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Chromosome 9 aberrations by fluorescence *in situ* hybridisation in bladder transitional cell carcinoma

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Abstract

To investigate the role of the monosomy 9 in bladder carcinogenesis, 96 cases of superficial bladder transitional cell carcinoma (TCC) were studied and followed periodically for around 3 years (mean±standard error of the mean (SEM); 3.46±0.34 years). Samples from bladder washings were analysed by fluorescent *in situ* hybridisation (FISH) to detect numerical anomalies of chromosome 9. Moreover, to evaluate the relative underrepresentation of this chromosome, we detected numerical changes of chromosome 8 and DNA ploidy by flow cytometric analysis (FCM). Chromosome 8 copy number were related to FCM DNA ploidy and both were related with tumour grade. Monosomy 9 did not correlate with tumour grade, stage, chromosome 8 aneuploidies and abnormal DNA content, but correlated with tumour progression. Comparing the results in the primary and subsequent tumours, we observed an increase in the frequency of aneuploidies by FCM, associated with an increase of chromosome 8 polysomies. The mean chromosome 9 copy number/nucleus remained nearly the same in most of the primary and invasive tumours. Our results confirm that monosomy 9 is an early event and that it is retained during tumour progression and invasion and that the loss occurs before the tetraploidisation process. The relationship between the presence of a sub-population with monosomy 9 and tumour progression suggests the presence of a region that could have a role in the progression of superficial bladder TCC.

Keywords: Monosomy 9; Chromosome 8; DNA ploidy; Tumour progression

1. Introduction

Transitional cell carcinoma (TCC) is the most common form of urinary bladder cancer. At presentation of disease, approximately 75% of TCCs are superficial, while the others are muscle invasive with a poor prognosis. Patients with a superficial bladder tumour have a favourable survival rate, but most of these show local recurrences and in a few cases (10–15%) tumour progression [1,2].

It is difficult to predict which superficial TCCs will recur and become invasive. Conventional pathological staging and grading cannot be considered predictive for this tumour. Therefore, it is necessary to find an accurate method of identifying patients at risk for disease progression.

Many recent studies have been carried out to determine new biological markers that will permit a more individualised prognosis and treatment according to the biological characteristics of the tumour [3–6]. Multiple molecular abnormalities have been identified in most human cancers studied to date, including bladder neoplasms [5–8].

The most common changes in bladder cancers are the loss of chromosome 9 and *TP53* mutation that probably represent two molecular pathways towards tumour development [7]. Inactivation of *TP53* occurs at a high frequency in carcinoma *in situ* [9] and is associated with high stage, while aberrations concerning chromosome 9 are found in TCCs of all grades and stages of papillary tumours [8,10,11].

Cytogenetic studies have evidenced a significant percentage of tumours with a monosomic subpopulation or with a lower number of chromosome 9 when compared with other chromosomes [10–12].

The loss of genetic material on chromosome 9, needed to inactivate tumour suppressor genes, are early events

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in the pathogenesis of bladder cancer, but may not be related to monosomy 9 [13], of which the role in bladder TCC is not known. We used a fluorescent *in situ* hybridisation (FISH) procedure to evaluate the relationship between numerical anomalies of chromosome 9 and tumour phenotype. In particular, we compared numerical chromosome 9 change and grade, Ta–T1 stage and FCM (flow cytometric measurement) ploidy. Moreover, the results for chromosome 9 with respect to chromosome 8 was investigated to determine the relationship between these chromosomes. These parameters were evaluated also in relation to tumour progression.

The study was carried out on samples from bladder washings that allow the alterations in cells derived from whole bladder to be monitored.

2. Patients and methods

2.1. Patients

A total of 96 patients with superficial (Ta, T1) bladder TCC cases were followed periodically for a mean of 41 months. They were 70 male and 26 female patients and their mean age was 61.5 ± 10.6 years (range 35.8–83.4 years). No sample of superficial TCC at the first presentation was excluded from the study. During the follow-up, 18 cases subsequently progressed to invade the bladder wall; the average follow-up time being 3.46 ± 0.34 years. In 12 of these 18 cases, it was possible to analyse both the primary and recurrent tumours.

