TRANSITIONAL CELL CARCINOMA OF THE BLADDER
Histopathological and Biological Factors and Prognosis

CIP GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Schapers, René Franciscus Maria

Transitional cell carcinoma of the bladder :
histopathological and biological factors and prognoses /
René Franciscus Maria Schapers. - Maastricht :
Universitaire Pers Maastricht. - Ill.
Proefschrift Rotterdam. - Met lit. opg. - Met samenvatting
in het Nederlands.
ISBN 90-5278-095-1
Trefw.: blaaskanker.

Production: Datawyse | Universitaire Pers Maastricht

This Project was financially supported by the "PRAEVENTIEFONDS", The Haque, projectnr. 002818760.
Financial support for the production of this thesis was given by:
- Stichting Ziekenhuizen Noord Limburg
- Crediet en Effectenbank, Utrecht

TRANSITIONAL CELL CARCINOMA OF THE BLADDER

Histopathological and Biological factors and Prognosis
(Blaaskanker, histopathologische en biologische factoren en prognose)

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus prof.dr. P.W.C. Akkermans M. Lit.
en volgens besluit van het College van Dekanen.
De openbare verdediging zal plaatsvinden op
donderdag 21 oktober 1993 om 13.30 uur

door

RENE FRANCISCUS MARIA SCHAPERS

geboren te Delft

PROMOTIECOMMISSIE

PROMOTOR:

Prof.dr. F.T. Bosman

OVERIGE LEDEN:

Prof.dr. F.H. Schröder

Prof.dr. A. Hofman

Prof.dr. F.J.W. ten Kate, Universiteit van Amsterdam

Ter herinnering aan mijn ouders

Aan Moon, Marieke, Pieter en Reinout

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

The incidence of carcinoma of the bladder is higher in industrialized countries than in underdeveloped regions such as Asia and Africa (excluding areas where schistosomiasis is endemic[1]).
In the Netherlands bladder cancer ranges fourth in the order of frequency in the occurrence of tumours in males (40.7 per 100000 person-years, standardized to the European standard population). In females bladder cancer is less frequent (twelfth most frequent; 8.5 per 100000 person-years). The male-female ratio is 4.8 to 1 (non-infiltrating and infiltrating bladder cancer together).[2] In other countries, including the USA, the male predominance is less pronounced.[3]
Bladder cancer typically affects older persons. Its peak incidence occurs in the 6th and 7th decades of life. Although the disease may develop in patients under 45 years of age, only approximately 2 percent of the cases occur in this age group.
The disease is at least in part attributable to exposure to carcinogens. Arylamines, used in various industrial settings, have been cited as agents involved in the development of bladder cancer.[4-7] Other factors include viruses, radiation and coffee intake. The most important factor however appears to be cigarette smoking.[8, 9] The incidence of bladder cancer in cigarette smokers is two to three times higher than in non smokers.

1.2 HISTOPATHOLOGICAL FACTORS

In the management of bladder cancer the "classic" prognostic parameters pathological stage and grade still play an important role next to clinical factors like clinical staging and cystoscopy. These two pathological factors have been subject to our investigations and are introduced under separate subheadings.

1.2.1 Histopathological staging

In the evaluation of patients with bladder cancer the determination of tumour stage has become an essential step. Bladder cancer stage is an important criterium for the clinician in planning treatment, predicts eventual outcome and helps to standardize patient groups which makes studies from different investigators more or less comparable. Current methods of staging rely primarily on bimanual palpation, cystoscopy and histopathological examination. The most widely accepted staging system is the TNM classification of malignant tumours.[10] In this staging system the distinction is made between non-papillary carcinoma in situ (Tis) and papillary non-infiltrating tumours (Ta). T1 tumours invade in the lamina propria. T2, T3 and T4 tumours show invasion of respectively the superficial parts of the muscularis, deep intramuscular and contiguous organs. In the USA the Marshall modification of the Jewett and Strong system[11, 12] is also in use. Although varying in details, both recognise the following features of urothelial carcinoma: (1) tumour behaviour is directly related to the depth of invasion into the bladder wall, (2) muscular invasion is the most accessible factor in determining tumour aggressiveness, (3) invasion in perivesical structures and metastases to lymphnodes or distant organs are grave prognostic signs, (4) non-invasive papillary lesions have a good prognosis. A disadvantage of the Jewett and Strong system of staging is that no difference is made between flat carcinomas in situ and non invasive papillary tumours, which are both classified as stage 0.

Unfortunately staging also has its problems.[13] It is sometimes difficult to assess infiltration in superficial tumours. The pathologist's interpretation of infiltration in Ta and T1 tumours is sometimes inconsistent and not always reproducible. To assess the exact infiltration depth of tumours an adequate biopsy is necessary, containing a part of the muscularis of the bladder wall.

1.2.2 Histopathological grading

The gross appearance of Transitional Cell Carcinoma (TCC) is extremely varied, ranging from delicately papillary to polypoid to flat and/or ulcerated. The growth pattern of TCC may be purely papillar, purely solid or both papillary and solid. Although gross examination is of obvious importance in guiding sampling, the most important information is obtained from histopathological examination. Several systems have been developed for the histopathological grading of carcinoma of the urinary bladder, [14-19] but most of them did not gain wide acceptance. The most commonly used system is the grading system proposed by the American Bladder Tumor Registry and the World Health Organisation (WHO).[16] The WHO recommends classification of epithelial tumours into four primary histological types: TCC, squamous cell carcinoma, adenocarcinoma and undifferentiated carcinoma. TCC constitute about 92 percent of the epithelial tumours, squamous cell carcinomas

6 to 7 percent, adenocarcinomas 0.5 to 2 percent and undifferentiated carcinomas less than 1 percent. Up to 20 percent of TCC contain areas of squamous cell differentiation, whereas glandular differentiation is present in 7 percent.
A major problem in grading is the high inter- and intra-observer variability. In most systems a sharp distinction between different grades cannot be obtained, mainly because of the many variables used. Although the WHO grading system is the most accepted system of grading, the problem of reproducibility also has been demonstrated.[20, 21] To compare the results of different studies using histopathological grading as a prognostic factor, grading systems should be available with high reproducibility. As long as this goal has not been achieved the results of histopathological grading as a prognostic factor should be interpreted prudently.

1.3 OBJECTIVE PROGNOSTIC PARAMETERS

Because histopathological factors have their problems in reproducibility, there is a need for objective other prognostic parameters. To reduce the grey area between low-grade malignant tumours and high-grade malignant TCC all efforts are aimed at creating a clear dichotomy between these extremes. The problem here, however, is of a biological nature, because low- and high-grade tumours are not separate and distinct entities, but extremes on a continuous scale. A number of possible prognostic factors have been analysed in the past. Already in the seventies cytogenetic factors[22] and bloodgroup antigen (BGA) [23] deletion were reported to have predictive value with respect to recurrence and progression. Later reports have contradicted the value of BGA deletion as prognostic factor.[24] Biochemical factors have been reported as being of possible clinical value in TCC like the autocrine motility factor, [25] epidermal growth factor, [26] tissue polypeptide antigen[27] and transferrin receptor.[28] PCNA[29] and Ki67[30] are used as proliferation markers with prognostic value. Further study is necessary to fully validate the additional value of all these factors in the management of TCC.
The factors with possible prognostic value which have been subject to our investigations are introduced under separate headings.

1.4 BASEMENT MEMBRANES

In all epithelial tissues basement membranes (BM) form the boundary between the epithelial compartment and adjacent stroma. BM are not static structures but are continuously catabolized and redeposited.[31] BM defects in neoplasm are caused by an imbalance between breakdown and production. Transition from in situ carcinoma to invasive carcinoma implies BM

dominance of breakdown and the possibility of passage of tumour cells through the interrupted BM.[32, 33] Visualisation of the BM could be helpful in assessing minimal infiltration.[33-35] Early attempts by Ozzello[36] to outline BM in breast cancer by PAS staining were frustrated by the occurrence of PAS reactive glycoproteins not only in BM but also in interstitial connective tissue. Electron microscopy appeared to be suitable for the study of BM,[37, 38] but this technique is too complicated and too expensive for routine application. The immunohistochemical localization of BM components has greatly expanded the possibilities to study the role of the BM in cancer invasion.[32-35, 39-41] BM deposition in cancer can also be regarded as an indicator of differentiation of a particular neoplasm.[40]
There are only a few reports concerning BM staining in bladder carcinomas.[42, 43] In one study a significant correlation was seen between BM continuity or discontinuity and progression.[42] In the other study bladder cancer patients with intact BM showed longer survival than patients with BM discontinuities.[43]

1.5 NUCLEAR MORPHOMETRY

The value of nuclear morphometry as a technique to discriminate between different histological grades in TCC has been investigated in several studies.[44-53] Some of the studies reported on the reproducibility of the methods,[21, 49] which is an essential requirement for broad application of such a method for prognostic purposes. Previous morphometric studies varied in their methodology. The methods of nuclear sampling varied as well the number of measured nuclei. In their report on the prognostic significance of morphometry in TCC Ooms et al.[46] described a method in which nuclei are measured in areas of nuclear crowding and in areas with the most pronounced nuclear enlargement. In such areas nuclear and cellular size were measured in 20 cells. They found that nuclear size was larger and nucleus-cell ratio smaller in non survivors.
Bjelkenkrantz et al. found an increase in nuclear size with grade. Their method of measurement was not described in detail.[44] Later, Ooms et al. described a new method of morphometric grading by measuring superficial and basal layer nuclei and the largest nuclei found in a TCC.[47] By plotting the mean nuclear area of the largest nuclei against the mean area of basal cell nuclei or the mean area of superficial cell nuclei, they could divide TCC in low, high and intermediate grades. The clinical value of these grades was not evaluated. A similar observation was reported by Monteroni et al.[48] but using different parameters than Ooms et al..[47] In several studies grade II TCC could be separated by morphometry into two groups of patients, with different clinical behaviour.[45, 50] Some studies analysed the clinical value of morphometry in TCC in comparison with classic parameters in relative small

groups of patients.[50-53] Blomjous et al. studied different methods of morphometric grading and found that selective measurement of the ten largest nuclei, after selection of the most atypical area of the TCC, gave the best results.[21]

1.6 DNA ANALYSIS

Deoxyribonucleic acid (DNA) ploidy analysis by flow cytometry (FCM) has steadily gained acceptance in recent years as a diagnostic and prognostic tool in the management of patients with TCC.[51, 54-60] Aneuploidy, as determined by FCM, appeared to be a parameter which can predict which tumours will progress.[56, 58-60] However, tumours with only a few cells with high DNA content against a background of abundant diploid tumour cells, might be classified as diploid by FCM and this approach therefore lacks in sensitivity. Image or static cytometry offers an alternative approach to DNA ploidy analysis.[61-70] By means of a video camera and computer, digitized images of cells are acquired from stained slides, viewed under a microscope. This technique permits DNA measurements to be performed under visual control. It has been shown by several authors that valuable prognostic information can be obtained from the nuclear DNA distribution pattern using this approach. In early, manually performed, image cytometric studies on imprints of fresh tumour tissue of TCC a correlation was found between DNA values and survival in univariate analyses.[61-65] Böcking et al. showed with the aid of multivariate analysis using age, tumour stage and tumour grade as covariates, that DNA grading was the only factor which contributed significantly to predicting survival.[64] Stöckle et al. also used automated DNA image cytometry and derived two parameters from the histograms (DNA ploidy and the number of cells above 5C) which correlated well with survival in patients with stage T1 to T4 TCC.[69]

1.7 CYTOGENETIC ANALYSIS

DNA content of tumour cells can be rapidly and objectively estimated by flow and image cytometry.[48, 51, 54-70] However these techniques only allow estimation of the total DNA content in large cell populations, but no specific information about chromosomal aberrations can be obtained. Karyotyping of tumours allows more precise determination of numerical and/or structural chromosome defects. One of the first reports on chromosome abnormalities in human tumours was published in 1960 by Nowell and Hungerford when they described an abnormal small chromosome, the so called Philadelphia chromosome, in patients with chronic myelogenous leukaemia.[71] Numerous chromosomal abnormalities in human cancer have been reported since

then.[72-75] With the introduction of banding techniques about 1970, [76] it became relatively easy to recognize the individual chromosomes and their structural abnormalities, at least when sufficient metaphases of adequate quality were obtained.
The first study on chromosomes in TCC was published in 1965 by Shigematsu.[77] He found a relation between the number of chromosomes and the histological type of TCC. In a study of Spooner and Cooper an increase in modal number of chromosomes was associated with histological grade and stage in TCC.[22] These authors found that well and moderately well differentiated tumours fall within the diploid range (42 to 49 chromosomes), whereas the majority of poorly differentiated cancers show a widespread distribution of chromosome number. In some studies a large number of structural abnormalities was found, including marker chromosomes, especially in poorly differentiated and invasive tumours.[78-81] Low-stage and low-grade carcinomas of the bladder were nearly always diploid or near diploid (42 to 49 chromosomes), although a few marker chromosomes have been observed.[79, 80] Pauwels et al. reported on modal chromosome number and range in relation to progression and recurrence and found that marker chromosomes had no predictive value.[82] The range of chromosome number appeared to be the best predictor for invasion. Smeets et al. found that monosomy of chromosome 9 is a frequent aberration in diploid TCC.[83] Karyotyping is only possible when fresh tumour tissue is available. Especially in tumours with a low number of mitoses it is sometimes difficult to obtain sufficient metaphases.
In situ hybridization (ISH) using a number of chromosome specific probes allows the detection of numerical chromosome aberrations in the interphase nucleus.[84-87] Most studies on ISH methods have dealt with the application on fresh TCC tissues.[87-90] A limited number of studies describe the application of this technique on routinely processed paraffin material.[91, 92]
This technique provides valid information about numerical chromosome aberrations in bladder cancer. This method offers the possibility to perform retrospective studies using paraffin blocks. Hybridization with different probes on the same tumour areas in parallel sections or double target ISH enables to study chromosome ratios and therefore the loss of some specific chromosomes.[91]

1.8 AIM OF THE STUDY

The main purpose of the studies reported in this thesis has been to determine the extent to which the behaviour of TCC can be predicted by histopathological and biological characteristics. The potential additional prognostic value of these factors was evaluated by combining them with other prognostic factors in multivariate analysis. In chapter 2 a two grade system of histological

grading, using simple histological criteria, is proposed. The interobserver variability of the WHO grading system and the two grade system is tested. The extent to which patient survival and progression free survival correlated with the two grade system, is evaluated. The additional value of grading is tested by combining it with other prognostic factors such as stage, age and mitotic index in multivariate analysis. In chapter 3 BM expression in TCC is described in an attempt to evaluate its use for the histopathological identification of microinvasion. Furthermore the usefulness of BM staining for the prediction of the clinical behaviour of TCC is assessed in comparison with grading, staging and ploidy. In chapter 4 the use of a two grade morphometrical grading system for prediction of the clinical behaviour of TCC is described. Also the heterogeneity in the WHO grade II tumours is evaluated using morphometry. In chapter 5 a study is reported in which it is determined whether image cytometry can provide useful parameters which can be used in the prediction of TCC behaviour. Special attention is given to the potential value of rare incidents, *e.g.* occasional cells with a very high DNA content. In chapter 6 a study is described concerning numerical chromosome aberrations in TCC as assessed by counting chromosomes in metaphase spreads. The modal number of chromosomes and the chromosomal range are used as potential prognostic factors in comparison with histological parameters. In chapter 7 "classical" metaphase chromosome counting is compared with interphase cytogenetics, especially in tumours having diploid and hyperdiploid DNA content. In chapter 8 The findings of these studies are discussed and general conclusions are drawn.

1.9 REFERENCES

1. EL-AAZAR, A.A. (1981). Aetiology of bilharzial bladder cancer in Egypt. In: *Bladder Cancer.* UICC Technical Reports Series, Skrabanek P, Walsh A (eds): vol 60, Geneva. 1. EL-AAZAR, A.A. (1981). Aetiology of bilharzial bladder cancer in
2. DE WINTER, G.A., COEBERGH, J.W.W., VAN LEEUWEN, F.E., SCHOUTEN, L.J. (1992). In: *Incidence of cancer in the Netherlands, 1989.* First report of the Netherlands cancer registry. Utrecht. LOK.
3. SILVERBERG, E., LUBERA, J. (1988). Cancer statistics. *CA-A Cancer Journal for Clinicians,* 38, 5-22.
4. REHN, L. (1895). Blasengeschwülste bei Fuchsin Arbeitern. *Arch. Klin. Chir.,* 50, 588-600.
5. CHOWANIEC, J. (1981). Epidemiological and experimental considerations. In: *Bladder Cancer* eds. Skrabanek, P. and Walsh, A.. Technical Report Series, Vol 60, Geneva.
6. LOWER, G.M. (1982). Concepts in causality: Chemically induced human urinary bladder cancer. *Cancer,* 49, 1056-1066.
7. HOOVER, R., COLE, P. (1973). Temporal aspects of occupational bladder carcinogenesis. *N. Engl. J. Med.,* 288, 1040-1043.
8. HOOVER, R., COLE, P. (1971). Population trends in cigarette smoking and bladder cancer. *Am. J. Epidemiol.,* 94, 409-418.
9. KABAT, G.C., DIECK, G.S., WYNDER, E.L. (1986). Bladder cancer in non-smokers. *Cancer,* 57, 362-367.
10. HERMANEK, B., SOBIN, L.H. (1987). In *T. N. M. Classification of Malignant Tumors,* Union International Contre le Cancer (UICC), ed. 4, pp. 121-144. New York: Springer-Verlag.
11. JEWETT, H.J., STRONG, G.H. (1946). Infiltrating carcinoma of bladder: relation of depth of penetration of bladder wall to incidence of local extension and metastases. *J. Urol.,* 55, 366-372.
12. MARSHALL, V.F. (1962). The relation of the preoperative estimate to the pathologic demonstration of the extent of vesicle neoplasm. *J. Urol.,* 68, 714-723.
13. REUTER, V.E. (1990). Pathology of Bladder Cancer: Assessment of Prognostic Variables and Response to therapy. *Seminars in Oncology,* 17, no 5, 524-532.
14. BRODERS, A.C. (1922).Epithelioma of the genito-urinary organs. *Annals of Surgery,* 75, 574-580.
15. BERGKVIST, A., LJUNGQVIST, G., MOBERGER, G. (1965). Classification of bladder tumours based on the cellular pattern. Preliminary report of a clinical-pathological study of 300 cases with a minimum follow-up of eight years. *Acta Chir. Scand.,* 130, 371-378.
16. MOSTOFI, F.K. (1973). In *Histological typing of Urinary Bladder Tumours,* International Histological classification of Tumours. No. 10, World Health Organization (WHO), Geneva.
17. FRIEDELL, G.H., BELL, J.R., BURNEY, S.W., SOTO, E.A., TILTMAN, A.J. (1976). Histopathology and classification of urinary bladder carcinoma. *Urol. Clinics North America,* 3: 53-70.
18. JORDAN, A.M., WEINGARTEN, J., MURPHY, W.M. (1987). Transitional cell neoplasms of the urinary bladder. *Cancer,* 60, 2766-2774.
19. PAUWELS, R.P.E., SCHAPERS, R.F.M., SMEETS, A.W.G.B., DEBRUYNE, F.M.J., GERAEDTS, J.P.M. (1988). Grading of superficial bladder cancer. *Br. J. Urol.,* 61, 129-134.
20. OOMS, E.C.M., ANDERSON, W.A.D., ALONS, C.L., BOON, M.E., VELDHUIZEN, R.W. (1983). Analysis of the performance of pathologists in the grading of bladder tumors. *Hum. Pathol.,* 14, 140-143.
21. BLOMJOUS, C.E.M., SMEULDERS, A.W.M., BAAK, J.P.A., VOS, W., VAN GALEN, E.M., MEIJER, C.J.L.M. (1989). A comparative study in morphometric grading of transitional cell carcinoma of the urinary bladder. *Anal. quant. cytol. histol.,* 11, 426-432.
22. SPOONER, M.E., COOPER, E.H. (1972). Chromosome constitution of transitional cell carcinoma of the urinary bladder. *Cancer,* 29, 1401-1412.

23. BERGMAN, S., JAVADPOUR, N. (1978). The cell surface antigen A, B, or O (H as an indicator of malignant potential in stage A bladder carcinoma: preliminary report. *J. Urol.*, 119, 49-51.
24. PAUWELS, R.P.E., SCHAPERS, R.F.M., SMEETS, A.W.G.B., JANSEN, L.E.G., DEBRUYNE, F.M.J., GERAEDTS, J.P.M. (1988). Blood group isoantigen deletion and chromosomal abnormalities in bladder cancer. *J. Urol.*, 140, 959-963.
25. GUIRGUIS, R., SCHIFFMANN, E., LIU, B., BIRKBECK, D., ENGEL, J., LIOTTA, L. (1988). Detection of autocrine motility factor in urine as a marker of bladder cancer. *J. Natl. Cancer Inst.*, 80, 1203-1211.
26. NEAL, D.E., SHARPLES, L., SMITH, K., FENNELLY, J., HALL, R.R., HARRIS, A.L. (1990). The epidermal growth factor receptor and the prognosis of bladder cancer. *Cancer*, 65, 1619-1625.
27. ADOLPHS, H.D., OEHR, P (1984). Significance of plasma tissue polypeptide antigen determination for diagnosis and follow up of urothelial bladder cancer. *Urol. Res.*, 12, 125-128.
28. SMITH, N.W., STRUTTON, G.M., WALSH, M.D., WRIGHT, G.R., SEYMOUR, G.J., LAVIN, M.F., GARDINER, R.A. (1990). Transferrin receptor expression in primary superficial human bladder tumours identifies patients who develop recurrences. *Br. J. Urol.*, 65, 339-344.
29. LIPPONEN, P.K., ESKELINNEN, M.J. (1992). Cell proliferation of transitional cell bladder cancer determined by PCNA/cyclin immunostaining and its prognostic value. *Br. J. Cancer*, 66, 171-176.
30. MELLON, K., NEAL, D.E., ROBINSON, M.C., MARCH, C., WRIGHT, C. (1990). Cell cycling in bladder carcinoma determined by monoclonal antibody Ki67. *Br. J. Urol.*, 66, 281-285.
31. KAFALIDES, N.A., ALPER, R., CLARK, C.C. (1979). Biochemistry and metabolism of basement membranes. *Int. Rev. Cytol.*, 61, 167-228.
32. ALBRECHTSEN, R., NIELSEN, M., WEWER, U., ENGVALL, E., RUOSLAHTI, E. (1981). Basement membrane changes in breast cancer detected by immunohistochemical staining for laminin. *Cancer. Res.*, 41, 5076-5081.
33. SIEGAL, G.P., BARSKY, S.H., TERRANOVA, V.P., LIOTTA, L.A. (1981). Stages of neoplastic transformation of human breast tissue as monitored by dissolution of basement membrane components. *Invasion Metastasis*, 1, 54-70.
34. BARSKY, S.H., SEIGAL, G.P., JANNOTTA, F., LIOTTA, L.A. (1983). Loss of basement membrane components by invasive tumors but not by their benign counterparts. *Lab. Invest.*, 49, 140-147.
35. VISSER, R., VAN DER BEEK, J.M.H., HAVENITH, M.G., CLEUTJENS, J.P.M., BOSMAN, F.T. (1986). Immunocytochemical detection of basement membrane antigens in the histopathological evaluation of laryngeal dysplasia and neoplasia. *Histopathology*, 10, 171-180.
36. OZELLO, L., SPEER, F.D. (1958). The mucopolysaccharides in normal and diseased breast: Their distribution and significance. *Am. J. Clin. Pathol.*, 34, 993-1009.
37. BOSMAN, F.T., HAVENITH, M.G., CLEUTJENS, J.P.M. (1985). Basement membranes in cancer. *Ultrastruct. Pathol.*, 8, 291- 304.
38. HAVENITH, M.G., DINGEMANS, K.P., CLEUTJENS, J.P.M., WAGENAAR, SJ.SC., BOSMAN, F.T. (1990). Basement membranes in bronchogenic squamous cell carcinoma: An immunohistochemical and ultrastructural study. *Ultrastruct.Pathol.*, 14, 51-63.
39. BURTIN, P., CHAVANEL, G., FOIDART, J.M., MARTIN, E. (1982). Antigens of the basement membrane and the peritumoral stroma in human colonic adenocarcinomas: An immunofluorescence study. *Int. J. Cancer*, 30, 13-20.
40. HAVENITH, M.G., ARENDS, J.W., SIMON, R.E.M., VOLOVICS, A., WIGGERS, T., BOSMAN, F.T. (1988). Type IV collagen immunoreactivity in colorectal cancer: Prognostic value of basement membrane deposition. *Cancer*, 62, 2207-2211.
41. WILLEBRAND, D., BOSMAN, F.T., DE GOEY, A.T.P.M. (1986). Patterns of basement membrane deposition in benign and malignant breast tumors. *Histopathology*, 10, 1231-1241.
42. CONN, I.G., CROCKER, J., WALLACE, D.M.A., HUGHES, M.A., HILTON, C.J. (1987). Basement membranes in urothelial carcinoma. *Br. J. Urol.*, 60, 536-542.

43. DAHER, N., ABOURACHID, H., BOVE, N., PETIT, J., BURTIN, P. (1987). Collagen IV staining pattern in bladder carcinomas: relationship to prognosis. *Br. J. Cancer*, 55, 665-671.
44. BJELKENKRANTZ, K., HERDER, A., GRONTOFT, O., STAL, O. (1982). Cytophotometric characterisation of the WHO grades of transitional cell neoplasms. *Pathol. Res. Pract.*, 174, 68-77.
45. HELANDER, K., HOFER, P.A., HOLMBERG, G. (1984). Karyometric investigations on urinary bladder carcinoma, correlated to histopathological grading. *Virchows Arch. A*, 403, 117-125.
46. OOMS, E.C.M., ESSED, E., VELDHUIZEN, R.W., ALONS, C.L., KURVER, P.H.J., BOON, M.E. (1981). The prognostic significance of morphometry in T1 bladder tumours. *Histopathology*, 5, 311-318.
47. OOMS, E.C.M., KURVER, P.H.J., VELDHUIZEN, R.W., ALONS, C.L., BOON, M.E. (1983). Morphomotric grading of bladder tumors in comparison with histologic grading by pathologists. *Hum. Pathol.*, 14, 144-150.
48. MONTIRONI, R., SCARPELLI, M., ANSUINI, E. MARINELLI, F., MARIUZZI, G. (1986). Quantitative evaluation of the progressive nuclear abnormalities in urothelial papillary lesions. *Appl. Pathol.*, 4, 65-73.
49. OOMS, E.C.M., BLOK, A.P.R., VELDHUIZEN, R.W. (1985). The reproducibility of a quantitative grading system of bladder tumors. *Histopathology*, 9, 501-509.
50. BLOMJOUS, C.E.M., SCHIPPER, N.W., VOS, W., BAAK, J.P.A., DE VOOGT, H.J., MEIJER, C.J.L.M. (1989). Comparison of quantitative and classic prognosticators in urinary bladder carcinoma. *Virchows Arch.*, 415, 421-428.
51. LIPPONEN, P.K., COLLAN, Y., ESKELINEN, M.J., PESONEN, E., SOTARAUTA, M., NORDLING, S. (1990). Comparison of morphometry and DNA flow cytometry with Standard Prognostic Factors in Bladder Cancer. *Br. J. Urol.*, 65, 589-597.
52. LIPPONEN, P.K., ESKELINEN, M.J., SOTARAUTA, M. (1990). Prediction of superficial bladder cancer by histoquantitative methods. *Eur. J. Cancer*, 26, no 10, 1060-1063.
53. QING-BEI YANG, YANG-ZHI XIA, ZHI-YOUNG WANG, GUANG-JUN WANG, SHU-ZHE DING, DIAN-ZHI SHI, WEN-DE LIU (1991). Morphometric diagnosis of bladder tumor. *Oncology*, 48, 188-193
54. WIJKSTRÖM, H., GRANBERG-ÖHMAN, I., TRIBUKAIT, B. (1984). Chromosomal and DNA patterns in transitional cell bladder carcinoma. A comparative cytogenetic and, flowcytofluorometric DNA study. *Cancer*, 53, 1718-1723.
55. FEITZ, W.F.J., BECK, H.L.M., SMEETS, A.W.G.B., DEBRUYNE, F.M.J., VOOIJS, G.P., HERMAN, C.J., RAMAEKERS, F.C.S. (1985). Tissue specific markers in flow cytometry of urological cancers: Cytokeratins in bladder carcinoma. *Int. J. Cancer*, 36, 349-356.
56. OLJANS, P.J., TANKE, H.J. (1986). Flow cytometric analysis of DNA content in bladder cancer: prognostic value of the DNA- index with respect to early tumour recurrence in G2 tumours. *World J. Urol.*, 4, 205-210.
57. SMEETS, A.W.G.B., PAUWELS, R.P.E., BECK, J.L.M., GERAEDTS, J.P.M., DEBRUYNE, F.M.J., LAARAKKERS L., FEITZ, W.F.J., VOOIJS, G.P., RAMAEKERS, F.C.S. (1987): Tissue specific markers in flow cytometry of urological cancers. III. Comparing chromosomal and flow cytometric DNA analysis of bladder tumors. *Int. J. Cancer*, 39, 304-310.
58. DEVERE WHITE, R.W., DEITCH, A.D., WEST, B., FITZPATRICK, J.M. (1988). The predictive value of flow cytometric information in the clinical management of stage 0 (Ta bladder cancer. *J.Urol.*, 139, 279-285.
59. BLOMJOUS, C.E.M., SCHIPPER, N.W., BAAK, J.P.A., VAN GALEN, E.M., DE VOOGT, H.J., MEIJER, C.J.L.M. (1988). Retrospective study of prognostic importance of DNA flow cytometry of urinary bladder transitional cell carcinoma. *J. Clin. Pathol.*, 41, 21-25.
60. SHAABAN, A.A., TRIBUKAIT, B., EL-BEDEIWY, A.A., GHONEIM, M.A. (1990). Prediction of lymph node metastases in bladder carcinoma with deoxyribonucleic acid flow cytometry. *J. Urol.*, 144, 884-887.

