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c-*myc* Copy Number Gains in Bladder Cancer Detected by Fluorescence *in Situ* Hybridization

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Amplification and overexpression of c-myc have been suggested as prognostic markers in human cancer. To assess the role of c-myc gene copy number alterations in bladder cancer, 87 bladder tumors were examined for c-myc aberrations by fluorescence in situ hybridization. Dual labeling hybridization with a repetitive pericentromeric probe specific for chromosome 8 and a probe for the c-myc locus (at 8q24) was performed to analyze c-myc copy number in relation to chromosome 8 copy number on a cell by cell basis. A clear-cut c-myc amplification (up to 40 to 150 copies per cell) was found in 3 tumors. There was a low level c-myc copy number increase in 32 of the remaining 84 tumors. There was no association of low level c-myc copy number increase with c-myc protein overexpression. This suggests that a c-myc gene copy number gain as detected by fluorescence in situ hybridization does not necessarily reflect a disturbed c-myc gene function but may indicate a structural chromosome 8 abnormality including gain of distal 8q. The strong association of low level c-myc (8q) gains with tumor grade (P < 0.0001), stage (P < 0.0001), chromosome polysomy (P < 0.0001), p53 protein expression (P = 0.0019), p53 deletion (P = 0.0403), and tumor cell proliferation (Ki67 labeling index; P = 0.0021) is consistent with a role of chromosome 8 alterations in bladder cancer progression. (Am J Pathol 1995, 146:1131–1139)

The c-*myc* gene, mapped to chromosome 8q24, encodes a nuclear phosphoprotein involved in transcriptional regulation. Its normal function has been linked to growth regulation, cell differentiation, and apoptosis.[1,2] Alterations in c-*myc* have been reported in a variety of human cancers, including breast,[3] renal cell,[4] head and neck,[5] and endometrium.[6] The few studies characterizing c-*myc* protein expression in bladder cancer have reported controversial results, showing overexpression associated with low stage tumors in one study[7] and with high grade tumors in another.[8]

To examine the role of c-*myc* genomic alterations in bladder cancer, we have applied dual labeling fluorescence *in situ* hybridization (FISH) with chromosome 8 and c-*myc* gene-specific probes to a set of superficial and invasive bladder tumors. This allowed identification of increased gene dosage on a cell by cell basis,[9] both as an absolute number of c-*myc* signals per cell and as a count relative to the number of copies of the chromosome 8 centromere. The biological significance of altered c-*myc* copy number was examined by correlations with c-*myc* protein expression, proliferation (Ki-67 labeling index), p53 alterations, and tumor grade and stage.

Materials and Methods

Patient Material

A total of 87 tumors (55 paraffin dissociated and 32 fresh touch preparations) from 87 patients were analyzed. Tumor stage and grade were defined according to International Union against Cancer[10] and World Health Organization[11] classifications. Because of the limitations of transurethral biopsies in accurately determining the depth of invasion of higher stage bladder cancer, all tumors showing muscle invasion were

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categorized into one group (pT2-T4). Of the 87 tumors, 27 were confined to the bladder mucosa (pTa), 22 showed invasion of the lamina propria (pT1), and 33 were muscle invasive (pT2-T4); 23 tumors were classified as grade 1, 30 were grade 2, and 28 were grade 3. Staging and grading could not unequivocally be performed in another 5 and 6 cases, respectively, because of inadequate biopsy size or mechanical damage. Paraffin-embedded tumor blocks chosen for analysis contained at least 60% tumor cells in all cases. Both fresh imprint preparations and cells dissociated from formalin-fixed paraffin-embedded thick sections (50 μ) were processed for analysis as previously described.[12]

Immunohistochemistry

Sections from formalin-fixed tumors were examined by immunohistochemistry by standard indirect immunoperoxidase procedures. Sections were stained for p53 expression with a polyclonal antibody CM1 (1:4000; Medac GmbH, Hamburg, Germany) as previously described.[13] Positive p53 staining was defined as at least 10% of tumor cells with distinct nuclear staining. The monoclonal antibody MIB1 (1:800; Dianova, Hamburg, Germany) was used for detection of Ki-67 protein in formalin-fixed tissue sections after antigen retrieval as previously described.[14] Nuclei were considered Ki67 positive if any nuclear staining was seen. Ki67 labeling index (Ki67 LI), defined as the percentage of Ki67-positive cells, was determined by scoring 500 cells in at least three well labeled areas.

