 1 hour (room temperature).
- With a pair of tweezers the pieces were put in 5 ml Hanks BSS (calcium and magnesium free) in a Petri dish and incubated for 1 hour (room temperature).
- A solution of 19 ml Hanks BSS and 6 ml colcemid was added, to obtain a final concentration of 2 μg colcemid/ml.
- The tissue was minced immediately in a collector tissue sieve (Bellco Glas Inc. N.Y.) and incubated for 30 minutes at 37°C.
- Centrifugation at 1000 rpm, 10 minutes, and aspiration of the supernatant.
- A prewarmed hypotonic solution of 6 ml foetal calf serum (FCS) (Gibco) and 24 ml 0,052 M Kl was slowly added and incubated for 15 minutes at 37°C.
- Centrifugation at 1000 rpm, 10 minutes, and aspiration of the supernatants.

– 30 ml fixative (acetic acid - methanol 3 : 7) was slowly added, mixed and incubated for 15 minutes.
– Centrifugation and aspiration of the supernatant were repeated several times.
– Ultimately a cell suspension in about 1 ml fixative remained for slide preparation.

Method II The specimens (collected in Earl's solution) were used for chromosomal analysis according to a modification of the direct technique as described by Atkin and Baker [7]. The modification implied the use of a collector tissue sieve.

For slide preparation cool wet slides were used. The preparations were gently flame dried. From each of the methods, initially three slides were made, stained with Giemsa and evaluated blindly, by two persons. After this check, more slides were prepared. In superficial tumors there is often not more than ± 3 mm^3 specimen available so a maximum of about 10 slides could be achieved. CBG-banding [8] was performed in all cases. GTG-banding [9] was done if the following criteria were fulfilled: if the chromosomes were not fuzzy, not overcontracted, not too elongated and when they were well spread. The age of the slides for GTG-banding was about 1 week; banding was performed with 0.1% trypsin (Trypsin 1 : 250, Difco).

Results

With the technique as described under I, recognizable metaphases were present in 25 of the 27 specimens (93%). Furthermore, in one of the remaining two cases metaphases were present, but these were not suitable for cytogenetic investigation (Table 1).
With the technique as described under II recognizable metaphases were present in 14 of the 27 cases (52%). Including the metaphases with insufficient quality for cytogenetic investigation, in 19 of the 27 cases (70%) metaphases were found.
Assuming that the cell suspensions of both methods were roughly equal in density, as seen microscopically, the highest metaphase frequency per slide was nearly always seen with method I. In two cases no quantitative differences were observed. In non-infiltrating tumors 2-5 metaphases per slide were present with method I, compared with 1-2 with method II. In deeply infiltrating tumors these counts were 5-10 and 2-5 respectively.
In general, the chromosomes obtained with method I were better spread and more elongated than with method II. C-banded chromosomes were obtained in 23 cases and G-banded chromosomes in 9 cases. In all of these 9 cases the best G-banded preparations were achieved with method I.
Detailed cytogenetic data are published as part of a larger group in a separate article [10].

Discussion

In a previous study we found recognizable metaphases more frequently in deeply infiltrating tumors than in superficial ones. However, taken together the presence of recognizable and unrecognizable metaphases, regardless of the frequency per slide or per biopsy in superficial and deeply infiltrating tumors, there is no significant difference, as we stated previously [2].
To obtain more recognizable metaphases we focused our attention on the observation that in order to increase the number of metaphases mitotic blocking agents were given

intravenously by some authors, before resection of the tumor [1, 11]. Therefore, the application of colcemid in two steps was introduced. Furthermore, an improvement of the direct technique as described by Atkin and Baker [7] was attempted. We used 0.5% sodium citrate with 0.5 μg/ml colcemid as collecting and transporting medium. The inclusion of colcemid in the sodium citrate solution appeared necessary in our technique, since in the absence of colcemid no recognizable metaphases were found. Before incubation with 2 μg/ml colcemid in Hanks BSS, the cells were incubated in pure Hanks BSS. This appeared to be an essential step.
To receive a cell suspension we preferred mechanical disaggregation of the tumor specimen, than with collagenase many tumor cells appeared to be degenerated [12].
In spite of the fact that with this method, recognizable metaphases were found in 93% of the specimens, G-banding remains problematic, especially in superficial tumors. The frequency of available metaphases per biopsy in non-infiltrating tumors, in general, is lower than in infiltrating ones. Besides the poor quality of the chromosomes is related to a low percentage of G-banding. Ill-defined, fuzzy chromosomes present a major obstacle in banding [13, 14].

This technique, compared with that of Atkin and Baker (method II), in our hands proved its value both with regard to quantity and quality of recognizable metaphases.
In conclusion, there are three essential steps in our technique: colcemid application in two steps, interruption of this treatment with Hanks BSS, and two separated hypotonic treatments. A possible explanation of augmentation of recognizable metaphases by a two-step colcemid treatment may be that a reasonable proportion of cells are arrested in metaphase by the first colcemid action. The colcemid is removed by application of Hanks BSS and a significant number of cells in interphase than enter mitosis. These newly dividing cells will be blocked by the second colcemid treatment. Both rounds of metaphase-arrest act cumulatively and therefore a fair amount of cells in metaphase is obtained. In addition to this quantitative improvement, the quality was significantly increased. Most probably this is due to the two hypotonic treatments. In particular this appeared to be beneficial for solid tumors.

In our opinion this quick and inexpensive method should be recommended since it gives a high yield of usable metaphases.