61. LEVI, P.E., COOPER, F.H., PHIL, D., ANDERSON, C.K., PATH, M.C., WILLIAMS, R.E. (1969). Analyses of DNA content, nuclear size and cell proliferation of transitional cell carcinoma in man. *Cancer*, 23, 1074-1085.
62. FOSSA, S.D., KAALHUS, O., SCOTT-KNUDSEN, O. (1977). The clinical and histopathological significance of Feulgen DNA- values in transitional cell carcinoma of the human urinary bladder. *Eur. J. Cancer*, 13, 1155-1162.
63. HOFSTÄDTER, F., JAKSE, G., LEDERER, B., MIKUZ, G. (1980). Cytophotometric investigations of DNA-content in transitional cell tumors of the bladder. Comparison of results with clinical follow up. *Path. Res. Pract.*, 167, 254-264.
64. BÖCKING, A., ADLER, C.P., COMMON, H.H., HILGARTH, M., GRANZEN, B., AUFFERMANN, W. (1984). Algorithm for DNA cytophotometric diagnosis and grading of malignancy. *Anal. Quant. Cytol.*, 6, 1-8.
65. HOFSTÄDTER, F., JAKSE, G., LEDERER, B., MIKUZ, G., DELGADO, R. (1984). Biological behavior and DNA cytophotometry of urothelial bladder carcinoma. *Br. J. Urol.*, 56, 289-295.
66. VAN DRIEL-KULKER, A.M.J., MESKER, W.E., VAN VELZEN, I., TANKE, H.J., FEICHTINGER, J., PLOEM, J.S. (1985). Preparation of monolayer smears from paraffin-embedded tissue for image cytometry. *Cytometry*, 6, 268-272.
67. AUFFERMANN, W., URQUARDT, M., RÜBBEN, H., WOHLTMANN, D., BÖCKING, A. (1986). DNA grading of urothelial carcinoma of the bladder. *Anticancer Res*, 6, 27-32.
68. PLOEM, J.S., VAN DRIEL-KULKER, A.M.J., GOYARTS-VELDSTRA, L., PLOEM-ZAAIJER, J.J., VERWOERD, N.P., VAN DER ZWAN, M. (1986).Image analysis combined with quantitative cytochemistry. *Histochemistry*, 84, 549-555.
69. STÖCKLE, M., TANKE, H.J., MESKER, W.E., PLOEM, J.S., JONAS, U., HOHENFELLNER, R. (1987). Automated DNA-image cytometry: a prognostic tool in infiltrating bladder carcinoma. *World J. Urol.*, 45, 127-132.
70. PLOEM, J.S., VAN DRIEL-KULKER, A.M.J., PLOEM-ZAAIJER, J.J. (1989). Automated cell analysis for DNA studies of large cell populations using the LEYTAS image cytometry system. *Path. Res. Pract.*, 185, 671-675.
71. NOWELL, P.C., HUNGERFORD, D.A. (1960). Chromosome studies on normal and leukemic human leucocytes. *J. Natl. Cancer Inst.*, 25, 85-109.
72. SANDBERG, A.A. (1986). Chromosome changes in bladder cancer: Clinical and other correlations. *Cancer Genet. Cytogenet.*, 19, 163-175.
73. TRENT, J., CRICKARD, K., GIBAS, Z., GOODACRE, A., PATHAK, S., SANDBERG, A.A., THOMPSON, F., WHANG-PENG, J., WOLMAN, S. (1986). Methodologic advances in the cytogenetic analysis of human solid tumors. *Cancer Genet. Cytogenet.*, 19, 57-66.
74. WOLMAN, S.R. (1984). Cytogenetics and cancer. *Arch. Path. Lab. Med.*, 108, 15-19.
75. WOLMAN, S.R. (1986). Cytogenetic heterogeneity: Its role in tumor evolution. *Cancer Genet. Cytogenet.*, 19, 129-140.
76. SANDBERG, A.A. (1980). The chromosomes in human cancer and leukemia. Elsevier North Holland, New York.
77. SHIGEMATSU, S. (1965). Significance of the chromosomes in vesical cancer. In: *Report of the XIII congress of the societé international d'urologie.* Vol. 2, pp 111-121, Livingstone, London.
78. SANDBERG, A.A. (1977). Chromosome markers and progression in bladder cancer. *Cancer Res.*, 37, 2950-2956.
79. FALOR, W.H. (1971). Chromosomes in noninvasive papillary carcinoma of the bladder. *J. A. M. A.*, 216, 791-794.
80. SUMMERS, J.L., COON, J.S., WARD, R.M., FALOR, W.H., MILLER, A.W. III, WEINSTEIN, R.S. (1983). Prognosis in carcinoma of the urinary bladder based upon tissue blood group ABH and Thomson- Friedenreich antigen status and karyotype of the initial tumour. *Cancer Res.*, 43, 934-939.

81. GRANBERG-ÖHMAN, I., TRIBUKAIT, B., WIJKSTRÖM, H. (1984). Cytogenetic analysis of 62 transitional cell bladder carcinomas. *Cancer Genet. Cytogenet.*, 11, 69-85.
82. PAUWELS, R.P.E., SMEETS, A.W.G.B., GERAEDTS, J.P., DEBRUYNE, F.M. (1987). Cytogenetic analysis in urothelial cell carcinoma, *J. Urol.*, 137, 210-215
83. SMEETS, W., PAUWELS, R., LAARAKKERS, L., DEBRUYNE, F., GERAEDTS, J. (1987). Chromosomal analysis of bladder cancer III. Nonrandom alterations. *Cancer Genet. Cytogenet.*, 29, 29- 41.
84. WILLARD, H.F., WAYE, J.S. (1987). Hierarchical order in chromosome-specific human alpha satellite DNA. *Trends Genet.*, 3, 192-198.
85. CREMER, T., TESSIN, D., HOPMAN, A.H.N., MANUELIDIS, L. (1988). Rapid interphase and metaphase assessment of specific chromsomal changes in neurectodermal tumor cells by in situ hybridization with chemically modified DNA probes. *Exp. Cell Res.*, 176, 199-220.
86. DEVILEE, P., THIERRY, R.F., KIEVITS, T., KOLLURI, R., HOPMAN, A.H.N., WILLARD, H.F., PEARSON, P.L., CORNELISSE, C.J. (1988). Detection of chromosome aneuploidy in interphase nuclei from human primary breast tumors using chromosome-specific repetitive DNA probes. *Cancer Res.*, 48, 5825-5830.
87. HOPMAN, A.H.N., RAMAEKERS, F.C.S., RAAP, A.K., BECK, J.L.M., DEVILEE, P., PLOEG VAN DER, M., VOOIJS, G.P. (1988). In situ hybridization as a tool to study numerical chromosome aberrations in solid bladder tumors. *Histochemistry*, 89, 307- 316.
88. HOPMAN, A.H.N., PODDIGHE, P.J., SMEETS, A.W.G.B., MOESKER, O., BECK, J.L.M., VOOIJS, G.P., RAMAEKERS, F.C.S. (1989). Detection of numerical chromosome aberrations in bladder cancer by in situ hybridization. *Amer. J. Pathol.*, 135, 1105-1118.
89. NEDERLOF, P.M., FLIER VAN DER, S., RAAP, A.K., TANKE, H.J., PLOEG VAN DER, M., KORNIPS, F., GERAEDTS, J.P.M. (1989). Detection of chromosome aberrations in interphase tumor nuclei by nonradioactive in situ hybridization. *Cancer Genet. Cytogenet.*, 42, 87-98.
90. HOPMAN, A.H.N., MOESKER, O., SMEETS, A.W.G.B., PAUWELS, R.P.E., VOOIJS, G.P., RAMAEKERS, F.C.S. (1991). Numerical chromosome 1, 7, 9, and 11 aberrations in bladder cancer detected by in situ hybridization. *Cancer Res.*, 51, 644-651.
91. HOPMAN, A.H.N., VAN HOOREN, E., VAN DE KAA, C.A., VOOIJS, G.P. RAMAEKERS, F.C.S. (1991). Detection of numerical chromosome aberrations using in situ hybridization in paraffin sections of routinely processed bladder cancers. *Modern Pathol.*, 4, 503-513.
92. VAN DE KAA, C.A., NELSON, K.A.M., RAMAEKERS, F.C.S., VOOIJS, G.P., HOPMAN, A.H.N. (1991). Interphase cytogenetics in parafin sections of routinely processed hydatiform moles and hydropic abortions. *J. Pathol.*, 165, 281-287.

CHAPTER 2

A SIMPLIFIED GRADING METHOD OF TRANSITIONAL CELL CARCINOMA OF THE URINARY BLADDER: Reproducibility, clinical significance, and comparison with other prognostic parameters.

R. F. M. SCHAPERS
R. P. E. PAUWELS
J. Th. M. WIJNEN
J. W. ARENDS
F. B. J. M. THUNISSEN
J. W. W. COEBERGH
A. W. G. B. SMEETS
F. T. BOSMAN

From the Departments of Pathology[RFMS] and Urology[RPEP], St. Ziekenhuizen Noord-Limburg, Venlo: the Department of Mathematics and Computing Science[JThMW], Eindhoven University of Technology: the Department of Clinical Pharmacy and Laboratory[AWGBS], Venray: the Department of Epidemiology[JWWC], Comprehensive Cancer Centre South, Eindhoven: the Departments of Pathology, University of Limburg, Maastricht[JWA, FBJMT] and Erasmus University, Rotterdam[FTB], the Netherlands.

Br. J. Urol (In press)

2.1 ABSTRACT

For histological grading of transitional cell carcinoma (TCC) of the urinary bladder, most pathologists prefer to use the three-grade system as proposed by the American Bladder Tumor Registry and the World Health Organization (WHO). However, interobserver agreement appears to be unacceptably low. We developed a simplified grading system in which only low-grade and high-grade tumours are distinguished and report on its reproducibility and predictive value for survival and progression. Reproducibility was good to excellent with a group Kappa value of 0.78.
The effect on survival and progression-free interval was measured in a group of 311 patients with bladder tumours. The survival of patients with low-grade tumours was significantly better than that of patients with high-grade tumours ($P < 0.0001$). The progression-free interval was also significantly longer in patients with low-grade tumours than in patients with high-grade tumours ($P = 0.0032$). Combining low-high grading, histological stage, mitotic index, and age, histological stage appeared to be the most important parameter in predicting survival and progression.
A reproducible and discriminating system such as our low-high grade system, is an important prognostic factor when stage cannot be established with certainty.

2.2 INTRODUCTION

The urinary bladder is the site of origin of a variety of benign and malignant neoplasms. The most important group of tumours consists of epithelial tumours of the transitional cell type. To provide a rational basis for follow-up and treatment, a generally accepted system of clinico-pathological classification is essential. The most widely accepted staging system is the TNM classification.[1] For histopathological classification the WHO system is generally in use.[2] In the WHO grading system a sharp demarcation between different grades cannot be attained. This results in considerable interobserver variability, and therefore poor agreement between diagnoses made by different pathologists.[3, 4] Unfortunately, staging has its problems as well. The pathologist's interpretation of infiltration in the Ta and T1 group of tumours may be inconsistent and not reproducible.[5] To assess the exact infiltration depth of tumours an adequate biopsy should be obtained, containing all layers of the bladder wall. Several efforts have been made to improve existing or to develop new grading systems, [6-12] but these have not gained wide acceptance. In some studies stage has proved to be the most discriminating variable to predict clinical outcome.[12-14] However in another study histological grading was claimed to be the best predictor of clinical outcome.[13] The purpose of the present study was to determine the extent to which the

biological potential of transitional cell neoplasms can be predicted by histological grading of the primary tumour in a two grade system using simple histological criteria. We also evaluated the additional value of grading when combined with other prognostic factors. The interobserver variability of the WHO grading and the two grade system was tested.

2.3 MATERIALS AND METHODS

The study included 311 patients with newly diagnosed bladder carcinomas (only TCC) seen at the St. Maartens Hospital, Venlo (community hospital serving the Northern Limburg area) between January 1979 and June 1990. Two hundred fifty six patients (82.3%) were male and fifty five (17.7%) were female. Age ranged from 17 to 92 years with a mean of 66 years. All patients were treated by transurethral resection (TUR) of the tumour. Seventy four of them received additional adjuvant intravesical therapy. Sixty seven patients were treated additionally by radiotherapy, cystectomy or both. Clinicopathological information and TUR specimen were collected prospectively. Staging was carried out according to the TNM system.[1]
The median follow-up was 38 months, with a maximum of 150 months (mean 46.2 months). Follow-up examinations were conducted at least semi-annually and included cystoscopy and urinary cytology. As follow-up criteria survival and progression-free interval were chosen. Progression, defined as increase in stage or death due to tumour, was assessed histologically by pathologists without knowledge of previous diagnostic information and clinical data. If a patient was lost to follow-up, survival was assessed by contacting municipal population registries.

2.3.1 Clinico-pathological methods

All tumours were classified according to a simplified method of grading. In this grading system only two grades are distinguished: low-grade and high-grade. The grading criteria are summarized in table 1 and illustrated in figure 1. We encountered some tumours with very small areas of abnormal polarization and moderate pleomorphism. These were mostly papillary tumours. We included them in the group of the low-grade tumours. Carcinoma in situ, pure squamous cell carcinoma, pure adenocarcinoma and undifferentiated carcinoma were not evaluated in this study.
Furthermore, we estimated the mitotic index (counts/10hpf), using the counting method as described by van Diest et al..[15] We counted 10 fields using a x10 ocular and a x40 objective with a numerical aperture of 0.75 and a field width of 450 µm. The criteria described by Baak were used to identify mitotic figures.[16]

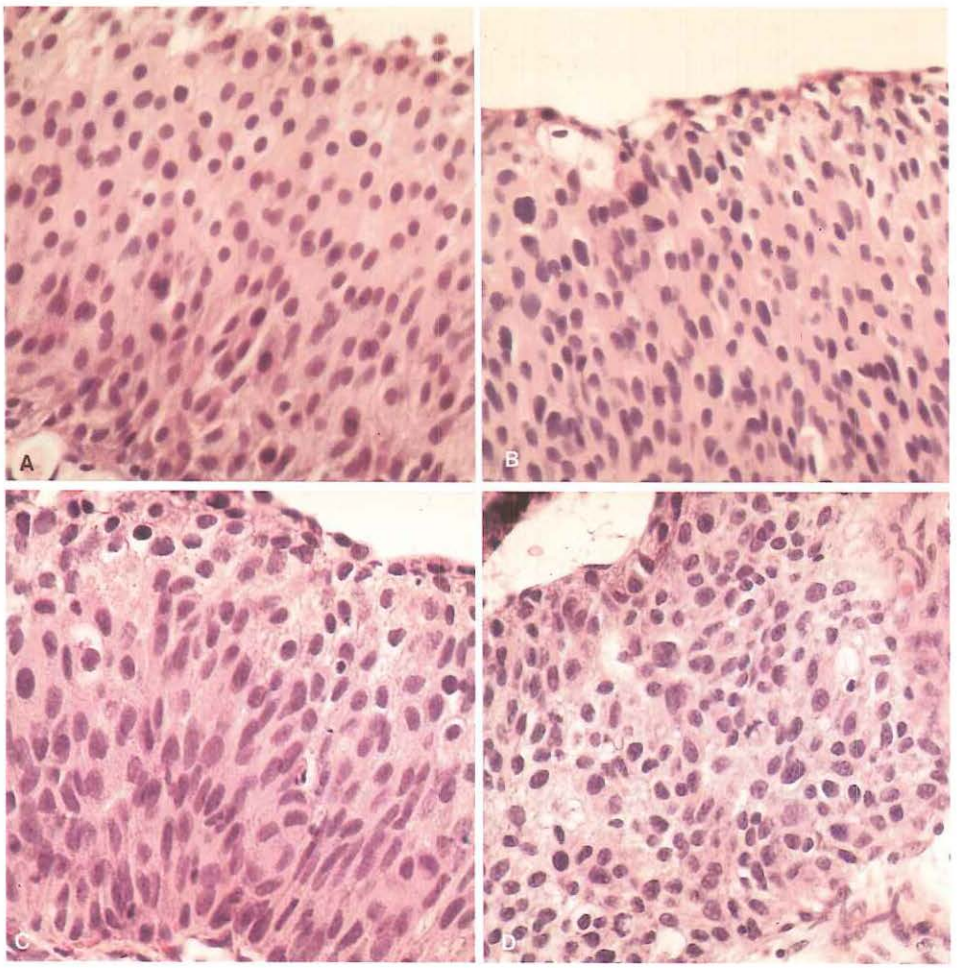

Figure 1. Low-grade TCC (A and B) with thickening of the epithelium, normal polarity of nuclei and none or slight pleomorphism (x250). High-grade TCC (C and D) with loss of polarity of nuclei and moderate to prominent pleomorphism (x250).

2.3.2 Statistical analysis

The following prognostic factors were included in the analysis: low/high grading, T category (Stage Ta; T1; T2 or more), mitotic index (less than 5 mitoses/10 hpf; 5 or more mitoses/10 hpf), age (less than 70 years; 70 years or more), and sex. For each prognostic factor Kaplan Meier survival curves[17] were compared by means of the log rank test[18] with regard to time interval to death and time interval to establishment of progression. A possible association between various prognostic factors was analysed using the Pearson chi square test.

Table 1. Grading criteria urothelial tumours

Papilloma:	a. Urothelium fewer than 7 layers in thickness. b. normal polarity of nuclei. c. No pleomorphism.
Low-grade:	a. Thickening of the urothelium (≥7 layers) b. *Normal polarity of nuclei in 95% or more of the tumour. c. *None or only slight pleomorphism in 95% or more of the tumour.
High-grade:	a. Loss of polarization of the nuclei. b. Moderate or prominent pleomorphism.

*In contrast to the WHO grading system some areas of the tumour may show a loss of polarization of the nuclei and/or moderate pleomorphism (<5% of the tumour).

For low/high grading the relative survival rate was calculated. The relative survival rate is the ratio of the observed survival rate in the group of patients to the expected survival rate in a group of people in the general population of the region, who are similar to the patients with respect to age, sex and calender time. Heterogeneity in withdrawal is taken into account. The regional mortality figures are derived from the Central Bureau of Statistics (CBS). We used the special computer programme of the Finnish Cancer Registry.[19]
Furthermore, multivariate survival analyses were performed using Cox proportional hazards model.[20] In these models, low/high grading was included together with the other above mentioned prognostic factors. The stepwise method was used to select the model, with use of the likelihood ratio test. A *P*-value of 0.05 was adopted as limit for entering and removing covariates. Of the prognostic factors, that contributed significantly to the model, the effect was calculated in terms of relative risk and the associated 95% confidence interval. The statistical analyses were performed with the SAS statistical package (SAS Inst Inc, Cary, North Carolina, USA).

2.3.3 Interobserver agreement

For evaluation of the interobserver variation four pathologists, three without special expertise in urothelial tumours, examined biopsy specimens of 88 consecutive cases of urothelial carcinoma from our files, without any knowledge of clinical outcome and prior histological grading. Both our simplified grading method and WHO system of grading[2] were used. Kappa statistics[21] were used to evaluate the degree of agreement between pairs of pathologists and among the group of pathologists.

Kappa was evaluated as described by Fleiss: [22] in general, Kappa values below 0.40 indicate poor agreement, kappa values between 0.40 and 0.75 indicate fair to good agreement and Kappa values above 0.75 indicate excellent agreement.

2.4 RESULTS

2.4.1 Prognostic factors

The frequency distribution for low/high grading, stage and mitotic index is shown in table 2. Strong association was seen between low/high grades and stage ($P < 0.0001$), and mitotic index ($P < 0.0001$).

Table 2. Association between low-high grading, histopathological stage and mitotic index.

		Grading		
		LOW	HIGH	Total
Stage	Ta	111	43	154
	T1	4	98	102
	T2+	0	55	55
Mitotic index	≤5	107	60	167
	>5	8	136	144
Total		115	196	311

2.4.2 Survival

For low/high grading we found a significant difference in survival ($P < 0.0001$) (fig. 2). Of the 115 patients with low-grade tumours, 2 died of tumour related disease and 29 died of (probably) unrelated causes of death. Of the 196 patients with high-grade tumours 45 died of tumour related disease and 70 died of (probably) unrelated causes of death. The survival of patients with low-grade TCC was almost the same as the expected survival of people in the general population of the region resulting in a relative survival of almost hundred percent. In patients with high-grade tumours the survival is worse than the expected survival (fig. 3).
Other strong predictors of survival were stage ($P < 0.0001$) (fig. 4) and mitotic index ($P < 0.0001$).

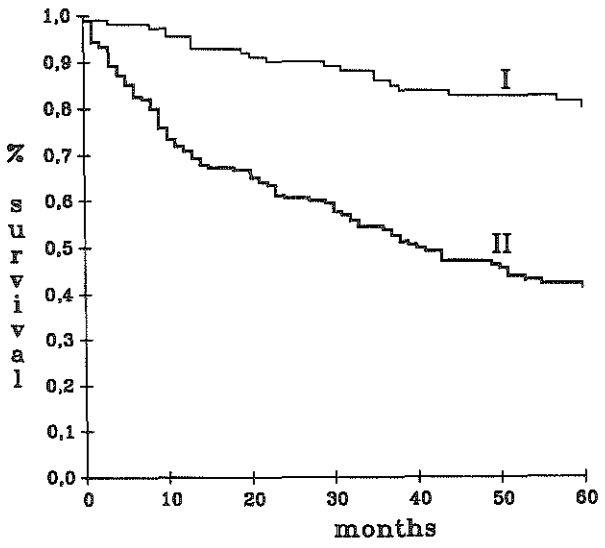

Figure 2. Overall survival according to low and high grades. I=low-grade (n=115), II=high-grade (n=196).
5 year survival: I=80.2% (95% CI, 72.3-88.1), II=41.2% (95% CI, 33.6-48.9).

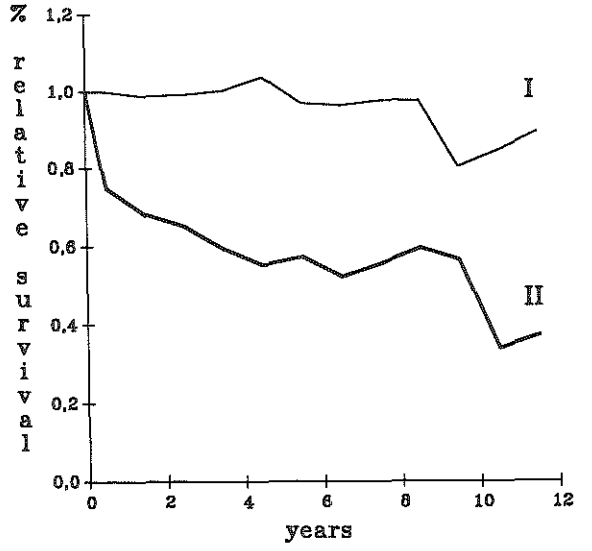

Figure 3. Relative survival rate according to low and high grades. I=low-grade (n=115), II=high-grade (n=196).

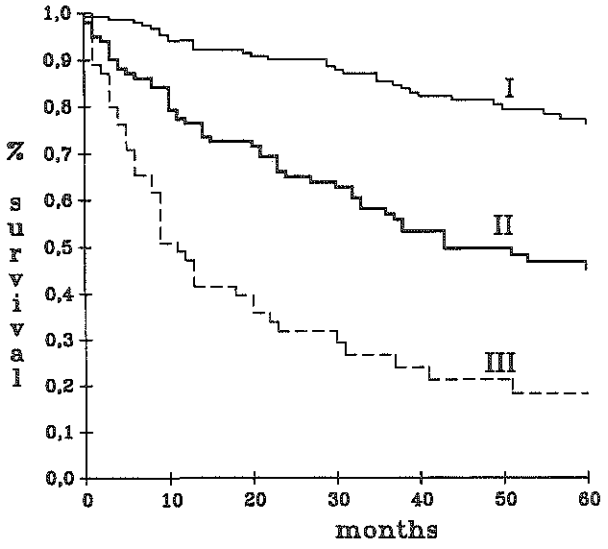

Figure 4. Overall survival according to tumour stage (T). I=Ta (n=154), II=T1 (n=102), III=T2 or more (n=55).
5 year survival: I=76.2% (95% CI, 68.7-83.7), II=45.2% (95% CI, 34.7-55.7), III=18.3% (95% CI, 6.7-29.8).

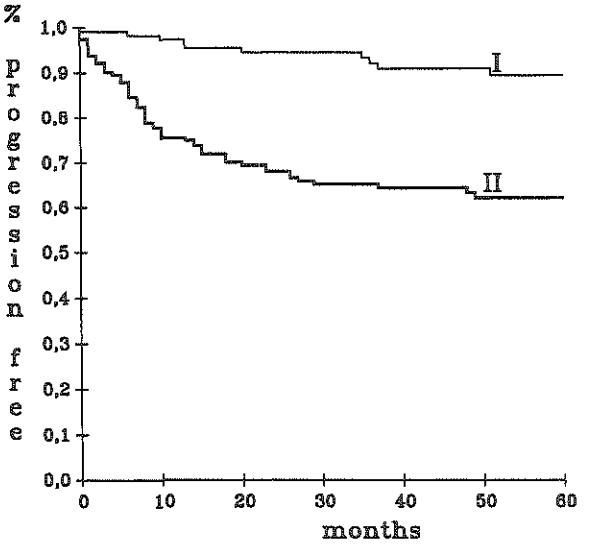

Figure 5. Progression-free survival according to low and high grades. I=Low-grade (n=115), II=High-grade (n=196).
5 year progression-free survival: I=89.3% (95% CI, 82.9-95.7), II=62.0% (95% CI, 54.3-69.7).

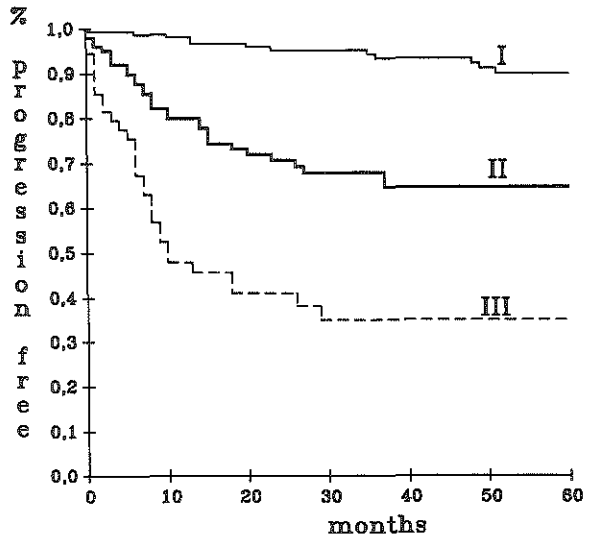

Figure 6. Progression-free survival according to tumour stage (T). I=Ta (n=154), II=T1 (n=102), III=T2 or more (n=55).
5 year progression free survival: I=89.9% (95% CI, 84.1-95.4), II=64.6% (95% CI, 54.2-74.9), III=34.9% (95% CI, 20.7-49.3).

In multivariate analysis, only stage ($P < 0.0001$) and age ($P = 0.0012$) were contributing significantly to the prediction of survival.

2.4.3 Progression

We found a significant association between low and high grades and progression-free interval ($P < 0.0001$) (fig. 5). Other strong predictors of progression were stage ($P < 0.0001$) (fig 6), and mitotic index ($P < 0.0001$) (fig 7).
In the multivariate analysis stage again was the most important prognostic parameter in predicting progression ($P < 0.0001$) next to the mitotic index ($P = 0.0005$). The other variables did not show a significant independent prediction.

2.4.4 Superficial TCC

For superficial tumours (TA+T1) the mitotic index appeared to be the most important parameter in predicting survival ($P < 0.0001$) and progression-free interval ($P < 0.0001$). However, when superficial TCC was divided into two separate groups of tumours, Ta and T1, stage appeared to be an independent parameter in the prediction of survival (in multivariate analysis), while mitotic index lost its importance. Stage also appeared to be the best predictor

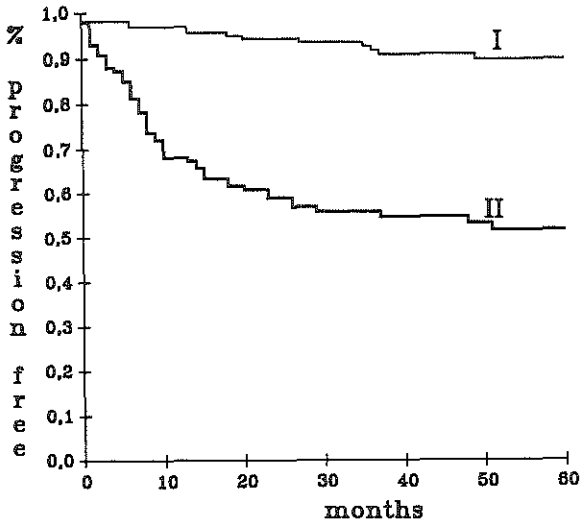

Figure 7. Progression-free survival according to mitotic index (MI). I= MI < 5 (n=167), II= MI ≥ 5 (n=144). 5 year progression free survival: I=89.8% (95% CI, 84.6-95.0), II=51.6% (95% CI, 42.2-61.0).

of progression in superficial tumours ($P < 0.0001$). Mitotic index is of borderline significant importance in the prediction of progression ($P = 0.0464$).

2.4.5 Interobserver agreement

The frequency distribution of low/high grading performed by four pathologists is presented in table 3. Kappa values for agreement between pairs of pathologists with the low/high grading system ranged from 0.71 to 0.85. The group kappa of the 4 pathologists was estimated at 0.78 (SE 0.05). With the WHO grading system kappa values ranged from 0.33 to 0.70 resulting in a group kappa was of 0.48 (SE 0.05).

Table 3. Interobserver agreement in low/high grading

Grading	Pathologist			
	1	2	3	4
low-grade	26	31	35	29
high-grade	62	57	53	59

Kappa values between pairs of pathologists ranged from 0.71 to 0.85. Group Kappa was 0.78 (SE 0.05).

2.5 DISCUSSION

Several systems have been proposed for histopathological grading of TCC of the urinary bladder.[2, 6-12] The most widely accepted system is the three grade WHO system.[2] However the criteria for the different grades are considered to be rather vague, resulting in considerable interobserver variability.[3, 4] Blomjous et al. reported kappa values for agreement between pairs of pathologists, using the WHO grading system, ranging from 0.38 to 0.63.[4] The same observation was made in our study. We found kappa values between pairs of observations, when using the WHO system, ranging from 0.33 to 0.70. Jordan et al. designed a detailed grading system, with many variables, in which 87% of TCC were classified as either low-grade (TCC-I) or high-grade (TCC-III).[11] Only 13% of cases fell into an intermediate category (TCC-II) and these closely followed the low-grade tumours with regard to survival and tumour progression. However, also these authors stated that inconsistency in the use of this system by different pathologists should be expected because of the many variables used.

In the presently proposed grading system only three criteria were employed to discriminate between low and high grades: epithelial thickness, nuclear polarity and pleomorphism.

Our working hypothesis was that the proposed low-high grading system would be reproducible, also when applied by pathologists without special expertise in urothelial tumours and would be informative with regard to clinical outcome.

We found that this simplified method of grading indeed shows good agreement between pathologists (group kappa = 0.78). This is mainly due to the simple criteria and the reduction in the number of categories.

As to the prediction of clinical outcome we have shown that patients with low-grade tumours have significantly longer overall survival and a longer progression-free survival than patients with high-grade tumours. Patients with low-grade tumours have approximately the life expectancy of the general population in the region. Our material was also analysed for a number of additional prognostic factors. Stage and mitotic index were found to have significant correlation with overall survival and the progression-free survival (age correlated, as expected, significantly with survival only).

The possible additional value of low-high grading in predicting clinical outcome was evaluated in multivariate analyses using stage, mitotic index and age as co-variate. Our results indicate that of all these variables, stage is the most important independent prognostic parameter for overall survival and progression. Due to the strong correlation between low/high grading and stage, the simplified grading provided no significant additional independent value. Mitotic index was the only parameter providing independent additional information concerning progression-free interval.

In conclusion, tumour stage remains the most important indicator for overall survival and tumour progression. The mitotic index provides important additional information. The division of superficial TCC in Ta and T1 tumours is useful, because these tumours did show a different behaviour independent of other parameters. Grading, preferably performed using a reproducible and discriminating system such as our low-high grade system, is important when stage cannot be established with certainty. The WHO grading system is not reproducible and as such not suitable for comparing results of epidemiological studies and results of therapy.