The mouse monoclonal antibody myc-1 6E10[15] was used for detection of c-*myc* expression (Paesel and Lorei, Frankfurt/M, Germany). Formalin-fixed cells of the colon cancer cell line COLO320HSR with known c-*myc* gene amplification and sections from 3 colon carcinomas known to overexpress c-*myc* were used as positive controls. At an antibody dilution of 1:500, which was recommended by others,[16] there was a strong cytoplasmic staining in the COLO320HSR cells as well as in all 3 colon carcinomas, as expected from previous reports.[16] All 51 bladder carcinomas examined and 3 normal urothelial biopsies (from patients without previous or simultaneous bladder tumors) showed a predominantly nuclear c-*myc* positivity at this antibody concentration. To identify those bladder tumors expressing c-*myc* protein beyond the level of normal urothelial (ie, overexpression), a dilution series was performed. It showed that all 3 normal urothelium biopsies were still positive at a dilution of 1:26,000 but were negative at 1:52,000. An antibody dilution of 1:52,000 was then used to examine 51 formalin-fixed bladder tumors.

DNA Probes for FISH

A probe specific for the chromosome 8 pericentromeric sequence (pJM128, courtesy of Tim Donlon) was used in combination with two contiguous phage probes at the c-*myc* locus (courtesy of Dr. Masaru Sakamoto, Tokyo University). A probe for the p53 locus on 17p (three contiguous cosmids, courtesy of Dr. Joe Gray, University of California at San Francisco) was used in combination with a probe specific for the chromosome 17 pericentromeric sequence (p17H8). The probe used for determination of chromosome 7 was p7αTET. c-*myc* and p53 DNA were labeled with digoxigenin-11-dUTP, and centromeric DNA was labeled with biotin-14-dATP by nick translation with standard protocols.

Fluorescence in Situ *Hybridization*

All slides were fixed in methanol/acetic acid (3:1) and subsequently air dried. FISH was performed as previously described[17] with modifications. Cells on slides were denatured in 70% formamide/2X standard saline citrate (SSC; 1X SSC is 0.15 mol/L NaCl, 0.015 mol/L sodium citrate), pH 7, at 75 C for 2.5 minutes. After dehydration in graded ethanol, formalin-fixed cells were treated with 2.0 μg/ml proteinase K (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline (pH 7.0) for 7 minutes at 37 C, followed by ethanol dehydration (proteinase K was reduced to 0.2 μg/ml for unfixed tumors). The hybridization mixture was denatured for 5 minutes at 75 C, allowed to re-anneal for 30 minutes at 37 C, and applied to denatured cells on slides. A 10-μl aliquot of hybridization mixture consisted of 10 ng of c-*myc* (or p53) probe, 30 ng of pJM128 (or 20 ng of p17H8), as well as 10 ng of unlabeled, sonicated (200 to 500 bp) human placental DNA (Sigma) in 50% formamide, 10% dextran sulfate, and 2X SSC (pH 7). Hybridization was overnight at 37 C. Immunostaining of hybridization signals was as described.[17] Amplification of the centromere signals was done with an additional layer of biotinylated anti-avidin followed by another layer of Texas Red if necessary. Lymphocyte controls were used to assure probe specificity. If hybridization signals were weak, presumably because of low hybridization efficiency, hybridization was repeated by using the same protocol with an increased proteinase K concentration (up to 10 μg/ml). Proteinase K concentration was reduced if excessive nuclear damage was observed.

Scoring of FISH Signals

For the present study, our goal was to detect increased c-*myc* copy number. Therefore, occasional split c-*myc* signals that were less than 0.5 μ apart (because of sister chromatids in S or G2 or because of gaps in the phage contig) were counted as one to avoid false signals. At least 50 paraffin-dissociated nuclei or 100 unfixed nuclei were counted from each tumor. Cell selection for scoring was as previously described.[12] Control lymphocyte hybridizations showed two centromere 8 signals and two *myc* signals in more than 95% of nuclei. c-*myc* copy gain was defined as more c-*myc* signals than centromere 8 signals in greater than 10% of cells. This was based on our findings in lymphocyte controls and cells from two normal bladder washes showing more c-*myc* signals than centromere 8 signals in less than 5% of cells. Increased copy number for chromosomes 7, 8, and 17 (polysomy) was defined as an average centromere 8 copy number above 2.3. Scoring of deletions for p53 was as previously reported.[17]

Statistics

Contingency table analysis was performed to examine the relationship of c-*myc* copy number alterations with grade, stage, p53 expression, 17p deletions, and polysomy. A Student's *t*-test was used to compare c-*myc* copy number alterations with Ki-67 LI. A Spearman Rank correlation test was performed to examine the relationship between the fraction of cells with a low level c-*myc* gain and the Ki-67 LI.