2.2. Samples

The analyses were carried out on exfoliated cells from a bladder washing at cystoscopy prior to biopsy.

Specimens for flow cytometry and for FISH analysis were processed in the same way, as previously described with minor modification in Refs. [14,15]. Briefly, bladder washing specimens were centrifuged for 10 min at 2500 rpm and treated with a hypotonic solution of 0.075 M KCl for 15 min at 37°C, and one aliquot of the samples was centrifuged and fixed in 70% ethanol.

2.3. Flow cytometry

Aliquots of the fixed cellular suspensions treated in 0.5% pepsin were stained with a solution containing 5 μg/ml ethidium bromide (Serva, Heidelberg, Germany), 12.5 mg/ml mithramicin (Pfizer Inc., New York, USA), and 7.5 mM $MgCl_2$ in 0.1 M Tris–buffer pH 7.4.

Samples were analysed by a PAS II flow cytometer (Partec, Arlesheim, Switzerland). Human lymphocytes were used as an internal standard of cytometrically diploid DNA.

The level of DNA content alteration was expressed in terms of DNA index (DI), calculated as the ratio of the $G_{1/0}$ modal values between the aneuploid and the diploid standard sub-populations.

Analysis of DNA histogram data was carried out to calculate the DNA index, the coefficient of variation and the relative frequencies of the different cellular compartments.

2.4. *Fluorescence* in situ *hybridisation*

The samples for FISH analysis, after treatment with hypotonic solution, were prefixed with 70% 0.075 M KCl in 3:1 methanol:acetic acid, followed by three fixations in 3:1 methanol:acetic acid and dropped onto microscope slides.

The slides were stored at −20°C. The fixed cellular suspensions were dehydrated. To improve DNA probe penetration, the slides were treated with 1% pepsin in HCl 0.1 N, dehydrated, and denatured for 2 min at 70°C in 70% formamide, 2×SSC (sodium saline citrate) pH 7, according to the procedure previously described in Ref. [15]. Biotinylated centromere-associated probes for chromosome 9 (classical satellite) and chromosome 8 (α satellite) kindly provided by M. Rocchi (Bari, Italy), was heat-denatured at 80°C for 8 min in a hybridisation mixture containing 2–4 ng/ml of probe DNA, 300 μg/ml of sonicated salmon sperm DNA, 0.1% of Tween 20, 10% dextran sulphate, and 2×SSC in 60% formamide. Two probes were applied to two adjacent areas on one slide. The hybridisation was performed overnight at 37°C in a humidified chamber. Detection of the biotinylated probes was accomplished with fluorescein isothiocyanate (FITC)-conjugated avidin. The DNA of the nuclei was counterstained with propidium iodide (0.2 mg/ml) to allow simultaneous observation of the total DNA and hybridisation signals. Fig. 1 shows representative areas with interphase nuclei hybridised with probes for chromosomes 9 and 8.

2.5. Scoring of FISH signals

The slides were viewed with a Zeiss Axiophot fluorescent microscope. Human lymphocytes, separated from peripheral blood, were used to optimise the specificity and sensitivity of the hybridisation protocol. Two hundred nuclei were scored for each chromosome and for each sample. FISH spots were interpreted as described by Hopman [16]. A conservative cut-off level of 20% aberrant cells was used to define clonal chromosome copy number aberrations. A tumour was considered disomic if no other sub-population with spot number/nucleus different from two had a frequency $>20\%$ of the total cells. A tumour was considered monosomic when a sub-population with one spot/nucleus exceeded 20% of the total cells and polysomic

when sub-populations with centromere copy number $\geqslant 3$ exceeded 20% of the total cells.

The mean chromosome copy number/nucleus was also calculated for each chromosome. Tumours that showed a mean chromosome copy number/nucleus between 1.8 and 2.2 were considered disomic.

