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Progression and Survival in Transitional Cell Bladder Cancer: a Comparison of Established Prognostic Factors, S-phase Fraction and DNA Ploidy

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DNA flow cytometric (FCM) data, histological features and clinical stage of bladder tumours in 222 patients were related to outcome during a mean follow-up of 10 years. Aneuploidy was detected in 82 (37%) of tumours and 140 (63%) were diploid. Intratumour heterogenity of DNA ploidy was found in 27% of 30 cases. Aneuploidy and high S-phase fraction (SPF) were related to clinical stage, histological grade and papillarity ($P < 0.0001$). Aneuploidy ($P < 0.0001$) and high SPF ($P < 0.0001$) predicted metastasis to pelvic lymph-nodes and to distant sites. T category ($P < 0.0001$), SPF ($P < 0.0001$), histological grade ($P < 0.0001$), papillarity ($P = 0.0021$), DNA aneuploidy ($P = 0.0094$) and G2 fraction ($P = 0.0340$) predicted cancer-related survival. Multivariate analysis showed DNA aneuploidy as the most important predictor of progression in T category ($P = 0.0003$) and T category was the most important predictor of lymph-node involvement ($P = 0.0083$) and metastasis ($P = 0.0015$), followed by FCM parameters. In Ta-T1 tumours SPF predicted progression independently ($P = 0.0153$). FCM offers more accurate prognostic information in Ta-T1 tumours than conventional methods. In invasive tumours, FCM offers quantitative prognostic information in terms of pelvic lymph-node metastasis and metastasis to distant sites.
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INTRODUCTION

AT PRESENT, decisions on treatment [1] of transitional cell tumours of the urinary bladder are based on clinical stage [2] and histological grade [3]. Both histological grading [4] and clinical staging [5] are variable due to the subjectivity of the assessments. Histoquantitative techniques [6–17] have been extensively studied to find reproducible grading methods for accurate selection of modes of therapy since the treatment results, in superficial tumours in particular, are unsatisfactory [1, 18]. Flow cytometry (FCM) is a rapid method to determine the DNA content and proliferative activity of tumours [6–10] and it is possible to use old paraffin-embedded starting materials [19]. Of special interest to histopathologists and urologists is whether DNA ploidy determination and estimation of SPF by means of FCM can help in prediction of progression in superficial tumours and in evaluating the risk of pelvic lymph-node involvement and metastasis to distant sites [1]. These questions were investigated and patient survival assessed by comparing clinical and flow cytometric data in 222 bladder cancer patients with a mean clinical follow-up period of 10 years.

PATIENTS AND METHODS

Patients, treatment and follow-up

265 bladder cancer patients were diagnosed, treated and followed up during 1965–1990 at Kuopio University Central Hospital, Finland. Of these patients, in 222 cases archival paraffin embedded tumour samples were suitable for FCM. The mean (S.D.) age of females ($n = 43$) at diagnosis was 69.6 (8.7) years and of males ($n = 179$) 65.9 (13.1) years. The follow-up period lasted for a mean (S.D.) of 10.8 (3.4) years (range 5–25 years). The diagnosis, treatment and follow-up investigations were carried out mainly by two urologists. The diagnosis, clinical staging (UICC 1978) [2] and follow-up were based on clinical examination, urine cytology, cystoscopy, bladder biopsy, ultrasound, computed tomography and pedal lymphography when adequate, and chest X-ray, bone X-ray and routine laboratory tests [1]. The tumours were treated as curatively as possible according to generally accepted principles [1]. The follow-up investigations were made at 3 month intervals during the first 2 years and thereafter at 6 month intervals. If a recurrent tumour was found the follow-up programme was started again. The progression of tumours was defined as an increase in T, N or M categories during the follow-up period. Many of the patients who died during the follow-up period underwent necropsy to assess the extent of bladder tumours and to confirm the cause of death.