Results

*Ki-67 and c-*myc *immunohistochemistry*

Ki-67 LI was analyzed in 55 cases and ranged from 0.5 to 48.9% (mean, 15.4%). As previously found in a larger series of tumors that included the present set, there was a significant trend for higher Ki67 LI in higher stage and higher grade tumors.[14]

An unequivocal c-*myc* positivity was seen in 37 of 51 tumors examined. These tumors were considered

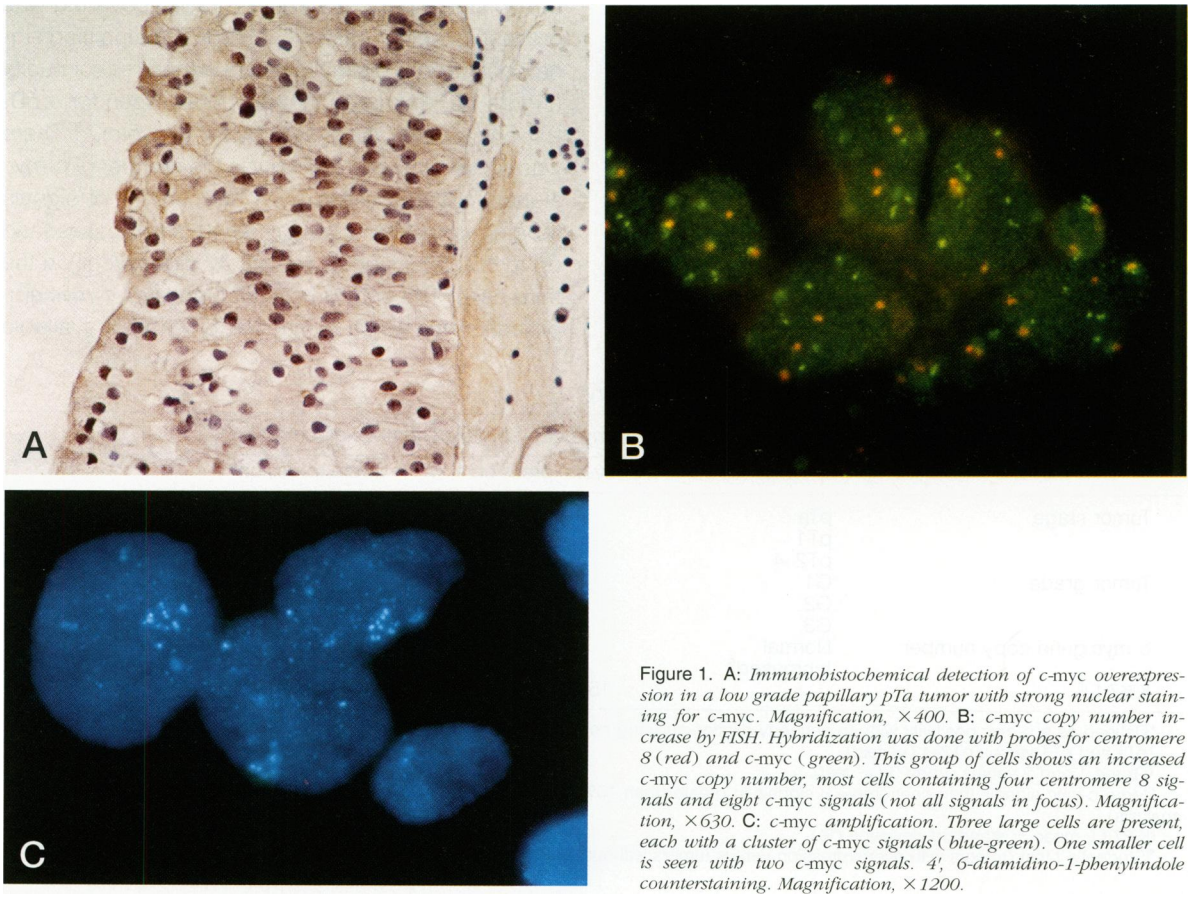

Figure 1. A: *Immunohistochemical detection of* c-myc *overexpression in a low grade papillary pTa tumor with strong nuclear staining for* c-myc. *Magnification,* ×*400.* B: c-myc *copy number increase by FISH. Hybridization was done with probes for centromere 8 (red) and* c-myc *(green). This group of cells shows an increased* c-myc *copy number, most cells containing four centromere 8 signals and eight* c-myc *signals (not all signals in focus). Magnification,* ×*630.* C: c-myc *amplification. Three large cells are present, each with a cluster of* c-myc *signals (blue-green). One smaller cell is seen with two* c-myc *signals. 4', 6-diamidino-1-phenylindole counterstaining. Magnification,* ×*1200.*

to overexpress c-*myc* as normal urothelium was completely negative at the selected antibody dilution (1: 52,000). c-*myc* positivity was predominantly nuclear (Figure 1A). An additional cytoplasmic c-*myc* staining was seen in most cases with overexpression. Detectable c-*myc* positivity was always focal, present in 5 to 50% of cells. The relationship between c-*myc* overexpression and tumor grade, stage, and Ki67 LI is shown in Table 1. Overexpression of c-*myc* was associated with low histological grade and with low stage. Overexpression of c-*myc* was seen in 5 of 12 grade 3 tumors but in 31 of 38 grade 1/2 tumors ($P = 0.0073$). There was also a tendency toward more frequent c-*myc* overexpression in pTa/1 tumors (79% positive) than in pT2–4 tumors (41% positive). This difference did not reach statistical significance, however ($P = 0.1204$). There was no significant difference in tumor cell proliferation between tumors with and without c-*myc* overexpression.

FISH Analysis

*Low Level c-*myc *Gene Copy Number Gains*