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Table 1.
Cytogenetical data of the different methods in relation to tumor stage.

tumor	T/G	Method I		Method II		HMF		modal
		a	b	a	b	I	II	number
1.	TaG2	+	+	–	–	+		46
2.	TaG2	+	+	+	–	+		46
3.	TaG2	+	+	+	+	+		46
4.	TaG2	+	+	+	+	+		45
5.	TaG2	+	+	+	+	+	+	78
6.	TaG2	+	+	–	–	+		43
7.	TaG2	+	+	–	–	+		42
8.	TaG1	–	–	–	–	–	–	–
9.	TaG2	+	+	+	+	+		45
10.	T1G2	+	+	+	–	+		72
11.	T1G2	+	+	+	+	+		87
12.	T1G2	+	+	–	–	+		88
13.	T1G2	+	+	+	–	+		43
14.	T1G2	+	–	–	–		–	–
15.	T1G2	+	+	+	–	+		84
16.	T1G2	+	+	+	+	+		44
17.	T1G2	+	+	+	+	+		47
18.	T1G2	+	+	+	+	+		87
19.	T1G2	+	+	+	+	+		80
20.	T1G2	+	+	–	–	+		95
21.	T1G2	+	+	+	–	+		43
22.	T2G2	+	+	+	+	+	+	85
23.	T2G3	+	+	–	–	+		68
24.	T2G3	+	+	+	+	+		61
25.	T3G3	+	+	+	+	+		73
26.	T3G3	+	+	+	+	+		73
27.	T3G3	+	+	+	+	+		115
Total + :	26	25	19	14				

Method I = the method as described in the materials and methods section.
Method II = method of Atkin and Baker [7]
a = recognizable plus unrecognizable metaphases (+ = present).
b = recognizable metaphases.
HMF = Highest Metaphase Frequency per slide.
(In case 5 and 22 no difference in metaphase frequency of method I and II was observed).

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Chapter VII

Chromosomal analysis of bladder cancer. III.

Nonrandom alterations.

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Abstract

Chromosome analysis using G- and C-banding was performed in thirteen primary transitional cell carcinomas of the bladder. The chromosome preparations were obtained by a direct method. In eight tumors with a (near) diploid modal chromosome number the most frequently observed chromosome aberrations were: (partial) monosomy chromosome # 9 in four cases, deletion of chromosome # 10q in two cases and partial trisomy of chromosome # 1 in two cases.
In five tumors with a modal chromosome number in the triploid or tetraploid range the chromosomes # 1, # 3, # 7, # 9, # 11 and # 17 were numerically and or structurally abnormal in at least four cases. In three out of ten males, the Y-chromosome was missing. These findings suggest that the loss of chromosome # 9, and possible also loss of 10q is a primary event in the karyotypic evolution of transitional cell carcinoma of the bladder.

Introduction

Chromosome analysis of transitional cell carcinoma (TCC) of the urinary bladder has revealed the involvement of a number of chromosomes. In one study, the presence of an isochromosome i(5p), an isochromosome i(8q), deletion and translocation of chromosome # 8, monosomy # 9 and interstitial deletion of # 13 were noticed [1].
Another study reported the involvement of chromosomes # 1 and # 11 and to a lesser degree chromosomes # 3 and # 17 [2]. Also alterations of chromosomes # 6, and # 13 have been published as non-random abnormalities [3]. Recently deletions of 10q24 and 21q22, trisomy # 7 and aberrations of # 9, # 13, # 15 and # 20 were added to the list of alterations [4].
Hitherto, some dozens of cases are described with successful banding of the tumor chromosomes
After the introduction of a new technique in our laboratory, G-banded chromosomes could be obtained much more frequently during the past [5]. In this report the chromosomal findings of 9 cases, studied with this method, are combined with the results of four patients studied at an earlier date.

Materials and methods

The tumor samples were collected and transported in 0.5% sodium citrate + 0.5 μg colcemid/ml and prepared as described elsewhere [5].
All tumors were primary tumors. None of the patients, before first resection, received chemotherapy or radiation therapy. Two of them recurred. Further data on the 13 cases are shown in Table 1.
Clinical staging of the tumors was done according to the rules of the Union International Contre le Cancer [6] and grading according to the WHO system [7]. In addition, intermediate tumors were divided in G2a and G2b. The latter is less differentiated than the former [8].
In addition to the cells with G- and C-banded chromosomes, a number of unbanded metaphases could be analyzed. Therefore the chromosomes of at least 15 metaphases of every tumor were counted in order to increase the reliability of the chromosome number.

Results

The tumors fell into two groups: those with near diploid modal chromosome numbers (cases 1-8) and those with near tri- or tetraploid modal numbers.

Case 1.: Mode 46. Range 43-90.
Two G-banded and two C-banded metaphases with 46 chromosomes were analyzed. A 10q- [del(10)(q22)] was present. There were some (near) tetraploid cells, which were unsuitable for chromosomal analysis.
The modal karyotype was: 46,XY, del(10)(q22).

Case 2.: Mode 46.
All cells counted showed 46 chromosomes.
Two G-banded and two C-banded metaphases were analyzed. One chromosome # 9 showed a deletion of the long arm 9q-[del(9)(q22?)]. One chromosome # 12 was missing and one marker chromosome, probably derived from a chromosome # 12, was present.
The modal karyotype was: 46, XY, 9q-, -12, + mar.

Case 3.: Mode 45. Range 44-45.
Five G-banded and two C-banded metaphases were analyzed. Consistent features were monosomy # 9 and 10q-[del(10)(q22)].
The modal karyotype was: 45,XY, -9, del(10)(q22).

Case 4.: Mode 47.
All cells, except one, had 47 chromosomes.
Seven G-banded and two C-banded metaphases were analyzed. Consistent features were 2q-, 9q+, +i(8q). Furthermore, the short arm of one chromosome # 8 was probably abnormal.
The single diploid cell showed no karyotypic abnormalities.
The modal karyotype was: 47,XY, 2q-, +i(8q), 9q+.

Case 5.: Mode 46. Range 42-46.
Seven G-banded and two C-banded metaphases were analyzed. Consistent features were an extra chromosome 1p-[del(1)(p22)] and a missing Y-chromosome.
The modal karyotype was: 46,X, -Y, +1p-.