2.6 REFERENCES

1. HERMANEK, B., SOBIN, L. H. (1987). In: *T.N.M. Classification of Malignant Tumours*, Union International Contre le Cancer (UICC), ed. 4, pp. 121-144. New York: Springer-Verlag.
2. MOSTOFI, F.K. (1973). In: *Histological typing of Urinary Bladder Tumours, International Histological classification of Tumours*. No. 10, World Health Organization (WHO), Geneva.
3. OOMS, E.C.M., ANDERSON, W.A.D., ALONS, C.L., BOON, M.E., VELDHUIZEN, R.W. (1983). Analysis of the performance of pathologists in the grading of bladder tumors. *Hum. Pathol.*, 14, 140-143.
4. BLOMJOUS, C.E.M., SMEULDERS, A.W.M., BAAK, J.P.A., VOS, W., VAN GALEN, E.M., MEIJER, C.J.L.M. (1989). A comparative study in morphometric grading of transitional cell carcinoma of the urinary bladder. *Anal. quant. cytol. histol.*, 11, 426-432.
5. REUTER, V.E. (1990). Pathology of Bladder Cancer: Assessment of Prognostic Variables and Response to therapy. *Seminars in Oncology*, 17, no 5, 524-532.
6. BRODERS, A.C. (1922). Epithelioma of the genito-urinary organs. *Annals of Surgery*, 75, 574-580.
7. BERGKVIST, A., LJUNGQVIST, G., MOBERGER, G. (1965). Classification of bladder tumours based on the cellular Pattern. Preliminary report of a clinical-pathological study of 300 cases with a minimum follow-up of eight years. *Acta Chir. Scand.*, 130, 371-378.
8. PUGH, R.C.B. (1973). The pathology of cancer of the bladder. An editorial overview. *Cancer*, 32, 1267-1274.
9. KOSS, L.G. (1975). Tumours of the urinary bladder. In: *Atlas of Tumour Pathology*, 2nd series, Fascicle 11, pp. 20-43. Washington DC: Armed Forces Institute of Pathology.
10. FRIEDELL, G.H., BELL, J.R., BURNEY, S.W., SOTO, E.A., TILTMAN, A.J. (1976). Histopathology and classification of urinary bladder carcinoma. *Urol. Clinics North America*, 3, 53-70.
11. JORDAN, A.M., WEINGARTEN, J., MURPHY, W.M. (1987). Transitional cell neoplasms of the urinary bladder. *Cancer*, 60, 2766-2774.
13. LIPPONEN, P.K., COLLAN, Y., ESKELINEN, M.J., PESONEN, E., SOTARAUTA, M., NORDLING, S. (1990). Comparison of morphometry and DNA flow cytometry with Standard Prognostic Factors in Bladder Cancer. *Br. J. Urol.*, 65, 589-597.
14. HENDRY W.F., RAWSON N.S.B., TURNEY L., DUNLOP A., WHITFIELD H.N., (1990). Computerisation of Urothelial Carcinoma Records: 16 Years Experience with the TNM System. *Br. J. Urol.*, 65, 583- 588.
15. VAN DIEST, P.J., BAAK, J.P.A., MATZE-COK, P., WISSE- BREKELMANS, E.C.M., VAN GALEN, C.M., KURVER, P.H.J., BELLOT, S.M., FIJNHEER, J., VAN GORP, L.H.M., KWEE, W.S., LOS, J., PETERSE, J.L., RUITENBERG, H.M., SCHAPERS, R.F.M., SCHIPPER, M.E.I., SOMSEN, J.G., WILLIG, A.W.P.M., ARIENS, A.TH. (1992). Reproducibility of mitosis counting in 2, 469 breast cancer specimens: Results from the multicenter morphometric mammary carcinoma project. *Hum. Pathol.*, 23, 603-607.
16. BAAK, J.P.A., OORT, J. (1983). Obtaining Quantitative Data. In: *A manual of morphometry in diagnostic pathology*. Chapter 3, pp. 18-20. New York: Springer-Verlag.
17. KAPLAN, E.L., MEIER, P. (1958). Non parametric estimation from incomplete observation. *J. Am. Stat. Assoc.*, 53, 457-481.
18. PETO, R., PIKE, M.C., ARMITAGE, P., BRESLOW, N.E., COX, D.R., HOWARD, S.V., MANTEL, N., MCPHERSON, K., PETO, J., SMITH, G. (1977). Design and analysis of randomised clinical trials requiring prolonged observation of each patient: II. Analysis and examples. *Br. J. Cancer*, 35, 1-39.
19. HAKULINEN, T., ABEYWICKRAMA, K.H. (1985). A computer program package for relative survivalanalysis. *Comput. Programs Biomed.*, 19, 197-207.
20. COX, D.R. (1972). Regression models and live tables. *J. Roy. Stat. Soc.*, 34, 187-220.

21. COHEN, J. (1960). A coefficient of agreement for nominal scales. *Educational and Psychological measurement*, 20, 37-46.
22. FLEISS, J.L. (1981). In: *Statistical methods for rates and proportions*, Second edition. New York: J. Wiley and S..

CHAPTER 3

PROGNOSTIC SIGNIFICANCE OF TYPE IV COLLAGEN AND LAMININ IMMUNOREACTIVITY IN UROTHELIAL CARCINOMAS OF THE BLADDER

R. F. M. SCHAPERS
R. P. E. PAUWELS
M. G. HAVENITH
A. W. G. B. SMEETS
P. A. van den BRANDT
F. T. BOSMAN

From the Departments of Pathology[RFMS]and Urology[RPEP], St. Ziekenhuizen Venlo-Venray, Venlo: the Departments of Epidemiology[PAvdB] and Pathology[MGH, FTB], University of Limburg, Maastricht: the Department of Clinical Pharmacy and Laboratory[AWGBS], Venray, the Netherlands.

Cancer, 66, 2583-2588, 1990

3.1 ABSTRACT

Invasion of a carcinoma involves the degradation and penetration of the subepithelial basement membrane (BM). This phenomenon might be used for histopathological evaluation of neoplasms of the bladder. The authors studied the clinicopathological data and tissue specimens of 125 cases of urothelial carcinomas, collected prospectively. Penetration of the BM was evaluated by immunohistochemical staining of the BM components laminin and type IV collagen. The use of this parameter as a prognostic indicator in bladder cancer was assessed. The 5-year survival rate of patients having tumours with an interrupted or absent BM was significantly lower than that of patients having tumours with an intact BM. The rate of progression was greater in tumours with an interrupted or absent BM than in tumours with an intact BM. No association was found between BM status and recurrence. However, a significant correlation between tumour stage and BM staining was found. A correlation was also found between ploidy and BM staining, as well as between histological grade and BM staining pattern.
When evaluating histological grade, stage, ploidy, age and BM score as prognostic parameters, the stage of bladder carcinomas turned out to be the most important factor in predicting the survival rate and the progression free survival. However, BM staining was found to be of value for early identification of microinvasion and is as such helpful for correct staging of urothelial carcinomas.

3.2 INTRODUCTION

The rapidly increasing knowledge in molecular biology and the new possibilities of biotechnology have opened new opportunities for the development of new approaches towards diagnosis and classification of neoplastic disease. Tumour invasion is an area that has benefitted remarkably from these developments. Invasive growth of a carcinoma is defined as the penetration of malignant neoplastic cells through a basement membrane (BM). In this view the BM serves as an important structural barrier to progression of the neoplasm. To infiltrate and metastasize an epithelial neoplasm has to penetrate one or more BM layers. BM degrading proteases such as type IV collagenase play an important part in this process.[1, 2] Recent studies indicate that BM are not static structures destined only for destruction, but they can also be deposited in tumour tissue.
The study of BM morphologic features in invasive cancer is not new. Earlier attempts to outline BM in breast cancer by periodic acid-Schiff (PAS) staining were frustrated by the occurrence of PAS-reactive glycoproteins not only in BM but also in interstitial connective tissue.[3] Immunohistochemical detection of BM components appears to be a more suitable approach towards the study

of invasive growth. Visualization of the BM by immunohistochemical techniques with BM-specific antibodies is specific and provides very high resolution. This technique is reliable and reproducible and now forms one of the essential tools in the study of the role of the BM in neoplasia. Both laminin and type IV collagen have been used as markers of BM in tumours.[4-7]
There are only a few reports published concerning BM staining in bladder carcinomas.[8, 9] In one study a significant correlation was seen between BM continuity or discontinuity and progression ($P = 0.036$).[8] In the other study bladder cancer patients with intact BM showed longer survival than patients with BM discontinuities ($P < 0.001$).[9] We studied BM expression in bladder carcinomas to evaluate its use for the histopathological identification of microinvasion. Furthermore this study was performed to assess the usefulness of BM staining in comparison with grading, staging and ploidy for the prediction of the biological behaviour of bladder tumours.

3.3 MATERIALS AND METHODS

The Department of Urology of the St. Maartens Gasthuis (Venlo, the Netherlands) provides a regional service for the Northern Limburg area. All patients diagnosed with primary bladder carcinoma between january 1979 and december 1988 were included in this study (n=140).
Clinicopathological information and transurethral resection specimens were collected prospectively. Staging was carried out according to the TNM system[10] and grading into two groups, low and high-grade, according to our criteria described elsewhere.[11] Complete follow-up data up to december 1989 or until the time of death were obtained for all patients. The median follow-up was 21 months (minimum, 1 month; maximum, 109 months). Follow-up was conducted at least semi-annually by cystoscopy.
Of the 125 evaluable cases 60 patients underwent only transurethral resection of the tumour and 34 patients received adjuvant intravesical therapy. The other 31 patients did receive radiotherapy, cystectomy or a combination of both.
As follow-up criteria we used survival, progression and recurrence. Progression and recurrence were assessed by pathologists without knowledge of any previous clinical data.

3.3.1 Immunohistochemistry

Routinely formalin fixed (10% formaldehyde in phosphate buffered-saline[PBS]) and paraffin embedded tissue blocks of the transurethral resections were sectioned at 4 µm. Sections were deparaffinized, rehydrated and pretreated with pepsin (Sigma Chemical Co., St. Louis, MO) (0.1% in 0, 1 Normal[N] hydrochloric acid[HCL] for 30 minutes at room temperature) to

enhance immunoreactivity. Endogenous peroxidase was blocked in 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min. After washing in PBS (3x5 min) the sections were incubated with polyclonal anti type IV collagen antiserum (diluted 1: 3000 in PBS with 1% bovine serum albumin[BSA]) and polyclonal anti laminin antiserum (diluted 1: 100 in PBS with 1% BSA) overnight at 4° C in a moist chamber. Both primary antisera were raised in rabbits. Their immunospecificity was documented previously.[12, 13]
After washing in PBS the sections were incubated with peroxidase- labelled swine anti-rabbit Ig antibodies (Dako, Copenhagen, Denmark) (diluted 1: 40 in PBS with 1% BSA) for 30 minutes at room temperature. After final washing with PBS a diaminobenzidin H_2O_2 substrate was used to visualize the immunoreactivity. In 125 cases satisfactory immunohistochemistry was accomplished, which was assessed using blood vessels as internal control. For statistical evaluation the immunoreactivity of BM at the tumour/stromal interface was scored semiquantitatively into two patterns as follows: (1) tumours with intact BM; and (2) tumours with patchy or absent BM.

3.3.2 Chromosomal analysis

For microscopic analysis of chromosomes, tumour samples were collected in 0.5 % sodium citrate with 0.5 μg/ml colcemid. After incubation for 1 hour at room temperature, the tissue was mechanically desegregated in 5 ml Hanks balanced sodium solution (Hanks BSS). Afterwards a solution of 19 ml Hanks BSS and 6 ml colcemid was added. After incubation for 30 min at 37°C, hypotonic treatment in a solution of 6 ml fetal calf serum (FCS) and 24 ml 0.052 mol/l potassium chloride was followed by fixation in methanol-acetic acid 7: 3. Chromosomes were routinely stained with Giemsa. This direct method has been described elsewhere in detail.[14]
Analyzable metaphases, at least five (mean 28) per case were photographed and underwent karyotyping according to the Paris nomenclature.[15] The tumours were classified according to their modal chromosome number and chromosome range. The latter classification distinguishes the tumours with diploid or hypodiploid (46 chromosomes or less) cells from those with hyperdiploid (more than 46 chromosomes) cells.

3.3.3 Statistical analysis

Prognostic factors that were included in the analyses, besides BM pattern, were as follows: age, sex, T category (stage), histological grade, ploidy and therapy.
Kaplan-Meier survival curves[16] for time to death, progression and recurrence were analysed by the log-rank test.[17] The association between the various prognostic parameters was analysed using a chi square-test. When a significant difference between the groups with a different pattern of BM deposi-

tion occurred, a multivariate analysis using Cox proportional hazards model[18] was executed, to determine the prognostic value of BM continuity after correction for the mentioned extraneous prognostic factors. All statistical analysis were conducted with the BMDP statistical package (BMDP statistical software Inc, Los Angeles, CA).

3.4 RESULTS

3.4.1 Immunohistochemical findings

Paraffin sections of 140 transitional cell carcinomas of the bladder were stained. Both antisera against type IV collagen and laminin showed strong staining of blood vessels and around muscle fascicles. Only when these internal controls stained properly the immunostaining was considered to be appropriate. In most cases laminin staining was somewhat weaker than staining with type IV collagen. In 125 cases reliable immunohistochemical evaluation could be performed. Cases with unreliable immunostaining were found to be evenly distributed over low- and high-grade malignant bladder carcinoma categories.

3.4.2 Clinicopathological data

Of the 125 lesions that could be analysed, 52 showed almost intact BM and 73 lesions showed patchy or absent BM (Figs. 1, 2 and 3). In these tumours the tumour stage could not be established with certainty in the HE stained sections in eight of 125 cases (6%). Of 46 tumours with intact BM staining 38 were non infiltrating in the H & E stained sections (82.6%). Of 71 tumours with patchy or absent BM staining 58 were infiltrating in H & E-stained sections (81.7%). A strong correlation was observed between T-category and BM staining (chi-square=48.6, $P < 0.0001$) (Table 1), as well as between grade

Table 1. BM expression versus stage of infiltration (T)

BM	Stage			
	Ta	T1	T2/T3	Total
BM I	38	8	0	46
BM II	13	39	19	71
Total	51	47	19	117

BM I=intact basement membrane; BM II=interrupted or absent basement membrane; chi-square=48.6, $P < 0.0001$.

Table 2.. BM expression versus tumour grade.

BM	Histological Grade		
	Low-grade	High-grade	Total
BM I	29	24	53
BM II	8	64	72
Total	37	88	125

BM I=intact basement membrane; BM II=interrupted or absent basement membrane; chi-square=28.8, $P < 0.0001$

Table 3. BM expression versus chromosomal numbers.

BM	Chromosomal number		
	Diploid	Hyperdiploid	Total
BM I	20	19	39
BM II	4	57	61
Total	24	76	100

BM I=intact basement membrane; BM II=interrupted or absent basement membrane; chi-square=27.9, $P < 0.0001$

and the BM staining pattern (chi-square=28.8, $P < 0.0001$). Of 37 low-grade lesions 29 showed intact BM (78.4%), whereas of 87 high-grade lesions only 24 showed intact BM (27.5%) (Table 2). A correlation was also found between ploidy and BM staining (chi-square=27.9, $P < 0.0001$). In 100 out of 125 cases chromosomal analysis could be successfully performed. Of the 24 diploid tumours 20 showed intact BM (83.3%), of the 76 hyperdiploid tumours 19 showed intact BM (25.0%) (Table 3).

The eight cases in which the stage could not be established with certainty were all of high-grade malignancy. In seven of them chromosomal analysis was performed. Of these cases six were hyperdiploid and one was diploid. With BM staining it was possible to establish the correct stage in all eight cases. Microinvasive growth was seen in four tumours while the other four were non-invasive. Of the 4 patients with infiltrating tumour, one showed progression and eventually died of metastatic disease after 14 months. Another of these patients died of unrelated disease after two months. One of the patients with a non-infiltrating tumour died after 30 months without any

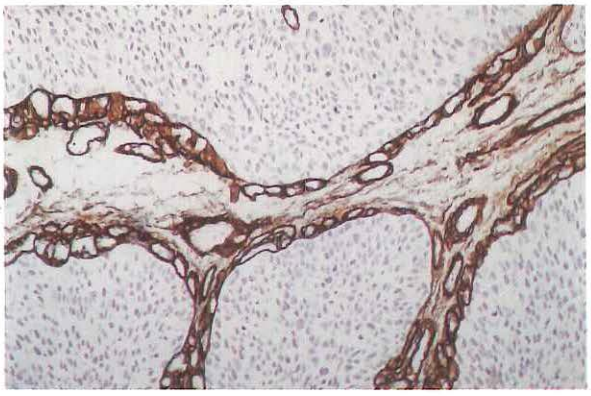

Figure 1. Transitional cell carcinoma with continuous BM staining.

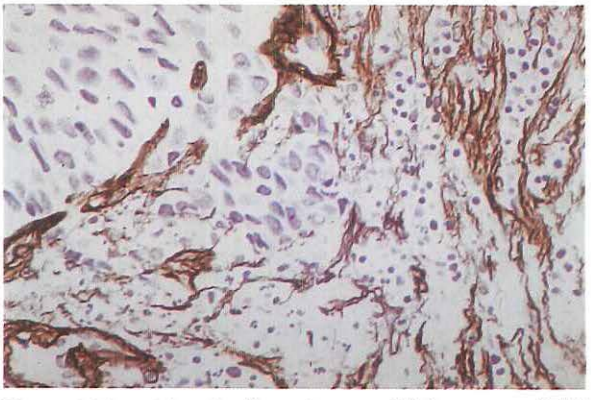

Figure 2. Transitional cell carcinoma with interrupted BM staining and micro-invasive growth in the lamina propria.

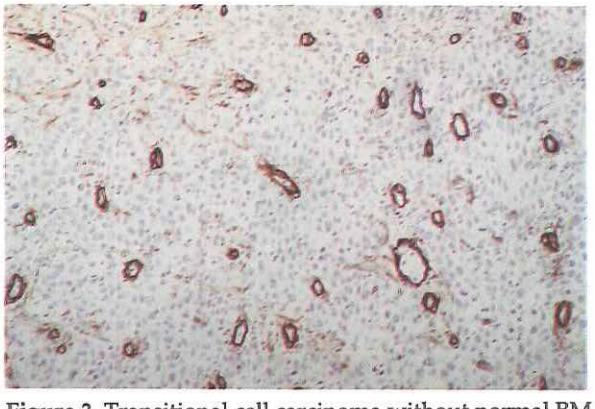

Figure 3. Transitional cell carcinoma without normal BM staining.

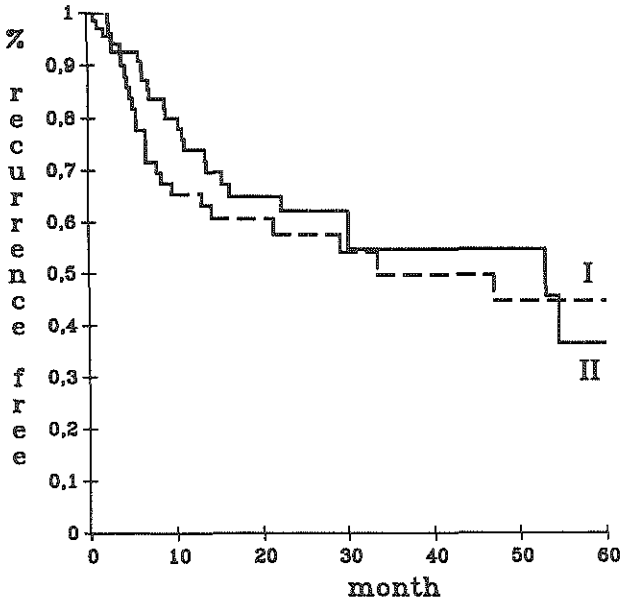

Figure 4. Recurrence free-survival. I=intact BM (n=52), II=patchy or absent BM (n=53). (Log rank test chi-square=0.350, P = 0.5542). 5 year recurrence free survival: BM I=44.7% (se 8.8), BM II=36.5% (se 11.8)

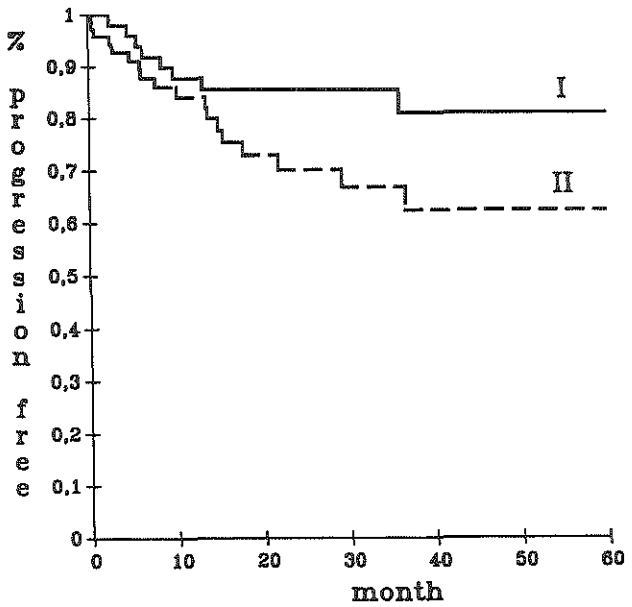

Figure 5. Progression-free survival. I=intact BM (n=52), II=patchy or absent BM (n=73). (Log rank test chi-square=3.906, P = 0.0481). 5 year progression-free survival: BM I=81.0% (se 6.5), BM II=62.3% (se 7.9)

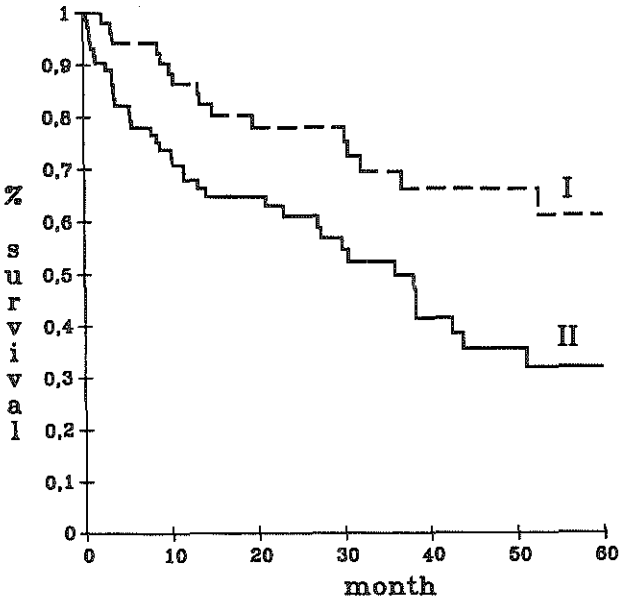

Figure 6. Survival data of intact versus patchy or absent BM expression. I=Intact BM (n=52), II=patchy or absent BM (n=73).
(Log-rank test chi-square=8.45, $P = 0.0037$). 5 year survival: BM I=61.1 (se8.4), BM II=32.0 (se 7.3).

sign of bladder tumour. All other five patients are alive without disease. The median follow-up for these 8 cases was 23 months (minimum, 2 months; maximum, 49 months).

The association between BM staining and the subsequent clinical course was further assessed. The BM staining pattern proved to be of no value for the prediction of recurrence-free survival (Fig 4). There was, however, a borderline significant correlation between BM staining pattern and progression-free survival (log-rank chi-square=3.906, $P = 0.048$). As is shown on actuarial survival curves (Fig.5) patients with tumours with intact BM showed less progression than patients with tumours with patchy or absent BM.

We also found a significant correlation between survival and BM staining patterns (log-rank chi-square=8.45, $P = 0.004$). Patients with tumours with intact BM showed a highly significant longer survival than those with tumours with interrupted or absent BM (Fig. 6).

In view of the effects on survival and progression-free survival, we studied the interrelation between different variables using Cox proportional hazards model.[16] We first calculated the univariate prognostic value for survival and progression-free survival regarding age, grade, stage, ploidy and therapy. Age did not influence the correlation between BM staining pattern and survival parameters. Stage, however, largely eliminated the value of BM expres-

sion as a prognostic indicator. Also, the other variables, such as grade, ploidy and therapy did not show a significant independent correlation with survival and progression-free survival in addition to tumour stage.
In the group of high-grade tumours (n=87) 26% showed intact and 74% showed patchy or absent BM staining. A slight difference in survival was observed, which appeared not to be statistically significant (chi-square=2.234, $P = 0.135$). The same was observed for progression-free survival (chi-square=1.533, $P = 0.216$).
When we evaluated the hyperdiploid group (tumours with more than 46 chromosomes per cell), 19 out of 76 tumours (25.0%) showed intact BM, while 57 tumours showed patchy or absent BM (75.0%). Between these groups we found no differences in survival or progression-free survival. A slight, but not significant difference in survival was seen between the two BM patterns in stage Ta (chi-square=1.754, $P = 0.1854$). In stage T1 tumours BM pattern did not correlate with survival. Likewise, the BM pattern did not correlate with progression-free survival in Ta or in T1 tumours.

3.5 DISCUSSION

In carcinomas, a dynamic interaction occurs at the interface between tumour cells and the surrounding mesenchymal stroma. Collagenases, including type IV-specific collagenase and other proteases, such as plasminogen activators, cathepsins and heparanases form a cascade system of enzymes facilitating extra cellular matrix breakdown.[1, 19, 20] Conversely, as a host reaction to the invading neoplasm, extracellular matrix components, including BM material and interstitial collagen, may also be deposited around tumour cells.[21, 22] A desmoplastic stromal reaction around tumour cells, involving myofibroblasts, might play a role in BM deposition at the tumour stromal interface. Numerous immunohistochemical studies have documented loss of continuity of BM in many different malignant neoplasms.[1, 5-9, 23-25]
The initial working hypothesis of these studies was that in benign (noninvasive) neoplasms an intact BM would be found, whereas in invasive malignant neoplasms, the BM would be interrupted or absent. This has proven to be an oversimplification, as also reflected in the results we obtained. On one hand some noninvasive bladder tumours showed discontinuities in the BM, whereas on the other hand some invasive bladder carcinomas showed intact BM. We then argued that a positive balance between deposition and breakdown of BM components, resulting in intact BM, could be a sign of competent host response to the neoplasm. This might correlate with a better prognosis.
In the present study a direct correlation between T category and BM loss was observed. A proportion of the Ta lesions (intraepithelial tumours) showed incomplete BM, suggesting that although invasion had not yet occurred, there was already significant discontinuity of the BM barrier. Conversely some of

the T1 lesions (tumours with invasion into the lamina propria) showed intact BM, reflecting the production of BM components even in invasive carcinomas. Conn et al. reported similar observations in their study on bladder carcinoma.[8] Daher et al. reported intact as well as interrupted BM in T1 tumours.[9] In their study no information was presented concerning Ta tumours.

Long-term follow-up studies are necessary to evaluate the biological significance of these observations. Statistical evaluation of clinical follow-up data in our study resulted in a highly significant correlation between BM deposition pattern and survival. Furthermore a borderline significant correlation between progression-free survival and BM deposition pattern was found. According to both survival parameters patients with tumours with intact BM had a better prognosis than patients with tumours with patchy or absent BM. No correlation was found with the recurrence- free survival, the mean number of recurrences per year and the recurrence rate.

These results are in concordance with the literature in regard of recurrence-free survival and progression-free survival.[8] As to survival, the only comparable data are found in the study of Daher at al..[9] They documented a significant difference in short-term survival between their two groups of BM staining pattern, which did not include Ta tumours.

Our material was also analysed for a number of additional parameters. For each parameter the prognostic value was calculated. All parameters with a significant correlation with survival or progression-free survival were further tested by a multivariate analysis. In the Cox proportional hazard model the additional value of BM deposition pattern was calculated after correction for other parameters. The following variables were found to have prognostic value: age, stage of the tumour, histological grade and ploidy. We found that tumour stage was the most important independent prognostic parameter for survival (Log-rank test chi-square=21.6, $P < 0.0001$) and progression-free survival (Log-rank test chi-square=9.88, $P = 0.0017$). The other variables did not add essential additional information.

In contrast with earlier studies, [8, 9] our results document that as a prognostic indicator BM staining is of limited additional value in comparison with grade and stage. Nevertheless, BM staining is of practical value for correct staging of bladder carcinomas because in 8 of 125 cases (6%), the tumour stage could not be established with certainty in the H & E stained sections. Basement membrane staining, which can be performed on routinely fixed and embedded material, facilitates the assessment of microinvasive growth of bladder cancer, which may be overlooked or only suspected in the H & E stained sections. As such this rather simple technique is indirectly important for prediction of survival and progression-free survival, because it helps to establish the correct stage of the neoplasm.

3.6 REFERENCES

1. BARSKY S.H., TOGO S, GARBISA S., LIOTTA L.A., (1983). Type IV collagenase immunoreactivity in invasive breast carcinoma. *Lancet*, 1, 296-297.
2. LIOTTA L.A., TRYGGVASON K., GARBISA S., HART I., FOLTZ C.M., SHAFIE S., (1980). Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature*, 284, 67-68.
3. OZELLO, L., SPEER, F.D. (1958). The mucopolysaccharides in normal and diseased breast: Their distribution and significance. *Am. J. Clin. Pathol.*, 34, 993-1009.
4. BARSKY, S.H., SEIGAL, G.P., JANNOTTA, F., LIOTTA, L.A. (1983). Loss of basement membrane components by invasive tumours but not by their benign counterparts. *Lab Invest*, 49, 140-147.
5. BOSMAN, F.T., HAVENITH, M.G., CLEUTJENS, J.P.M. (1985). Basement membranes in cancer. *Ultrastruct. Pathol.*, 8, 291- 304.
6. VISSER, R., VAN DER BEEK, J.M.H., HAVENITH, M.G., CLEUTJENS, J.P.M., BOSMAN, F.T. (1986). Immunocytochemical detection of basement membrane antigens in the histopathological evaluation of laryngeal dysplasia and neoplasia. *Histopathology*, 10, 171- 180.
7. HAVNITH, M.G., ARENDS, J.W., SIMON, R.E.M., VOLOVICS, A., WIGGERS, T., BOSMAN, F.T. (1988). Type IV collagen immunoreactivity in colorectal cancer: Prognostic value of basement membrane deposition. *Cancer*, 62, 2207-2211.
8. CONN, I.G., CROCKER, J., WALLACE, D.M.A., HUGHES, M.A., HILTON, C.J. (1987). Basement membranes in urothelial carcinoma. *Br. J. Urol.*, 60, 536-542.
9. DAHER, N., ABOURACHID, H., BOVE, N., PETIT, J., BURTIN, P. (1987). Collagen IV staining pattern in bladder carcinomas: relationship to prognosis. *Br. J. Cancer*, 55, 665-671.
10. HERMANEK, B., SOBIN, L. H. (1987). In: *T.N.M. Classification of Malignant Tumours*, Union International Contre le Cancer (UICC), ed. 4, pp. 121-144. New York: Springer-Verlag.
11. PAUWELS, R.P.E., SCHAPERS, R.F.M., SMEETS, A.W.G.B., DEBRUYNE, F.M.J., GERAEDTS, J.P.M. (1988). Grading of superficial bladder cancer. *Br. J. Urol.*, 61, 129-134.
12. HAVENITH, M.G., CLEUTJENS, J.P.M., BEEK, C., V.D.LINDEN, E., DE GOEIJ, A.F.P.M., BOSMAN, F.T. (1987). Human specific anti- type IV collagen monoclonal antibodies, characterization and immuno histochemical application. *Histochemistry*, 87, 123-128.
13. HAVENITH, M.G., DINGEMANS, K.P., CLEUTJENS, J.P.M., WAGENAAR, SJ.SC., BOSMAN, F.T. (1989). Basementmembranes in bronchogenic squamous cell carcinoma: An immunohistochemical and ultrastructural study. *Ultrastruct.Pathol.*, 14, 51-63.
14. SMEETS, W., PAUWELS, R., LAARAKKERS, L., DEBRUYNE, F., GERAEDTS, J. (1987). Chromosomal analysis of bladder cancer III. Nonrandom alterations. *Cancer Genet. Cytogenet.*, 29, 29- 41.
15. ISCN (1978). An international system for human cytogenetic nomenclature. In: *Birth Defects*: Original Article Series, 14, 8 (The International Foundation, New York): also in: *Cytogenetic Cell Genetic* (1978), 21, 309-404.
16. KAPLAN, E.L., MEIER, P. (1958). Non parametric estimation from incomplete observation. *J. Am. Stat. Assoc.*, 53, 457- 481.
17. PETO, R., PIKE, M.C., ARMITAGE, P., BRESLOW, N.E., COX, D.R., HOWARD, S.V., MANTEL, N., MCPHERSON, K., PETO, J., SMITH, G. (1977). Design and analysis of randomised clinical trials requiring prolonged observation of each patient: II. Analysis and examples. *Br. J. Cancer*, 35, 1-39.
18. COX, D.R. (1972). Regression models and live tables. *J. Roy. Stat. Soc.*, 34, 187-220.
19. THORGEIRSSON U.P., TURPEENNIEMI-HUJANEN T, LIOTTA, L.A. (1985). Cancer cells, components of basement membranes and proteolytic enzymes. *Int. Rev. Exp. Pathol.*, 27, 203-234.
20. LIOTTA L.A., RAO N., WEWER U.M., (1986). Biochemical interactions of tumour cells with the basement membrane. *Ann. Rev. Biochem.*, 55, 1037-1057.