The c-*myc* hybridization was clear and bright in all cases examined. A relative gain of c-*myc* copy number was determined by comparing the number of c-*myc* signals with the number of chromosome 8 centromeric signals in each tumor cell analyzed. Three tumors averaged more than two c-*myc* signals per centromere 8 and were considered to have high level amplification (see below). For the remaining cases, the fraction of cells with more c-*myc* signals than chromosome 8 signals was calculated (Figure 2). Tumors cells frequently showed a small number of additional c-*myc* signals relative to centromere 8 signals. In normal lymphocyte controls and in cells from normal bladder washes, the fraction of cells showing more c-*myc* signals than chromosome 8 signals was always less than 5%. A threshold of 10% cells with increased c-*myc* copy number was thus used to define c-*myc* gene copy number gain at twice the maximal 5% value seen in normal urothelium or lymphocyte controls. The fraction of cells showing more c-*myc* signals than chromosome 8 signals was calculated for each tumor (Figure 2). Only 13 tumors showed greater than 75% of nuclei with a low level increase of c-*myc* signals, 3 had 51 to 75%, 5 had 26 to 50%, 11 had 11 to 25%, and 52 had fewer than 11% such nuclei. Cells of a representative case are shown in Figure 1B. Low level c-*myc* gene copy number gains were less frequent in tumors with c-*myc* protein overexpression than in tumors without overexpression (Table 1). Copy number gains were seen in only 7 of 35 tumors with c-*myc* overexpression but in 10 of 14 tumors without c-*myc* overexpression ($P = 0.0006$).

*High Level c-*myc *Amplifications*

A clear-cut c-*myc* amplification with an average c-*myc* signal per centromere 8 ratio above 6.0 was present in 3 of 87 cases (Table 2). The amplified signals occurred in distinct clusters in these tumor nuclei (Figure 1C), similar to that previously seen for *erb*B-2[12] and epidermal growth factor receptor.[18] There was significant heterogeneity in the number of c-*myc* signals in these cases, with the number of signals ranging from 10 to as many as 40 in two cases, and up to 150 total signals (mean, 47 signals/cell) in the third case. The only tumor with high level c-*myc* amplification for which a formalin block was available