Case 6.: Mode 44. Range 41-72.
Three G-banded and two C-banded metaphases were analyzed. Missing were one copy of # 9, # 11, # 14 and the Y-chromosome.
Two structurally abnormal chromosomes were present: 1p-[probably del(1)(p13)] and an 11p+ chromosome.
There were some (near) triploid cells, which were unsuitable for chromosomal analysis.
One diploid cell showed no karyotypic abnormalities.
Eleven months after the first resection a biopsy specimen of a recurrent tumor was obtained which showed the same modal karyotype.
The modal karyotype of both resections was: 44,X, -Y, +1p-, -9, 11p+, -14.

Case 7.: Mode 47. Range 44-86.
Three G-banded and two C-banded metaphases were analyzed. In all cells examined trisomy # 20 was present.
Six months after the first resection, a biopsy specimen of a recurrent tumor at the primary site was obtained which showed the same karyotype.
There were a few near tetraploid cells, which were unsuitable for chromosomal analysis.
The modal karyotype of both resections was: 47,XY, + 20.

Case 8.: Mode 42. Range 41-86.
Ten G-banded and two C-banded metaphases were analyzed. Missing chromosomes were one copy of # 3, # 6, # 7, # 17, one X-chromosome and both copies of # 9. A dicentric translocation, tdic (7;9)(p11;p11)[7qter→p11:: 9p11→qter] and two marker chromosomes were present. Mar 1, a large marker, was composed of most of a chromosome # 3 and the short arm (including the centromere) of # 6. Between these parts a homogeneously staining region (HSR) was present. With C-banding the presence of the two centromeres was demonstrated. The composition of this marker is probably: 3qter→p13;HSR;6q13→pter. Mar 2 is a derivative 9 chromosome and is probably a t(9;17)(q34;q21?) [9pter→ q34::17q21? → qter?].
A cell with 57 chromosomes was also fully karyotyped. The HSR containing marker and two of the dicentric translocation chromosomes as described were present. Besides, a small metacentric marker chromosome was seen.
In the metaphases with about 85 chromosomes, present in 8 out of 60 cells, two HSR containing markers were present. Further karyotyping was impossible.
The modal karyotype was: 42,X, -X, -3, -6, -7, -9, -9, + t(7;9), -17, + mar 1, + mar 2.

Case 9.: Mode 60. Range 52-63.
Six G-banded and two C-banded metaphases were analyzed. Consistent features were: a translocation t(1;6)(p12;p12) [6pter--->p12::1p12 --->1qter], del 2(q12), 4p+, del 9(q13), 11p+, 12p+, two abnormal derivative 13 and 14 chromosomes, 17q+, 19q+, 20q+ and three different markers. Chromosomes # 1, # 3, # 7, and # 17 were present in triplicate. Chromosomes # 2, # 10, # 12, # 14, were present in single copies. No normal # 13 and # 14 were seen.
The modal karyotype was most probably: 60,XY,+X, + 1, + t(1;6) (p12;p12), 2q-, +3, +4q+, +7, +9q-, -10, +11p+, 12p+, 13p+, 13p+, 14p+, 14p+, +17, +17q+, +19q+, +20q+, + mar 1, + mar 2, + mar 3.

Case 10.: Mode 83. Range 64-85.
Four G-banded and two C-banded metaphases were analyzed. Consistent features were 5 structurally abnormal chromosomes, three of which could be identified to some extent: 12q+, 6q-, 21p+?. The remaining two were qualified as markers.
Therefore, the modal karyotype was most probably: 83,XX,+X, +1, +2, +3, +3, +4, +5, +6, +6q-, +7, +8, +8, +9, +10, +10, +11, + 11, +12, +12q+, +13, +14, +15, +16, +17, +17, +18, +19, +19, +20, +20, +21, +21, +21p+?, +22, +22, + mar 1, + mar 2.

Case 11.: Mode 77. Range 46-87.
Four G-banded and two C-banded metaphases were analyzed. Consistent features were: an extra 1q- [del (1)(q12?)], a large 11p+ chromosome composed of most of chromosome # 11 and the long arm of # 1, including the centromere. Between these two chromosome parts, a HSR was present. The probable composition of this marker was: 11qter→p14?;HSR;1p12?→ 1qter.
Eleven markers varying in length from the A- to G-group were present.
Most chromosomes were present in triplicate, except for # 2, # 4, # 8, # 10, which were normal, and # 22 showing four copies.
The modal karyotype was most probably: 77,XX, +1, +1q-, +3, +5?, +6, +7, +9, +11p+, +12, +13, +14, +15, +16, +17, +18, +19, +20, +21, +22, + 22, + mar 1, + mar 2, + mar 3, + mar 4, + mar 5, + mar 6, + mar 7, + mar 8, + mar 9, + mar 10 + mar 11.

Case 12.: Mode 71. Range 53-76.
Two metaphases were completely and three partially karyotyped after G-banding. Two C-banded cells were available. Striking features were: 1q-(q22), 2q-(q14), 5p+, 8p+, 10p+, 12p+.
Fourteen marker chromosomes, varying in length from the A- to G-group were present.
The modal karyotype was most probably: 71,XY, +Xq+, +1, +1q-, +2q-, +3, -4, 5p+, +7, 8p+, +10p+, +11, 12p+, +13?, +17, +17, +21, + mar 1, + mar 2, + mar 3, + mar 4, + mar 5, + mar 6, + mar 7, + mar 8, + mar 9, + mar 10, + mar 11, + mar 12, + mar 13 + mar 14.