21. BARSKY, S.H., GOPALAKRISHNA, R. (1987). Increased invasion and spontaneous metastasis of BL6 melanoma with inhibition of the desmoplastic response in C57BL/6 mice. *Cancer Res.*, 47, 1663- 1667.
22. LAGACE, R., GRIMAUD, J.A., SCHURCH, W., SEEMAYER, T.A. (1985). Myofibroblastic stromal reaction in carcinoma of the breast: Variations of collagenous matrix and structural glycoproteins. *Virchow Arch. (Pathol. Anat.)*, 408, 49-59.
23. ALBRECHTSEN, R., NIELSEN, M., WEWER, U., ENGVALL, E., RUOSLAHTI, E. (1981). Basement membrane changes in breast cancer detected by immunohistochemical staining for laminin. *Cancer Res.*, 41, 5076-5081.
24. SIEGAL, G.P., BARSKY, S.H., TERRANOVA, V.P., LIOTTA, L.A. (1981). Stages of neoplastic transformation of human breast tissue as monitored by dissolution of basement membrane components. *Invasion Metastasis*, 1, 54-70.
25. WILLEBRAND, D., BOSMAN, F.T., DE GOEY, A.T.P.M. (1986). Patterns of basement membrane deposition in benign and malignant breast tumours. *Histopathogy*, 10, 1231-1241.

CHAPTER 4

MORPHOMETRIC GRADING OF TRANSITIONAL CELL CARCINOMA OF THE URINARY BLADDER

RENE F. M. SCHAPERS
RUUD P. E. PAUWELS
JACK Th. M. WIJNEN
A. WIM G. B. SMEETS
FRE T. BOSMAN

From the Departments of Pathology[RFMS] and Urology[RPEP], St. Ziekenhuizen Venlo-Venray, Venlo: the Department of Mathematics and Computing Science[JThMW], Eindhoven University of Technology: the Department of Clinical Pharmacy and Laboratory[AWGBS], Venray: the Department of Pathology[FTB], Erasmus University, Rotterdam, the Netherlands.

J. Urol., Pathol. (Accepted for publication)

4.1 ABSTRACT

Of 294 patients with primary transitional cell carcinoma (TCC) of the urinary bladder, tumour samples were studied by selective nuclear morphometry as a quantitative measure of nuclear grade. We found a strong association with the "classic" parameters stage, grade and mitotic index. In WHO grade II tumours morphometric heterogeneity could be demonstrated. There was a significant difference in survival between morphometrically determined low-grade malignant cases and high-grade malignant cases, the presence of large nuclei (>95μm^2) indicating poor prognosis. The morphometrically determined grade was also found to be a strong predictor of tumour progression. In multivariate analysis, combining histopathological and morphometric factors, only pathological stage and mitotic index proved to be independent predictive factors.
It is concluded that grading by means of morphometry, as a single factor, is correlated with survival and tumour progression and therefore this parameter is useful in addition to the classical parameters stage and grade, especially in WHO grade II TCC.

4.2 INTRODUCTION

The urinary bladder is the site of origin of a variety of benign and malignant neoplasms. The most important group of tumours consists of epithelial tumours of transitional cell type. One of the most keenly awaited advances in the management of bladder cancer is a means of assessing the biological potential of a tumour at, or soon after, the patients initial presentation. At present the decision on treatment of bladder tumours is based on the TNM classification, [1] with an emphasis on staging and on histological grading using the WHO system.[2] However in the WHO grading system a sharp demarcation between different grades cannot be attained. This results in considerable interobserver variability and therefore poor agreement between diagnoses made by different pathologists.[3, 4] Unfortunately, also staging has its problems. The pathologist's interpretation of infiltration in the Ta and T1 group of tumours is sometimes inconsistent and not always reproducible, [5] notably when the biopsy does not contain all layers of the bladder wall.
Therefore, other potential predictors of disease evolution have been searched for. Flow cytometry, [6-9] image cytometry, [10] mitotic activity[11] and nuclear morphometry[4, 12, 13] have been used to grade bladder cancer, with promising results. The purpose of the present study was to determine the extent to which the biological potential of TCC, in terms of survival and progression-free survival, can be predicted by morphometrical grading of the primary tumour in a two grade system, especially when compared and combined with other prognostic factors like stage, grade, mitotic index and age.

4.3 MATERIALS AND METHODS

This study was based on clinical follow-up of 294 consecutive patients with TCC of the bladder. These were all primary tumours diagnosed between 1979 and 1991. Of these patients 53 (18%) were female (mean age 70 years) and 241 (82%) were male (mean age 66 years). These patients were treated and followed up during 1979-1992. The median follow-up was 46 months, with a maximum of 163 months (mean follow-up 54 months). Follow-up examinations were conducted at least semi-annually and included cystoscopy and urinary cytology. As follow-up criteria survival and progression-free interval were chosen. Progression was defined as increase in stage or death due to tumour. If a patient was lost to follow-up, survival was assessed by contacting municipal population registries or family doctors.
Staging was carried out according to the UICC criteria[1] and grading according to the WHO criteria.[2]
We furthermore estimated the mitotic index, using the counting method as described elsewhere.[14] We counted 10 fields using a x10 ocular and a x40 objective with a numerical aperture of 0.75 and a field width of 450 μm (counts/10 hpf). The criteria described by Baak were used to identify mitotic figures.[15]

4.3.1 Morphometry

For morphometric measurements we used the "VIDEOPLAN" image analyzer (Kontron, Munich, Germany). The most atypical fields were selected in approximately 5 low power fields and subsequently 10 nuclei were selected on account of their large size. This method has shown satisfactory intra- and interobserver reproducibility.[4] The patient group was subdivided into two groups on account of the mean nuclear area. The low-grade malignant group had small nuclei (mean nuclear area $\leq 95\mu m^2$) and the high-grade malignant group had large nuclei ($>95\mu m^2$).

4.3.2 Statistical analysis

Prognostic factors that were included in the analysis were: Morphometric grading (low-grade; high-grade), WHO grading (grade I; II; III), T category (stage Ta; T1; T2 or more), mitotic index (5 or less mitoses/10 hpf; more than 5 mitoses/10 hpf), age (70 or less years; more than 70 years) and sex. For each prognostic factor Kaplan Meier survival curves[16] were analysed by means of the log-rank test[17] with regard to time interval to death and time interval to establishment of progression. A possible association between various prognostic factors was analysed using the Pearson chi- square test.
Furthermore, multivariate survival analyses were performed using Cox proportional hazards model.[18] In these models, low/high grading was in-

cluded together with the mentioned other factors. The stepwise method was used to select the model, with use of the likelihood ratio test. A *P*-value of 0.05 was adopted as limit for entering and removing covariates. Of the prognostic factors, that contributed significantly to the model, the effect was calculated in terms of relative risk and the associated 95% confidence interval. The statistical analyses were performed with the SAS statistical package (SAS Inst Inc, Cary, North Carolina, USA).

4.4 RESULTS

According to the morphometric values 108 TCC were classified as low-grade malignant and 186 TCC as high-grade.
Strong association was observed between morphometric grades, WHO grades, stage and mitotic index ($P < 0.0001$) (Table 1).

4.4.1 survival

The WHO grade II tumours could be divided by morphometry into two groups with significantly different survival ($P = 0.02$) (fig. 1).
For morphometric grading we found a significant difference in survival ($P < 0.0001$) (fig. 2). Of the 108 patients with morphometric low-grade tumours, 5 died of tumour related disease and 31 died of unrelated causes of death. Of the 186 patients with high-grade tumours 44 died of tumour related disease and 65 died of unrelated causes of death. Other strong predictors of survival were stage ($P < 0.0001$) (fig. 3), age ($P < 0.0001$) and mitotic index ($P < 0.0001$). In multivariate analysis, stage ($P < 0.0001$) was the most important factor. The other factors (except age) did not contribute significantly to the prediction.

4.4.2 progression

The WHO grade II tumours could be separated by morphometry into two groups with a significantly different tendency for progression ($P = 0.0115$) (fig. 4).
We found a significant association between morphometric grades and progression ($P < 0.0001$) (fig. 5). Other strong predictors of progression were stage ($P < 0.0001$) (fig. 6), mitotic index ($P < 0.0001$) and WHO grades ($P < 0.0001$).
In the multivariate analysis stage was again the most important prognostic parameter in predicting progression ($P < 0.0001$) next to the mitotic index ($P = 0.0022$). The other variables did not show significant independent predictive value.

Table 1. Association between morphometric grading, WHO-grading, stage and mitotic index.

		morphometric grading		
		LOW	HIGH	Total
WHO grading	I	54	14	68
	II	54	132	186
	III	1	39	40
Stage	Ta	89	59	148
	T1	18	77	95
	T2+	2	49	51
Mitotic index	≤ 5	87	69	156
	>5	22	116	138
Total		109	185	294

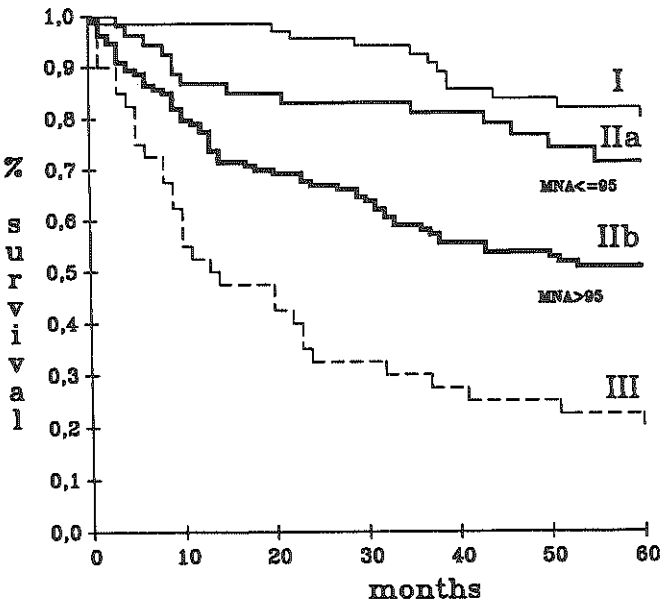

Figure 1. Overall survival according to WHO grades. WHO grade II tumours are devided into two different prognostic subgroups (IIa and IIb) on account of the mean nuclear area; I=WHO (n=68); II= WHO II (n=186); III= WHO III (n=40); 5 year survival: I=79.9% (95% CI, 69.7-90.2), IIa=71.3% (95% CI, 58.3-84.3)(n=53), IIb=50.9% (95% CI, 42.1-59.6)(n=133), III=19.7% (95% CI, 7.2-32.1).

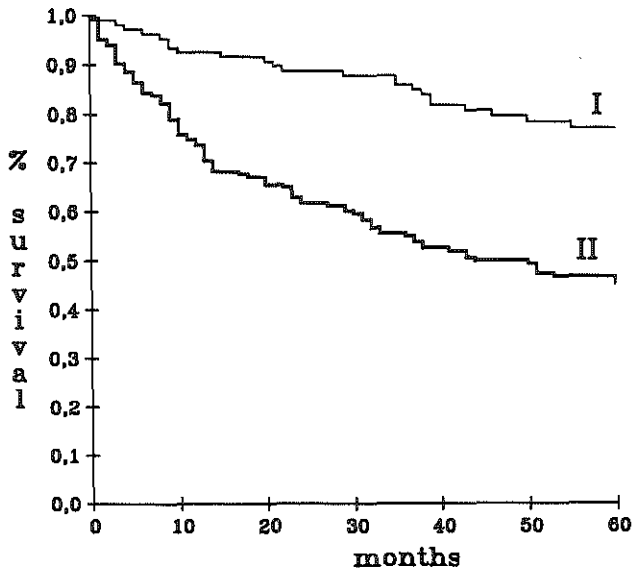

Figure 2. Overall survival according to morphometric grades. I=low-grade (n=108), II=high-grade (n=186). 5 year survival: I=77.1% (95% CI, 68.7-85.4), II=45.2% (95% CI, 37.8-52.7).

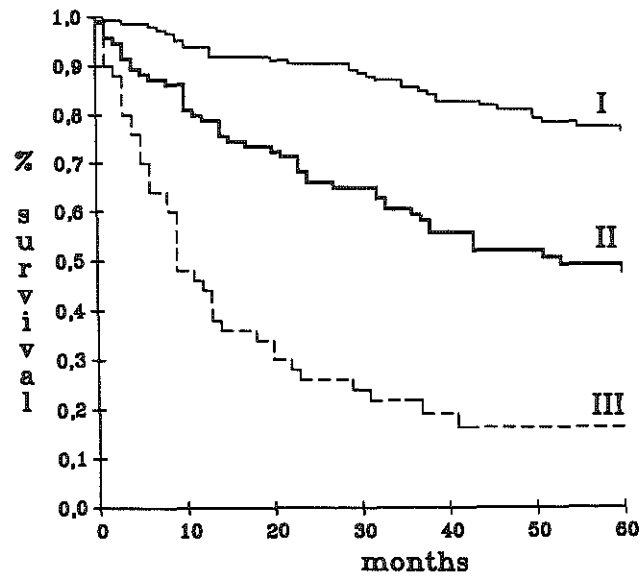

Figure 3. Overall survival according to tumour stage (T). I=Ta (n=147), II=T1 (n=94), III=T2 or more (n=50); 5 year survival: I=76.4% (95% CI, 69.1-83.6), II=47.6% (95% CI, 37.1-58.2), III=16.4% (95% CI, 5.5-27.2).

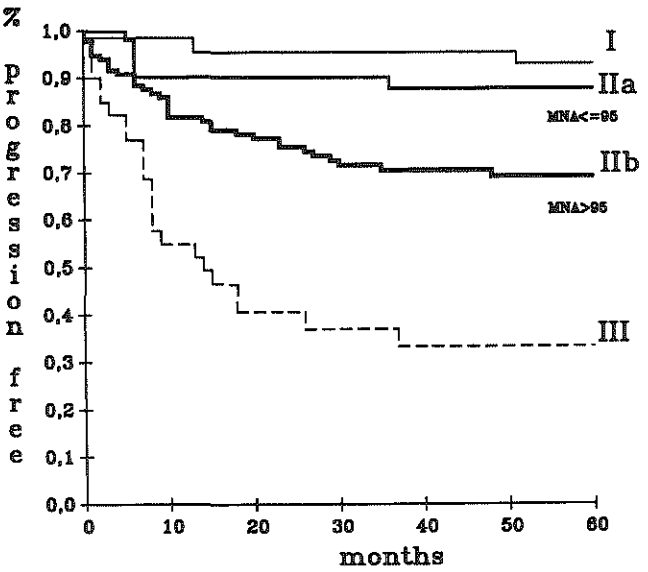

Figure 4. Progression-free survival according to WHO grades. WHO grade II tumours separated into different subgroups (IIa and IIb) on account of mean nuclear area. I=WHO I (n=68); II=WHO II (n=186); III=WHO III (40); 5 year progression-free survival: I=93.2% (95% CI, 86.5-99.8), IIa=87.9% (95% CI, 78.9-97.0)(n=53), IIb=69.3 (95% CI, 60.8-77.8)(n=133), III=33.3% (95% CI, 17.3-49.4).

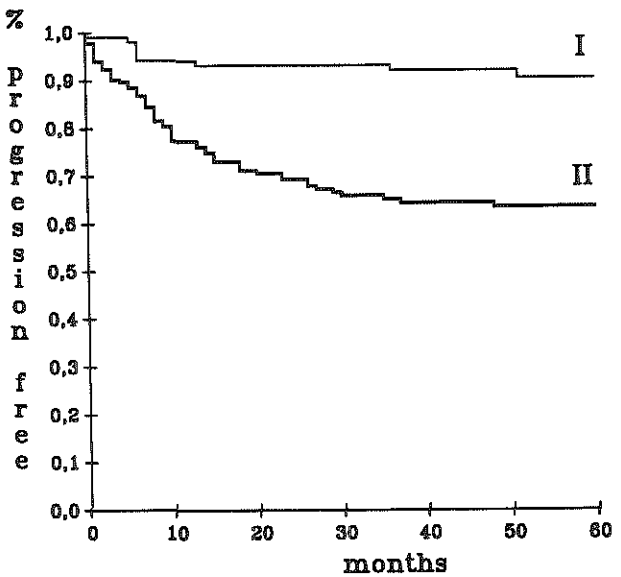

Figure 5. Progression-free survival according to morphometric grades. I=Low-grade (n=108), II=High-grade (n=186); 5 year progression-free survival: I=90.6% (95% CI, 84.6-96.6), II=63.5% (95% CI, 56.0-70.9).

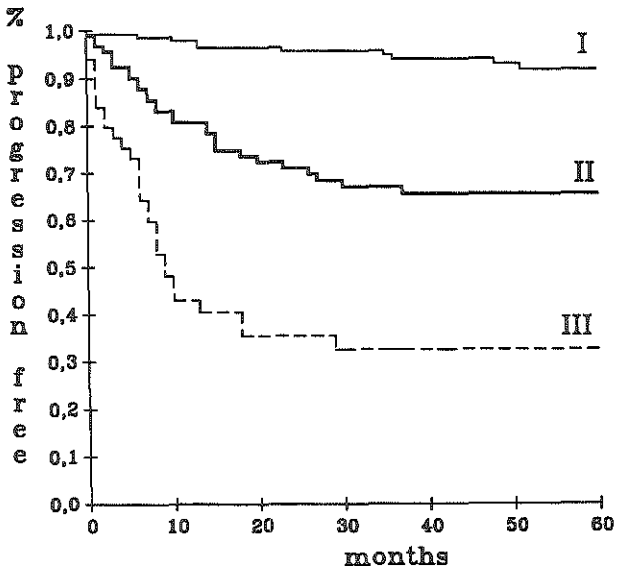

Figure 6. Progression-free survival according to tumour stage (T). I=Ta (n=147), II=T1 (n=94), III=T2 or more (n=50); 5 year progression-free survival: I=91.9% (95% CI, 87.0-96.8), II=65.6% (95% CI, 55.3-75.8), III=32.5% (95% CI, 18.2-46.8).

4.5 DISCUSSION

Bladder cancer has an unpredictable clinical course. Treatment is still generally based on histopathological grade and stage. The most widely accepted histopathological grading system is the three grade system proposed by the WHO.[2] However, the criteria for the different grades are considered to be rather vague, resulting in considerable intra- and interobserver variability.[3, 4] Staging, especially in superficially infiltrating tumours, also is not sufficiently reproducible. The resolution of these problems can be assisted by using immuno-staining techniques to stain the basement membrane, which helps in assessing infiltrative growth.[19] Nevertheless, when the biopsy is inadequate and does not contain all layers of the bladder wall, staging cannot be performed with certainty.

For adequate clinical management of TCC it would be useful to have a parameter (s) that would allow early recognition of those patients who have a high risk for tumour progression and consequently for dying of the tumour. For the pathologist, such a test ideally should be simple and reproducible. Morphometry appears to satisfy most of these criteria as has been pointed out by different authors in showing the usefulness of nuclear morphometry for the objective assessment of tumour grade in bladder carcinoma.[3, 4, 20] Morphometric grading was found to be of prognostic significance in the predic-

tion of survival and of tumour progression.[4, 13, 21-23] However, in these studies these parameters were not tested for their prognostic value when compared with the conventional prognostic factors. Blomjous et al. described a method of morphometry in which, after selection of the most atypical areas of the tumour, only the ten largest nuclei were measured.[4] Two morphometric grades were proposed based on mean nuclear area.

In the present study, these morphometric grades showed a highly significant association with the histopathological parameters (stage, grade and mitotic index). As to the prediction of clinical outcome, morphometric grading showed a significant correlation with survival and tumour progression. The additional value of this parameter was tested by multivariate analysis, also taking into account other parameters, that are significantly correlated with survival and tumour progression *i.e.* stage, grade and mitotic index.

The results indicate that stage is the only independent histopathological parameter significantly correlated with survival. Morphometric grading has no additional independent value in this respect. Also in predicting tumour progression, stage is the most significant parameter, the mitotic index being the only other independent predictor.

In conclusion, morphometry appears to be an objective method in grading TCC. A high morphometric grade is correlated with aggressive tumour behaviour. However, as shown by multivariate analyses, tumour stage remains the most important predictor of survival and tumour progression. When histopathological staging cannot be performed reliably morphometry can be of use in separating tumour groups with different clinical behaviour especially in WHO grade II tumours. When a TCC has a high morphometrical grade infiltration is very likely.

4.6 REFERENCES

1. HERMANEK, B., SOBIN, L. H. (1987). In *T.N.M. Classification of Malignant Tumours*, Union International Contre le Cancer (UICC), ed. 4, pp. 121-144. New York: Springer-Verlag.
2. MOSTOFI, F.K. (1973). In: *Histological typing of Urinary Bladder Tumours, International Histological classification of Tumours*. No. 10, World Health Organization (WHO), Geneva.
3. OOMS, E.C.M., ANDERSON, W.A.D., ALONS, C.L., BOON, M.E., VELDHUIZEN, R.W. (1983). Analysis of the performance of pathologists in the grading of bladder tumours. *Hum. Pathol.*, 14, 140-143.
4. BLOMJOUS, C.E.M., SMEULDERS, A.W.M., BAAK, J.P.A., VOS, W., VAN GALEN, E.M., MEIJER, C.J.L.M. (1989). A comparative study in morphometric grading of transitional cell carcinoma of the urinary bladder. *Anal. quant. cytol. histol.*, 11, 426-432.
5. REUTER, V.E. (1990). Pathology of Bladder Cancer: Assessment of Prognostic Variables and Response to therapy. *Seminars in Oncology*, 17, no 5, 524-532.
6. OLJANS, P.J., TANKE, H.J. (1986). Flow cytometric analysis of DNA content in bladder cancer: prognostic value of the DNA- index with respect to early tumour recurrence in G2 tumours. *World J. Urol.*, 4, 205-210.
7. BLOMJOUS, C.E.M., SCHIPPER, N.W., BAAK, J.P.A., VAN GALEN, E.M., DE VOOGT, H.J., MEIJER, C.J.L.M. (1988).Retrospective study of prognostic importance of DNA flow cytometry of urinary bladder transitional cell carcinoma. *J. Clin. Pathol.*, 41, 21-25.
8. DEVERE WHITE, R.W., DEITCH, A.D., WEST, B., FITZPATRICK, J.M. (1988). The predictive value of flow cytometric information in the clinical management of stage 0 (Ta bladder cancer. *J. Urol.*, 139, 279-285.
9. SHAABAN, A.A., TRIBUKAIT, B., EL-BEDEIWY, A.A., GHONEIM, M.A. (1990). Prediction of lymph node metastases in bladder carcinoma with deoxyribonucleic acid flow cytometry. *J. Urol.*, 144, 884-887.
10. STÖCKLE, M., TANKE, H.J., MESKER, W.E., PLOEM, J.S., JONAS, U., HOHENFELLNER, R. (1987). Automated DNA-image cytometry: a prognostic tool in infiltrating bladder carcinoma. *World J. Urol.*, 5, 127-132.
11. VASCO, J., MALMSTRÖM, P-U., TAUBE, A., WESTER, K., BUSCH, C. (1991). Prognotic value of a systematized method for determination of mitotic frequency in bladder cancer assisted by computerized image analysis. *J. Urogenital Pathol.*, 1, 53- 59.
12. NIELSON, K., ORNTOFT, T., WOLF, H. (1989). Stereological estimates of nuclear volume in non-invasive bladder tumours (Ta correlated with the recurrence pattern. *Cancer*, 64, 2269-2274.
13. LIPPONEN, P.K., COLLAN, Y., ESKELINEN, M.J., PESONEN, E., SOTARAUTA, M., NORDLING, S. (1990). Comparison of morphometry and DNA flow cytometry with Standard Prognostic Factors in Bladder Cancer. *Brit. J. Urol.*, 65, 589-597.
14. VAN DIEST, P.J., BAAK, J.P.A., MATZE-COK, P., WISSE- BREKELMANS, E.C.M., VAN GALEN, C.M., KURVER, P.H.J., BELLOT, S.M., FIJNHEER, J., VAN GORP, L.H.M., KWEE, W.S., LOS, J., PETERSE, J.L., RUITENBERG, H.M., SCHAPERS, R.F.M., SCHIPPER, M.E.I., SOMSEN, J.G., WILLIG, A.W.P.M., ARIENS, A.TH. (1992). Reproducibility of mitosis counting in 2, 469 breast cancer specimens: Results from the multicenter morphometric mammary carcinoma project. *Hum. Pathol.*, 23, 603-607.
15. BAAK, J.P.A., OORT, J. (1983). Obtaining Quantitative Data. In: *A manual of morphometry in diagnostic pathology*, pp. 18-20. Berlin: Springer-Verlag.
16. KAPLAN, E.L., MEIER, P. (1958). Non parametric estimation from incomplete observation. *J. Am. Stat. Assoc.*, 53, 457- 481.

17. PETO, R., PIKE, M.C., ARMITAGE, P., BRESLOW, N.E., COX, D.R., HOWARD, S.V., MANTEL, N., MCPHERSON, K., PETO, J., SMITH, P.G. (1977). Design and analysis of randomised clinical trials requiring prolonged observation of each patient: II. Analysis and examples. *Br. J. Cancer*, 35, 1-39.
18. COX, D.R. (1972). Regression models and live tables. *J. Roy. Stat. Soc.*, 34: 187-220.
19. SCHAPERS, R.F.M., PAUWELS, R.P.E., HAVENITH, M.G., SMEETS, A.W.G.B., VAN DEN BRANDT, P.A., BOSMAN, F.T. (1990). Prognostic significance of type IV collagen and laminin immunoreactivity in urothelial carcinomas of the bladder. *Cancer*, 66, 2583- 2588.
20. OOMS, E.C.M., BLOK, A.P.R., VELDHUIZEN, R.W. (1985). The reproducibility of a quantitative grading system of bladder tumours. *Histopathology*, 9, 501-509.
21. DE SANCTIS, P.N., CONCEPCION, N.B., TANNENBAUM, M., OLSSON, C. (1987). Quatitative morphometry measurements of transitional cell bladder cancer nuclei as indicator of tumour aggression. *Urology*, 29, 322-324.
22. MONTIRONI, R., SCARPELLI, M., ANSUINI, E. MARINELLI, F., MARIUZZI, G. (1986). Quantitative evaluation of the progressive nuclear abnormalities in urothelial papillary lesions. *Appl. Pathol.*, 4, 65-73.
23. QING-BEI YANG, YANG-ZHI XIA, ZHI-YOUNG WANG, GUANG-JUN WANG, SHU-ZHE DING, DIAN-ZHI SHI, WEN-DE LIU (1991). Morphometric diagnosis of bladder tumour. *Oncology*, 48, 188-193.

CHAPTER 5

IMAGE CYTOMETRIC DNA ANALYSIS IN TRANSITIONAL CELL CARCINOMA OF THE BLADDER

RENE F. M. SCHAPERS
JOKE J. PLOEM-ZAAIJER
RUUD P. E. PAUWELS
A. WIM G. B. SMEETS
PIET A. van den BRANDT
HANS J. TANKE
FRE T. BOSMAN

From the Departments of Pathology[RFMS] and Urology[RPEP], the Stichting Ziekenhuizen Venlo-Venray, Venlo: the Department of Cytochemistry and Cytometry, University of Leiden[JJP-Z, HJT]: the Department of Clinical Pharmacy and Laboratory[AWGBS], Venray, the Department of Epidemiology[PAvdB], University of Limburg, Maastricht: the Department of Pathology[FTB], Erasmus University, Rotterdam, The Netherlands.

Cancer, 72, 182-189, 1993

5.1 ABSTRACT

Background. The current study was initiated to investigate measurable objective and reproducible characteristics that might have prognostic significance in bladder cancer.
Methods. Tumour samples from 91 patients with primary transitional cell carcinoma (TCC) of the urinary bladder, were studied by DNA image cytometry and cytogenetic analysis. Image cytometry is a more sensitive method of determining ploidy than flow cytometry, especially in tumours with a low number of aneuploid cells.
Results. There was a significant difference in survival between DNA image cytometry-determined diploid and nondiploid cases. The presence of nuclei with a high DNA content indicated poor prognosis. The 2C deviation index (2CDI) also was an indicator of survival. Image cytometry-determined factors also were found to be strong predictors of progression-free survival. In multivariate analysis, 2CDI was the only cytometric parameter with an independent but weak correlation with survival. In multivariate analysis, none of the cytometric parameters had an important contribution to prediction of progression-free survival.
In superficial tumours (Ta and T1), 2CDI appeared to be the most important independent predictor of survival. With respect to progression-free survival, tumours with a high mitotic index proved to have a worse prognosis.
Conclusions. Parameters determined by DNA image cytometry appear to be valuable in predicting survival and progression-free survival and may be useful in addition to the classic parameters of stage and grade, especially in superficial TCC.

5.2 INTRODUCTION

Urothelial cancer displays unpredictable variations in biological behaviour. Histopathological characteristics of transitional cell carcinoma (TCC) have a limited predictive value with respect to survival and progression. Therefore, other potential predictors of disease evolution have been searched for. The current study was initiated in 1979 to investigate measurable objective and reproducible characteristics that might have prognostic significance in bladder cancer.
During the last decades, many studies have focused on the DNA content of bladder cancer, using image cytometry[1-6] as well as flow cytometry (FCM).[7-10] It has been shown by several authors that valuable prognostic information can be obtained from the nuclear DNA distribution pattern using these techniques. Aneuploidy, as determined by FCM, appeared to be a parameter that could predict which tumours will progress;[9] however, tumours with only a few cells with high DNA content against a background of abundant

diploid tumour cells might be classified as diploid by FCM and this approach therefore lacks sensitivity.
The purpose of the current study therefore was to determine the potential use of image cytometry in providing parameters that might be useful in predicting the biological behaviour of the neoplasm. Special attention was given to the potential value of rare incidents (*e.g.*, occasional cells with a very high DNA content). The cytometric parameters were compared to those from conventional cytogenetic analysis and other prognostic factors.