Relative underrepresentation of chromosome 9 was considered when the average number for chromosome 9 differed more than 0.3 from the average number for chromosome 8.

2.6. Statistical analysis

The χ^2 test was used to determine the correlation between the different parameters (age, follow-up time, tumour stage and grade) and the clinical outcome. It was also used to evaluate the significance of association between monosomy 9 and grade, polysomy 9 and grade and aneuploidies of chromosome 8 and grade. Moreover, this test was used to evaluate the relationship between FCM aneuploidies and monosomy and trisomy of chromosome 8 and 9 as detected by FISH $P<0.01$ was considiered significant. The progression-free survival was analysed using Kaplan–Meier analysis with the log-rank test.

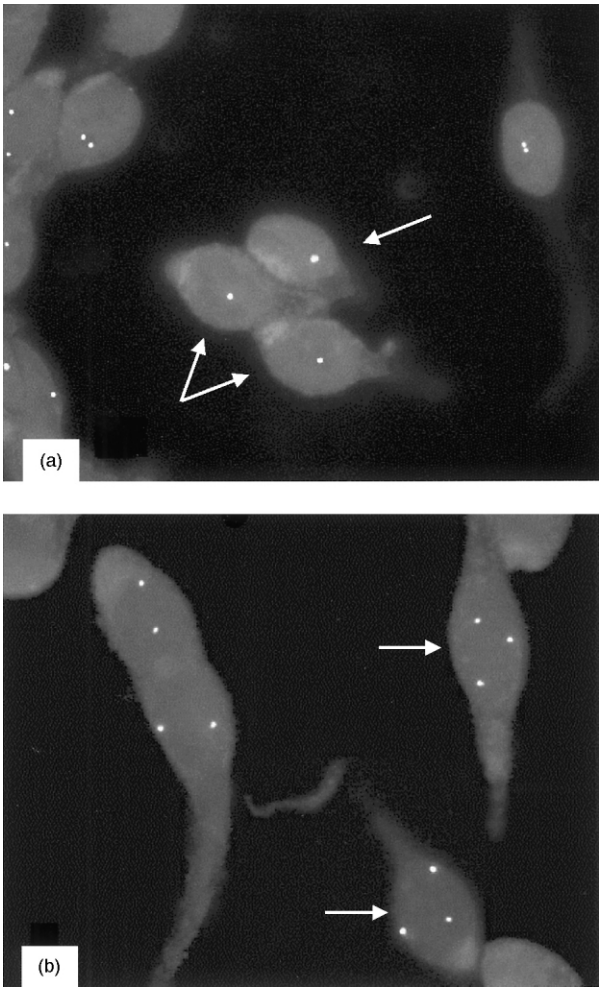

Fig. 1. Fluorescent *in situ* hybridisation (FISH) on isolated cells from a bladder washing in a case of bladder transitional cell carcinoma (TCC). (a) FISH with probes for chromosome 9 shows disomic and monosomic cells The frequency of monosomic cells in this case was higher than 20%; (b) FISH with probes for chromosome 8 shows disomic and trisomic cells. The frequency of trisomic cells in this case was higher than 20%.

3. Results

We investigated 96 superficial bladder TCC. 60 (63%) were confined to the bladder mucous (pTa) and 36 (38%) showed invasion of lamina propria (pT1). The cases were subdivided on the basis of the histological grading; 14 G_1 (15%), 65 G_2 (68%) and 17 G_3 (18%).

During follow-up, 18 of 96 cases (19%) subsequently progressed. Progression was defined as an increase in stage during follow-up. No significant differences in patient age were observed between the group of that did not progress and those that did. In fact, the mean age at diagnosis was similar in the first and second groups 61.0 ± 10.96 years compared with 62.1 ± 3.64 years, respectively. Similar mean values were found for the follow-up time in the two groups: 3.47 ± 0.33 years in patients without progression and 3.49 ± 0.39 years in those with progression. Between these two groups, no significant differences were found in distributions of stage ($P=0.22$) and grade ($P=0.44$).