Case 13.: Mode 73. Range 63-79.
Five G-banded and two C-banded metaphases were analyzed. Consistent features were two, and in some cells three, 9q+ chromosomes. It appeared that these derivative chromosomes showed two centromeres, one of which was suppressed [psu dic (9)]. An isochromosome i(2q) and two markers were present.
The modal karyotype was most probably: 73,X,+X,-Y, +1, +1, -2, +i(2q), +6, +6q-, +7, +8, +8, +psu dic (9), +psu dic (9), +10, +11, +11, +12, +12, +13, +13, +14, +14, +15, + 15, +17, +18, +18, +19, +20, +mar 1, +mar 2.

Discussion

As reported in a previous study, non-infiltrating bladder tumors mostly have a modal chromosome number and a chromosomal range in the near diploid region, whereas infiltrating cancers mostly show hyperdiploidy [9].

In this study representative karyotypes of the modal chromosome numbers or 13 G-banded tumors are given.

In three (cases 5, 6 and 13) of the ten males the Y chromosome was missing. In bone marrow this is frequently observed and could be an age dependent phenomenon [10]. The ages of our three patients where 54, 73 and 90 years, respectively. In three of four primary gastric cancers, recently published, the Y chromosome was missing. The age of these patients was 27, 68, 54 years, respectively, whereas in the fourth patient, age 74 the Y

chromosome was present [11]. Our data suggest that the loss of the Y chromosome is not merely related to age.
The autosomes most frequently involved in structural and/or numerical aberrations are in numerical sequence:
Chromosome # 1: This chromosome was involved in structural rearrangements in 6 cases (Table 2) (Fig. 1). Also, in published series of bladder cancer, chromosome # 1 frequently underwent structural changes [2, 3, 12-14]. Furthermore, abnormalities of # 1 are reported as occurring in various other types of human neoplasia [15-19]. However, there is much variation in the position of the breakpoints. The eleven recognized breakpoints in the cases of bladder cancer, cited above and in our cases, are between 1p22 and 1q25, i.e. in the proximal regions of the chromosome arms, consisting of approximately of half of the entire chromosome.

Chromosome # 2 showed structural changes in four cases: in three a deletion of part of the q-arm and in one an i(2q) were observed. The involvement of chromosome # 2 is not striking in the reports of previous authors dealing with bladder tumors. On the other hand in malignant melanoma structural aberrations of # 2 are frequent [19].

Chromosome # 3 was involved in a structural change in one case and in four other cases it was present in triplicate.

Chromosome # 6 was frequently involved. In case 9 a translocation, t(1;6) (p12;p12), and in case 8 a translocation t(3;?;6)(p13;HSR;q13), were seen. Trisomy 6 was present in four near triploid cases. In the study of Gibas et al. [3] chromosome # 6 was affected in five of the nine tumors, mostly as a deletion of the long arm. Deletions of the long arm of chromosome # 6 are often found in malignant disorders [20]. Involvement of chromosome # 6 in structural or numerical alterations of the karyotype, was accompanied by other chromosomal changes in all cases including ours. Thus we agree with other authors [3, 21]. that abnormalities of chromosome # 6 most likely are secondary changes.

Chromosome # 7 was involved in structural rearrangements in one case and in the five near triploid tumors as an additional chromosome. Trisomy # 7 has been reported as a possible primary change in TCC of the ureter [4]. In bladder cancer this change was present in two near triploid cases of Atkin and Baker [2] and in one near diploid and one near triploid case of Gibas et al. [1, 3]. Because trisomy # 7 was absent in our near diploid cases and absent in all (except one) near diploid bladder tumors of the studies cited above, we tend to consider trisomy # 7 as a secondary change.

Chromosome # 8 was involved in structural changes in two cases. An isochromosome i(8q), as in our case 4, has been reported before by Gibas et al. [1, 3] in two cases.

Chromosome # 9 was involved in five of the eight near diploid cases: (9q-; -9; 9q+; -9; -9, -9, +t(7;9) respectively). Atkin and Baker [2] found a missing # 9 in three of six near diploid bladder tumors and in one of four strongly hyperdiploid ones.
Gibas et al. [1] reported monosomy # 9 in three of six near diploid tumors and in one of three near tetraploid cases. In the following study of this authors [3] monosomy # 9 has been reported in one of four near diploid cases.
Berger et al. [4] found a missing # 9 in two of six near diploid cases. The case described by Kovacs [13] and the diploid case examined by Vanni et al. [22] also showed a missing # 9. Taken together, the cited studies and our data, in 14 of 32 near diploid bladder tumors

chromosome # 9 was missing and in two other cases a structural aberration was recognized (Table 3). It is more and more likely that loss of chromosome # 9 is a primary change in bladder tumor.

In two cases a deletion of the long arm of # 10, breakpoint at q22, was present. This is very interesting since Gibas et al. [1] and Berger et al. [4] each reported a patient with a deletion of 10q(24). The deletion in one of our cases was the sole abnormality, in the other it was accompanied by monosomy # 9.
Deletion of # 10 could be a primary change, since it was found in two near diploid tumors.

Chromosome # 11 was involved at least three times in structural rearrangements; in one case with a near diploid chromosome count and in two near triploid cases. Thus, it appears to us, that chromosome # 11 is preferentially associated with invasiveness and that it is not involved in the initial steps of carcinogenesis of the bladder. Atkin and Baker [2] reported frequent structural changes of # 11, including short arm deletions. This could be of interest since the oncogene c-Ha-ras 1 is located on 11p14 [23]. In our cases we were unable to recognize the breakpoint in # 11 rearrangements.
Deletions of 11p are well known to occur in tumor cells of patients with Wilms' tumor [24].
Two of our cases (cases 8 and 11) contained homogeneously staining regions (HSR). This phenomenon has been described in a variety of tumors and is related to gene amplification [25, 26]. It may be a reaction of exposure to drugs [27]. However, our cases were primary tumors and the patients did not receive chemotherapy before resection of the tumors mentioned.
Atkin and Baker [2], using a direct technique, revealed involvement of chromosome # 11 as structural change in all six near diploid cases. In the eight cases examined by Berger et al. [4], data obtained after enzymatical disaggregation of the specimens and culturing the malignant cells, no involvement of # 11 was seen. Our results show an abnormal chromosome # 11 in one of the near diploid tumors.
The simultaneous application of both methods, to more tumors is necessary to draw conclusions about the significance of chromosome # 11 in bladder carcinogenesis.