5.3 MATERIALS and METHODS

Between January, 1979 and May, 1991 bladder carcinoma was diagnosed for the first time in 399 patients. Over this period, 156 specimens were subjected to cytogenetic analysis. Representative paraffin blocks of 143 of these 156 specimens were available for image cytometric DNA analysis. To reduce the influence of therapy on survival, only patients undergoing transurethral resection of the tumour, without any other radical therapy (radiation therapy, cystectomy, or both), were included in this study (n=91). Clinicopathological information and transurethral resection specimens were collected prospectively. Staging was carried out according to the TNM system, [11] and grading into two groups, low and high-grade, was done according to criteria described elsewhere[12] (low-grade tumours are grade 1 and 2a; high-grade tumours are grade 2b and 3). We also estimated the mitotic index, which expresses the number of mitotic figures per 10 high-power fields (hpf, x40) of neoplastic tissue.[13] The criteria of counting mitoses described by Baak and Oort were used.[14]
The median follow-up was 30 months, with a maximum of 112 months (mean follow-up, 36.7 months). Follow-up examinations were conducted at least semiannually in all patients and included cystoscopy and urinary cytological analysis. Survival and progression-free survival were chosen as follow-up criteria. Progression, defined as increase in stage at recurrence or death, was assessed histologically by pathologists without knowledge of previous diagnostic information and clinical data. If a patient was lost to follow-up, survival was assessed by contacting municipal population registries.

5.3.1 DNA Image Cytometry

Automated image analysis was performed with Leytas (Leica, Wetzlar, Germany) on slides made from nuclear suspensions obtained from 50-µm sections, cut after each section from a paraffin-embedded tumour block used for histological analysis. The procedure for the preparation of nuclear smears from paraffin-embedded tissue has been described in detail elsewhere.[15]

Briefly, the sections were dewaxed with xylene, rehydrated in decreasing concentrations of ethanol, digested in a solution of 0.05% pronase in phosphate- buffered saline at 37°C for 30 minutes and resuspended mechanically. The cell suspension was diluted to a value of about 20, 000 epithelial cells/ml and monolayer preparations were obtained by cytocentrifugation. DNA staining was performed with acriflavine-Feulgen. Automated image analysis was carried out with Leytas, as described in detail previously.[16-18] Briefly, Leytas consists of an automated microscope (Autoplan, Leica) interfaced to an image analysis computer (MIAC). The Autoplan includes one objective and two cameras, one for low and one for high magnification. The low-magnification channel was used for automated cell selection on the basis of grey level (intensity thresholding) and size. The selection procedure included extensive artifact rejection to remove overlapping cells or artifacts. The settings for the cell selection criteria were adapted such that first 250 epithelial cells were selected randomly and then the preparation was screened for 70 cells with higher density and size.

A high-magnification image was obtained of each selected cell for measurement of cellular parameters. To obtain histograms consisting of single cells only, a visual step was included at the end of the measurement procedure to remove artifacts not rejected by the automated procedure. This visual evaluation was done by inspection of stored grey value images of the selected objects on a television monitor (Fig. 1).

DNA histograms were obtained by converting measured densities into C values with the aid of reference cells (normal epithelial cells or leukocytes). When two populations were present in the integrated optical density (IOD) histogram, it was assumed that the first population consisted of normal cells. The mean IOD of these populations also was used to estimate the 2C value.

To obtain a reproducible DNA histogram classification, the DNA histograms were classified into four categories on the basis of 2C and 5C exceeding rates. The first group consists of purely diploid cases with less than 5% of the cells outside the 2C population. The second category is referred to as probably diploid and includes cases that have up to 10% of cells outside the 2C population. As in the diploid category, few cells over 5C are present. The third category contains cases with a maximum of 15% cells outside the 2C population. In addition, the cells outside the 2C population present a higher proportion of cells above 5C (*i.e.*, more than 0.05%). The fourth category includes all histograms in which more than 15% of the cells have a DNA content higher than the 2C cell population. Typical examples are given in Figure 2.

In addition the number of cells exceeding 5C in the total smear (400 microscopic fields) was calculated from the histogram. A third parameter was the 2C deviation index (2CDI), which calculates the sum of all deviation squares of the differences of the DNA values from the 2C value divided by the number of measured cells.[19] The 2CDI value thus represents a numerical

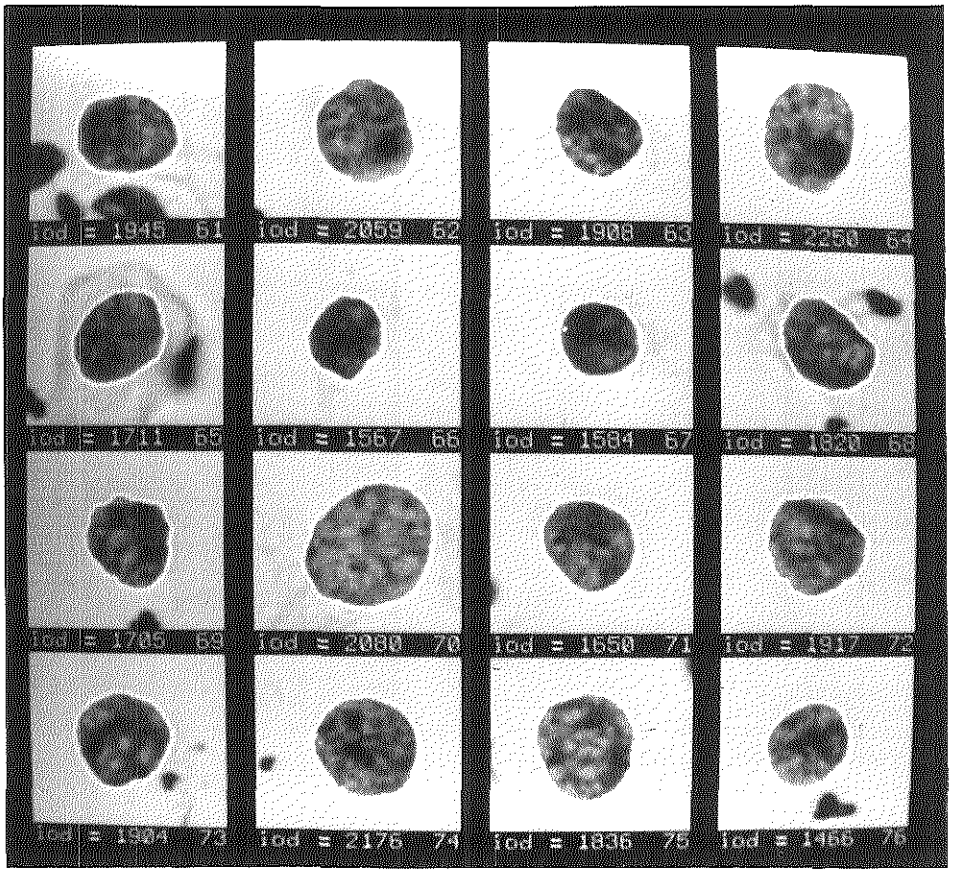

Figure 1. Stored grey value images of the selected objects on a TV monitor.

expression of the deviation of the DNA content of the tumour cell population from the mean DNA content of a nonproliferating diploid cell population.

5.3.2 Chromosomal analysis

A direct method was used for microscopic analysis of chromosomes, as described elsewhere in detail.[20] Tumour samples were collected in 0.5 % sodium citrate with 0.5 µg/ml colcemid. After incubation for 1 hour at room temperature in 5 ml Hanks balanced sodium solution (Hanks BSS), the tissue was mechanically desegregated. Next, a solution of 19 ml Hanks BSS and 6 ml colcemid was added. After incubation for 30 minutes at 37°C, hypotonic treatment in a solution of 6 ml fetal calf serum (FCS) and 24 ml 0.052 mol/l potassium chloride was followed by fixation in methanol-acetic acid 7: 3.

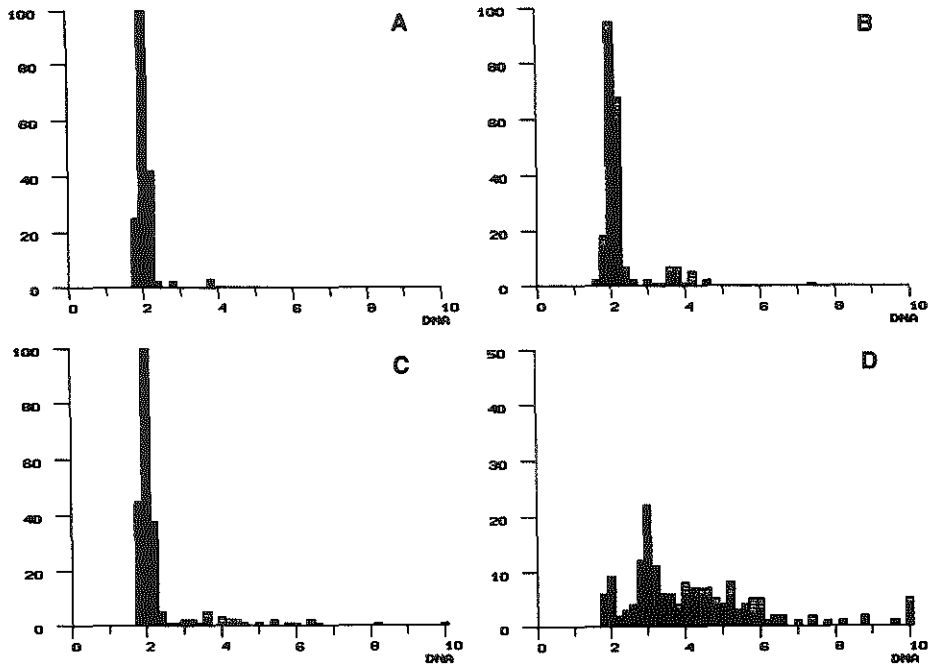

Figure 2. Examples of DNA-histograms of TCC in four ploidy classes.
a. diploid, b. probably diploid, c. probably aneuploid, d. aneuploid.

Chromosomes were routinely stained with Giemsa. If possible, C- and G-banding was performed. According to the modal chromosome number (CM), the tumours were classified into diploid (46 chromosomes or less) and hyperdiploid (more than 46 chromosomes).

5.3.3 Statistical analysis

Prognostic factors that were included in the analysis were: image cytometric ploidy class (four categories), the number of cells exceeding 5C (≤ 10 cells or > 10 cells per 400 microscopic fields), 2CDI (0.2 or less, between 0.2 and 2.0, 2.0 or more), CM (diploid, hyperdiploid), low-high grading, T category (Stage Ta; T1; T2+), mitotic index (≤5 mitoses/10 hpf; > 5 mitoses/10 hpf), age (younger than 70 years; 70 years or older) and sex. For each prognostic factor, Kaplan-Meier survival curves[21] were analysed by means of the log-rank test[22] with regard to time interval to death and time interval to progression. A possible correlation between various prognostic factors was analysed using the Pearson chi-square test.

In addition, multivariate survival analyses were performed using the Cox proportional hazards model.[23] Age was entered as a continuous variable in these models. Covariates were deleted in a stepwise manner (backward elimination), with use of the likelihood ratio test. A P-value of 0.10 was adopted as limit for deletion of a covariate. For the prognostic factors that contributed significantly to the model, the effect was calculated in terms of relative risk and the associated 95% confidence interval. The survival analyses were performed with the BMDP statistical package (BMDP Statistical Software Inc, Los Angeles, CA).

5.4 RESULTS

Of the 91 patients meeting our selection criteria, 71 (78%) were men and 20 (22%) were women. Age ranged from 26 to 99 years, with a mean of 74 years.

5.4.1 Correlation between prognostic factors

Strong correlations were found between stage, histological grade, the cytogenetic parameter CM and the cytometrically determined parameters: DNA cytometric ploidy class (Table 1), number of cells above 5C (Table 2) and 2CDI (Table 3; all $P < 0.0001$).

Table 1. Tumour stage, grade, modal number of chromosomes(CM) in relation to image cytometric ploidy class.

		Image DNA Ploidy				
		1	2	3	4	Total
Stage:	Ta	9	13	11	10	43
	T1	1	1	3	32	37
	T2+	0	1	0	10	11
Grade:	Low	6	12	6	6	30
	High	4	3	8	46	61
CM:	Diploid	9	14	12	16	51
	Hyperdiploid	1	1	2	36	40
Total		10	15	14	52	91

image DNA ploidy class 1 = diploid; 2 = probably diploid; 3 = probably aneuploid; 4 = aneuploid

Table 2. Tumour stage, grade, modal number of chromosomes(CM) in relation to number of cells >5c.

		Number of Cells > 5c		
		≤ 10	>10	Total
Stage:	Ta	33	10	43
	T1	6	31	37
	T2+	1	10	11
Grade:	Low	25	5	30
	High	15	46	61
CM:	Diploid	36	15	51
	Hyperdiploid	4	36	40
Total		40	51	91

Table 3. Tumour stage, grade, modal number of chromosomes(CM) in relation to the 2c deviation index.

		2c deviation index			
		≤0.2	0.2><2.0	≥2.0	Total
Stage:	Ta	28	12	4	44
	T1	3	14	19	36
	T2+	1	5	5	11
Grade:	Low	18	11	1	30
	High	14	20	27	61
CM:	Diploid	28	21	2	51
	Hyperdiploid	4	10	26	40
Total		32	31	28	91

5.4.2 Survival

In univariate analyses, the parameters correlating with survival were stage (log-rank chi-square=15.9; $P = 0.0003$), mitotic index (log-rank chi-square=5.3; $P = 0.0208$), age (log-rank chi-square = 6.8; $P = 0.0093$), CM (log-rank chi-square=12.4; $P = 0.0004$), DNA cytometric ploidy class (log-rank chi-square=11.7; $P = 0.0085$; Fig. 3a), number of cells above 5C (log-rank

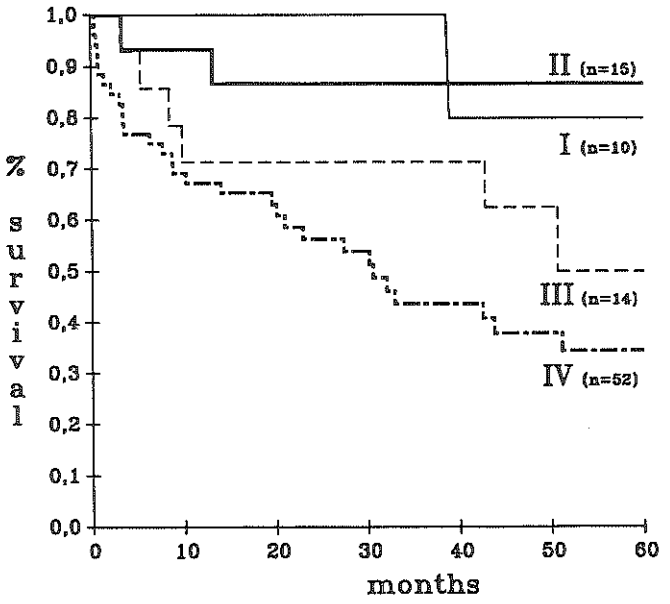

Figure 3a. Survival according to the cytometric ploidy class: I=diploid, II=probably diploid, III=probably aneuploid, IV= aneuploid.

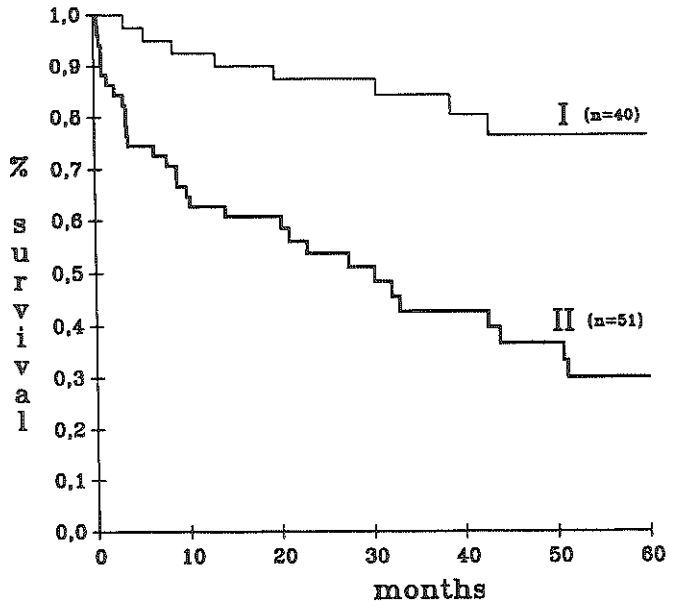

Figure 3b. Survival according to the number of cells above 5c: I=10 cells or less, II=more than 10 cells.

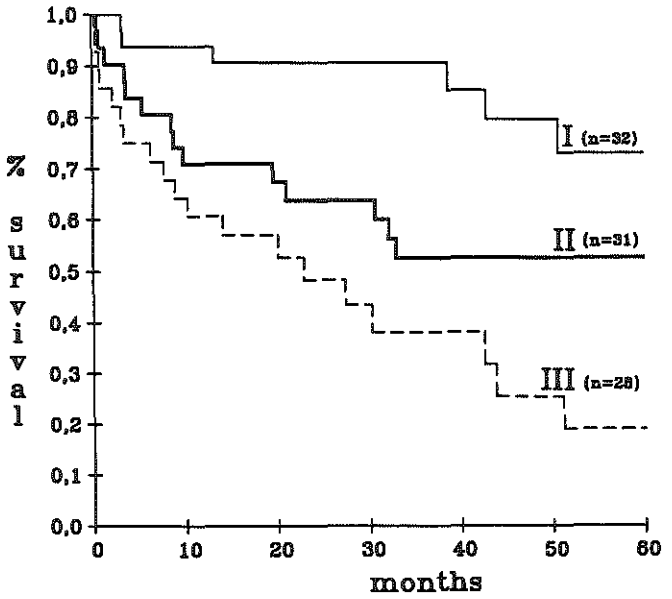

Figure 3c. Survival according to the 2C deviation index: I = ≤ 0.2, II = between 0.2 and 2, III = ≥ 2.

chi-square=16.1; $P < 0.0001$; Fig. 3b) and 2CDI (log-rank chi- square=16.4; $P = 0.0003$; Fig. 3c).
In multivariate analyses, age (likelihood ratio chi-square=14.3; $P = 0.0002$) and stage (likelihood ratio chi-square=12.2; $P = 0.0022$) were the only parameters correlating significantly with survival. The 2CDI was of borderline significance in this respect (likelihood ratio chi-square= 5.3; $P = 0.0698$; Table 4).

5.4.3 Progression

Stage (log-rank chi-square=7.4; $P = 0.0250$), mitotic index (log-rank chi-square=6.3; $P = 0.0124$), CM (log-rank chi-square=8.6; $P = 0.0034$), number of cells above 5C (log-rank chi-square=4.3; $P = 0.0387$; Fig. 4, a) and 2CDI (log-rank chi-square=7.9; $P = 0.0190$; Fig. 4, b) were parameters correlating with progression-free survival.
In multivariate analyses, CM was the parameter with the highest correlation with progression (likelihood ratio chi-square=4.4; $P = 0.0370$).
Mitotic index was the only additional parameter that contributed with borderline significance to the model (likelihood ratio chi-square=2.9; $P = 0.0851$). All other variables did not show a significant independent correlation (Table 5).

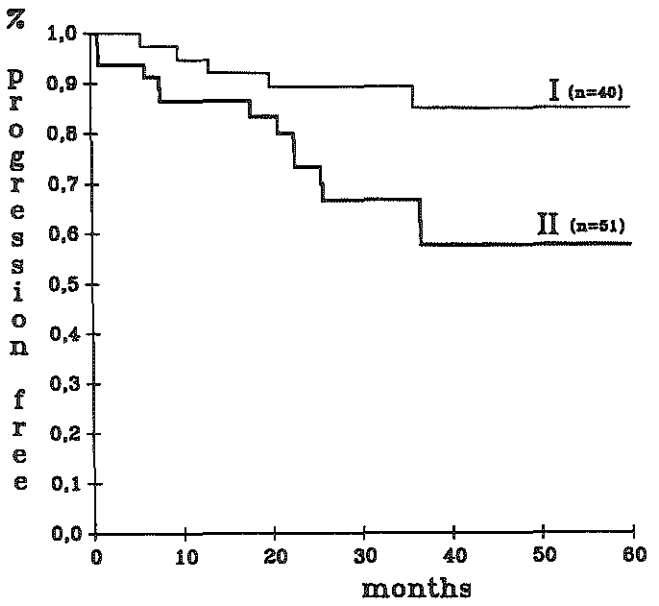

Figure 4a. Progression-free survival according to the number of cells above 5C: I= 10 cells or less, II=more than 10 cells.

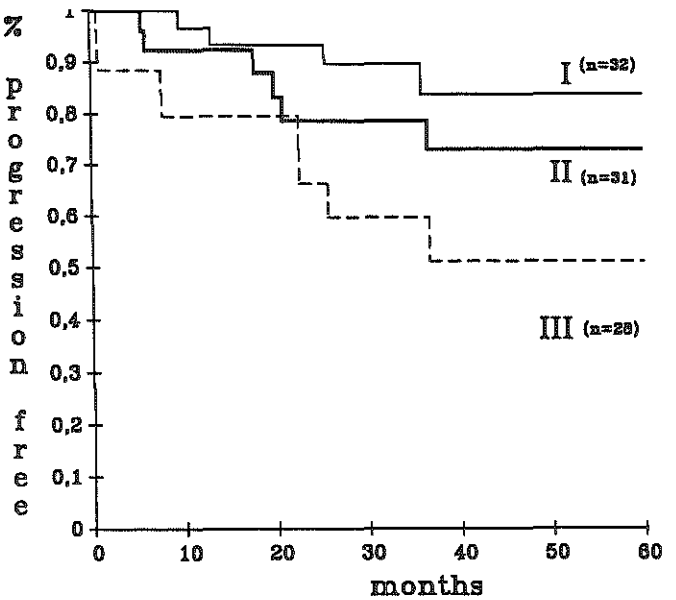

Figure 4 b. Progression-free survival according to the 2C deviation index: I = ≤0.2, II = between 0.2 and 2.0, III = ≥2.0.

Table 4. Multivariate analysis of effect on survival

Prognostic factor		RR	95% CI	P-value
Histological grade		_	NS	0.4659
Age		1.06	(1.03, 1.10)	0.0002
Sex		_	NS	0.1424
Stage	T1 vs Ta	1.87	(0.84, 4.14)	
	T2+ vs Ta	4.22	(1.48, 12.05)	0.0332
MI		_	NS	0.3490
2CDI	≥2 vs ≤0.2	2.04	(0.80, 5.18)	0.0698

RR, relative risk for covariates; CI, confidence Interval; NS, not significant; MI, mitotic index; 2CDI, 2C deviation index

Table 5. Multivariate analysis of the effect on Progression

Prognostic factor		RR	95% CI	P-value
grade		_	NS	0.1629
Age		_	NS	0.7429
Sex		_	NS	0.0838
Stage	T1 vs Ta	-	NS	
	T2+ vs Ta	-	NS	0.1237
MI	>5 vs ≤5	2.40	(0.84, 6.87)	0.0851
CM	>46 vs ≤46	2.62	(1.04, 6.61)	0.0370

RR, relative risk for covariates; CI, confidence Interval; NS, not significant; MI, mitotic index; CM, modal chromosome number

Table 6. Multivariate analysis of effect on survival in superficial TCC

Prognostic factor		RR	95% CI	P-value
Histological grade		_	NS	0.4002
Age		1.06	(1.02, 1.10)	0.0012
MI		_	NS	0.1017
2CDI	0.2><2.0 vs ≤0.2	1.76	(1.00, 3.09)	
	>2.0 vs ≤0.2	7.63	(1.64, 35.57)	0.0062

RR, relative risk for covariates; CI, confidence Interval; NS, not significant; MI, mitotic index; 2CDI, 2C deviation index

Table 7. Multivariate analysis of the effect on Progression in superficial TCC

Prognostic factor		RR	95% CI	P-value
grade		_	NS	0.1629
Age		_	NS	0.9487
MI	>5 vs ≤5	2.99	(0.92, 9.70)	0.0571
2CDI		-	NS	0.0856

RR, relative risk for covariates; CI, confidence Interval; NS, not significant; MI, mitotic index; 2CDI, 2C deviation index

5.4.4 Survival and progression in superficial TCC

In patients with superficial tumours only (Stages Ta, T1; n=80) the same analyses were performed. In multivariate analyses, 2CDI (likelihood ratio chi-square=10.2; P = 0.0062) and age appeared to be parameters independently correlating with survival in patients with superficial tumours (table 6). The presence of higher 2CDI correlated significantly with increased risk of death (relative risk for covariates, RR > 1). In the multivariate analyses of progression-free survival, mitotic index appeared to be the only independent parameter (likelihood ratio chi-square=3.6; P = 0.0571; table 7). Elevated risks for progression were found in TCC patients with a high mitotic index (RR > 1). The 2CDI was only of borderline significance.

5.5 DISCUSSION

Bladder cancer has an unpredictable clinical course. The most widely used prognostic factors are grade and stage. Unfortunately, both lack in reproducibility. Using FCM, it is possible to assess DNA ploidy in bladder cancer as a rough measure of chromosomal content. Some studies have shown that there is an association between FCM DNA ploidy and clinical behaviour, in the sense that aneuploidy predicts which tumours will progress.[7-10] When FCM is performed on cell samples with large numbers of cells with a diploid DNA content and only a small number of cells with a higher DNA content, however, these tumours might be classified as diploid, and this approach therefore lacks in sensitivity [7]. The advantage of image cytometry as compared to FCM lies in the possibility of selectively measuring the DNA content of single nuclei of epithelial origin. Artifacts such as cell doublets or degenerated cells as well as most inflammatory and stromal cells can be excluded from the analyses. This justifies the interpretation of relatively low numbers of very abnormal cells (DNA content higher than 5C). In early, manually performed image cytometric studies on imprints of fresh tumour tissue of TCC, a correlation was found between DNA values and

survival in univariate analyses.[1-4] In a cytometric study on 25 patients, using sections stained with haematoxylin and eosin, a DNA malignancy grade was calculated from the 2CDI. In multivariate analyses using age, tumour stage and tumour grade as covariate, DNA grading proved to be the only factor that contributed significantly to predicting survival.[5]

The advantage of automated cytometry with Leytas is that all available cells are subjected to image analysis, in contrast to other studies, in which only 100 selected cells were measured.[1-5] In the current study, the 2C DNA indices were found to be lower than reported in the study of Auffermann et al., [5] which might be due to differences in cell selection procedures. Stöckle et al. also used automated DNA image cytometry and derived two parameters from the histograms (DNA ploidy and the number of cells above 5C) that correlated well with survival in 38 patients with stages T1 to T4 TCC.[6] Based on their results, we began the current study of 91 patients with predominantly low-stage tumours, using the same two parameters. In addition, the 2CDI was calculated. This parameter was used to arrive at a numerical expression of the deviation of the DNA content of the tumour cells from the mean DNA content of a nonproliferating diploid cell population.

In the current study, the cytometric parameters showed a highly significant correlation with the modal number of chromosomes as derived from cytogenetic analyses and with the histopathological parameters (stage, grade and mitotic index). As to prediction of clinical outcome, all cytometric parameters showed a significant correlation with survival and progression-free survival. The clinical relevance of these findings was tested using multivariate analyses with a number of other parameters that are significantly correlated with survival and progression-free survival (*i.e.*, stage, grade, mitotic index and modal number of chromosomes).

Our results indicate that only 2CDI has borderline significance in the prediction of survival, in addition to stage and age. None of the cytometric variables was significant in predicting progression-free survival, whereas modal number of chromosomes and mitotic index are significant in this respect. In superficial TCC (Stages Ta and T1), 2CDI appears to be a significant independent predictor of survival and has borderline significance in the prediction of progression-free survival.

In conclusion, automated DNA image cytometry appears to be an objective method for grading TCC. DNA aneuploidy, high 2CDI and number of cells above 5C are parameters correlated with aggressive tumour behaviour. In superficial TCC, 2CDI is an important predictor of survival. This parameter might be used to select a group of patients with superficial TCC that needs to be treated more aggressively.

5.6 REFERENCES

1. LEVI, P.E., COOPER, F.H., PHIL, D., ANDERSON, C.K., PATH, M.C., WILLIAMS, R.E. (1969). Analyses of DNA content, nuclear size and cell proliferation of transitional cell carcinoma in man. *Cancer*, 23, 1074-1085.
2. FOSSA, S.D., KAALHUS, O., SCOTT-KNUDSEN, O. (1977). The clinical and histopathological significance of Feulgen DNA- values in transitional cell carcinoma of the human urinary bladder. *Eur. J. Cancer*, 13, 1155-1162.
3. HOFSTÄDTER, F., JAKSE, G., LEDERER, B., MIKUZ, G. (1980). Cytophotometric investigations of DNA-content in transitional cell tumours of the bladder. Comparison of results with clinical follow up. *Path. Res. Pract.*, 167, 254-264.
4. HOFSTÄDTER, F., JAKSE, G., LEDERER, B., MIKUZ, G., DELGADO, R. (1984). Biological behaviour and DNA cytophotometry of urothelial bladder carcinoma. *Br. J. Urol.*, 56, 289-295.
5. AUFFERMANN, W., URQUARDT, M., RÜBBEN, H., WOHLTMANN, D., BÖCKING, A. (1986). DNA grading of urothelial carcinoma of the bladder. *Anticancer Res.*, 6, 27-32.
6. STÖCKLE, M., TANKE, H.J., MESKER, W.E., PLOEM, J.S., JONAS, U., HOHENFELLNER, R. (1987). Automated DNA-image cytometry: a prognostic tool in infiltrating bladder carcinoma. *World J. Urol.*, 5, 127-132.
7. OLJANS, P.J., TANKE, H.J. (1986). Flow cytometric analysis of DNA content in bladder cancer: prognostic value of the DNA- index with respect to early tumour recurrence in G2 tumours. *World J. Urol.*, 4, 205-210.
8. BLOMJOUS, C.E.M., SCHIPPER, N.W., BAAK, J.P.A., VAN GALEN, E.M., DE VOOGT, H.J., MEIJER, C.J.L.M. (1988). Retrospective study of prognostic importance of DNA flow cytometry of urinary bladder transitional cell carcinoma. *J. Clin. Pathol.*, 41, 21-25.
9. DEVERE WHITE, R.W., DEITCH, A.D., WEST, B., FITZPATRICK, J.M. (1988). The predictive value of flow cytometric information in the clinical management of stage 0 (Ta bladder cancer. *J. Urol.*, 139, 279-285.
10. SHAABAN, A.A., TRIBUKAIT, B., EL-BEDEIWY, A.A., GHONEIM, M.A. (1990). Prediction of lymph node metastases in bladder carcinoma with deoxyribonucleic acid flow cytometry. *J. Urol.*, 144, 884-887.
11. HERMANEK, B., SOBIN, L. H. (1987). In *T.N.M. Classification of Malignant Tumours*, Union International Contre le Cancer (UICC), ed. 4, pp. 121-144. New York: Springer-Verlag.
12. PAUWELS, R.P.E., SCHAPERS, R.F.M., SMEETS, A.W.G.B., DEBRUYNE, F.M.J., GERAEDTS, J.P.M. (1988). Grading of superficial bladder cancer. *Br. J. Urol.*, 61, 129-134.
13. HAAPASALO, H., COLLAN, Y. (1989). Volume corrected mitotic index (M/V-Index the standard of mitotic activity in neoplasms. *Path. Res. Pract.*, 185, 551-557.
14. BAAK, J.P.A., OORT, J. (1983). Obtaining Quantitative Data. In: *A manual of morphometry in diagnostic pathology*, pp. 18-20. Berlin: Springer-Verlag.
15. VAN DRIEL-KULKER, A.M.J., MESKER, W.E., VAN VELZEN, I., TANKE, H.J., FEICHTINGER, J., PLOEM, J.S. (1985). Preparation of monolayer smears from paraffin-embedded tissue for image cytometry. *Cytometry*, 6, 268-272.
16. CORNELISSE C.J., VAN DRIEL-KULKER A.M.J., (1985). DNA image cytometry on machine-selected breast cancer cells and a comparison between flow cytometry and scanning cytophotometry. *Cytometry*, 6: 471-477.
17. PLOEM, J.S., VAN DRIEL-KULKER, A.M.J., GOYARTS-VELDSTRA, L., PLOEM-ZAAIJER, J.J., VERWOERD, N.P., VAN DER ZWAN, M. (1986). Image analysis combined with quantitative cytochemistry. *Histochemistry*, 84, 549-555.
18. PLOEM, J.S., VAN DRIEL-KULKER, A.M.J., PLOEM-ZAAIJER, J.J. (1989). Automated cell analysis for DNA studies of large cell populations using the LEYTAS image cytometry system. *Path. Res. Pract.*, 185, 671-675.