Flow cytometry and histological grading

The samples used for FCM were transurethral or peroperative biopsy specimens from the primary tumours, fixed in 10% puffered formalin (pH = 7.0) and embedded in paraffin. In 30 cases (randomly chosen) FCM was done from 2–5 samples from the same tumour to detect intratumour heterogenity of DNA ploidy. Samples for DNA analysis were prepared using a modification [9] of the method described by Hedley *et al.* [19]. Briefly, 50 μm thick sections were treated with 10 μg/ml proteinase K (Sigma) for 30 min at room temperature. After filtration, the

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Table 1. Distribution of patients into clinical stage groups and histological grades

Histological grade		Clinical stage				
		Ta	T1	T2	T3	T4
I	($n = 81$)	2	60	13	4	2
II	($n = 97$)		42	34	17	4
III	($n = 44$)		8	12	13	11
Total	($n = 222$)	2	110	59	34	17

The relation between clinical stage and histological grade is significant ($\chi^2 = 62.3, P < 0.0001$).

nuclei were treated with 10 μl/ml RNase and stained with 25 μg/ml ethidium bromide (Sigma) for at least 1 hour. The DNA was determined by FCM (FACS IV and FACS Star, Becton Dickinson) using an emission at 488 nm at 200 mW, and the total emission above 560 nm was recorded. As the staining intensity of fixed nuclei varied from one sample to another, no internal standard was included. The lowest peak was given a DNA index (DI) value of 1 and the DI of other peaks were calculated with this as a reference. The S-phase fraction (SPF) was calculated using the built-in computer program. SPF and G2% could be determined in 193/222 (87%) of the samples. Tumours with DI of 1.00–1.049 were recorded as diploid and tumours with DI > 1.05 were considered aneuploid. Tumours with DI between 1.75–2.00 were considered near tetraploid or tetraploid. In cases DNA analysis showed intratumour heterogenity, the aneuploid DI value and corresponding SPF and G2% values were used in further analysis. The mean (S.D.) of CV (coefficient of variation) of the measurements was 7.5 (4.6)%.

The histological grading of tumours was done by a board-certified pathologist, according to WHO criteria [3]. The growth type of the tumours was recorded and tumours were divided into papillary or flat types. The grouping of patients, subdivided according to clinical stage (T category) and histological grade, is shown in Table 1. Statistical analysis was done by the SPSS/PC+ program package [20] in an Amstrad PC1640HD20 computer. The statistical tests used and mean (S.E.) of continuous variables are indicated where appropriate. The survival analysis was done according to life table method with Lee–Desu statistics [21] in a VAX-computer at the University of Kuopio. The material was tested in two phases: firstly, the whole material was analysed, and thereafter Ta-T1 tumours were separately analysed.

RESULTS

All cases

140 (63%) tumours had a diploid DNA content and 82 (37%) were aneuploid (2 multiploid, 35 tetraploid or near tetraploid). Intratumour heterogenity of DI was found in 8/30 (27%) of tumours whereas 16/30 (53%) of tumours were diploid and 6/30 (20%) were aneuploid in all samples. The grouping of patients according to DNA ploidy, clinical stage, histological grade and papillarity is shown in Table 2.

66% of grade III tumours and 57% of T3-T4 tumours were aneuploid whereas 75% of Ta-T1GI-II tumours were diploid. High grade T3-T4 tumours as well as flat tumours showed significantly higher SPF and G2% values than superficial papillary grade I tumours (Table 3). 26 tumours presenting with pelvic lymph-node metastasis at the diagnosis had significantly higher mean (S.E.) values of SPF values [16.2 (2.1)], than tumours confined to the bladder wall [8.1 (0.6)], (*t* test, $P < 0.0001$). The mean (S.E.) values of SPF were higher in aneuploid tumours [16.7 (1.2)] than in diploid ones [5.2 (0.4)] (*t* test, $P < 0.0001$; Pearson, $r = 0.625$). The mean (S.E.) values of G2% were higher in aneuploid tumours [6.1 (0.7)] than in diploid tumours [3.4 (0.2)%] (*t* test, $P < 0.0001$; Pearson, $r = 0.336$).