Chromosome # 17 had undergone structural aberrations in two cases and was present in three or more copies in all near triploid cases. The involvement of this chromosome has been reported before [2].

In one case an additional chromosome # 20 was seen as a sole abnormality. In a resection specimen obtained six months after the first, the same karyotype was present. Atkin and Baker [2] reported on the presence of one or more extra # 20, together with other chromosomal changes. Berger et al. [4] reported this in two bladder tumors, but an extra # 20 was even more frequently found in renal tumors. They interpreted trisomy # 20 as a possible secondary change.
Our findings confirm those of others in some cases, but it is also clear that differences were observed. The most obvious example is the absence of i(5p). This isochromosome was reported by Gibas et al. [1, 3] in 7 out of 16 tumors. It cannot be established with certainty that i(5p) was missing in our study. For instance, in case 8 a small metacentric chromosome was present which resembles i(5p) or a deletion of 5q at q13. However, both copies of chromosome # 5 present were normal.

In some cases normal diploid cells were present. Presumably, they represent normal stromal, epithelial or muscle cells.

The number of translocations found in this study was low. Furthermore, different breakpoints in different chromosomes were involved. Therefore, specific translocations were not observed.
Reciprocal translocations characterise several forms of leukemia and lymphoma and contribute to the process of neoplastic transformation by activating oncogenes situated close to the breakpoints [21, 28, 29]. Solid tumors in contrast, generally present unbalanced chromosomal changes, rather than reciprocal translocations. This results in duplication or loss of (parts of) chromosomes [30]. This mechanism is relevant for the acquisition of cancer genes in hemizygous form. As suggested by Knudson [31] invisible mutations - which can lead to homozygoty for recessive cancer genes - or loss of genetic material by deletion of (a part of) the chromosomes - which leads to hemizygoty of the recessive cancer gene - can produce cancer, as demonstrated in Wilms' tumor and retinoblastoma. Further support for Knudson's model has been obtained at the DNA level [32].

To find chromosomal changes which are specific or directly related to the origin of the tumors, near diploid tumors are needed, with preferably a few or ideally one chromosome abnormality. In spite of the problem of many chromosome abnormalities in poorly differentiated tumors, it is worthwhile to examine these tumors as well, since possible specific rearrangements could be found, which are indicators for aggressiveness of the tumor [28].
To establish the primary chromosome change, direct cytogenetic examination offers the best chance of success, in spite of the fact that after culturing the cells, banding of the chromosomes is better. Chromosomal changes in vitro and growth advantage of a special cell line include the risk of contradictory data.
This study stresses loss of chromosome # 9 as a probable primary change. Deletion of chromosome 10q at band q22 is possibly another primary change.
If more well differentiated transitional cell carcinomas of the bladder are chromosomally analyzed, it might be possible to divide bladder cancer in subgroups analogous to the situations in the acute leukemias.
Prospectives in the field of therapeutic approaches and clinical behavior of the tumors also may be improved.

Table 1.

Summary of the clinical data, range and modal chromosome number.

case	age	sex	stage/grade	chromosomes range	chromosomes m.n.
1.	74	M	TaG2b	43-90	46
2.	64	M	TaG2a	46	46
3.	80	M	TaG2a	44-45	45
4.	33	M	TaG2b	47	47
5.	73	M	Ta(m)G2a	42-46	46
6A.	54	M	T1(m)G2a	41-72	44
B.	55	M	TaG2b	41-75	44
7A.	74	M	T1G2b	44-86	47
B.	74	M	T1G2b	45-83	47
8.	87	F	T2G2b	41-86	42
9.	74	M	T2G3	52-63	60
10.	79	F	T2G2b	64-120	83
11.	67	F	T3G3	46-87	77
12.	69	M	T3G3	53-76	71
13.	90	M	T3G3	63-79	73

m.n. = modal number of chromosomes.
(m) = multiple tumors

Table 2.

Summary of the most frequently involved chromosomes and their abnormalities.

case	m.n.	1	3	7	9	10	11	17
1.	46					10q−		
2.	46				9q−			
3.	45				−9	10q−		
4.	47				9q+			
5.	46	+1p−						
6.	44	+1p−			−9		11p+	
7.	47							
8.	42		−3	−7	−9			
			t(3;H;6)	t(7;9)	−9			
					t(9;17)			−17
9.	60	+t(1;6)	+3	+7	+9q−	−10	+11p+	+17
		+						+17q+
10.	83	+1	+3	+7	+9	+10	+11	+17
			+3			+10	+11	+17
11.	77	+1q−	+3	+7	+9		+t(11;H;1)	+17
		+1						
12.	71	+1q−	+3	+7		+10p+	+11	+17
		+1						+17
13.	73	+1		+7	+9q+	+10	+11	+17
		+1			+9q+		+11	

Table 3.

Summary of the near diploid tumors and the most frequently involved chromosomes.