19. BÖCKING, A., ADLER, C.P., COMMON, H.H., HILGARTH, M., GRANZEN, B., AUFFERMANN, W. (1984). Algorithm for DNA cytophotometric diagnosis and grading of malignancy. *Anal. Quant. Cytol.*, 6, 1-8.
20. SMEETS, W., PAUWELS, R., LAARAKKERS, L., DEBRUYNE, F., GERAEDTS, J. (1987). Chromosomal analysis of bladder cancer II. A practical method. *Cancer Genet. Cytogenet.*, 29, 23-28.
21. KAPLAN, E.L., MEIER, P. (1958). Non parametric estimation from incomplete observation. *J. Am. Stat. Assoc.*, 53, 457-481.
22. PETO, R., PIKE, M.C., ARMITAGE, P., BRESLOW, N.E., COX, D.R., HOWARD, S.V., MANTEL, N., MCPHERSON, K., PETO, J., SMITH, P.G. (1977). Design and analysis of randomised clinical trials requiring prolonged observation of each patient: II. Analysis and examples. *Br. J. Cancer*, 35, 1-39.
23. COX, D.R. (1972). Regression models and live tables. *J. Roy. Stat. Soc.*, 34: 187-220.

CHAPTER 6

CYTOGENETIC ANALYSIS IN TRANSITIONAL CELL CARCINOMA OF THE BLADDER

Numerical chromosomal abnormalities, its clinical significance and comparison with other prognostic factors.

R. F. M. SCHAPERS
A. W. G. B. SMEETS
R. P. E. PAUWELS
P. A. van den BRANDT
F. T. BOSMAN

From the Departments of Pathology[RFMS] and Urology[RPEP], St. Ziekenhuizen Venlo-Venray, Venlo: the Departments of Epidemiology[PAvdB] and Pathology[FTB], University of Limburg, Maastricht: the Department of Clinical Pharmacy and Laboratory[AWGBS], Venray, the Netherlands.

Br. J. Urol. (In Press)

6.1 ABSTRACT

The potential use of numerical chromosomal abnormalities as predictive factor for the clinical behaviour of Transitional Cell Carcinoma (TCC) was investigated. The effect on survival and progression-free survival was measured in a group of 91 patients with TCC treated by transurethral resection. The survival rate of patients having tumours with a diploid chromosomal modal number was significantly better than of patients having tumours with hyperdiploid chromosomal modal number. The survival rate of patients having TCC with diploid cells only was also significantly better than of patients having TCC with diploid and hyperdiploid cells. Progression-free survival was significantly higher in patients having TCC with a diploid modal number of chromosomes than in patients with a hyperdiploid modal number.
Simultaneous evaluation of modal chromosome number or chromosomal range and histological grade, stage, mitotic index, age and sex as prognostic factors in multivariate analyses showed that the histological stage of bladder carcinomas is the most important factor in predicting the survival rate. In patients with superficial tumours (stage Ta and T1) the modal chromosome number is the most important factor in predicting survival. In multivariate analyses concerning progression-free survival, the modal chromosome number appeared to be the most important factor.
We conclude that modal chromosome number in TCC is useful in predicting survival in patients having superficial tumours (stages Ta and T1) and in predicting progression-free survival in tumours of all stages.

6.2 INTRODUCTION

Histopathological characteristics of transitional cell carcinoma (TCC) have a limited predictive value for survival and progression. Therefore, other potential predictors of disease evolution have been sought. The present study was initiated in 1979 in search of measurable characteristics that might have prognostic relevance. We reported on cytogenetic analysis, [1, 2] ABH blood group deletion, [3] and basement membrane expression in TCC.[4] In an earlier study an increase in modal number of chromosomes was associated with histological grade and stage in TCC.[5] Spooner and Cooper found that well and moderately well differentiated tumours fell within the diploid range (42 to 49 chromosomes), whereas the majority of poorly differentiated cancers showed a widespread distribution of chromosome number.[5] In some studies a large number of structural abnormalities was found, including marker chromosomes, especially in poorly differentiated and invasive tumours.[6-10] Low stage and low-grade carcinomas of the bladder were nearly always diploid or near diploid (42 to 49 chromosomes), although a few marker

chromosomes have been observed.[7, 8] Pauwels et al. reported on modal chromosome number and range in relation to progression and recurrence and found that marker chromosomes had no predictive value.[1] The range of chromosome number appeared to be the best predictor for invasion.
The purpose of the present study was to determine the extent to which the biological potential of TCC, in terms of survival and progression-free survival, could be predicted by numerical chromosomal aberrations (modal number and/or range).
We also compared our cytogenetic findings with other prognostic factors like stage, grade, mitotic index and age.

6.3 MATERIALS AND METHODS

Between January 1979 and May 1991, in 399 new patients TCC was diagnosed. The operation specimen of 201 of these primary TCC's were selected at random by the urologist directly after the operation, without knowledge of stage and grade and a part of the tumour was subjected to cytogenetic analysis. Recognizable metaphases were found in 156 out of 201 specimens (78%). Most of the tumour specimens in which no metaphases were found, were small, showed a low-grade histology and showed no infiltration (these patients all showed a benign clinical behaviour). To reduce the influence of therapy on survival, in this study only patients undergoing transurethral resection (TUR) of the tumour, without any other radical therapy (radiotherapy, cystectomy or both), were included (n=91). Clinicopathological information and TUR specimen were collected prospectively. Staging was carried out according to the TNM system[11] and grading (low and high-grade), according to a slightly adapted WHO system described elsewhere.[12] Briefly, low-grade tumours are grade 1 and 2a tumours, high-grade tumours are grade 2b and 3 tumours. We furthermore estimated the mitotic index, which expresses the number of mitotic figures per 10 high power (x 40) fields (hpf) of neoplastic tissue.[13] As criteria of counting mitoses we used the recommendations of Baak and Oort.[14]
The mean follow-up was 36.7 months with a maximum of 122 months (median follow-up 30 months). Follow-up examinations were conducted at least semi-annually in all patients and included cystoscopy and urinary cytology. As parameters for outcome, survival and progression-free survival were chosen. Progression (increase in stage and/or the appearance of metastases) was assessed histologically without knowledge of previous diagnostic information or clinical data. If a patient was lost to follow-up, survival was assessed by contacting municipal population registries or family doctors.

6.3.1 Chromosomal analysis

For microscopic analysis of chromosomes a direct method was used as described elsewhere in detail.[15] Briefly, tumour samples were collected in 0.5% sodium citrate with 0.5 μg/ml colcemid. After incubation for 1 hour at room temperature in 5 ml Hanks balanced sodium solution (Hanks BSS), the tissue was mechanically desegregated. Afterwards a solution of 19 ml Hanks BSS and 6 ml colcemid was added. After incubation for 30 min at 37°C, hypotonic treatment in a solution of 6 ml Foetal Calf Serum (FCS) and 24 ml 0.052 M potassium chloride was followed by fixation in methanol-acetic acid 7: 3. Chromosomes were routinely stained with Giemsa. If possible C- and G-banding was performed.
According to the chromosome number the tumours were classified with regard to: a. the modal number of chromosomes (CM) into diploid (46 chromosomes or less) or hyperdiploid (more than 46 chromosomes). b. the chromosomal range (CR) into tumours with cells only in the diploid range (46 chromosomes or less) or tumours with cells also in the hyperdiploid range (more than 46 chromosomes).

6.3.2 Statistical analysis

Prognostic factors that were included in the analysis were: CM (46 or less, more than 46), CR (46 or less, more than 46), grade (low- versus high-grade), T category (stage Ta; T1; T2+), mitotic index (less than 5 mitoses/10 hpf; 5 or more mitoses/10 hpf), age (less than 70 years; 70 years or more) and sex. Possible correlations between various prognostic factors were evaluated using the Pearson chi-square test. In the univariate analyses for each prognostic factor Kaplan-Meier survival curves[16] were analysed by means of the log-rank test[17] with regard to time interval to death and time interval to progression.
Furthermore, multivariate survival analyses were performed using Cox proportional hazards model.[18] In these models, either CM or CR was included together with the other factors. Age was entered as a continuous variable in these models. Covariates were deleted in a stepwise manner (backward elimination), with use of the likelihood ratio test. A *P*-value of 0.10 was adopted as limit for deletion of a covariate. Of the prognostic factors that contributed significantly to the model, the effect was calculated in terms of relative risk and the associated 95% confidence interval. The survival analyses were performed with the BMDP statistical package (BMDP statistical software Inc, Los Angeles, CA).

6.4 RESULTS

Of the 91 patients meeting our selection criteria, 71 (78%) were male and 20 (22%) were female. Age ranged from 26 to 99 years with a mean of 74 years. A strong correlation was found between CM and stage (chi-square=22.4, $P < 0.0001$) and between CM and grade (chi-square=20.9, $P < 0.0001$). The same was observed between CR and stage (chi-square=32.3, $P < 0.0001$) and between CR and grade (chi-square=21.7, $P < 0.0001$) (Table 1).

Table 1. Tumour stage and grade in relation to modal number of chromosomes and chromosomal range in individual cells

		Chromosome number				
		CM		CR		Total
		≤46	>46	≤46	>46	
Stage:	Ta	36	8	25	19	44
	T1	12	24	1	35	36
	T2+	3	8	0	11	11
Grade:	Low	27	3	18	12	30
	High	24	37	8	53	61
Total		51	40	26	65	91

CM, modal number of chromosomes; CR, chromosomal range

6.4.1 Survival

CM and CR correlated significantly with survival (log-rank chi-square=12.4, $P = 0.0004$ and log-rank chi-square=6.9, $P < 0.0083$ respectively, fig. 1 and 2). Significant predictors of survival in univariate analyses were stage (log-rank chi-square=15.9; $P < 0.0003$), grade (log-rank chi-square=6.1; $P = 0.0134$), mitotic index (log-rank chi-square=5.3; $P < 0.0208$) and age (log-rank chi-square=6.8; $P = 0.0093$).

In multivariate analyses age (likelihood ratio chi-square=14.33, $P = 0.0002$) and stage (likelihood ratio chi-square=12.2, $P = 0.0022$) were the only factors contributing significantly to the prediction of survival (Table 2). For age the relative risk (RR) of dying is 1.06 (95% confidence interval [CI]; 1.03-1.09), which means that the risk of dying increases by 6% per year of age. The RR of dying of patients having stage T1 tumours versus patients having stage Ta tumours is 2.27 (95% CI; 1.17-4.38). The RR of dying in patients with stage T2 (or more) tumours is 5.21 compared to patients with stage Ta tumours (95% CI, 1.99-13.61).

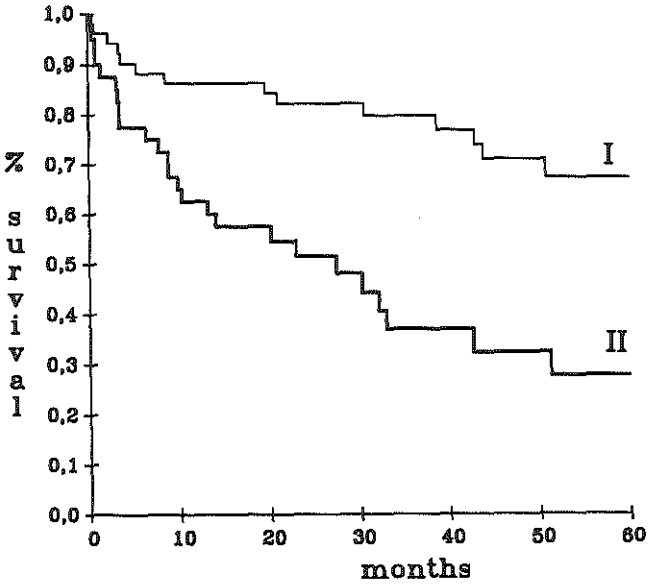

Figure 1. Survival according to modal number of chromosomes.
I=CM ≤ 46 (n=51), II=CM >46 (n=40). 5 year survival: I=67.2% (95% CI; 52.5, 81.9), II=27.7% (95% CI; 9.2, 46.2).

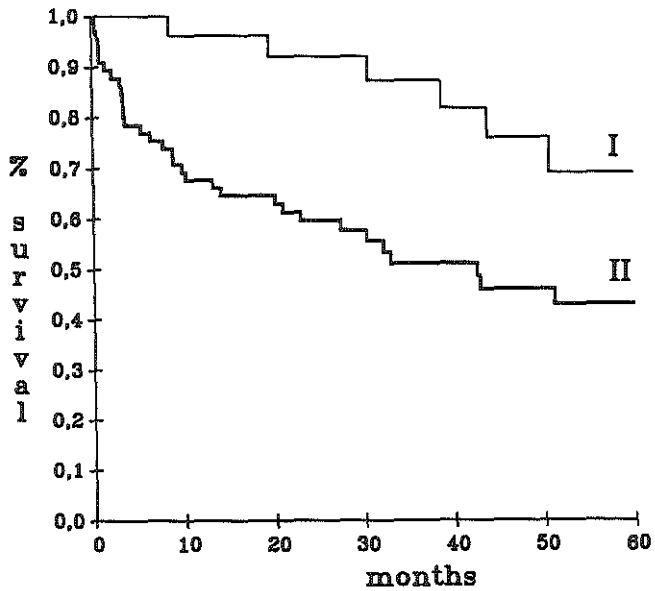

Figure 2. Survival according to chromosomal range in individual cells. I=CR ≤ 46 (n=26), II=CR >46 (n=65). 5 year survival: I=69.0% (95% CI; 47.6, 90.4), II=42.9% (95% CI; 29.1, 56.7).

Table 2. Multivariate analysis of effect on survival

Prognostic factor	RR	95% CI
Histological grade	_	NS
Age	1.06	(1.03, 1.09)
Sex	_	NS
Stage		
T1 vs Ta	2.27	(1.17, 4.38)
T2+ vs Ta	5.21	(1.99, 13.61)
Mitotic index	_	NS
CM	-	NS

RR, relative risk for covariates; CI, confidence interval; NS, not significant; CM, modal number of chromosomes

6.4.3 Progression

CM showed a significant correlation with progression-free survival (log-rank chi-square=8.6, $P = 0.0034$) (fig. 3).
Other significant univariate predictors of progression-free survival were stage (log-rank chi-square=7.4; $P = 0.0250$) and mitotic index (log-rank chi-square=6.3; $P = 0.0124$).
The CR appeared to be only of borderline significance in this respect (chi-square=3.4; $P = 0.0670$) (fig. 4).
In multivariate analyses CM was the most important prognostic factor in the prediction of progression (likelihood ratio chi-square=4.4, $P = 0.0370$) (Table 3). The risk of progression for patients having hyperdiploid tumours is 2.62 higher than for patients having diploid tumours (95% CI; 1.04, 6.61).
Mitotic index was the only additional factor that contributed borderline significantly to the model (likelihood ratio chi-square=2.9, $P = 0.0851$) (Table 3).
All other variables did not show a significant independent prediction.

6.4.4 Patients with superficial tumours

In patients with only superficial tumours (stage Ta, T1) (n=80) similar univariate and multivariate analyses were performed. In multivariate analyses CM is (next to age) the only independent factor in predicting survival in patients with superficial tumours (RR= 2.4; 95% CI: 1.22, 4.66). In predicting progression-free survival CM appears to be the only independent factor (RR=3.26 with 95%CI 1.36, 8.49) in the multivariate analyses.

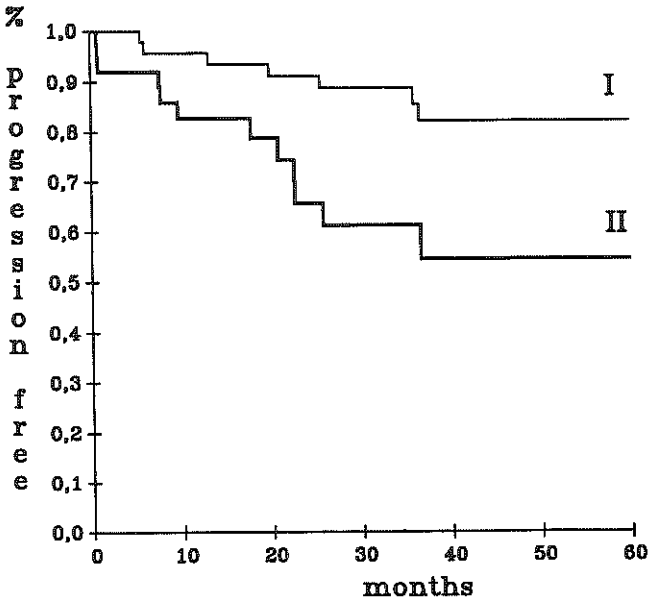

Figure 3. Progression-free survival according to modal number of chromosomes. I=CM ≤ 46 (n= 51), II=CM >46 (n=40). 5 year progression-free survival: I=82.2% (95% CI; 69.9, 94.5), II=54.5% (95% CI; 33.6, 75.4).

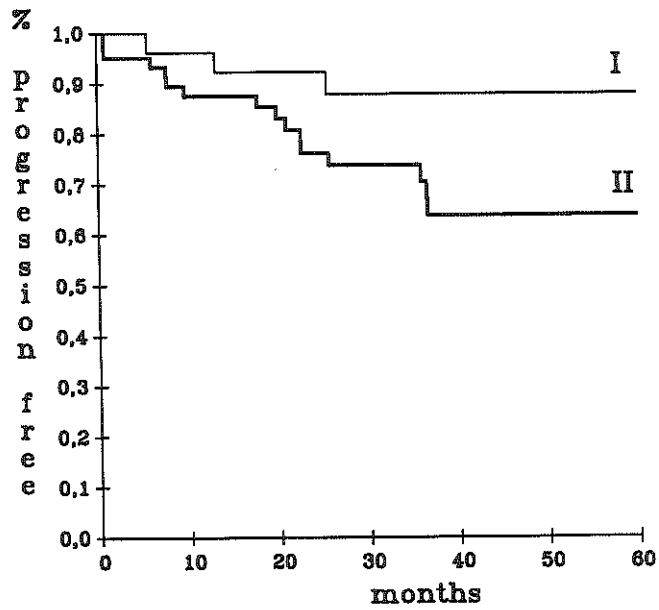

Figure 4. Progression-free survival according to chromosomal range in individual cells. I=CR ≤ 46 (n=26), II=CR >46 (n=65). 5 year progression-free survival: I=87.9% (95% CI; 75.0, 100), II=63.8% (95% CI; 49.7, 78.9).

Table 3. Multivariate analysis of the effect on progression

Prognostic factor	RR	95% CI
grade	_	NS
Age	_	NS
Sex	_	NS
Stage		
T1 vs Ta	-	NS
T2+ vs Ta	-	NS
Mitotic index		
≤5 vs <5	2.40	(0.84, 6.87)
CM	2.62	(1.04, 6.61)

RR, relative risk for covariates; CI, confidence interval; NS, not significant; CM, modal number of chromosomes

6.5 DISCUSSION

Bladder cancer has an unpredictable clinical course. The most widely used prognostic factors are grade and stage. Unfortunately both have their problems in reproducibility. Using flow cytometry (FCM) it is possible to assess DNA-ploidy in bladder cancer as a rough measure of chromosomal content. Some studies have shown that there is an association between DNA-ploidy and clinical behaviour, in the sense that aneuploidy is a feature which predicts which tumours will progress.[19-22] However, when FCM is performed on cell samples with large numbers of cells with a diploid DNA content and only a small number of cells with a higher DNA content, these tumours might be classified as diploid[19] and this approach therefore lacks in sensitivity. Karyotyping is another possibility. Specific chromosomal aberrations of chromosome 1, 7, 9, 11 and 17 have been described using conventional cytogenetic and chromosome specific in situ hybridization studies.[23, 24] As yet the number of patients in these studies is too small and follow-up times are too short to assess the clinical relevance of these findings. Numerical abnormalities in TCC are rather simple to establish when analyzable metaphases can be obtained in a tumour (in 78% of our specimen), in contrast to full karyotyping which is a much more complicated procedure. In search of additional prognostic factors we therefore studied the clinical relevance of numerical chromosomal abnormalities.

With regard to the prediction of clinical outcome we have shown that patients with diploid tumours have a significantly longer survival (5-year survival 67.2% versus 42.9%) and progression-free survival (5-year progression-free survival 82.2% versus 54.5%) than patients with hyperdiploid tumours.

Our material was also analysed for additional, potentially useful prognostic factors. Stage, grade and mitotic index were found to have significant correlation with overall survival and progression-free survival (age only had significant correlation with survival). Multivariate analyses were performed and the possible additional value of CM in predicting clinical outcome was evaluated. Our results indicate that of all the prognostic factors, stage and age are the most important independent predictors of overall survival (Table 2). With regard to progression-free survival, CM appears to be the most important predictor (Table 3).

In superficial TCC (stage Ta and T1) CM and age are the only independent predictors of survival in multivariate analyses. In this group of patients CM appeared to be the only factor with predicting value for progression-free survival.

In conclusion, tumour stage appears to be the most important indicator for overall survival in TCC. Our findings suggest that CM may be an important factor to predict progression, in terms of more aggressive behaviour of TCC, independent of stage and grade. CM could be of help to establish more effective treatment protocols for this disease.

6.6 REFERENCES

1. PAUWELS, R.P.E., SMEETS, A.W.G.B., GERAEDTS, J.P., DEBRUYNE, F. M. (1987). Cytogenetic analysis in urothelial cell carcinoma, *J. Urol.*, 137, 210-215.
2. PAUWELS, R.P.E., SMEETS, A.W.G.B., SCHAPERS, R.F.M., GERAEDTS, J.P.M., DEBRUYNE, F.M.J. (1988). Grading in superficial bladder cancer. (2 Cytogenetic Classification. *Br. J. of Urol.*, 61, 135-139.
3. PAUWELS, R.P.E., SCHAPERS, R.F.M., SMEETS, A.W.G.B., JANSEN, L.E.G., DEBRUYNE, F.M.J., GERAEDTS, J.P.M. (1988). Blood group isoantigen deletion and chromosomal abnormalities in bladder cancer. *J. Urol.*, 140, 959-963.
4. SCHAPERS, R.F.M., PAUWELS, R.P.E., HAVENITH, M.G., SMEETS, A.W.G.B., VAN DEN BRANDT, P.A., BOSMAN, F.T. (1990). Prognostic significance of type IV collagen and laminin immunoreactivity in urothelial carcinomas of the bladder. *Cancer*, 66, 2583-2588.
5. SPOONER, M.E., COOPER, E.H. (1972). Chromosome constitution of transitional cell carcinoma of the urinary bladder. *Cancer*, 29, 1401-1412.
6. FALOR, W. H. (1971). Chromosomes in noninvasive papillary carcinoma of the bladder. *J. A. M. A.*, 216, 791-794.
7. SANDBERG, A.A. (1977). Chromosome markers and progression in bladder cancer. *Cancer Res.*, 37, 2950-2956.
8. SUMMERS, J.L., COON, J.S., WARD, R.M., FALOR, W.H., MILLER, A.W. III, WEINSTEIN, R.S. (1983). Prognosis in carcinoma of the urinary bladder based upon tissue blood group ABH and Thomson-Friedenreich antigen status and karyotype of the initial tumour. *Cancer Res.*, 43, 934-939.
9. GRANBERG-ÖHMAN, I., TRIBUKAIT, B., WIJKSTRÖM, H. (1984). Cytogenetic analysis of 62 transitional cell bladder carcinomas. *Cancer Genet. Cytogenet.*, 11, 69-85.
10. WIJKSTRÖM, H., GRANBERG-ÖHMAN, I., TRIBUKAIT, B. (1984). Chromosomal and DNA patterns in transitional cell bladder carcinoma. A comparative cytogenetic and flow-cytofluorometric DNA study. *Cancer*, 53, 1718-1723.
11. HEMANEK, B., SOBIN, L.H. (1987). In *T.N.M. Classification of Malignant Tumours*, Union International Contre le Cancer (UICC), ed. 4, pp. 121-144. New York: Springer-Verlag.
12. PAUWELS, R.P.E., SCHAPERS, R.F.M., SMEETS, A.W.G.B., DEBRUYNE, F.M.J., GERAEDTS, J.P.M. (1988). Grading of superficial bladder cancer. *Br. J. Urol.*, 61, 129-134.
13. HAAPASALO, H., COLLAN, Y. (1989). Volume corrected mitotic index (M/V-Index the standard of mitotic activity in neoplasms. *Path. Res. Pract.*, 185, 551-557.
14. BAAK, J.P.A., OORT, J. (1983). In: *A manual of morphometry in diagnostic pathology*, pp. 18-20. Berlin: Springer-Verlag.
15. SMEETS, W., PAUWELS, R., LAARAKKERS, L., DEBRUYNE, F., GERAEDTS, J. (1987). Chromosomal analysis of bladder cancer II. A practical method. *Cancer Genet. Cytogenet.*, 29, 23-27.
16. KAPLAN, E.L., MEIER, P. (1958). Non parametric estimation from incomplete observation. *J. Am. Stat. Assoc.*, 53, 457-481.
17. PETO, R., PIKE, M.C., ARMITAGE, P., BRESLOW, N.E., COX, D.R., HOWARD, S.V., MANTEL, N., MCPHERSON, K., PETO, J., SMITH, P.G. (1977). Design and analysis of randomised clinical trials requiring prolonged observation of each patient: II. Analysis and examples. *Br. J. Cancer*, 35, 1-39.
18. COX, D.R. (1972). Regression models and live tables. *J. Roy. Stat. Soc.*, 34, 187-220.
19. OLJANS, P.J., TANKE, H.J. (1986). Flow cytometric analysis of DNA content in bladder cancer: prognostic value of the DNA- index with respect to early tumour recurrence in G2 tumours. *World J. Urol.*, 4, 205-210.
20. BLOMJOUS, C.E.M., SCHIPPER, N.W., BAAK, J.P.A., VAN GALEN, E.M., DE VOOGT, H.J., MEIJER, C.J.L.M. (1988). Retrospective study of prognostic importance of DNA flow cytometry of urinary bladder transitional cell carcinoma. *J. Clin. Pathol.*, 41, 21-25.

21. DEVERE WHITE, R.W., DEITCH, A.D., WEST, B., FITZPATRICK, J.M. (1988). The predictive value of flow cytometric information in the clinical management of stage 0 (Ta bladder cancer. *J. Urol.*, 139, 279-285.
22. SHAABAN, A.A., TRIBUKAIT, B., EL-BEDEIWY, A.A., GHONEIM, M.A. (1990). Prediction of lymph node metastases in bladder carcinoma with deoxyribonucleic acid flow cytometry. *J. Urol.*, 144, 884-887.
23. TSAI, Y.C., NICHOLS, P.W., HITI, A.L., WILLIAMS, Z., SKINNER, D.G., JONES, P.A. (1990). Allelic losses of Chromosomes 9, 11 and 17 in human bladder cancer. *Cancer Res.*, 50, 44-47.
24. HOPMAN, A.H.N., MOESKER, O., SMEETS, A.W.G.B., PAUWELS, R.P.E., VOOIJS, G.P., RAMAEKERS, F.C.S. (1991). Numerical chromosome 1, 7, 9 and 11 aberrations in bladder cancer detected by in situ hybridization. *Cancer Res.*, 51, 644-651.

CHAPTER 7

HETEROGENEITY IN BLADDER CANCER AS DETECTED BY CONVENTIONAL CHROMOSOME ANALYSIS AND INTERPHASE CYTOGENETICS

R. F. M. SCHAPERS
A. W. G. B. SMEETS
A. H. N. HOPMAN
R. P. E. PAUWELS
J. P. GERAEDTS
F. S. C. RAMAEKERS

From the Departments of Pathology[RFMS] and Urology[RPEP] of the St. Ziekenhuizen Venlo-Venray, Venlo: the Hospital Pharmacy and Laboratory for Clinical Chemistry[AWGBS], Venray: the Department of Molecular Cell Biology & Genetics[AHNH, JPG, FSCR], University of Limburg, Maastricht.

Cancer genet. cytogenet. (In Press)

7.1 ABSTRACT

Thirty TCC's of the bladder were examined by classical chromosome counting to establish range, modal number and percentage of metaphases with 2n, 3n, 4n and ≥5n chromosomes. In addition fluorescence in situ hybridization (FISH) was applied to interphase nuclei to detect the percentage of tumour cells showing polyploidization and chromosome imbalance. In FISH centromere specific DNA probes for chromosomes 1, 7, 9, and 11 were used. The tumours were analysed flow cytometrically to determine the DNA index (DI).
Fourteen out of 21 cases (67%) having a DI = 1 showed, after classical chromosome counting, in addition to a diploid modal number, some cells with a 3n and 4n chromosome count. With FISH 8 cases (38%) showed a low percentage of cells with multiple signals for each of the probes, thus indicating polyploidization. In 13 (62%) cases an imbalance between different chromosomes was detected. In 9 tumours, having a DI of 1.6 to 1.9, classical chromosome counting showed low percentages of ≥5n cells in 4 cases, in addition to a triploid modal number. With FISH in 6 cases a low percentage of cells showed five or more signals for each of the chromosomes, indicating polyploidization. In all the cases a chromosome imbalance was detected.
With classical chromosome counting not all tumours can be analysed. With FISH small percentages of polyploid cells are not recognized. Both methods complement each other in that chromosome counting allows readier detection of heterogeneity in DNA-diploid tumours after polyploidization, whereas FISH allows efficient recognition of the chromosomes involved in the process of imbalance.

7.2 INTRODUCTION

Transitional cell carcinomas (TCC's) of the bladder comprise a heterogeneous group of tumours. Apart from the heterogeneity that is observed for specimens of a defined histopathological category, an intra-lesional heterogeneity also exists. Heterogeneity may explain differences in the behaviour of tumours with identical classifications.[1]
With respect to prognostic parameters, in addition to histopathological characteristics, DNA content,[2-4] as well as cytogenetic data, obtained on the basis of classical metaphase spreads[1] or interphase cytogenetics,[4,5] are mentioned as useful predictors. Flow cytometric detection of the DNA content has, however, the disadvantage that small variations in DNA content cannot be detected. Karyotyping of bladder cancers to assess numerical and structural chromosomal defects, is not always successful due to limitations of the cancer specimen, such as small number of metaphases, the quality of the chromosomes, the complexity of the karyotypes and in case of culture, a

selection of cell types.[6, 7] However, the evaluation of the chromosome number in a few dozens of tumour cells can still give relevant information. In such studies the presence of some hyperdiploid cells, among predominantly diploid ones, is found much more frequently in invasive than in non-invasive bladder tumours.[1, 8] Intra-lesional heterogeneity can also be analysed by fluorescence in situ hybridization (FISH). FISH, also called interphase cytogenetics, allows the analysis of several hundreds of (tumour) cells, with many different DNA probes. As a result the detection of minor cell populations, as well as an imbalance in chromosome copy numbers within one tumour has become possible.[4] In a recent study it appeared that in 14 out of 24 flow cytometrically diploid, non-invasive TCC's, an imbalance between chromosome copy numbers was detected. In addition to this imbalance, 3 out of 24 cases showed a considerable amount of cells with multiple copies for several chromosomes, suggesting polyploidization.[4] This process has also been described for other solid tumours.[9, 10, 15]
This study was undertaken to detect intra-tumoral heterogeneity by classical chromosome counting and by FISH, in bladder tumours having a diploid or a hyperdiploid DNA content.