Table 2. Patients categorised by clinical stage, histological grade, papillarity and DNA ploidy

Feature	Number	Diploid	Aneuploid	χ^2	*P*
Clinical stage					
Ta	2	2	0		
T1	110	84	26		
T2	59	34	25	23.3	0.0001
T3	34	18	16		
T4	17	4	13		
Papillarity					
Papillar	182	130	52		
Flat	40	12	28	22.6	< 0.0001
Histological grade					
I	81	68	13		
II	97	59	38	31.5	< 0.0001
III	44	15	29		

Progression in clinical stage (T) was related significantly to initial clinical stage (T) ($\chi^2 = 36.5$, $P < 0.0001$), histological grade ($\chi^2 = 12.4$, $P = 0.0020$), papillarity ($\chi^2 = 4.1$, $P = 0.0412$) as well as to flow cytometric variables (Table 4). Progressing tumours had higher SPF values and G2% values, too (Table 5). In a multivariate regression analysis of progression aneuploidy, SPF and clinical stage had prognostic value (Table 6).

The clinical stage (Fig. 1), WHO grade (Fig. 2) and papillarity ($\chi^2 = 20.1$, $P = 0.0021$) predicted disease-related survival. The significant differences in disease-related survival subdivided

Table 3. Mean (S.E.) values of SPF and G2% in clinical stages, histological grades and in flat or papillary tumours

Feature	Number	SPF	*P*	G2%	*P*
Stage					
Ta	2	10.0 (4.1)		4.3 (0.3)	
T1	97	6.0 (0.7)		3.4 (0.3)	
T2	52	9.5 (1.2)	< 0.0001*	4.7 (0.8)	0.0450*
T3	27	13.7 (1.8)		5.7 (0.8)	
T4	15	19.4 (2.7)		5.7 (0.9)	
Grade					
I	70	4.5 (0.6)		2.6 (0.2)	
II	87	9.1 (0.9)	< 0.0001*	4.1 (0.3)	< 0.0001*
III	36	18.1 (1.6)		7.6 (1.1)	
Papillarity					
Papillar	160	8.5 (0.6)		7.2 (1.3)	
Flat	33	17.0 (1.6)	< 0.0001†	3.6 (0.2)	0.0100†

SPF and G2% was available in 193 cases.
*Two-tailed analysis of variance, †Student's *t* test.

Table 4. Progression in T, N and M categories related with flow cytometric data

Category	Diploid	Aneuploid	SPF ≤10%	SPF >10%	G2 ≤4.7%	G2 >4.7%
T category						
No progression	109	33	103	24	96	31
Progression	48	32	38	28	44	22
	$P = 0.0131$		$P = 0.0009$		$P = 0.2031$	
N category						
No progression	112	30	106	21	98	29
Progression	39	41	30	36	36	30
	$P < 0.0001$		$P < 0.0001$		$P = 0.0013$	
M category						
No progression	112	30	105	22	96	31
Progression	39	41	31	35	38	28
	$P < 0.0001$		$P < 0.0001$		$P = 0.0110$	

SPF and G2% were available in 193 cases.
P values were calculated by the χ^2 test.

according to DNA ploidy (Fig. 3), SPF (Fig. 4) and G2% (Fig. 5) are presented graphically.

Ta-T1 tumours

Using χ^2 statistics (same group limits as in Table 4), progression in T category was related to DNA ploidy ($\chi^2 = 4.1$, $P = 0.0425$) and SPF ($\chi^2 = 5.5$, $P = 0.0190$) whereas papillarity ($P = 0.13$), histological grade ($P = 0.50$) and G2% ($P = 0.35$) were weak predictors. 34% (9/26) of aneuplopid tumours progressed whereas 16% (14/86) of diploid tumours showed progression in T category. Of tumours having SPF >10%, 39% showed progression in T category. Progressing tumours had higher mean (S.E.) of SPF [10.0 (1.9)] than non-progressing tumours [5.2 (0.7)%] (t test, $P = 0.0051$). Similarly progressing tumours showed higher mean (S.E.) of G2% [4.3 (0.8)], than non-progressing tumours [2.5 (0.3)] (t test $P = 0.0301$). DNA ploidy ($\chi^2 = 4.4$, $P = 0.0356$) and SPF ($\chi^2 = 3.4$, $P = 0.0664$) predicted progression in N and M categories. A multivariate analysis revealed SPF as the most important independent predictor of progression (Table 6). In survival analysis DNA ploidy ($\chi^2 = 4.0$, $P = 0.0433$) and SPF ($\chi^2 = 4.7$, $P = 0.0301$) predicted disease-related survival.