Table 1. c-myc *Protein Expression by Immunohistochemistry*

		c-*myc* copy protein (*number of tumors*)		
		No overexpression	Overexpression*	
Tumor stage	pTa	3	18	
	pT1	4	9	
	pT2–4	7	10	P = ***0.1204***†
Tumor grade	G1	2	18	
	G2	5	13	
	G3	7	5	***P*** = 0.0073‡
c-***myc*** gene copy number	Normal	4	28	
	Increased§	10	7	***P*** = 0.0006
Ki67 LI‖		15.4 ± 9.9	14.0 ± 10.1	***P*** = 0.67¶

*Defined as a c-*myc* positivity with an antibody dilution showing no expression in normal urothelium (see Materials and Methods).
†pTa and pT1 versus pT2–4 (χ^2 *test*).
‡Grade 1 and 2 versus grade 3 (χ^2 *test*).
§More c-myc signals than centromere 8 signals in greater than 10% of cells (three cases with high level amplification excluded from this analysis).
‖Ki67 LI; mean ± standard deviation (%).
¶Student's *t*-test (tumors with overexpression versus tumors without overexpression).

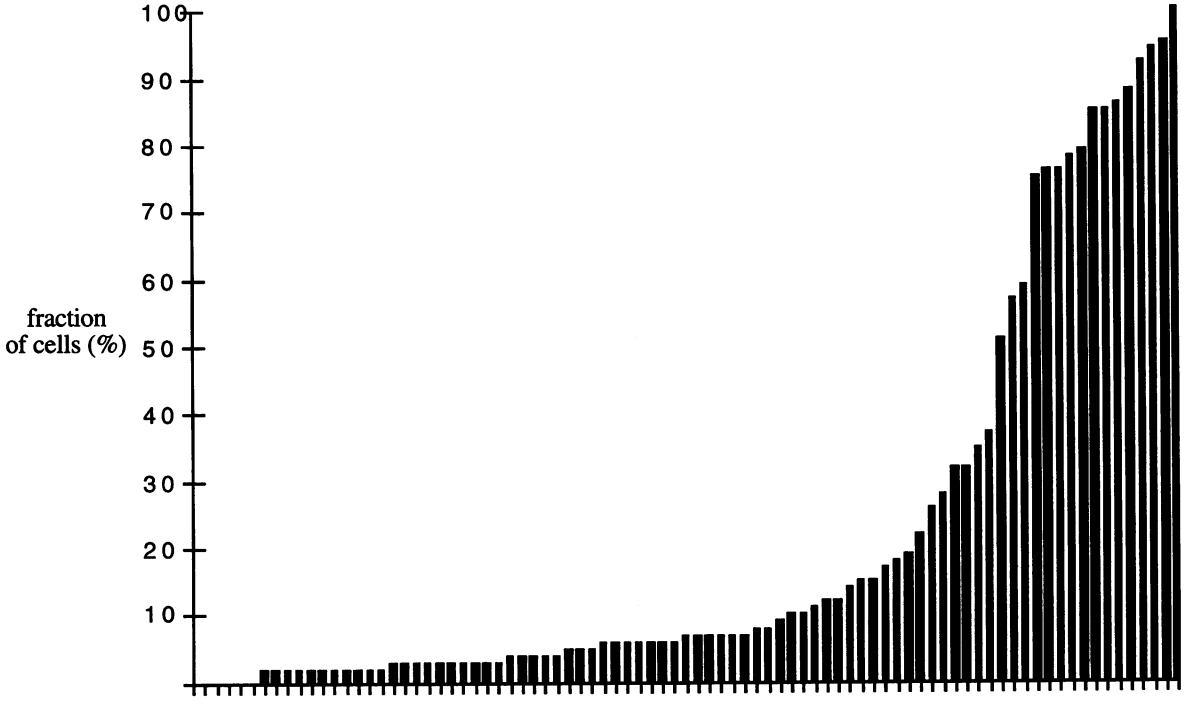

Figure 2. *Fraction of tumor cells with increased* c-myc *copy number by FISH. Each bar represents one case. Increased* c-myc *copy number was defined as more* c-myc *signals than chromosome 8 centromeric signals. Tumors were defined as having a* c-myc *copy number increase when greater than 10% of tumor cells showed more* c-myc *signals than chromosome 8 centromeric signals.*

Table 2. c-myc *Amplified Cases*

Case	Average centromere 8 copy number per cell	Average c-myc signal number per cell	Range of c-*myc* signal numbers per cell	Average c-*myc* signals per centromere 8 signal per cell	Amplified cells (%)*
210	3.47	47.1	6–150	13.8	73
244	3.01	18.9	4–40	6.4	71
17117	2.12	11.9	2–45	6.0	42
Lymphocyte control	2.02	2.1	1–4	1.03	0

*Number of cells with c-*myc* signals/centromere 8 > 2.

showed c-*myc* overexpression by immunohistochemistry.