7.3 MATERIALS AND METHODS

7.3.1 Tumour tissue samples

Thirty cases of unfixed fresh TCC's were mechanically desegregated as described before[12] and the cell suspensions were used to determine chromosome counts in at least 20 cells. FISH was applied as well on these samples for 4 chromosomes. The DNA content was established flow cytometrically. Staging was done according to the TNM classification and grading according to a modification of the WHO system.[11]

7.3.2 Classical chromosome counting

For microscopic chromosome analysis a direct method has been used as described earlier.[12] The percentage of metaphases of a defined tumour being diploid ($2n = 46 \pm 11$), triploid ($3n = 69 \pm 11$) and so on is calculated. Chromosomal range and modal number are determined as well.

7.3.3 Fluorescence in situ hybridization (FISH)

200 cells were evaluated after application of centromere specific probes for chromosomes 1, 7, 9 and 11.[4, 13] Aberrations in the chromosome numbers were expressed in terms of tetraploidization, polyploidization and chromosome imbalance.

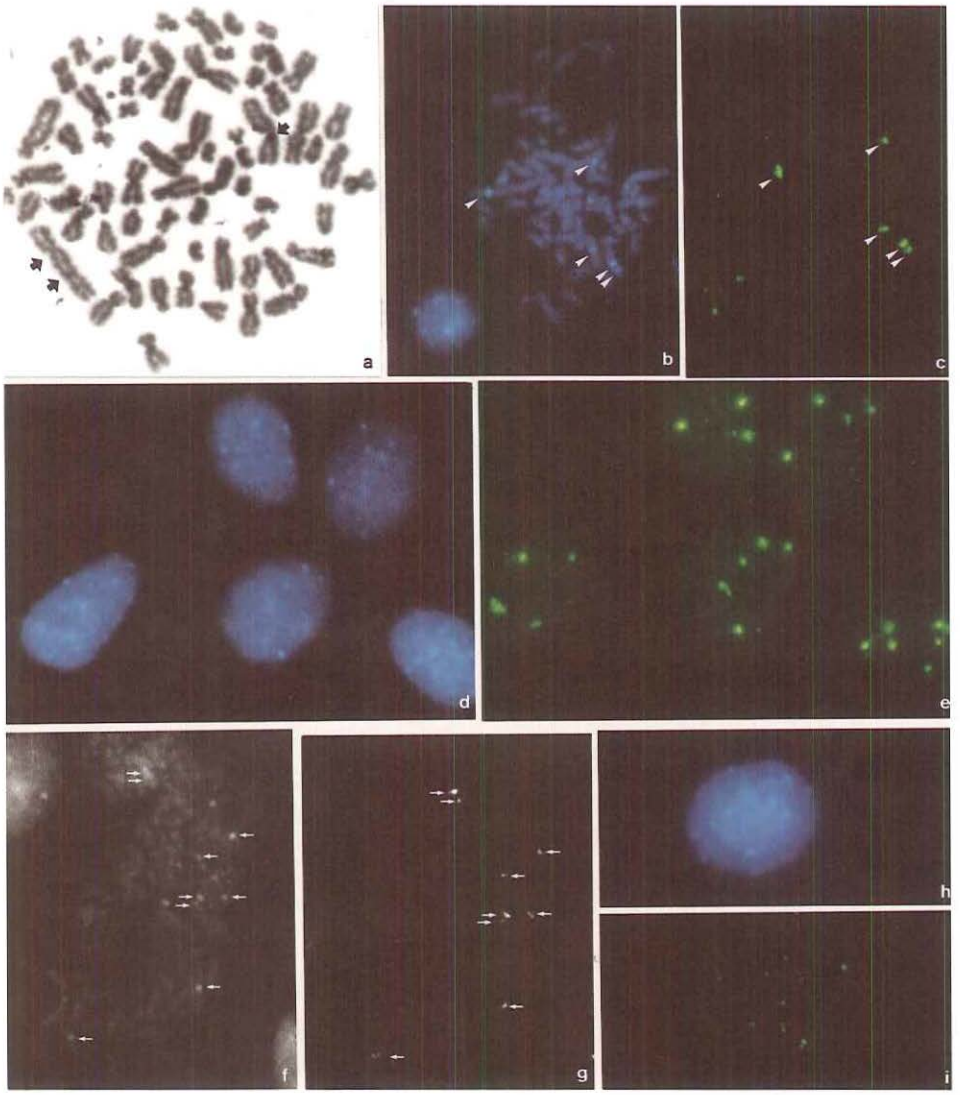

Figure 1. C-banded metaphase (a) and FISH for chromosome 1 (b-i) of case nr. 30
a Arrows indicate heterochromatine, probably of chromosome 1. Double arrow indicates 1q+; b FISH in a metaphase of this TCC after DAPI staining. Arrows indicate heterochromatin staining based on preferential AT staining by DAPI (double arrow indicate 1q+); c FISH signals, after using a probe for the heterochromatin; d, e FISH in interphase nuclei showing heterogeneity in copy numbers; f, g Polyploidization (>5n) shown by FISH in a metaphase after DAPI staining. Two copies of 1q+ indicate polyploidization; h, i Interphase nucleus stained with DAPI (h) and after FISH (i) showing multiple copies for chromosome 1.

In case of tetraploidization four fluorescent signals were observed for all the probes used, in a significant part of the cells above a certain threshold value set based on criteria summarized before.[4, 13, 16] Among these criteria are the low percentages of multiploid cells detected in normal epithelium of the urinary bladder. In this series of experiments a cell population was indicated as tetraploid/polyploid when more than 3-7% of the total cell population contained a duplicated number of FISH signals. The threshold level is dependent on nuclear morphology, cell clumping, an even distribution of DNA staining in the nuclei, discreetness of FISH signals and the percentage of cells with no FISH reactivity. In the diploid tumours, most cells showed two signals for the individual chromosomes.
Polyploidization was considered real when a fraction of cells above the arbitrary threshold showed ≥ 5 signals for all the probes used. In the triploid/tetraploid tumours, most cells showed 3 or 4 signals for the chromosomes tested.
Chromosome imbalance is defined as a difference in the number of signals for individual chromosomes detected with the probes used. For instance a single signal for chromosome 9, three signals for chromosome 7 and two signals for chromosome 1 and 11, in a significant percentage of cells of the tumour, is interpreted as an imbalance. This particular pattern would thus show an underrepresentation of chromosome 9 and an overrepresentation of chromosome 7.
Intra-tumoral heterogeneity is expressed in case of chromosome imbalance and/or tetra- or polyploidization, as determined by FISH.
Heterogeneity is also expressed when cells have a ploidy count deviating from the modal ploidy count as determined by classical chromosome counting.

7.3.4 Flow Cytometry (FCM)

The FCM procedure was described in detail before.[6, 14] Briefly, the samples of mechanically desegregated cells were stained with propidium iodide (PI) for DNA estimation. Cell analysis was performed using a cytofluorograph 5OH (Ortho Instruments, Westwood, MA). PI was excited at 488 nm with an argon ion laser. Fluorescence was measured using a 630 nm filter. Chicken red blood cells were used as an internal standard, while human lymphocytes were used as an external standard. Samples with a DI of 1 ± 0.12 were interpreted as normal diploid.[6]

7.4 RESULTS

7.4.1 DNA-Diploid tumours (n=21)

Fourteen tumours out of 21, with a DI = 1, revealed triploid (3n) and/or tetraploid (4n) cells by means of classical chromosome counting (table 1). Heterogeneity was thus detected in 67% of the tumours.
After FISH, 8 tumours (38%) showed tetraploidization. Imbalance of chromosomes was seen in 13 (62%) cases in which a loss or gain of chromosomes was detected. The chromosomes involved were presented earlier.[4] When the aberrant cases showing tetraploidization and/or imbalance were combined, 14 out of 21 cases (67%) showed intra-lesional heterogeneity.

Table 1. Summary of the data of 21 patients with DNA diploid TCC's.

nr	age	stage	grade	chromosomes					FISH	
						% cells with				
				range	mn	2n	3n	4n	tet	imb
1	74	pTa	G2b	40-83	44	95		5	-	+
2	68	pT1	G2b	40-90	43	77	10	13	+	+
3	80	pT1	G2b	38-49	46	100			-	-
4	68	pTa	G2b	39-65	45	98	2		-	-
5	76	pTa	G2b	42-87	46	67		33	+	-
6	73	pTa	G2a	45-46	46	100			-	-
7	50	pTa	G2a	43-46	46	100			-	+
8	63	pTa	G2b	45-46	46	100			-	-
9	78	pTa	G2b	42-100	46	90		10	-	-
10	65	pTa	G2b	46	46	100			+	+
11	53	pTa	G2b	45-90	46	96		4	-	+
12	78	pTa	G2b	46-90	46	84		16	+	+
13	72	pT2	G2b	35-65	46	95	5		-	-
14	61	pTa	G2b	46-100	47	94		6	+	+
15	78	pTa	G2a	46	46	100			-	-
16	66	pTa	G2a	45-46	45	100			-	+
17	63	pT1	G2b	36-90	43	94		6	-	+
18	56	pTa	G2b	42-80	44	68		32	+	+
19	74	pTa	G3	42-90	46	86		14	+	+
20	71	pT2	G3	37-80	42	84	8	8	+	+
21	70	pT1	G2b	37-85	46	97		3	-	+

Table 2. Summary of data of 9 patients with DNA aneuploid TCC (DI 1.6 - 1.9)

nr	age	stage	grade	chromosomes						FISH	
						% cells with					
				range	mn	2n	3n	4n	≥ 5n	poly	imb
22	64	T2	G3	66-69	69		100			+	+
23	90	T2	G3	55-65	65	10	90			+	+
24	80	Ta	G2b	60-120	69		88		12	-	+
25	66	T1	G2b	47-69	62	23	77			-	+
26	41	T1	G2b	75-140	80		95		5	+	+
27	60	T3	G2b	48-80	75	7	93			+	+
28	91	T2	G3	45-78	70	7	93			-	+
29	65	T2	G2b	65-130	75		97		3	+	+
30	72	T1	G3	50-250	70	3	92		5	+	+

poly = polyploidization

A considerable overlap was noticed between the cases with tetraploidization as detected by FISH and the cases with 3n and or 4n cells as detected by classical chromosome counting. Seven out of eight cases having four fluorescent signals also had cells with a 3n and or 4n chromosome count. In one case (nr 10) no numerical aberration was found using classical cytogenetics, whereas FISH showed an imbalance as well as tetraploidization.
Comparing chromosome counting and FISH with regard to their feasibility to detect (near-) tetraploid cells, classical chromosome counting gave a better score than FISH.

7.4.2 DNA-Aneuploid tumours (n=9)

Eight tumours out of 9, with a DI= 1.6-1.9 revealed 2n and/or ≥ 5n cells by means of classical chromosome counting (table 2). Heterogeneity was thus present in 89% of the tumours. In 4 of these cases one or more ≥ 5n cells were found. All cases showed a triploid modal chromosome count.
After FISH 6 out of 9 cases (67%) showed polyploidization and all cases showed chromosome imbalance. Thus, heterogeneity was detected in all tumours. One example is given in Fig 1.

7.5 DISCUSSION

Tetraploidization is a generally accepted step in the process of tumour progression and accepted as an indicator of poor prognosis when cells, resulting from this process are detected in a malignant disease.[4, 10, 15] For recognition of tetraploidization by means of classical cytogenetic procedures, metaphases are needed. However, dividing cells may be scanty in solid tumours; in our hands the metaphase frequency in well-differentiated TCC's, without previous culture, amounts to a few odd usable metaphases between about 10.000 non-dividing or non-metaphase cells per slide. In normal bladder tissue the metaphase frequency is even lower. Hence, (nearly) all metaphases originate from tumour cells. This means that for analysis of 20 metaphases at least 100.000 cells must be checked. Earlier studies have shown that the analysis of 200 interphases by means of FISH could be performed in a routine setting.[4, 16, 17] However, the risk exists that abnormal cells, when present in low frequencies, are missed as a result of large amounts of normal epithelial or stromal cells. Analysing more than 200 cells per sample without computer help, is difficult to do. Moreover, it is open to discussion whether more information can be gathered by counting more than 200 cells from clinical material, on account of the threshold level. In stimulated lymphocytes from a healthy donor, up to 2% of the cells were identified as having three or four signals and 10% showed only one signal for the probes of chromosomes 1, 7, 10 and 18 used.[18] In bladder tumour material it appeared that up to 3% of the cells did not react with the probes and up to 10% of the cells of diploid tumours showed one, three or four signals with the probes for chromosomes 1, 7, 9 and 11. The aberrant copy number must therefore exceed a certain threshold before being interpreted as abnormal. Such thresholds depend on the quality of the FISH procedure, the presence of split spots, morphology of nuclei or signal intensities.[4, 13] For the analysis of polyploidization a high quality of the cell preparations is required.[4] For classical cytogenetics standard tables at different confidence limits, specific to the problem of presence or absence of mosaicism in a cell sample, are available.[19] For FISH such tables are not yet available.

Comparing the power of classical chromosome counting with the FISH procedure for the detection of heterogeneity in tumour cell populations, it appeared that FISH could not recognize most cases in which the percentage of 3n/4n cells was under 10%. On the other hand, in 13 out of 21 DNA-diploid cases a chromosome imbalance was detected with FISH. However, in interpreting these differences in the sensitivity of both methods, it has to be kept in mind that classical chromosome counting is based on the analysis of dividing cells and that no information is obtained about non-dividing cells. Therefore, FISH procedures are non-selective, in contrast to the classical chromosome counting procedure. This may explain some of the discrepancies between these methods.

Invasive tumours (pT1-pT3) more frequently showed one or more hyperdiploid cells as compared to non-invasive lesions.[1]
It has been reported that during tumour progression chromosomal deletions precede the process of tetraploidization.[4, 10] As examined previously, in about 40% of superficial tumours chromosome 9 was lost.
It may therefore be of clinical importance to detect the deletion and the following tetra- and polyploid cells as early as possible in the course of the disease.
The tumours with a DI between 1.6 and 1.9 all showed a modal chromosome count of 3n. In 4 out of 9 cases ≥ 5n metaphases were also detected, whereas by FISH in 6 out of 9 cases polyploidization was observed. The problem with chromosome counting is that, generally, chromosome counts in the hexaploid region show so many overlaps that counting is arbitrary. With FISH, the evaluation of such cells can be done more successfully.
It is striking that all 9 cases showed a near triploid modal chromosome number (mean 70.5), whereas a tetraploid number was expected. Tetraploidization is probably followed by loss of chromosomes as a result of progression. A similar situation was described for colorectal tumours, where a mean chromosome number of 71.5 was described in 23 cases.[10] The biological implications of polyploidization for tumour evolution are still unknown.
The flow cytometric estimation of the DI, as used in this study, could not detect low frequencies of tetraploid and polyploid cells. The presence of variable admixtures of non-epithelial, non-tumour cells may be one of the reasons. To eliminate these cells, labelling for cytokeratins has been suggested. However a substantial alteration of the DI was not obtained in the past.[6]
Classical chromosome counting is not always successful. In our hands, about 65% of non-invasive TCC specimens provided us with enough metaphases for numerical analysis. Heterogeneity was detected in 67% of DNA-diploid and 89% of DNA-hyperdiploid cases after chromosome counting. FISH could be applied successfully in more than 90% of the tumours. This latter procedure however has limitations with respect to the threshold and the restricted number of DNA probes that can be applied in a routine setting. Heterogeneity was detected in 38% of DNA-diploid cases regarding tetraploidization and in 67% of the cases regarding imbalance. In DNA-hyperdiploid tumours in 67% of the cases heterogeneity was seen as polyploidization by FISH and in 100% as chromosome imbalance. Both methods complement each other in the sense that chromosome counting allows a better detection of heterogeneity after polyploidization, whereas FISH allows the characterization of the specific chromosomes involved in the process of imbalance.

7.6 REFERENCES

1. PAUWELS, R.P.E., SMEETS, A.W.G.B., GERAEDTS, J.P., DEBRUYNE, F.M. (1987). Cytogenetic analysis in urothelial cell carcinoma, *J. Urol.*, 137, 210-215
2. DEVERE WHITE, R.W., DEITCH, A.D., WEST, B., FITZPATRICK, J.M. (1988). The predictive value of flow cytometric information in the clinical management of stage 0 (Ta bladder cancer. *J. Urol.*, 139: 279-285.
3. PERUCCA D., SZEPETOWSKI P., SIMON M-P., GAUDRAY P., (1990). Molecular genetics of human bladder carcinomas. *Cancer Genet. Cytogenet.*, 49, 143-156.
4. HOPMAN, A.H.N., MOESKER, O., SMEETS, A.W.G.B., PAUWELS, R.P.E., VOOIJS, G.P., RAMAEKERS, F.C.S. (1991). Numerical chromosome 1, 7, 9 and 11 aberrations in bladder cancer detected by in situ hybridization. *Cancer Res.*, 51, 644-651.
5. WALDMAN, F.M., CARROLL, P.R., KERSCHMANN, R., COHEN, M.B., FIELD, F.G., MAYALL, B.H. (1991). Centromeric copy number of chromosome 7 is strongly correlated with tumour grade and labelling index in human bladder cancer. *Cancer Res.*, 51, 3807-3813.
6. SMEETS, A.W.G.B., PAUWELS, R.P.E., BECK, J.L.M., GERAEDTS, J.P.M., DEBRUYNE, F.M.J., LAARAKKERS L., FEITZ, W.F.J., VOOIJS, G.P., RAMAEKERS, F.C.S. (1987): Tissue specific markers in flow cytometry of urological cancers. III. Comparing chromosomal and flow cytometric DNA analysis of bladder tumours. *Int. J. Cancer*, 39, 304-310.
7. TEYSSIER, J.R. (1989). The chromosomal analysis of human solid tumours. A triple challenge. *Cancer Genet. Cytogenet.*, 37, 103-125.
8. SANDBERG, A.A. (1986). Chromosome changes in bladder cancer: Clinical and other correlations. *Cancer Genet.Cytogenet.*, 19, 163-175.
9. REMVIKOS, Y., MULERIS, M., VIELH, PH., SALMON, R.J., DUTRILLAUX, B. (1988). DNA content and genetic evolution of human colorectal adenocarcinoma. A study by flow cytometry and cytogenetic analysis. *Int. J. Cancer*, 42, 539-543.
10. MULERIS, M., DELATTRE, O., OLSCHWANG, S., DUTRILLAUX, A-M., REMVIKOS, Y., SALMON, R-J., THOMAS, G., DUTRILLAUX, B. (1990). Cytogenetic and molecular approaches of polyploidization in colorectal adenocarcinomas. *Cancer Genet. Cytogenet.*, 44, 107- 118.
11. PAUWELS, R.P.E., SCHAPERS, R.F.M., SMEETS, A.W.G.B., DEBRUYNE, F.M.J., GERAEDTS, J.P.M. (1988). Grading of superficial bladder cancer. *Br J of Urology*, 61, 129-134.
12. SMEETS, W., PAUWELS, R., LAARAKKERS, L., DEBRUYNE, F., GERAEDTS, J. (1987). Chromosomal analysis of bladder cancer II. A practical method. *Cancer Genet. Cytogenet.*, 29, 23-27.
13. HOPMAN, A.H.N., RAMAEKERS, F.C.S., RAAP, A.K., BECK, J.L.M., DEVILEE, P., PLOEG VAN DER, M., VOOIJS, G.P. (1988). In situ hybridization as a tool to study numerical chromosome aberrations in solid bladder tumours. *Histochemistry*, 89, 307- 316.
14. FEITZ, W.F.J., BECK, H.L.M., SMEETS, A.W.G.B., DEBRUYNE, F.M.J., VOOIJS, G.P., HERMAN, C.J., RAMAEKERS, F.C.S. (1985). Tissue specific markers in flow cytometry of urological cancers: Cytokeratins in bladder carcinoma. *Int. J. Cancer*, 36, 349-356.
15. SHACKNEY, S.E., SNITH, C.A., MILLER, B.W., BURHOLT, D.R., MURTHA, K., GILES, H.R., KETTERER, D.M., POLLICE, A.A. (1989). Model for the genetic evolution of human solid tumours. *Cancer Res.*, 49, 3344- 3354.
16. HOPMAN, A.H.N., PODDIGHE, P.J., SMEETS, A.W.G.B., MOESKER, O., BECK, J.L.M., VOOIJS, G.P., RAMAEKERS, F.C.S. (1989). Detection of numerical chromosome aberrations in bladder cancer by in situ hybridization. *Amer. J. Pathol.* 135, 1105-1118.
17. PODDIGHE, P.J., RAMAEKERS, F.C.S., SMEETS, A.W.G.B., VOOIJS, G.P., HOPMAN, A.H.N. (1992): Structural chromosome 1 aberrations in transitional cell carcinoma of the bladder. Interphase cytogenetics combining a centromeric, telomeric and library DNA-probe. *Cancer Res.*, 52, 4929-4934.

18. NEDERLOF, P.M., FLIER VAN DER, S., RAAP, A.K., TANKE, H.J., PLOEG VAN DER, M., KORNIPS, F., GERAEDTS, J.P.M. (1989). Detection of chromosome aberrations in interphase tumour nuclei by nonradioactive in situ hybridization. *Cancer Genet. Cytogenet.*, 42, 87-98.
19. HOOK, E.B. (1977). Exclusion of chromosomal mosaicism: Tables of 90%, 95% and 99% confidence limits and comments on use. *Am. J. Hum. Genet.*, 29, 94-97.

CHAPTER 8

GENERAL DISCUSSION

8.1 INTRODUCTION

The majority of patients who develop Transitional Cell Carcinoma (TCC) of the urinary bladder are between the ages of 50 and 70. Especially in the older age groups, the disease does not significantly affect the overall survival of these patients.[1] These patients seem to be at such low risk of dying from the tumour, that overtreatment should be avoided. In some patients the TCC progresses rapidly and these patients may die form this cancer. For the clinical management of TCC therefore, methods are necessary that accurately predict the behaviour of the tumour in individual patients at an early stage of the disease process. In this final chapter we will discuss the results of the studies, aimed at defining such parameters, from a more general point of view.

8.2 HISTOPATHOLOGICAL FACTORS

8.2.1 Histopathological Staging

Clinical tumour staging is based on cystoscopy, bimanual palpation and echoscopy. However the most exact and objective method is histopathological staging. Of course, also histopathological staging has its problems.[2] The biopsy material should be representative for the tumour and should contain all layers of the bladder wall. In superficial TCC the pathologist has to differentiate between Ta and T1 TCC, also in case of micro-invasion. Basement membrane (BM) immunostaining can be of help in some cases to distinguish these superficial stages, as was described in chapter 3. Also muscle invasion is an important prognostic sign. In some tumours a considerable fibromuscular reaction is seen underneath the tumour. Invasive growth between these structures should not be considered as muscular invasion. BM immunostaining can be of help in finding small groups of tumour cells between muscle fibres because smooth muscle fibres show a distinct BM. Pathological stage is a very important single prognostic parameter, also in superficial TCC there is a clear difference in progression and survival between stage Ta and T1 tumours (chapter 2-6).

8.2.2 Histopathological Grading

All systems of grading distinguish one or several intermediate grades in addition to low and high-grades as extremes.[3-8] The most frequently used grading system is that of the World Health Organisation (WHO).[5] The criteria used for determining anaplasia in this system are rather vague. As shown by others[9, 10] as well in our own study (chapter 2), the interobserver reproducibility in grading TCC is only about 60% in this system. Furthermore, WHO grade II tumours appear to be heterogenous, as is shown in chapter 4 by morphometric analysis and comprise low- and high-grade tumours. Jordan et al. designed a system in which WHO grade I tumours are classified as papilloma and the other tumours as either low-grade or high-grade.[7] Even then a few intermediate grade tumours did not fit into either group. These authors also stated that a considerable interobserver variation could be expected due to the many variables used. In the grading system we described in chapter 2, only three variables were used: epithelial thickness, nuclear polarity and pleomorphism, resulting in a high interobserver agreement (80% of the tumours were correctly graded by different pathologists). The high interobserver agreement would be an advantage in comparing results of therapy and for epidemiological studies. The described low-high grading appeared to be a significant single predictor of survival and progression. The survival of patients with low-grade tumours was significantly better than that of patients with high-grade tumours. The progression-free interval was also significantly longer in patients with low-grade tumours than in patients with high-grade tumours.

8.3. BASEMENT MEMBRANES

It is a generally accepted concept, that invasion of a carcinoma involves the degradation and penetration of the subepithelial BM. This phenomenon might be used for histopathological evaluation of neoplasms of the bladder. Conventional staining techniques for visualisation of the BM, like reticulin silver impregnation and PAS staining, are not specific for BM alone.[11] Other structures are stained at the same time. Immunostaining techniques, using antibodies specific for collagen type IV and laminin, allow visualization of specific BM constituents.[12-21] The study of the BM in TCC is simplified by this technique. TCC's show various amounts of BM structures. Some TCC's show a continuous BM, some show a patchy BM and others show no BM structures at all. As is shown in chapter 3 the BM pattern as a single prognostic factor conveys significant information. We did find a significant correlation between tumour stage and BM staining. A correlation was also found between ploidy and BM staining, as well as between histological grade and BM staining pattern.

Survival could be predicted significantly, however progression could only be predicted borderline significantly. We found no relation between the presence or absence of BM structures and recurrence rate. BM visualization by immunohistochemical techniques can facilitate recognition of tissue architecture. As such this technique is of importance in the demonstration of microinvasion in superficial TCC and in demonstrating muscle invasive growth.

8.4 NUCLEAR MORPHOMETRY

High-grade tumours are characterized by larger nuclei and more variation in nuclear size.[22] By morphometry, measurement of individual nuclei is easily performed on histological slides with high accuracy.[23] The methods of morphometry are relatively simple and reproducible.[9, 24] We applied a simple technique of morphometric grading to test whether it could divide TCC in low- and high-grade malignant tumours.[25] In chapter 4 the clinical relevance of morphometry is described. Tumours with relatively small nuclei appeared to have a significantly better clinical behaviour in respect to survival and progression than tumours with large nuclei. Morphometry could be especially useful when the WHO system of grading is used, because by nuclear size WHO grade II tumours can be divided in two groups of TCC, with significantly different clinical behaviour. Patients with WHO grade II tumours with relative small nuclei have a significantly better prognosis than patients with WHO grade II tumours with large nuclei.
The counting of mitotic figures in a histological section has been described as a useful diagnostic procedure, yielding prognostic information.[26-28] The counting of mitoses can be highly reproducible when a standardized method is used as was shown in a large multicenter study.[29] The mitotic index was used as a separate prognostic factor in our analyses, as described in the chapters 2 to 6. The mitotic index appears to be an important single prognosticator in respect of survival and progression. Patients having TCC with a low mitotic count have a better prognosis than patients with a high number of mitosis in their tumour.

8.6 DNA ANALYSIS

The clinical use of the measurement of nuclear DNA content in TCC has been described in many papers.[30-46] Many of the studies in this field describe flow cytometry (FCM) as a method to determine DNA ploidy and stress the clinical relevance of this method.[30-37] FCM has the advantage that it can rapidly measure many cells but it has the disadvantage that tumours with predominance of cells with a diploid DNA content and only very few cells

with a high DNA content, might be classified as diploid. The image cytometry method that is described in chapter 5 has the additional value that the DNA content of individual cells can be measured and that all cell images are stored in computer files, which allows visual control of the measured nuclei. The parameters we calculated from DNA cytometric analyses and used for statistical evaluation of their prognostic value were: ploidy class, the number of cells exceeding 5C and the 2C deviation index (2CDI). There was a significant difference in survival between DNA image cytometrically determined diploid and non-diploid cases. The presence of nuclei with a high DNA content indicated poor prognosis. The 2CDI was also an indicator of survival. Image cytometrically determined factors were also found to be strong predictors of progression-free survival.

8.7 CYTOGENETIC ANALYSIS

In chapter 6 and 7 "classical" metaphase and "modern" interphase cytogenetic analyses are described. In the classical cytogenetic analysis chromosomes are identified in the metaphase of the cell cycle. Numerical and structural abnormalities can be recognized. The disadvantage of the method is that it can be only be performed on fresh tumour tissue. Furthermore, sampling may be a problem because not all the tissue can be used for chromosome analysis and only a fraction of the cells will yield analyzable metaphases. Interphase cytogenetics allows assessment of numerical and structural chromosome abnormalities through the use of probes which specifically bind to chromosome specific DNA sequences in the chromosomal centromeric region.[47-52] This technique can be applied on fresh tumour samples[47-50] but also on routinely processed paraffin sections.[51, 52] As is described in chapter 6, with the classical chromosome counting enough cases were analysed to allow testing for clinical relevance of the method. The parameters derived from chromosome counting include the range of the number of chromosomes in different metaphases and the modal number of chromosomes in all cells counted. Both parameters appear to have significant prognostic impact. The survival rate of patients having tumours with a diploid chromosomal modal number was significantly better than that of patients having tumours with a hyperdiploid chromosomal modal number. The survival rate of TCC patients with diploid cells only, was also significantly better than of patients having TCC with diploid and hyperdiploid cells. Progression-free survival was significantly higher in patients having TCC with a diploid modal number of chromosomes than in patients with a hyperdiploid modal number.
The fluorescence in situ hybridization (FISH) method was applied successfully on more than 90% of the TCC tissue samples, compared with a success rate of about 75% for the classical cytogenetic method. In chapter 7 the results of

classical cytogenetic analysis are compared with those of interphase cytogenetics using probes for chromosomes 1, 7, 9 and 11. With regard to their feasibility to detect (near-) tetraploid cells between predominantly diploid cells, chromosome counting in metaphases gave a better score than FISH in tumours with a FCM diploid DNA index. The presence of small percentages of tetraploid or polyploid cells is interpreted as an indicator of progression. In tumours aneuploid by FCM, intralesional heterogeneity could be detected better by FISH than by means of the classical method. Both methods complement each other in the sense that chromosome counting allows better detection of heterogeneity following tetra- or polyploidization, whereas FISH allows the characterization of the specific chromosomes involved in the process of imbalance.

8.8 MULTIVARIATE ANALYSIS

In the chapters 2 to 6 multivariate analysis is applied in order to evaluate the relative contributions of the tested parameters to the prediction of prognosis. Several parameters were found to be significant single prognostic indicators of survival and progression. Between all these parameters strong correlations were invariably found.
To eliminate the effect of correlation between parameters multivariate analysis can be applied successfully.[37, 53-56]
In a multiparameter model with the tested parameters stage, grade, BM content, chromosomal counting, morphometric factors and cytometric parameters, stage always proved to be the most important factor. Because of the strong correlation between different parameters most other parameters could not add much to the prediction of survival and progression. The only parameters that had additional value were mitotic index, 2C-deviation index and modal number of chromosomes, especially in the prediction of progression. These parameters could be used in TCC patients to plan the therapeutic strategy. However it is evident that in individual patients the behaviour of TCC cannot be predicted by single parameter or even by multiparameter analysis. If a patient has for instance a superficial TCC, a high mitotic count can still be the main factor that determines a bad prognosis.