3.1. FCM analysis

Flow cytometric analysis showed a diploid distribution in 53 of 96 (55%) superficial cases, while the remaining 43 cases (45%) were characterised by at least one aneuploid cell sub-population. No significant differences were found between the aneuploid frequency in the pTa (42%) and in pT1 (50%) tumours ($P=0.42$). A statistically significant correlation was found between FCM aneuploidies and grade ($P=0.004$). In fact, the aneuploidy frequencies increased with grade from 7% in G_1, 48% in G_2, and 65% in G_3.

However, no difference was found between patients with different clinical outcomes ($P=0.308$); the frequency of aneuploidy was 42% in cases without progression and 55% in those that progressed.

3.2. Chromosome 8 analysis by FISH

Disomy of chromosome 8 was observed in 55/96 samples (57%), while in the remaining 41 (43%) cases, cell sub-populations with numerical chromosome abnormalities were detected. In particular, a sub-population with one FISH spot for chromosome 8 was detected in 13/96 (14%) and sub-population with three or more spots in 28/96 (29%) of cases.

Correlation was found between chromosome 8 polysomies and histological grade ($P = 0.001$) and with FCM aneuploidies ($P < 0.001$), while no correlation was observed between chromosome 8 aberrations and the clinical outcome ($P = 0.600$) (Table 1).

The results of chromosome 8 ploidy in relation to chromosome 9 are shown in the Tables 1 and 2. FISH analysis was performed also in 12 recurrent cases that progressed. The results are shown Table 3.

3.3. Chromosome 9 analysis by FISH

Monosomy of chromosome 9 was observed in 46% of samples (44/96). The frequencies of monosomic subpopulations were between 21 and 94%, with an average of 31%. Relative loss of chromosome 9 (fewer centromere copies than copies of chromosome 8) was present in 11 additional cases (11%) of which 8 were disomic and 3 were polysomic for chromosome 9.

Table 1
Results of FISH analyses with probes for centromere 8 in relation to tumour stage and grade, FCM ploidy, numerical aberrations in chromosome 9 and progression

		Chromosome 8 (tumour cases)			Total
		Monosomic[a]	Disomic[b]	Polysomic[c]	
Stage	pTa	8	34	18	60
	pT1	5	21	10[d]	36
Grade	1	5	9	0	14
	2	8	39	18	65
	3	0	7	10[e]	17
FCM	Diploid	8	41	4	53
	Aneuploid	5	14	24[f]	43
Chromosome 9	Monosomic[a]	4	28	12	44
	Disomic[b]	9	20	10	39
	Polysomic[c]	0	7	6[d]	13
Outcome	No progression	11	46	21	78
	Progression	2	9	7[d]	18

[a] >20% cells with one centromeric signal.
[b] <20% cells with 1 or ⩾3 centromeric signals.
[c] 20% cells with three or more centromeric signal.
[d] Non significant.
[e] $P = 0.001$ for polysomic versus disomic and monosomic.
[f] $P < 0.001$ for polysomic versus monosomic and disomic.

Table 2
Results of FISH analyses with probes for centromere 9 in relation to tumour stage and grade, FCM ploidy, numerical aberrations in chromosome 8 and progression

		Chromosome 9 (tumour cases)			Total
		Monosomic[a]	Disomic[b]	Polysomic[c]	
Stage	pTa	24	28	8	60
	pT1	20	10	6[d]	36
Grade	1	8[d]	6	0	14
	2	25	32	8	65
	3	11	0	6[e]	17
FCM	Diploid	25[d]	24	4	53
	Aneuploid	19	15	9[f]	43
Chromosome 8	Monosomic[a]	4	9	0	13
	Disomic[b]	28	20	7	55
	Polysomic[c]	12	10	6[d]	28
Outcome	No progression	29	37	12	78
	Progression	15	2	1[g]	18

[a] >20% cells with one centromeric signal.
[b] <20% cells with 1 or ⩾3 centromeric signals.
[c] 20% cells with three or more centromeric signals.
[d] Non significant.
[e] $P = 0.05$ for polysomic versus disomic and monosomic.
[f] $P = 0.07$ for polysomic versus disomic and monosomic.
[g] <0.001 for monosomic versus disomic and polysomic.