Chromosome 8 Copy Number

Chromosome 8 polysomy (ie, average centromere 8 copy number per tumor cell greater than 2.3) was found in 41 of 87 cases. Considerable heterogeneity was present in chromosome 8 copy number within these tumors. Monosomy 8 was not observed in the 87 cases examined. The fraction of cells with only one centromere 8 signal ranged from 0 to 13.2% (average, 2.6%).

The average centromere 8 copy number was higher in tumors without c-*myc* overexpression (3.57 ± 1.60) than in overexpressing tumors (2.37 ± 0.77; $P = 0.0004$). Polysomy 8 was strongly associated with tumor grade and stage ($P < 0.0001$ each, three-group contingency table analysis). Polysomy 8 was seen in 5 of 27 pTa tumors, 9 of 22 pT1 tumors, and 24 of 33 pT2–4 tumors. Polysomy 8 was found in 1 of 23 grade 1 tumors, 15 of 30 grade 2 tumors, and 22 of 28 pT2–4 tumors. There was a strong association between polysomy 8 and polysomies of chromosomes 7 and 17. All tumors showing polysomy 8 also showed polysomy for either 7 or 17.

*Association of c-*myc *Gene Copy Number Gains and Tumor Phenotype*

Associations between c-*myc* gene copy number increases and tumor stage, grade, and Ki67 LI are shown in Table 2. In contrast to c-*myc* overexpression, c-*myc* gene copy number gains were signifi-

cantly associated with malignant tumor phenotype. Increased c-*myc* copy number was generally more frequent in tumors with advanced stage and higher grade. There was a strong association between c-*myc* copy number increase and tumor stage when all stages were compared ($P < 0.0001$). Significant differences of increased c-*myc* copy number were also present between pTa and pT1 tumors alone ($P = 0.038$) and between pT1 and pT2–4 tumors ($P = 0.0267$). Similarly there was a strong association between c-*myc* copy number increase and tumor grade when all grades were analyzed ($P < 0.0001$). Differences in c-*myc* copy number increase were present between grade 1 and grade 2 tumors alone ($P = 0.0043$) and between grade 2 and grade 3 tumors ($P = 0.042$).

Increased c-*myc* copy number was strongly associated with polysomies of chromosomes 7, 8, and 17 ($P < 0.0001$ each). The fraction of proliferating cells (Ki67 LI) was higher in tumors with c-*myc* copy number increases compared with tumors without a c-*myc* copy number increase, but this relationship did not achieve statistical significance ($P = 0.0766$). However, there was a strong association between the fraction of Ki67-positive cells and the proportion of cells with extra c-*myc* copies ($P = 0.0021$).

p53 Alterations

The relationship of p53 gene alterations with c-*myc* and centromere copy number is shown in Tables 3 and 4. There was a positive staining for p53 in 24 of 54 tumors examined and a p53 deletion (defined as less p53 signals than centromere 17 signals in at least 40% of cells) in 25 of 73 tumors analyzed. A c-*myc* copy number increase was associated with both positive p53 protein expression ($P = 0.0019$) and the presence of p53 deletions ($P = 0.0403$). Increases in c-*myc* copy number were particularly frequent in tumors with both p53 protein expression and p53 deletion (c-*myc* copy number increase in 8 of 10 tumors). This was more frequent than in tumors with either p53 protein expression or p53 deletion alone (7 of 19 tumors; $P = 0.0271$) or in tumors without a detectable p53 alteration (4 of 23 tumors; $P = 0.0002$). There was also a strong association between p53 alterations and polysomies of chromosomes 7, 8, and 17 ($P < 0.005$ each). Polysomy for all three chromosomes was present in all 10 tumors with both p53 protein expression and 17p deletion ($P < 0.0001$), whereas polysomy for any of the three chromosomes examined was present in only 4 of 24 tumors without p53 alteration. There was no significant association between p53 alterations and c-*myc* protein expression ($P = 0.3420$; three-group contingency table analysis).