8.9 THE FUTURE

The ongoing developments in the molecular biology of cancer will deepen our understanding of the process of oncogenesis. New techniques, developed on the basis of the results of fundamental research, such as the interphase cytogenetics, could prove to be of clinical importance. Specific chromosome aberrations, numerical or structural, could valuable for the prediction of

survival, progression and recurrence in TCC. Interphase cytogenetic analysis could be combined with more conventional techniques including light microscopy, morphometry, DNA cytometry and blood group deletion assay to develop powerful prognostic algorithms. Ideally one should ultimately find a combination of variables that can distinguish tumours with an aggressive and those with a relatively benign nature. The nature of the multistep and multifactorially determined process of carcinogenesis, however, renders it unlikely that parameters will be found, which distinguish patients with absolute certainty.

8.10 REFERENCES

1. KIEMENEY, L.A.L.M., COEBERGH, J.W.W., KOPER, N.P., VAN DER HEIJDEN, L.H., PAUWELS, R.P.E., SCHAPERS, R.F.M., VERBEEK, A.L.M. (1993). Bladder cancer incidence and survival in the south-eastern part of the Netherlands. *Eur. J. Cancer*, in press.
2. REUTER, V.E. (1990). Pathology of Bladder Cancer: Assessment of Prognostic Variables and Response to therapy. *Seminars in Oncology*, 17, no 5, 524-532.
3. BRODERS, A.C. (1922).Epithelioma of the genito-urinary organs. *Annals of Surgery*, 75, 574-580.
4. BERGKVIST, A., LJUNGQVIST, G., MOBERGER, G. (1965). Classification of bladder tumours based on the cellular pattern. Preliminary report of a clinical-pathological study of 300 cases with a minimum follow-up of eight years. *Acta Chir. Scand.*, 130, 371-378.
5. MOSTOFI, F.K. (1973). In *Histological typing of Urinary Bladder Tumours*, International Histological classification of Tumours. No. 10, World Health Organization (WHO), Geneva.
6. FRIEDELL, G.H., BELL, J.R., BURNEY, S.W., SOTO, E.A., TILTMAN, A.J. (1976). Histopathology and classification of urinary bladder carcinoma. *Urologic Clinics of North America*, 3, 53-70.
7. JORDAN, A.M., WEINGARTEN, J., MURPHY, W.M. (1987). Transitional cell neoplasms of the urinary bladder. *Cancer*, 60, 2766-2774.
8. PAUWELS, R.P.E., SCHAPERS, R.F.M., SMEETS, A.W.G.B., DEBRUYNE, F.M.J., GERAEDTS, J.P.M. (1988). Grading of superficial bladder cancer. *Br. J. Urol.*, 61, 129-134.
9. OOMS, E.C.M., ANDERSON, W.A.D., ALONS, C.L., BOON, M.E., VELDHUIZEN, R.W. (1983). Analysis of the performance of pathologists in the grading of bladder tumours. *Hum. Pathol.*, 14, 140-143.
10. BLOMJOUS, C.E.M., SMEULDERS, A.W.M., BAAK, J.P.A., VOS, W., VAN GALEN, E.M., MEIJER, C.J.L.M. (1989). A comparative study in morphometric grading of transitional cell carcinoma of the urinary bladder. *Anal. quant. cytol. histol.*, 11, 426-432.
11. OZELLO, L., SPEER, F.D. (1958). The mucopolysaccharides in normal and diseased breast: Their distribution and significance. *Am. J. Clin. Pathol.*, 34, 993-1009.
12. ALBRECHTSEN, R., NIELSEN, M., WEWER, U., ENGVALL, E., RUOSLAHTI, E. (1981). Basement membrane changes in breast cancer detected by immunohistochemical staining for laminin. *Cancer Res.*, 41, 5076- 5081.
13. SIEGAL, G.P., BARSKY, S.H., TERRANOVA, V.P., LIOTTA, L.A. (1981). Stages of neoplastic transformation of human breast tissue as monitored by dissolution of basement membrane components. *Invasion Metastasis*, 1, 54-70.
14. BURTIN, P., CHAVANEL, G., FOIDART, J.M., MARTIN, E. (1982). Antigens of the basement membrane and the peritumoral stroma in human colonic adenocarcinomas: An immunofluorescence study. *Int. J. Cancer*, 30, 13-20.
15. BARSKY, S.H., SEIGAL, G.P., JANNOTTA, F., LIOTTA, L.A. (1983). Loss of basement membrane components by invasive tumours but not by their benign counterparts. *Lab. Invest.* 49, 140-147.
16. VISSER, R., VAN DER BEEK, J.M.H., HAVENITH, M.G., CLEUTJENS, J.P.M., BOSMAN, F.T. (1986). Immunocytochemical detection of basement membrane antigens in the histopathological evaluation of laryngeal dysplasia and neoplasia. *Histopathology*, 10, 171- 180.
17. WILLEBRAND, D., BOSMAN, F.T., DE GOEY, A.T.P.M. (1986). Patterns of basement membrane deposition in benign and malignant breast tumours. *Histopathogy*, 10, 1231-1241.
18. CONN, I.G., CROCKER, J., WALLACE, D.M.A., HUGHES, M.A., HILTON, C.J. (1987). Basement membranes in urothelial carcinoma. *Br. J. Urol.*, 60, 536-542.
19. DAHER, N., ABOURACHID, H., BOVE, N., PETIT, J., BURTIN, P. (1987). Collagen IV staining pattern in bladder carcinomas: relationship to prognosis. *Br. J. Cancer*, 55, 665-671.

20. HAVENITH, M.G., ARENDS, J.W., SIMON, R.E.M., VOLOVICS, A., WIGGERS, T., BOSMAN, F.T. (1988). Type IV collagen immunoreactivity in colorectal cancer: Prognostic value of basement membrane deposition. *Cancer*, 62, 2207-2211.
21. HAVENITH, M.G., DINGEMANS, K.P., CLEUTJENS, J.P.M., WAGENAAR, SJ.SC., BOSMAN, F.T. (1989). Basement membranes in bronchogenic squamous cell carcinoma: An immunohistochemical and ultrastructural study. *Ultrastruct.Pathol.*, 14, 51-63.
22. LIPPONEN, P.K., COLLAN, Y., ESKELINEN, M.J., PESONEN, E., SOTARAUTA, M. (1990). Morphometry in human transitional cell bladder cancer. *Eur. Urol.*, 17, 155-160.
23. LIPPONEN, P.K., COLLAN, Y., ESKELINEN, M.J., SIMPANEN, H., PESONEN, E., SOTARAUTA, M. (1989). Potential of morphometry in grading transitional cell carcinoma of the urinary bladder. *Path. Res. Pract.*, 185, 617-620.
24. OOMS, E.C.M., KURVER, P.H.J., VELDHUIZEN, R.W., ALONS, C.L., BOON, M.E. (1983). Morphometric grading of bladder tumours in comparison with histologic grading by pathologists. *Hum. Pathol.*, 14, 144-150.
25. BLOMJOUS, C.E.M., SCHIPPER, N.W., VOS, W., BAAK, J.P.A., DE VOOGT, H.J., MEIJER, C.J.L.M. (1989). Comparison of quantitative and classic prognosticators in urinary bladder carcinoma. *Virchows Arch.*, 415, 421-428.
26. SILVERBERG, S.G. (1976). Reproducibility of the mitosis count in the histologic diagnosis of smooth muscle tumours of the uterus. *Hum. Pathol.*, 7, 451-454.
27. ZUCKMAN, M.H., WILLIAMS, G., LEVIN, H. (1988). Mitosis counting in seminoma: An exercise of questionable significance. *Hum. Pathol.*, 19, 329-335.
28. VASCO, J., MALMSTRÖM, P-U., TAUBE, A., WESTER, K., BUSCH, C. (1991). Prognotic value of a systematized method for determination of mitotic frequency in bladder cancer assisted by computerized image analysis. *J. Urogen. Pathol.*, 1, 53-59.
29. VAN DIEST, P.J., BAAK, J.P.A., MATZE-COK, P., WISSE-BREKELMANS, E.C.M., VAN GALEN, C.M., KURVER, P.H.J., BELLOT, S.M., FIJNHEER, J., VAN GORP, L.H.M., KWEE, W.S., LOS, J., PETERSE, J.L., RUITENBERG, H.M., SCHAPERS, R.F.M., SCHIPPER, M.E.I., SOMSEN, J.G., WILLIG, A.W.P.M., ARIENS, A.TH. (1992). Reproducibility of mitosis counting in 2469 breast cancer specimens: Results from the multicenter morphometric mammary carcinoma project. *Hum. Pathol.*, 23, 603-607.
30. LEVI, P.E., COOPER, F.H., PHIL, D., ANDERSON, C.K., PATH, M.C., WILLIAMS, R.E. (1969). Analyses of DNA content, nuclear size and cell proliferation of transitional cell carcinoma in man. *Cancer*, 23, 1074-1085.
31. FOSSA, S.D., KAALHUS, O., SCOTT-KNUDSEN, O. (1977). The clinical and histopathological significance of Feulgen DNA-values in transitional cell carcinoma of the human urinary bladder. *Eur. J. Cancer*, 13, 1155-1162.
32. HOFSTÄDTER, F., JAKSE, G., LEDERER, B., MIKUZ, G. (1980). Cytophotometric investigations of DNA-content in transitional cell tumours of the bladder. Comparison of results with clinical follow up. *Path. Res. Pract.*, 167, 254-264.
33. BÖCKING, A., ADLER, C.P., COMMON, H.H., HILGARTH, M., GRANZEN, B., AUFFERMANN, W. (1984). Algorithm for DNA cytophotometric diagnosis and grading of malignancy. *Anal. Quant. Cytol.*, 6, 1-8.
34. HOFSTÄDTER, F., JAKSE, G., LEDERER, B., MIKUZ, G., DELGADO, R. (1984). Biological behaviour and DNA cytophotometry of urothelial bladder carcinoma. *Br. J. Urol.*, 56, 289-295.
35. WIJKSTRÖM, H., GRANBERG-ÖHMAN, I., TRIBUKAIT, B. (1984). Chromosomal and DNA patterns in transitional cell bladder carcinoma. A comparative cytogenetic and flow-cytofluorometric DNA study. *Cancer*, 53, 1718-1723.
36. FEITZ, W.F.J., BECK, H.L.M., SMEETS, A.W.G.B., DEBRUYNE, F.M.J., VOOIJS, G.P., HERMAN, C.J., RAMAEKERS, F.C.S. (1985). Tissue specific markers in flow cytometry of urological cancers: Cytokeratins in bladder carcinoma. *Int. J. Cancer*, 36, 349-356.

37. AUFFERMANN, W., URQUARDT, M., RÜBBEN, H., WOHLTMANN, D., BÖCKING, A. (1986). DNA grading of urothelial carcinoma of the bladder. *Anti Cancer Res.*, 6, 27-32.
38. OLJANS, P.J., TANKE, H.J. (1986). Flow cytometric analysis of DNA content in bladder cancer: prognostic value of the DNA-index with respect to early tumour recurrence in G2 tumours. *World J. Urol.*, 4, 205-210.
39. PLOEM, J.S., VAN DRIEL-KULKER, A.M.J., GOYARTS-VELDSTRA, L., PLOEM-ZAAIJER, J.J., VERWOERD, N.P., VAN DER ZWAN, (1986). Image analysis combined with quantitative cytochemistry. *Histochemistry*, 84, 549-555.
40. SMEETS, A.W.G.B., PAUWELS, R.P.E., BECK, J.L.M., GERAEDTS, J.P.M., DEBRUYNE, F.M.J., LAARAKKERS L., FEITZ, W.F.J., VOOIJS, G.P., RAMAEKERS, F.C.S. (1987): Tissue specific markers in flow cytometry of urological cancers. III. Comparing chromosomal and flow cytometric DNA analysis of bladder tumours. *Int. J. Cancer*, 39, 304-310.
41. STÖCKLE, M., TANKE, H.J., MESKER, W.E., PLOEM, J.S., JONAS, U., HOHENFELLNER, R. (1987). Automated DNA-image cytometry: a prognostic tool in infiltrating bladder carcinoma. *World J. Urol.*, 5, 127-132.
42. BLOMJOUS, C.E.M., SCHIPPER, N.W., BAAK, J.P.A., VAN GALEN, E.M., DE VOOGT, H.J., MEIJER, C.J.L.M. (1988). Retrospective study of prognostic importance of DNA flow cytometry of urinary bladder transitional cell carcinoma. *J. Clin. Pathol.*, 41, 21-25.
43. DEVERE WHITE, R.W., DEITCH, A.D., WEST, B., FITZPATRICK, J.M. (1988). The predictive value of flow cytometric information in the clinical management of stage 0 (Ta bladder cancer. *J. Urol.*, 139: 279-285.
44. PLOEM, J.S., VAN DRIEL-KULKER, A.M.J., PLOEM-ZAAIJER, J.J. (1989). Automated cell analysis for DNA studies of large cell populations using the LEYTAS image cytometry system. *Path. Res. Pract.*, 185, 671-675.
45. LIPPONEN, P.K., COLLAN, Y., ESKELINEN, M.J., PESONEN, E., SOTARAUTA, M., NORDLING, S. (1990). Comparison of morphometry and DNA flow cytometry with standard prognostic factors in bladder cancer. *Brit. J. Urol.*, 65, 589-597.
46. SHAABAN, A.A., TRIBUKAIT, B., EL-BEDEIWY, A.A., GHONEIM, M.A. (1990). Prediction of lymph node metastases in bladder carcinoma with deoxyribonucleic acid flow cytometry. *J. Urol.*, 144, 884-887.
47. HOPMAN, A.H.N., RAMAEKERS, F.C.S., RAAP, A.K., BECK, J.L.M., DEVILEE, P., PLOEG VAN DER, M., VOOIJS, G.P. (1988). In situ hybridization as a tool to study numerical chromosome aberrations in solid bladder tumours. *Histochemistry*, 89, 307-316.
48. HOPMAN, A.H.N., PODDIGHE, P.J., SMEETS, A.W.G.B., MOESKER, O., BECK, J.L.M., VOOIJS, G.P., RAMAEKERS, F.C.S. (1989). Detection of numerical chromosome aberrations in bladder cancer by in situ hybridization. *Amer. J. Pathol.* 135, 1105-1118.
49. NEDERLOF, P.M., FLIER VAN DER, S., RAAP, A.K., TANKE, H.J., PLOEG VAN DER, M., KORNIPS, F., GERAEDTS, J.P.M. (1989). Detection of chromosome aberrations in interphase tumour nuclei by nonradioactive in situ hybridization. *Cancer Genet. Cytogenet.*, 42, 87-98.
50. HOMAN, A.H.N., MOESKER, O., SMEETS, A.W.G.B., PAUWELS, R.P.E., VOOIJS, G.P., RAMAEKERS, F.C.S. (1991). Numerical chromosome 1, 7, 9 and 11 aberrations in bladder cancer detected by in situ hybridization. *Cancer Res.*, 51, 644-651.
51. HOPMAN, A.H.N., VAN HOOREN, E., VAN DE KAA, C.A., VOOIJS, G.P. RAMAEKERS, F.C.S. (1991). Detection of numerical chromosome aberrations using in situ hybridization in paraffin sections of routinely processed bladder cancers. *Modern Pathol.*, 4, 503-513.
52. VAN DE KAA, C.A., NELSON, K.A.M., RAMAEKERS, F.C.S., VOOIJS, G.P., HOPMAN, A.H.N. (1991). Interphase cytogenetics in parafin sections of routinely processed hydatiform moles and hydropic abortions. *J. Pathol.*, 165, 281-287.
53. BAAK, J.P.A., VAN DOP, H., KURVER, P.H.J., HERMANS, J., (1985). The value of morphometry to classic prognosticators in breast cancer. *Cancer*, 6, 374-382.

54. MONTERONI, R., SCARPELLI, M., PISANI, E., ANSUINI, E., COLLINA, G., MARIUZZI, G.M., COLLAN, Y. (1986). Multivariate classifications of transitional cell tumours of the bladder. Nuclear abnormality index and pattern recognition analysis. *Appl. Pathol.*, 4, 48-54.
55. BLOMJOUS, C.E.M., SCHIPPER, N.W., BAAK, J.P.A., VOS, W., DE VOOGT, H.J., MEIJER, C.J.L.M. (1989). The value of morphometry in addition to classic prognosticators in superficial urinary bladder carcinoma. *Am. J. Clin. Pathol.*, 91, 243-248.
56. ESKELINEN, M., COLLAN, Y., PAJARINEN, P., PESONEN, E., KETTUNEN, K., NORDLING, S. (1990). Improving the prognostic value of axillary nodal status in breast cancer by combining DNA ploidy and tumour size in a prognostic index. *Acta Chir. Scand.*, 156, 521-527.

SUMMARY

In this thesis a study of objective and reproducible characteristics, that might have prognostic significance in bladder cancer, is described. In the Netherlands bladder cancer is fourth in the order of frequency in the occurrence of tumours in males. In females bladder cancer is less frequent (twelfth most frequent). The male-female ratio is 4.8 to 1. In other countries the male predominance is less pronounced. The most used parameters in the management of bladder cancer are stage and grade. However reproducibility problems are well known from the literature. We have described a grading system for bladder cancer in which only low-grade and high-grade malignant tumours are distinguished. Reproducibility proved to be good. The survival of patients with low-grade tumours was significantly better than that of patients with high-grade tumours. The progression-free interval was also significantly longer in patients with low-grade tumours than in patients with high-grade tumours.

Penetration of the basement membrane (BM) was studied by immunohistochemical staining of some BM components. The survival rate of patients having tumours with an interrupted or absent BM was significantly lower than that of patients having tumours with an intact BM. The rate of progression was greater in tumours with an interrupted or absent BM than in tumours with an intact BM. No association was found between BM status and recurrence.

Selective nuclear morphometry was used as a quantitative measure of nuclear grade. In WHO grade II (histological grading) tumours morphometric heterogeneity could be demonstrated. There was a significant difference in survival between morphometrically determined low-grade malignant cases and high-grade malignant cases, the presence of large nuclei indicating poor prognosis. The morphometrically determined grade was also found to be a strong predictor of tumour progression.

DNA content of nuclei was studied by DNA image cytometry. By image cytometry the DNA content in individual cells is measured. This method is more sensitive in determining ploidy than flow cytometry, especially in tumours with a low number of aneuploid cells. There was a significant difference in survival between DNA image cytometrically determined diploid and non-diploid cases. The presence of nuclei with a high DNA content indicated poor prognosis. The 2C deviation index (2CDI) was also an indicator of survival. Image cytometrically determined factors were also found to be strong predictors of progression. We also investigated the potential use of numerical chromosomal abnormalities as predictive factor for the

clinical behaviour of TCC. The survival of patients having tumours with a diploid chromosomal modal number was significantly better than of patients having tumours with hyperdiploid chromosomal modal number. Progression was seen more frequently in patients having TCC with a hyperdiploid modal number of chromosomes.
Chromosome counting in metaphase nuclei was compared with fluorescence in situ hybridization (FISH). In FISH centromere specific DNA probes for chromosomes 1, 7, 9, and 11 were used.
Both methods complement each other in that sense that chromosome counting allows better the detection of heterogeneity after tetra- or polyploidization, while FISH allows the detection of the involvement of specific chromosomes in karyotypic abnormalities. With classical chromosome counting not all tumours can be analysed. With FISH small numbers of cells with tetra- or polyploidization, between many diploid cells, are not recognized.
Combining multiple parameters from our studies, stage appeared to be the most important prognostic factor in all our studies. The mitotic index appeared to be a factor that has independent value in predicting progression. In superficial bladder cancer the modal chromosome number and one of the cytometrically determined factors (2CDI) appeared to be important parameters in predicting survival and progression. FISH on interphase nuclei has to be further investigated regarding its clinical value.

SAMENVATTING

In dit proefschrift wordt een onderzoek beschreven naar objectieve en reproduceerbare kenmerken van blaaskanker die mogelijk betekenis hebben voor de prognose. In Nederland is blaaskanker de vierde meest voorkomende kwaadaardige tumor bij mannen. Bij vrouwen komt blaaskanker minder voor (twaalfde plaats). Bij mannen komt blaaskanker 4.8 maal zo veel voor als bij vrouwen. In andere landen is het verschil tussen mannen en vrouwen minder duidelijk. De meest gebruikte parameters voor het tumor gedrag bij de behandeling van blaaskanker zijn stadium en graad. Echter uit de literatuur is bekend dat er problemen bestaan met de reproduceerbaarheid van het vaststellen van deze parameters. Wij hebben een graderingssysteem voor blaaskanker beschreven waarbij alleen laag- en hooggradig kwaadaardige tumoren worden onderscheiden. De reproduceerbaarheid bleek goed te zijn. Patiënten met laaggradige tumoren leven significant langer dan patiënten met hooggradige tumoren.

Het doordringen van de basaalmembraan werd bestudeerd door immuun histochemische kleuring van enkele basaalmembraan componenten. Bij patiënten waarbij in de blaastumor de basaalmembraan onderbroken of afwezig was, bleek de overleving significant korter dan bij patiënten met een tumor waarvan de basaalmembraan intact was. Ook was de progressievrije periode van patiënten met een tumor waarvan de basaalmembraan onderbroken of afwezig was korter dan van patiënten met een tumor met een intakte basaalmembraan. Er werd geen samenhang gezien met het optreden van recidieven.

Het selectief meten van kernen (morfometrie) werd gebruikt als een kwantitatieve maat voor kern gradering. Bij WHO graad II tumoren (histologische gradering) kon een morfometrische diversiteit worden aangetoond. Er werd een verschil gevonden in overleving tussen morfometrisch bepaalde laag- en hooggradig kwaadaardige gevallen. De aanwezigheid van grote kernen wees op een slechte prognose. Deze morfometrische gradering kon ook de progressie voorspellen.

Het DNA gehalte van kernen werd bestudeerd met behulp van DNA beeld cytometrie. Bij DNA beeld cytometrie wordt het DNA van individuele kernen gemeten. Deze methode is gevoeliger om de ploïdie vast te stellen dan flow cytometrie, speciaal in gezwellen met een laag aantal aneuploïde cellen in een overmaat van diploïde cellen. Er werd een significant verschil in overleving gevonden tussen DNA beeld cytometrisch vastgestelde diploïde en niet diploïde gezwellen. De aanwezigheid van een groot aantal cellen met een hoog DNA gehalte voorspelde een slechte prognose. Het aantal kernen dat een

DNA gehalte had dat afweek van het diploïde gehalte (2CDI) bleek eveneens een indicator voor overleving. Deze beeld cytometrische factoren bleken eveneens progressie te kunnen voorspellen.
Wij onderzochten ook het mogelijk nut van numerieke chromosoom afwijkingen in metafase kernen als voorspellende factor van het klinische gedrag van blaastumoren. De overleving van patiënten met een diploïd modaal aantal chromosomen was beter dan van patiënten met een tumor met een hyperdiploïd aantal chromosomen. Progressie werd eveneens meer gezien bij patiënten met een hyperdiploïd aantal chromosomen.
De beoordeling van chromosomen in metafase kernen werd vergeleken met chromosoom onderzoek met behulp van fluorescentie in situ hybridisatie op interfase kernen. Bij deze techniek werden specifieke nucleïnezuur "probes" voor de chromosomen 1, 7, 9 en 11 gebruikt. Beide methoden vullen elkaar aan omdat het klassieke chromosoom onderzoek beter in staat stelt diversiteit vast te stellen na tetra- en polyploïdisatie, terwijl in situ hybridisatie het mogelijk maakt om de betrokkenheid van specifieke chromosomen te bepalen bij karyotypische afwijkingen. Het klassieke chromosoom onderzoek lukt niet bij alle tumoren, in situ hybridisatie meestal wel.
Bij het analyseren van combinaties van de verschillende parameters uit ons onderzoek bleek het stadium de belangrijkste factor te zijn voor het bepalen van de prognose. De mitosefrequentie bleek een factor die onafhankelijk van het stadium, aanvullende informatie geeft voor het voorspellen van progressie. Bij oppervlakkige blaastumoren bleken het modaal aantal chromosomen en een van de beeld cytometrische factoren (2CDI) het meest belangrijk voor het bepalen van overleving en progressie. Chromosoom onderzoek met behulp van in situ hybridisatie op interfase kernen moet nog verder onderzocht worden op zijn klinische bruikbaarheid.

DANKWOORD

Het onderzoek voor dit proefschrift werd voor een belangrijk deel uitgevoerd in het kader van het praeventiefonds project "Preventie van over- en onderbehandeling van blaastumoren: Ontwikkeling van een prognostische index". Het onderzoek heeft plaatsgevonden in het laboratorium voor pathologie van de stichting ziekenhuizen Noord-Limburg, Venlo, het klinisch chemisch laboratorium van de stichting ziekenhuis apotheek en laboratorium, Venray, het Sylvius laboratorium van de Universiteit van Leiden en de afdeling moleculaire biologie van de Universiteit Limburg. Er werd bovendien intensief samengewerkt met de vakgroepen Pathologie van de Universiteit van Limburg, Maastricht, en van de Erasmus Universiteit, Rotterdam.

Het voorbereiden en schrijven van een proefschrift is bijna nooit het product van één of enkele personen. Velen hebben dan ook bijgedragen aan het totstandkomen van dit boekje, deels zelfs onbewust. Het is dan ook bijna ondoenlijk een ieder bij name te noemen. Om te voorkomen dat ik iemand vergeet, wil ik allen die mij tot steun zijn geweest bij het verrichten van dit werk, hartelijk bedanken.

Een aantal mensen wil ik toch in het bijzonder noemen.

In 1978 werd op initiatief van Ruud Pauwels het onderzoek naar blaastumoren gestart in het St. Maartens Gasthuis Venlo. Samen met Wim Smeets ontstond een hecht "Urologisch-Genetisch-Pathologisch" pact.
Ruud en Wim, jullie hebben mij in 1987 onder het genot van een glas wijn, gesuggereerd de reeds verrichte inspanningen op het gebied van de blaastumoren uit te breiden tot een promotiewaardig onderzoek.
Jullie enthousiasme en doorzettingsvermogen was voor mij een stimulans om het werk aan te vangen en af te ronden.

Prof. Dr. F.T. Bosman, beste Fré, jij hebt je in december 1987 bereid verklaard op te treden als mijn promotor. In je brief van toen toonde je je verheugd over het feit dat een perifere patholoog zich weer op het smalle pad van de wetenschap durfde begeven. Je gaf aan dat je dit onderzoek wel zag zitten. Jou snelle en deskundige adviezen aangaande de inhoud van de afzondelijke hoofdstukken en uiteindelijk het hele manuscript waren voor mij van onschatbare waarde.

De leden van de beoordelingscommissie dank ik voor de voortvarende wijze waarop zij het manuscript hebben getoetst.

De diverse co-auteurs van de afzondelijke hoofdstukken hadden ieder op hun vakgebied gewaardeerde inbreng.

Piet van den Brandt, afdeling epidemiologie van de Universiteit Limburg, en Jack Wijnen, afdeling wiskunde en informatica van de technische Universiteit Eindhoven, worden bedankt voor hun hulp bij de statistische bewerking van de gegevens.
Jan Willem Arends en Eric Thunnissen, afdeling Pathologie van de Universiteit Limburg en Jan Willem Coebergh, epidemiloog en adviseur van het Intergraal Kankercentrum Zuid, dank ik voor hun medewerking en totstandkoming van hoofdstuk 2.

Miek Havenith, destijds afdeling pathologie van de Universiteit Limburg, tegenwoordig afdeling pathologie Enschede, wil ik danken voor de inbreng van zijn expertise op het gebied van de basaal membranen en voor het tot stand komen van hoofdstuk 3.

Hoofdstuk 5 kwam tot stand in nauwe samenwerking met het Sylvius Laboratorium van de Universiteit Leiden. Joke (Ploem) en Hans (Tanke) jullie inzet en enthousiasme voor de Leidse cytometrie was zeer stimulerend. Irma Ouwerkerk wil ik bedanken voor de technische bewerking van het materiaal in Leiden. Zonder jullie hulp was hoofdstuk 5 niet tot stand gekomen.

De vergelijking van het conventionele chromosoom onderzoek met het ISH onderzoek op interfase kernen, zoals beschreven in hoofdstuk 7 kwam tot stand in samenwerking met de afdeling moleculaire biologie en genetica van de Universiteit Limburg. Ton Hopman, Frans Ramaekers en Joep Geraedts worden bedankt voor hun deskundige hulp bij het tot stand komen van dit hoofdstuk.

René Retera, mijn associé, bedankt voor je belangstelling en steun bij dit onderzoek. Zonder enig probleem werd door jou een deel van mijn werk overgenomen als ik weer eens naar de statisticus, de promotor of andere wetenschappers moest.

Collega Jan Scheepers, Uroloog, wil ik bedanken voor het beschikbaar stellen van zijn patientenbestand en voor de nauwgezette follow-up van zijn blaascarcinoom patiënten.

Natuurlijk moet ik ook alle medewerkers van het laboratorium voor Pathologie van de stichting ziekenhuizen Noord Limburg bedanken voor de extra inspanningen die geleverd moesten worden voor mijn onderzoek.
Met name Wies Hamstra wil ik bedanken. Wies jou nauwgezet bewaken van het patiënten materiaal, de immunohistochemie en het prepareren van het materiaal voor image cytometrie waren van groot belang voor het onderzoek.
Wouter Tiggelovend dank ik voor het meten van vele kernen. Deze meetgegevens werden gebruikt in hoofdstuk 4.
De dames van het secretariaat dank ik voor het tot vervelens opzoeken van patientengegevens.

Jan van de Kerkhof wil ik bedanken voor het tekenen van de grafieken uit de diverse hoofdstukken.

Dank ook aan Anja van Gulick voor het verzorgen van litteratuur gegevens.

De medewerkers van de afdeling ABC van het St. Maartens Gasthuis dank ik voor de vele praktische tips die ik van hen kreeg als ik weer eens even was vastgelopen met de PC.

Sue Muysers-Godfrey dank ik voor het kritisch doorlezen en corrigeren van de tekst van het manuscript.

Het bestuur en de directie van de Stichting Ziekenhuizen Noord-Limburg en van de stichting Ziekenhuis en Apotheek Venray, wil ik bedanken voor de mogelijkheden die geschapen werden om onderzoek te verrichten in een perifeer ziekenhuis.

Wekenlang werd door Moon elke avond besteed aan het vastleggen van alle gegevens van het onderzoek in het patiëntenbestand op de PC. Hiermee werd de basis voor dit proefschrift gelegd.

CURRICULUM VITAE

1946	Geboren te Delft
1965	Eindexamen H.B.S. B, St. Stanislas College, Delft
1965-1972	Studie Geneeskunde Rijksuniversiteit Utrecht. Als assistent arts werkzaam op de afdelingen Pathologie (Prof. dr. G. Bras) en Anaesthesiologie (Prof. dr. B. Smalhout)
1973	Arts assistent afdeling chirurgie Zuiderziekenhuis, Rotterdam (Opleider: Dr. G.A.A. Olthuis)
1974-1977	Opleiding tot Patholoog, Dijkzicht Ziekenhuis, Rotterdam (opleiders: Prof. dr. R.O. v.d. Heul; Prof. dr. G. Wielenga)
1977-	Patholoog, werkzaam in het St. Elisabeth Ziekenhuis, Venray en het St. Maartens Gasthuis, Venlo (verenigd in de Stichting Ziekenhuizen Noord-Limburg)