A. Culture method:

author(s)	all tum.	dipl. tum.	case	m.n.	chromosome # involved 1	5	6	8	9	10	11
Gibas '84	9	6	1	48		+i(5p)		i(8q)			
			2	45	p+		q–		–9	p+	–11
			3	44				p–		q+	
			5	47					–9		
			6	47		+i(5p)	q–			q–	
			7	45					–9		
Gibas '86	7	4	1	45	q+				–9		
			2	47							
			3	43	p+;q–	i(5p)	q–				p–;p+
			5	48		q–	t(6;X)				t(11;13) q–
Berger '86	8	6	1	46				p–	–9;i(9q)		
			2	45							
			3	46							
			4	46						q–	
			6	46					–9		
			7	47							

B. Direct method:

author(s)	all tum.	dipl. tum.	case	m.n.	chromosome # involved 1	5	6	8	9	10	11
Vanni '85	2	1	1	49	q–				–9		p+
Kovacs '85	1	1	1	46	+p–				–9		
Atkin '85	10	6	1	42/3							i(11q)
			2	42/5	+p–		+6		–9		t(11;14)
			3	44/5	q+		q–				–p
			4	46				–8	–9	–10	q+
			5	46	+i(1q)			p–	–9	–10	p–
			6	46	q+						r(11)
This study	13	8	1	46						q–	
			2	46					q–		
			3	45					–9	q–	
			4	47				i(8q)	q+		
			5	46	+p–						
			6	44	+p–				–9		p+
			7	47							
			8	42			–6 t(3;H;6)		–9;-9; t(7;9) t(9;17)		

All tum. : patients examined; near diploid and strongly hyperdiploid tumors.
dipl. tum.: number of tumors with a near diploid chromosome number.
m.n. : modal number.

	chromosome # 1	chromosome # 9	chrom # 10
Case 1			10 10 q-
Case 2		9 9 q-	
Case 3			10 10 q-
Case 4		9 9 q+	
Case 5	1 1 p-		
Case 6	1 1 p-		

Figure 1
Structural abnormal G-banded chromosomes derived from # 1, # 9, # 10 and two HSR containing chromosomes. In a number of cases the C-banded chromosomes are placed on the right side.

	chromosome # 1	chromosome # 9	HSR
Case 8		7 t (7;9) t (9;17)	6 3 t (3;H;6)
Case 9	6 1 t (1;6)	9 9 q-	
Case 11	1 1 q-		1 11 t (11;H;1)
Case 12	1 1 q-		
Case 13		9 9q+ 9q+	

Structural abnormal G-banded chromosomes derived from # 1, # 9, # 10 and two HSR containing chromosomes. In a number of cases the C-banded chromosomes are placed on the right side.

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Chapter VIII

General discussion

The aim of the present study was to obtain more information about the biology of bladder cancer on the basis of chromosomal analysis and DNA estimations.

Improvements of chromosomal preparations.

For chromosomal analysis, different steps in the preparative procedure leading to metaphase spreads were tested and improved. Three techniques for cytogenetic examination of neoplastic tissues can be distinguisted: direct technique [6, 19, 26]; short term culture [18, 23] and long term culture [9]. Each of these techniques has a variety of modifications, developed in a period of years. In the initial phase of our study all three techniques were applied showing that in 42% of all tumors examined recognizable chromosomes could be obtained. In superficial tumors this was the case in 37%, while in deeply infiltrating lesions 62% of the samples gave recognizable chromosomes. If all metaphases were considered, irrespective of the quality, in 52% to 94% of the specimens, depending on the technique applied, chromosomes were found. When the direct technique and the short term culture of the same tumor specimens were compared, more metaphases were found with the latter technique. However, despite the higher metaphase frequency after short term culture, we prefer the direct method, since, the risk of chromosomal changes and selection of certain cell types during culture is present [11, 14, 30].
A requirement for the direct method is the preparation of a single cell suspension, for which currently two methods are in use, i.e. mechanical disaggregation [1, 14, 29] and enzymatical treatment, with for instance collagenase II [3, 30]. Wake et al. [30] found that the use of the latter method yielded more metaphases after culturing the cells for at least 60 hrs compared with mechanical treatment of the tissue. However, no bladder tissue was used in their study. Our results with bladder tumor cells and a culture time between 24 - 48 hrs were quite the opposite. Besides, after examination for cell morphology, it appeared that the enzymatic method had a degenerative effect on the tumor cells.
On the first international workshop on chromosomes in solid tumors [27] , it appeared that most of the laboratories are using enzymatic tissue disaggregation and afterwards cell culture. However, none of the techniques presented there was completely successful in obtaining analyzable chromosomes. The general consensus of the workshop was that both direct and short term culture must be used.
Owing to technical improvements, at present 93% of the tumor specimens received, show recognizable and countable chromosomes with our direct technique. Three conditions appear essential to obtain this result: colcemid application in two steps, interruption of colcemid treatment with Hanks BSS solution and two separated hypotonic treatments. Nevertheless, G-banding, or another banding technique with adequate resolution remains a problem. As stated by Trent [27] all workers in the field of cytogenetics have this problem.

The relation between aneuploidy and tumor behavior.

The 92 newly diagnosed tumor specimens examined between 1980 - 1986, which yielded recognizable chromosomes, were related to the stage and the grade of the tumor. Of the non-invasive (Ta) tumors, 88% had a modal number of ≤ 49 chromosomes. In T1 tumors this figure was 43% and in T2-T4 tumors only 19%.
Taking the chromosomal range as criterion it appeared that all infiltrating (T1-T4) tumors showed cells with more than 49 chromosomes, compared to 22% of the non-infiltrating tumors. These differences in chromosome number and chromosome range between non-invasive and invasive tumors were significant. Of the grade 1 tumors 97% had a modal chromosome number ≤ 49, compared to 49% of the G2 and 8% of the G3 tumors. The differences in chromosome number and chromosome range between G1 tumors on the one hand and G2 plus G3 tumors on the other hand were also significant. Besides, significantly more G3 tumors showed a modal number > 49 as compared to G2-lesions. Tumors with a chromosomal range of ≤ 49 recur much later than tumors with > 49 chromosomes. Progression occurs significantly more frequently in tumors with a chromosome range > 49.
In previous examinations it was found that in high graded bladder tumors much more chromosomes per cell were present as compared to low graded tumors [26]. In invasive tumors the modal chromosome number varied from hypodiploid to hypertetraploid and frequently these tumors contained marker chromosomes [7, 24].

Marker chromosomes.