Table 3
A comparison of FISH results and DNA ploidy by FCM in primary and successive invasive tumours

CASE	FCM		Mean chromosome 8 copy number/nucleus		Mean chromosome 9 copy number/nucleus	
	Primary	Invasive	Primary	Invasive	Primary	Invasive
1	Diploid	Diploid	2.02	2.20	1.55	1.60
2	Diploid	Diploid	2.10	1.98	1.70	1.65
3	Diploid	Aneuploid	2.05	2.81	1.77	1.80
4	Diploid	Aneuploid	2.05	2.20	1.68	1.75
5	Diploid	Aneuploid	2.20	2.34	1.72	1.73
6	Aneuploid	Diploid	2.24	2.29	1.70	1.68
7	Aneuploid	Aneuploid	1.79	2.54	1.77	1.79
8	Aneuploid	Aneuploid	2.27	2.52	2.01	1.80
9	Aneuploid	Aneuploid	2.54	2.63	1.97	1.71
10	Aneuploid	Aneuploid	2.56	2.51	1.76	1.69
11	Aneuploid	Aneuploid	2.56	3.54	1.74	2.32
12	Aneuploid	Aneuploid	2.73	2.91	1.77	2.00

The number of monosomic, disomic and polysomic tumours are shown in Table 2.

Monosomy of chromosome 9 was found in 40% of pTa (24/60) and in 55% of pT1 tumours (20/36). This difference was non significant ($P=0.183$) (Table 2).

The frequencies of monosomic 9 tumours in the G_1, G_2 and G_3 groups was not significantly different ($P=0.101$). No significant correlation was found between tumours with relative chromosome 9 loss and DNA ploidy monitored by FCM ($P=0.770$) (Table 2) and also with chromosome 8 numerical change ($P=0.132$). Relationships between polysomic 9 cases and G_3 ($P=0.07$) and FCM aneuploidy ($P=0.005$) tumours were found ($P=0.007$); while no association was observed with Ta and T1 stages ($P=0.183$). A significant difference was found between patients with different clinical outcomes ($P<0.001$); 15 of 18 cases with tumour progression showed monosomy 9, in the remaining three, the chromosome 9 were relatively underrepresented with respect to chromosome 8.

Kaplan–Meier survival analysis was carried out to assess the probability of remaining progression-free in relation to the chromosome 9 number (Fig. 2). The log-rank test displayed a significant difference between the group with monosomy 9 and the group without ($P<0.0001$).

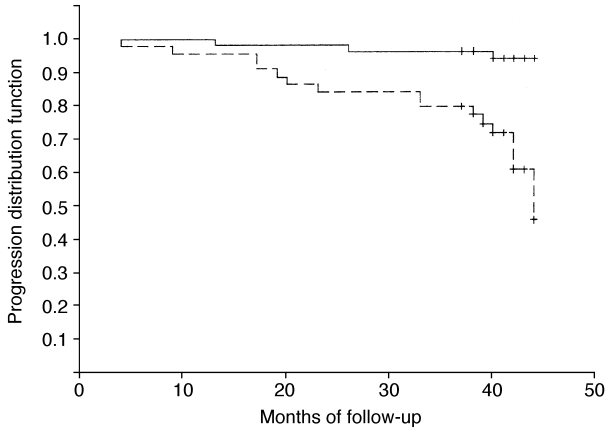

Fig. 2. A Kaplan–Meier disease-specific survival curve, according to chromosome 9 status. Smooth line: cases with monosomy 9; dotted line: cases without monosomy 9. $P<0.0001$, log-rank test.