Discussion

The results of this study show that both c-*myc* protein overexpression and an increased c-*myc* gene copy number are frequent in bladder cancer. Although overexpression is associated with low grade and early stage tumors, a low level c-*myc* gene copy number gain is more frequent in advanced tumors. The lack of association between low level c-*myc* gene copy number gain and protein overexpression suggests that a detectable low level gene copy number gain represents a structural chromosome 8 alteration rather than a c-*myc*-specific phenomenon.

Initial immunohistochemical analysis showed that c-*myc* expression was present in normal urothelium

Table 3. c-myc *Copy Number and Phenotype/Genotype*

		c-*myc* copy number (*number of tumors*)*		
		Normal†	Increased	
Tumor stage	pTa	23	2	
	pT1	15	7	
	pT2–4	12	20	P < 0.0001
Tumor grade	G1	22	1	
	G2	18	11	
	G3	9	17	*P* < 0.0001
Chromosome 7	Disomic‡	24	2	
	Polysomic	13	21	*P* < 0.0001
Chromosome 8	Disomic‡	42	4	
	Polysomic	10	28	*P* < 0.0001
Chromosome 17	Disomic‡	39	7	
	Polysomic	8	22	*P* < 0.0001
Ki67 LI§		13.5 ± 11.5	19.2 ± 9.9	*P* = 0.0766

*Three cases with high level amplification excluded from this analysis.
†More c-*myc* signals than centromere 8 signals in less than 10% of cells.
‡Average centromere copy number <2.3.
§Ki67 LI; mean ± standard deviation (*%*).

Table 4. *p53 Alterations and Phenotype*

		Number of cases											
		c-*myc* copy number*			Chromosome 7			Chromosome 8			Chromosome 17		
		Normal†	Increased		Disomic‡	Polysomic		Disomic‡	Polysomic		Disomic‡	Polysomic	
p53 protein	Negative	24	5		16	6		25	5		26	4	
	Positive	10	14	$P = 0.0019$	5	16	$P = 0.0013$	6	18	$P < 0.0001$	6	18	$P < 0.0001$
p53 FISH	Absent	33	15		18	17		32	17		36	12	
deletion	Present	11	14	$P = 0.0403$	6	16	$P = 0.0721$	9	17	$P = 0.0111$	7	17	$P = 0.0002$
No p53 expression, no p53 deletion		19	4		12	4		20	4		22	2	
Either p53 expression or deletion		12	7		8	9		10	9		9	10	
p53 expression and p53 deletion		2	8	$P = 0.0028$	0	9	$P = 0.0015$	0	10	$P < 0.0001$	0	10	$P < 0.0001$

*Three cases with high level amplification excluded from this analysis.
†More c-*myc* signals than centromere 8 signals in less than 10% of cells.
‡Average centromere copy number <2.3.

as well as in all 51 bladder tumors examined. An antibody dilution series allowed us to identify tumors with an expression level beyond the level of normal urothelium (ie, overexpression), and c-*myc* overexpression was more frequent in early stage/low grade bladder cancers than in the more advanced tumors. Although differing with the results of Kotake et al,[8] our data are in complete agreement with the results of Masters et al,[7] who used the same antibody (*myc*-1 6E10) for flow cytometric quantitation of nuclear c-*myc* expression in archival bladder cancer tissue and showed a higher expression level in superficial than in muscle-invasive tumors. The observation of a higher c-*myc* expression level in well differentiated than in undifferentiated tumors is analogous to previous findings in breast,[19] colon,[20] anal,[7] and testicular tumors.[21] On the basis of these findings it has been hypothesized that c-*myc* protein may play a role in differentiation control.[19] Alternatively, it could be speculated that decreased c-*myc* expression may contribute to inhibition of apoptosis, as recent studies have shown that c-*myc* expression is required for induction of apoptosis under specific conditions.[22]