Some authors [7, 24] have concluded that, if marker chromosomes are present in non-invasive cancers, recurrence will take place in 90%. When marker chromosomes are absent recurrence will take place in less than 5%. The 5-years survival rate was 40% when markers were present, as compared to 95% when markers were absent in non-invasive cancers [24]. It was proposed that the triad markers, tetraploidy and submucosal invasion in moderately well differentiated tumors appeared to carry such a poor prognosis that radical resection of the bladder was indicated [7].
However, recurrence was not limited to tumors with markers or aneuploidy [5]. Granberg-Öhman [14] postulated that the occurrence of marker chromosomes is related to the histopathological pattern. In our study, the predicting value of marker chromosomes as established by routine techniques, appeared not to be better than the predicting value based on chromosome numbers.
It is my opinion, that the significance of markers has been exaggerated in the past. Only one of the thirteen cases examined for this thesis did not show a structural abnormal banded chromosome. The other cases showed at least one, but sometimes many abnormal chromosomes, which without banding would have been called marker chromosomes, if recognized at all.
The low frequency of abnormal chromosomes in cells of well differentiated tumors as reported in the past may be explained by technical shortcomings of chromosome identification.
Possibly primary changes related with bladder cancer are a loss of chromosome # 9 and deletion 10q22 [25], and isochromosome i(5p) as mentioned by Gibas [11, 12]. Other frequently involved chromosomes in near diploid bladder cancers are # 11 [1] and # 1 [17, 29]. It is striking that, unlike the situation in several forms of leukemia and

lymphoma, in bladder cancers no consistent reciprocal translocations are found. The loss or duplication of (parts of) chromosomes is probably relevant for the acquisition of homo- or hemizygoty for recessive cancer genes, as suggested by Knudson [15].

Flow cytometry.

Flow cytometric DNA estimations are regarded as an important method for examination of bladder cancer [13]. The mass of DNA can be used as an additional parameter in patient treatment [28, 31]. To obtain single cell suspensions, mechanical and enzymatical disaggregation of the tissue were compared with respect to DNA index and percentage of diploid cells in the resulting suspensions. Few studies comparing different disaggregation techniques and their usefulnes for FCM are available [4, 10]. After mechanical treatment, all the cases were suitable for DNA estimation, compared to 88% of the enzymatically disaggregated tumors (collagenase II). The DI after mechanical and enzymatical disaggregation was identical. More diploid cells were present in the sample after enzymatical treatment, possibly due to release of stromal cells.
For discriminating epithelial from non-epithelial cells we applied antibodies to cytokeratin in order to increase the discriminating power of flow cytometry [8, 22]. Thus, the percentage of epithelial cells in S- and G_2M phase could be estimated more accurately. Although labeling with antibodies did increase the sensitivity of the FCM technique in detecting tumors with an abnormal DNA mass or abnormal proliferative fractions, the discriminating power of chromosomal analysis appeared to be greater than that of FCM. Tumors with a near diploid chromosome count, translocations, or small aberrations, which do not change the mass of DNA rigorously, can not be recognized by flow cytometry.

Application of chromosomal analysis in daily urological practise.

When the aim of chromosomal analysis of bladder cancer in daily practise is to determine the modal chromosome number and range, these data can be obtained in about 90% of all specimens. When the aim is to karyotype the tumor specimen after adequate chromosomal banding, this can be achieved in only 30% of the cases
In well- and moderately well differentiated superficial tumors chromosomal data are informative with respect to tumor behavior. These chromosome data show that tumors having a chromosome range ≤ 49, recur later than tumors with > 49 chromosomes. These latter tumors more often show progression. This could imply that after endo-resection of the tumors with ≤ 49 chromosomes, chemotherapy could be omitted and that the patient could be checked after a longer interval. Tumors having > 49 chromosomes need extra attention of the clinician. In classifying tumors there is a discrepancy in grading among pathologists [16, 20, 21]. Chromosome numbers as counted and photographed are objective criterions. When a discrepancy in chromosomal count and histopathological data is found, reexamination of the tissue slide is recommended.

Prospectives of future studies.

The sensitivity of flow cytometry can be increased by application of monoclonal antibodies and other improvements to achieve reliable DNA estimations and proliferative data of the tumor cells. Patients with superficial bladder cancers must be followed over years and the clinical parameters such as multiplicity, recurrence rate and progression should be

correlated with flow cytometric data. Combination of these data with clinical behavior could result in a malignancy index which may be helpful to obtain a correct prognosis [2]. In search of primary changes of the chromosomes only some dozens of bladder cancers are examined after adequate chromosome banding so far. Already this small number gave indications that some chromosomal aberrations are primary changes. To get certainty, it is necessary to augment the number of cases analyzed. Besides, this gives the possibility to divide bladder cancers in subgroups, depending on their chromosomal abnormalities. In future studies, chromosomal aberrations have to be correlated with the clinical follow-up of the individual patients with the aim the ultimate use of such chromosomal changes as prognostic factors.

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Chapter IX

Summary

Transitional Cell Carcinoma (TCC) of the urinary bladder has an unpredictable natural history with respect to recurrence and progression. In the daily urological practice the most important diagnostic and prognostic factors of this cancer are its stage and grade as established by histopathological examinations. More information, especially about the prognosis, is required. Chromosomal analysis and DNA-flow cytometry are considered as important methods for a further elucidation of the biological properties of bladder cancer. In this study investigations on chromosomal changes and DNA content of bladder tumor cells are described. The first goal was to develop a useful technique to obtain recognizable chromosomes from as many tumor specimens as possible. Initially in only 42% of the tumor specimens examined, chromosomes were recognized, mainly after short term culture. A more reliable technique is described in this thesis resulting in recognizable chromosomes in 93% of the tumors.

To obtain single cell suspensions from the solid tumor specimens, for flow cytometry as well as for chromosomal analysis, mechanical treatment appeared better than enzymatical disaggregation.