3.4. Invasive recurrent tumour

It was possible, in 12 of 18 (14%) cases that progressed during the follow-up period, to also analyse the subsequent tumour. All these tumours became invasive (T2–4). Table 3 summarises the results of the FCM and FISH analyses in the primary and subsequent tumours. Comparing the results in the primary and subsequent tumours, we observed an increase in the frequency of aneuploidies by FCM associated with an increase in chromosome 8 polysomies. In contrast chromosome 9 copy number/nucleus remained almost the same in most of the primary and subsequent invasive tumours.

4. Discussion

Aberration of chromosome 9 is one of the most frequent changes observed in superficial bladder TCC using different techniques [10,11]. Molecular methods, such as the loss of heterozygosity (LOH), evidence the loss of chromosome 9 regions [17,18]. Loss of the distal long arm of chromosome 9 (9q34) is observed with a frequency between 22 and 60% [17–19]. A distinct region has been identified at 9q22, and, more recently, a loss has been found at 9p21. This region contains a gene, Multiple Tumour Suppressor 1 (*MTS1*), that encodes a previously identified inhibitor (p16) of cyclin-dependent kinase 4. *MTS1* is homozygously deleted at high frequency in cell lines derived from tumours of the

lung, breast, brain, bone, skin, bladder, kidney, ovary and lymphocyte [20]. Molecular mapping of the common deletion regions on chromosome 9 has shown several independent tumour suppressor loci [21,22]. It was demonstrated that the loss needed for inactivation of tumour suppressor genes on this chromosome does not depend on the loss of the whole chromosome [13] or from the loss of the centromere region as is revealed by FISH in numerous studies [10,23]. Hence, monosomy 9 and deletions of tumour suppressor genes may have a different significance [13].

Recently, studies in which genetic alteration were related to patient clinical outcome proposed that chromosome 9 monosomy detected by FISH could be a possible marker for disease recurrence [24,25].

In this paper, we evaluated numerical chromosome 9 aberrations by FISH in 96 patients with superficial bladder TCC, at the first manifestation of the disease, the average follow-up time being 3.46 ± 0.34 years.

The analyses were carried out on specimens from bladder washings. These samples, that are often obtained as part of a routine follow-up, allow the analysis of cells from the whole bladder thereby allowing the multifocality of disease to be monitored. Moreover, this approach allowed us to also study those cases in which the tumour samples were too small. It has been demonstrated that FISH on cells isolated from bladder washings is a powerful technique for early cancer detection, monitoring treatment outcome, and predicting recurrences of disease [23].

The FISH method used in this study to determine chromosome 9 and 8 copy number was validated by appropriate scoring criteria. Samples from bladder washings contain bladder cells, as well as inflammatory and squamous cells; therefore, on the basis of morphological characteristics these last two types of cells were recognised and not scored.

We found underrepresentation of chromosome 9 in 46% of cases. Our data are consistent with those reported from other authors [10,11,14,16], that observed a frequency of monosomic cases between 31 and 50%.

A similar frequency of monosomy was observed in all grades and in Ta, T1 tumours and is consistent with other reports [11,24] and suggests that the chromosome 9 loss is an early event in bladder cancer development and that this loss is retained.

Overrepresentation of chromosome 9 observed in 13% of the TCC analysed was found prevalently associated with high grade tumours. This is not surprising and is in agreement with other authors [11,14] that showed an association of this aberration with high grade, high stage and p53-positivity, suggesting that polysomy 9 is a late event.

In addition, the relationship between cytometric ploidy and chromosome 9 is interesting as it demonstrates that underrepresentation of chromosome 9 is an independent event with respect to DNA ploidy. Instead, overrepresentation of chromosome 9 was strongly associated with FCM aneuploidy.

Analysis of DNA flow cytometry has been widely utilised in studies of bladder TCCs on histological and cytological specimens [26–30]. In agreement with some authors [26,27,29] we evidenced the occurrence of cytometric aneuploidy in 45% (43/96) of samples with a significant relationship with grade ($P = 0.01$), but no differences were found in the frequency distribution between Ta and T1 tumours. It was previously reported that combined use of histological and flow cytometric parameters may offer additional information regarding the clinical outcome for bladder cancer patients [31].