Our FISH examination revealed three tumors with high level c-*myc* gene amplification. The clustered arrangement of c-*myc* signals in these tumors is suggestive of an intrachromosomal amplification (homogeneously staining regions), as a nonrandom distribution of amplified signals could not be explained by an extrachromosomal amplification (double minutes). It has previously been shown that the results of amplification analysis by FISH and by Southern blot are strongly correlated.[9] The presence of c-*myc* overexpression in the one amplified tumor for which sections were available for immunohistochemical examination indicates that overexpression can be caused by gene amplification. The lack of amplification in most tumors with overexpression, however, suggests that gene amplification is an unusual mechanism of c-*myc* overexpression in bladder cancer. This is similar to recent reports, showing that mechanisms of overexpression other than gene amplification are present in the majority of bladder tumors with epidermal growth factor receptor[18,23] and *erb*B-2 overexpression.[12,24]

The selected dual labeling FISH approach with probes for the c-*myc* gene at 8q24 and the centromere region of chromosome 8 allows the determination of subtle c-*myc* gene copy number aberrations on a cell by cell basis. This enabled us to identify a low level increase in c-*myc* signals in more than 10% of tumor cells in 32 of 87 tumors examined. Such a low level gene copy number gain was not associated with protein overexpression. This shows that a gain of a few extra c-*myc* gene copies is not sufficient to cause a detectable overexpression. The lack of a positive association between a low level increase of c-*myc* copy number and c-*myc* protein overexpression was not unexpected, as our previous study applying comparative genomic hybridization (CGH) to primary bladder tumors had revealed a number of cases with a gain of large fragments of the long arm of chromosome 8, often including c-*myc*.[25] Such a gain of the entire 8q arm would lead to a low level copy number increase for all loci on 8q, including c-*myc*. It is therefore likely that the c-*myc* copy number increase detected by our approach is not c-*myc* specific but rather represents a gain of a large fragment of 8q.

There are several arguments that our findings may represent formation of isochromosome 8q in many of these cases. First, isochromosome 8q has repeatedly been described in cytogenetic reports on bladder cancer.[26] Second, in our previous CGH study we found a concomitant loss of 8p in a number of bladder tumors with gains of large segments of chromosome

8q, a finding that is also consistent with isochromosome 8q.[25] Indirect evidence for this hypothesis is also seen in a recent report of Knowles et al,[27] showing an association between loss of heterozygosity for both 8q and 8p. The authors of this study suggested that their finding may represent monosomy 8. However, we have observed neither monosomy 8 nor deletions of 8q24. It is therefore possible that allelic imbalance on 8q is not caused by allelic loss but by allelic gain in their study. If c-*myc* copy number gains do represent isochromosome 8q, then these gains presumably involve most genes on chromosome 8, only one of which is c-*myc*. The fact that several 8q gains did not include the c-*myc* locus in our CGH study, and a high level amplification at 8q21 in one case, is suggestive of other relevant genes on chromosome 8q.[25] Considering the high frequency of 8p losses observed by cytogenetics,[26] restriction fragment length polymorphism analysis,[27] and CGH,[25] it is also possible that 8p is the site of a yet unknown tumor suppressor gene in bladder cancer.

Failure of cellular mechanisms involved in the preservation of genomic integrity may be associated with the genomic alteration resulting in a detectable increase in c-*myc* gene copy number. The observed strong association between increased c-*myc* copy number and polysomies of all examined chromosomes suggests that the same mechanisms may apply for development of both aberrations. The p53 gene has recently been suggested as a key gene for preservation of genomic stability.[28,29] p53 inactivation is frequently due to deletion of one allele and mutation of the other allele. In this study we have used two different approaches to study p53 gene alterations, both with inherent limitations. It is well known that sensitivity and specificity of immunohistochemistry for detection of p53 mutations is not satisfactory.[30] Also, we have previously shown that most but not all allelic losses can be detected by FISH.[17,31] It appears likely, however, that tumors with both a p53 deletion detected by FISH and positive p53 immunostaining represent a classical p53 inactivation, with deletion of one allele and mutation of the other allele. The observed high prevalence of polysomies and increased c-*myc* copy number in these tumors is consistent with an important role of p53 for genomic stability.

In summary, these data show that c-*myc* protein overexpression is associated with low grade, early stage bladder cancer. c-*myc* gene amplification is a rare cause of c-*myc* overexpression. A gain of a few extra c-*myc* gene copies is not related to c-*myc* overexpression and represents most likely a structural chromosome 8 abnormality. The association of this finding with advanced grade and stage as well as with tumor cell proliferation is evidence for a significant role of chromosome 8 alterations in bladder cancer biology.

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