Table 5. Mean (S.E.) values of SPF and G2% in progressing and non-progressing tumours in T, N and M categories

Category	Number	SPF(S.E.)	P	G2%(S.E.)	P
T category					
No progression	141	7.7 (0.7)		3.9 (0.3)	
Progression	52	12.8 (1.4)	0.0005	5.4 (0.7)	0.0350
N category					
No progression	136	6.6 (0.6)		3.5 (0.3)	
Progression	57	15.1 (1.4)	< 0.0001	5.9 (0.6)	0.0020
M category					
No progression	136	6.8 (0.6)		3.7 (0.3)	
Progression	57	14.6 (1.4)	< 0.0001	5.5 (0.6)	0.0059

SPF and G2% were available in 193 cases.

Table 6. The significant independent predictors of progression in T, N, and M categories according to logistic multivariate regression analysis

Features contributing to progression	β	S.E.	Significance
All cases			
T category			
DNA index	1.336	0.3669	0.0003
N category			
Clinical stage (T)	0.5472	0.2072	0.0083
SPF	0.0575	0.0220	0.0090
WHO grade	0.7502	0.3092	0.0153
M category			
Clinical stage (T)	0.6350	0.2004	0.0015
WHO grade	0.8272	0.2990	0.0057
DNA index	1.0249	0.4100	0.0124
Ta-T1 tumours			
T category			
SPF	0.0850	0.0351	0.0153
N category			
SPF	0.0867	0.0360	0.0161
M category			
SPF	0.0796	0.0351	0.0234

The coefficient of the regression model β, the standard error (S.E.) of β and the significance of the coefficients are shown. The flow cytometric variables were entered as continuous variables in the analysis. All cases ($n = 193$) and Ta-T1 tumours ($n = 99$) were separately analysed. The significance level was 0.055.

DISCUSSION

Much experience has been gained on the use of FCM in bladder cancer [6–13, 22, 23]. The results obtained by using old paraffin-embedded materials have been comparable to those obtained from fresh tissues [22, 23]. However, some criticism

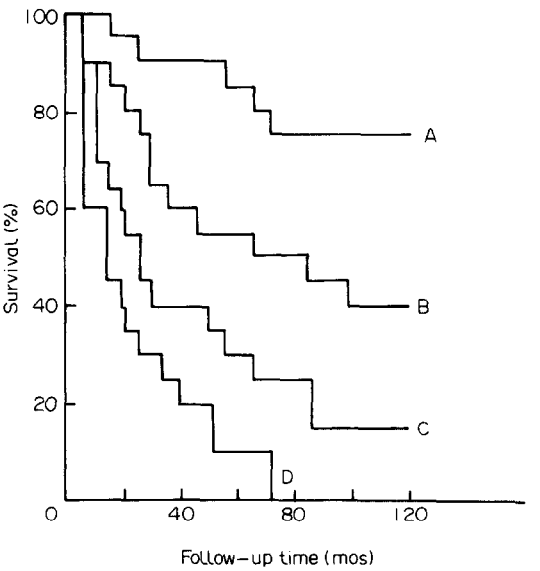

Fig. 1. Disease-related survival of patients categorised by T-category. The difference in survival among the curves is significant ($\chi^2 = 42.6$, $P < 0.0001$). A: Ta-T1, $n = 112$; B: T2, $n = 59$; C: T3, $n = 34$; D: T4, $n = 17$.