Our data showed no significant difference between the patients with different clinical outcome ($P = 0.308$).

Alterations of chromosome 8, including deletions of 8p, occur frequently in many epithelial-derived tumours [32,33]. Two genes (arylamine N-acetyltransferase types 1 and 2, *NAT1* and *NAT2*), which are known to metabolise bladder carcinogens, are located on chromosome band 8p22. We found monosomy 8 in 14% of cases particularly associated with low grade (36% G_1 and 12% in G_2), while polysomies of this chromosome observed in 29% of cases were strongly associated with high grade. These results confirm that an increased copy number of chromosome 8, prevalently associated with high stage as observed by other authors that found also associations with tumour recurrence [34]. Moreover, our data show chromosome 8 copy number was related to DNA ploidy by FCM suggesting that chromosome 8 centromere probe can reflect the ploidy of tumour.

We compared the mean centromere copy number (MCCN) between chromosomes 8 and 9. 50 cases displayed higher MCCN of chromosomes 8 compared with MCCN of chromosome 9. It is interesting to note that MCCN of chromosome 9 in 3 of these cases was > 2.2 and in 10 comprised between 1.8 and 2.2. So, in an additional 14% (13/96) of cases, chromosome 9 were underrepresented if considered in relation to chromosome 8. Our data confirm that centromere 9 loss is frequent and that it can also occur in low grade, low stage TCCs, and in FCM diploid tumours. The relative loss of chromosome 9 with respect to chromosome 8 indicated that the loss of one copy occurred before the polyploidisation process that seems to be a late event in bladder carcinogenesis.

A major problem in the management of superficial TCCs is the identification of those cases with the highest risk of progression, but routine histopathological parameters, such as tumour stage and grade, cannot predict which superficial bladder tumours will recur and become invasive. To evaluate the clinical relevance of monosomy 9 we have followed a group of patients for 3 or more years. We found a correlation between monosomy 9 and tumour progression ($P < 0.001$). Monosomy 9 was observed in 15 of 18 primary tumour cases that

progressed during the follow-up period. Two of the remaining cases were disomic and one was polysomic: all these cases showed a chromosome 9 loss with respect to chromosome 8 and total DNA content.

The relationship between the presence of sub-populations with monosomy 9 and tumour progression suggests the presence of a region that could have a role in the development of superficial bladder TCC. Furthermore, a difference in the progression rate was found in patients with a monosomic 9 tumour and those without monosomy 9 using Kaplan–Meier analysis.

The comparison of the results obtained in primary and the subsequent tumours that developed in the cases that progressed, adds further to these results. We observed an increase of aneuploid frequency by FCM associated with an increase of chromosome 8 polysomies and with a higher mean of chromosome 8 copy number/nucleus in most of the tumours examined. This finding supports the hypothesis that process of polyploidisation is a late event. The change observed in tumour 6 from aneuploid in the primary tumour to diploid in the subsequent tumour could be related to the treatment of the tumour. Regarding chromosome 9, the mean of the chromosome 9 copy number/nucleus remained nearly the same in the primary and invasive tumours, only in one case did we observe a consistent increase (from 1.74 to 2.32). This case showed a tetraploidisation of chromosome 8 and FCM revealed the presence of sub-population having a DNA index near tetraploid value. This result confirms that the losses of chromosome 9 persist during tumour progression and invasion and that the loss occurs before the tetraploidisation process. The finding that the chromosome 9 underrepresentation is consistent in TCCs from the same patient many months apart is in agreement with the result found by Bartlett [24]. It is surprising that MCCN of chromosome 9 does not change in the subsequent tumour from the value in the primary tumour. Perhaps, there is no mechanism of selection for cells that lose chromosome 9, but loss of this chromosome might allow further genetic changes related to progression.

In conclusion, our data confirm that monosomy 9 is an early frequent event in bladder TCC and it seems reasonable to suggest that it may have a role in bladder cancer progression.

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