The chromosomal data were correlated with the histopathological classification of the tumor and with the clinical course of the patient. In non-invasive tumors (Ta) almost exclusively cells in the near diploid region (≤ 49 chromosomes) were found, in contrast to invasive (T1-T4) lesions, which showed strongly hyperdiploid chromosome numbers (> 49). In well differentiated tumors (G1) the vast majority of cells was in the near diploid region, and in poorly differentiated (G3) tumors in the strongly hyperdiploid region. In intermediate tumors (G2) both groups were present. Near diploid tumors recur much later than the hyperdiploid cases. Progression of the tumor is observed significantly more often in tumors with hyperdiploid cells than in near diploid lesions.

The discriminating power of flow cytometry after labeling the cells with antibodies to cytokeratin was better than without labeling. After use of these antibodies the percentage of cells in the different phases of the cell cycle could be estimated reliably. However, the tumors with an abnormal karyotype but with a chromosome number in the near diploid range, could not be discriminated by flow cytometric estimations.

Non-random chromosome aberrations were established after banding the chromosomes of the tumor cells. The loss of chromosome #9 and deletion of a part of chromosome #10 seem primary alterations in the process of bladder oncogenesis. Aberrations in number or structure of the chromosomes #1, #7, #11 and #17 are possibly secondary steps in the development of bladder cancer.

Samenvatting

Zowel het recidiveren als de progressie in groei van kanker van het overgangsepitheel van de urineblaas is onvoorspelbaar. In de urologische praktijk worden tumorstadium en tumorgraad, zoals vastgesteld door histopathologisch onderzoek, als belangrijkste diagnostische en prognostische factoren gehanteerd. Met name met het oog op verbetering van de prognose is het verwerven van meer gegevens van groot belang. In dit verband moeten chromosomenonderzoek en DNA-doorstroomcytometrie worden genoemd, als belangrijke methoden om tot opheldering van de biologische eigenschappen van blaaskanker te komen. In deze studie worden de gegevens, verkregen uit chromosomenonderzoek en meting van het DNA-gehalte van de tumorcel, beschreven. Eerste doelstelling was het ontwikkelen van een bruikbare techniek om chromosomen van zoveel mogelijk blaastumor-biopsieen te kunnen bestuderen.
In het begin van het onderzoek konden, voornamelijk na kortdurende celkweek, in 42% van de tumoren beoordeelbare chromosomen worden verkregen. Een later ontwikkelde en meer betrouwbare methode, eveneens in deze studie beschreven, leverde in 93% van de tumoren beoordeelbare chromosomen op. Verder bleken voor gebruik in doorstroomcytometrie en chromosomenonderzoek tumoren die mechanisch tot celsuspensies waren bewerkt geschikter dan enzymatisch verkregen celsuspensies.
De gegevens, verkregen uit chromosomenonderzoek, werden gecorreleerd aan gegevens over het tumorstadium en de tumorgraad, alsmede met gegevens over het klinisch verloop van de tumor. In niet-infiltrerende tumoren (Ta) werden voornamelijk cellen met een nagenoeg diploid chromosomenaantal (≤ 49 chromosomen) aangetroffen. Daarentegen werden in infiltrerende tumoren (T1-T4) voornamelijk hyperdiploide chromosomenaantallen (> 49) aangetroffen.
In goed gedifferentieerde tumoren (G1) waren bijna alle cellen nagenoeg diploid (≤ 49), terwijl deze in slecht gedifferentieerde tumoren (G3) bijna allen hyperdiploid (> 49) waren. In de groep der G2-tumoren, waren zowel diploide als hyperdiploide chromosomenaantallen aanwezig. Vervolgens bleek dat tumoren met een nagenoeg diploid chromosomenaantal veel later recidiveren dan tumoren die hyperdiploid zijn. Progressie komt significant vaker voor bij hyperdiploide dan bij nagenoeg diploide tumoren.
Tijdens het onderzoek bleek ook dat het oplossend vermogen van doorstroomcytometrie verbeterde als aan de te onderzoeken tumorcellen anti-lichamen tegen cytokeratine werden gehecht. Bovendien kon door aanhechting van deze anti-lichamen het percentage cellen in de onderscheiden fasen van de celcyclus betrouwbaar vastgesteld worden. Helaas konden tumorcellen met een abnormaal karyotype, maar met een (nagenoeg) diploid chromosomenaantal, met behulp van doorstroomcytometrie niet van normale cellen onderscheiden worden.
Door bandering konden non-random chromosomenafwijkingen in de tumorcellen worden vastgesteld. Het verlies van chromosoom nummer 9 en het verlies van een deel van chromosoom nummer 10 lijken primaire chromosomenafwijkingen in de ontwikkeling van de blaastumor. Afwijkingen in aantal of structuur van de chromosomen nummers 1, 7, 11 en 17 zijn mogelijk secundaire gebeurtenissen in de ontwikkeling van kanker van de urineblaas.

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Curriculum vitae

The author of this thesis was born in Limbricht, the Netherlands on January 30th 1941.
He attended High School and afterwards the Agriculture College in Roermond where he took his finals in 1960.
He started studying Biology at Nijmegen University and graduated as a biologist in 1966.
In 1965 he started teaching biology, first in Nijmegen and from 1966 onwards at the Grammar School "Jerusalem" Venray.
From 1967 he combined his teaching profession with work as a cytogeneticist with the "Stichting Ziekenhuisapotheek en Klinisch Laboratorium Venray".
In 1971 he continued his studies and graduated at Utrecht University in 1973. His main subject was genetics and his minors were general botany and endocrinology.
In 1980, in combination with his activities as mentioned before, he started working on the present thesis in the laboratories of the "Stichting Ziekenhuisapotheek en Klinisch Laboratorium Venray" and with facilities of the Catholic University Nijmegen and the State University Limburg.