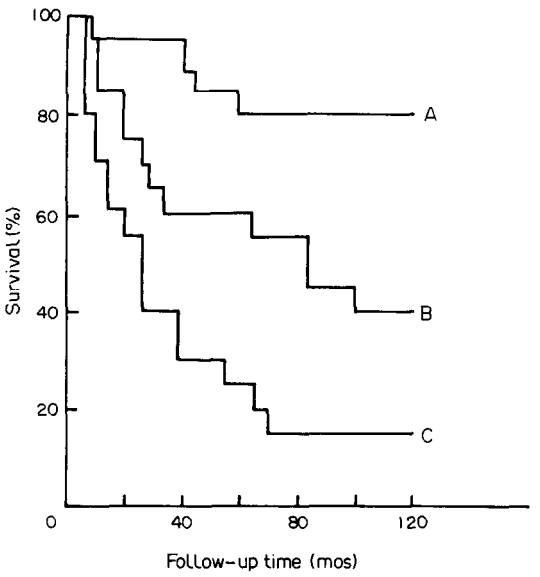

Fig. 2. Disease-related survival of patients categorised by WHO grade. The difference in survival among the curves is significant ($\chi^2 = 30.8$, $P < 0.0001$). A: grade 1, $n = 81$; B: grade 2, $n = 97$; C: grade 3, $n = 44$.

has been raised against SPF determinations from paraffin blocks [23]. In the present series the technical performance of FCM was good and 87% of DNA histograms were interpretable for SPF calculation.

There was a significant correlation between aneuploidy, high SPF, high G2%, clinical stage, histological grade and growth pattern of bladder tumours. The majority of flat tumours as well as of grade II-III T3-T4 tumours were aneuploid, which concurs with previous reports [6–13, 24]. However, higher as well as lower percentages of aneuploid grade III tumours and T3-T4 tumours than in the present series have been reported [6–13, 24]. The differences in results are probably due to presence of non-tumour cells, e.g. leucocytes [10], in samples leading to

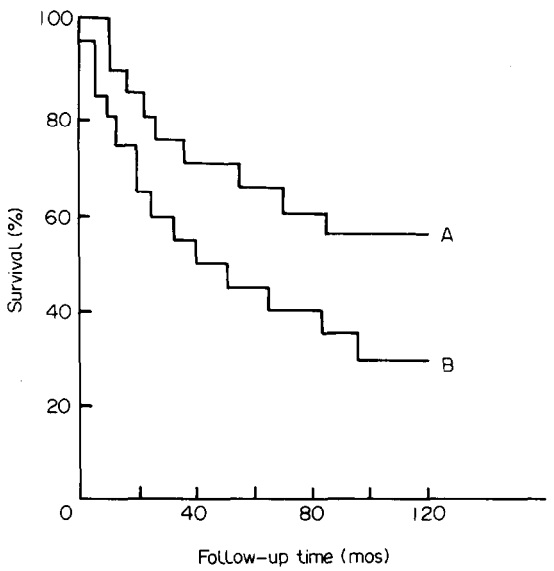

Fig. 3. Disease-related survival of patients categorised by DNA ploidy. The difference in survival between the curves is significant ($\chi^2 = 7.4$, $P = 0.0094$). A: diploid, $n = 142$; B: aneuploid, $n = 80$.

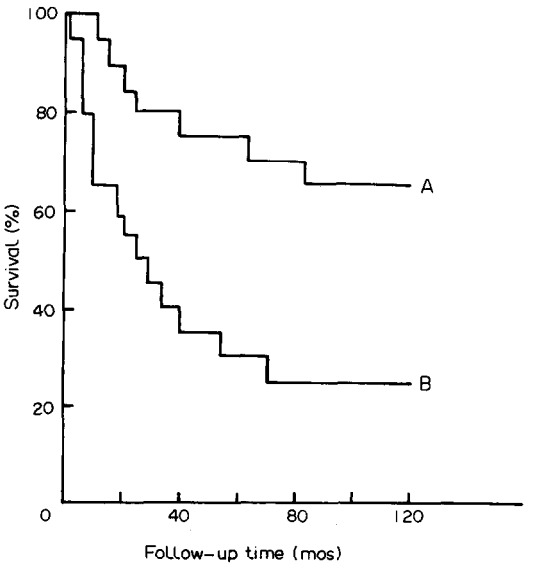

Fig. 4. Disease-related survival of patients categorised by SPF. The difference in survival between the curves is significant ($\chi^2 = 21.5$, $P < 0.0001$). A: SPF ≤ 10.0%, $n = 127$; B: SPF > 10.0%, $n = 66$.

false diploid DI in FCM. To avoid the false interpretation of FCM data due to admixture of non-cancer cells in the sample, methods have been devised to identify the cancer cells [10, 25]. In the present series no such labelling was done.

There is a heterogenity of DNA ploidy in bladder cancer. In the present series 27% of the tumours where multiple samples were measured showed intratumour heterogenity. The percentage is close to that observed in other cancers. About 25% of ovarian and breast tumours have heterogenous DNA indexes [26]. In lung tumours the heterogenity has been reported to be much higher (90%) [27]. Therefore, it is obvious that at least in some of the diploid tumours where only one sample was measured there may have been undetected aneuploid cell populations. Image analyser studies claim that FCM is insensitive to detect small aneuploid cell populations [28].

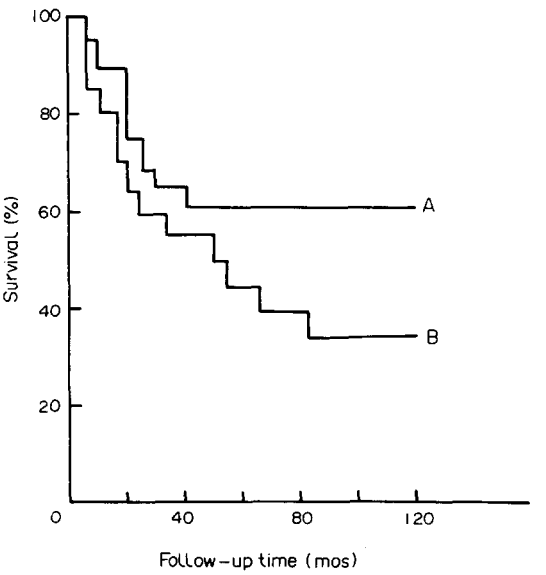

Fig. 5. Disease-related survival of patients categorised according to G2%. The difference in survival between the curves is significant ($\chi^2 = 4.5$, $P = 0.0340$). A: G2% ≤ 4.7, $n = 127$; B: G2% > 4.7, $n = 66$.

The FCM data were also analysed by separating the tetraploid or near tetraploid tumours into a separate group. However, there was no improvement in the prognostic results. The conclusions were similar when the diploid tumours with a CV above 15% were considered as aneuploid tumours. In one study [12], patients with tetraploid tumours had better prognosis than those with non-tetraploid aneuploid tumours.

The results showed that patients with aneuploid tumours and a SPF of 10% or more had a decreased expectance of survival, which agrees with other studies [7, 8, 11, 12]. DNA ploidy and SPF predicted survival in non-invasive tumours better than in muscle-invasive cancers, at least in some cases, related to intratumour heterogenity of DNA ploidy. The chances to analyse a diploid region in usually large T2-T4 tumours is increased in comparison to smaller superficial tumours when the analysis is based on one sample only. In survival analysis clinical stage was the best predictor followed by WHO grade and FCM parameters. The present result is in agreement with the results by Blomjous *et al.* [7, 8] who found that only in superficial tumours was aneuploidy superior to clinical stage in predicting prognosis. Moreover, SPF was a better predictor of progression than WHO grade in univariate analysis as well as in multivariate analysis. Consequently FCM parameters predicted disease related survival like morphometric variables [17] in the same clinical material. Of special interest to urologists are the results which show that tumours with pelvic lymph-node metastasis have significantly higher SPFs than tumours without pelvic lymph node metastasis. Aneuploidy predicted metastasis significantly as well. These results are in agreement with those of Saaban *et al* [13] who showed a clear relation between lymph-node metastasis, high SPF and aneuploidy. The progression in T category was related to aneuploidy and SPF in the present series as well. The results of morphometry are similar [15, 16], particularly in superficial tumours [17].

We conclude that FCM offers prognostic information beyond that of classic predictors in superficial tumours whereas in advanced cancers the benefits of this technique are more limited. However, in advanced cancers FCM offers quantitative data to evaluate the risk of pelvic lymph-node metastasis and metastasis to distant sites. Intratumour heterogenity of DNA ploidy may interfere with the result when only one tumour sample is measured.

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