

Chromosomal aneuploidies in bladder cancer, chronic cystitis and normal urothelium detected by fluorescence *in situ* hybridization

Aneuploidias cromossômicas em câncer da bexiga, cistite crônica e urotélio normal detectadas por hibridização in situ fluorescente

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Abstract

The objective of present work is to investigate the occurrence of numerical alterations in lesions and bladder carcinomas that may be helpful in early diagnosis. Fluorescence *in situ* hybridization (FISH) was applied to identify such alterations in chromosomes 7, 9 and 17, in interphase nuclei of 14 fresh bladder tumor specimens (13 transitional cell carcinomas, TCC, and 1 undifferentiated anaplastic carcinoma, UAC), and 5 specimens derived from the TCC patients (2 chronic cystitis, UCC, and 3 macroscopically normal urothelium biopsies, MNU). The most frequent anomalies in malignant tumors of the bladder were trisomy/tetrasomy 7 (6/14=43%), trisomy/tetrasomy 17 (7/14=50%) and monosomy 9 (4/14=29%). The two chronic cystitis samples showed monosomy 9, whereas one macroscopically normal urothelium sample presented similar findings (polysomy 7, 9, and 17) observed in the matched tumoral tissue. Two carcinomas, an invasive grade IV (TCC13) and an invasive primary (UAC14), presented trisomy/tetrasomy 7, 9 and 17 suggesting that the cells were polyploidy. These results strengthens the involvement of chromosomes 7, 9, and 17 in urothelial carcinogenesis. The alterations of chromosomes 7 and 9 are related to the initiation process; and of chromosome 17, to tumoral progression and recurrence. Moreover, the results also support the hypothesis that chronic cystitis and normal urothelium from patients with bladder carcinoma can carry important chromosome abnormalities which may predict risk for tumor progression and recurrence. Thus, interphase FISH may be a useful tool for early diagnosis in patients at risk of disease and for follow-up in cases of recurrence and metastase.

Key words: bladder neoplasms; carcinoma; cystitis; aneuploidy; fluorescence in situ hybridization; chromosomes.

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Resumo

O presente trabalho investigou a ocorrência de anomalias cromossômicas numéricas em lesões da bexiga que possam auxiliar no diagnóstico precoce. A técnica de Híbridização in situ Fluorescente (FISH) foi aplicada para identificar tais anomalias nos cromossomos 7, 9 e 17 em núcleos interfásicos de 14 amostras de tumores da bexiga frescos (13 carcinomas de células transitórias, TCC, e 1 carcinoma anaplásico indiferenciado, UAC), e 5 amostras derivadas de pacientes com TCC (2 cistites crônicas e 3 biopsias de urotélio macroscopicamente normais). As anomalias mais frequentes nos tumores malignos da bexiga foram trissomia/tetrassomia 7 (6/14=43%), trissomia/tetrassomia 17 (7/14=50%) e monossomia 9 (4/14=29%). As duas amostras de cistites crônicas apresentaram monossomia 9, uma das amostras de urotélio macroscopicamente normal exibiu resultado semelhante (polissomia 7, 9 e 17) ao do tecido tumoral correspondente. Dois carcinomas, um grau IV invasivo (TCC13) e um primário invasivo (UAC14), exibiram trissomia e tetrassomia 7, 9 e 17 sugerindo a ocorrência de poliploidia. Tais resultados reforçam o envolvimento dos cromossomos 7, 9 e 17 na carcinogênese urotelial. As alterações dos cromossomos 7 e 9 estão relacionadas com os processos de iniciação e do cromossomo 17 com progressão e recorrência tumoral. Os resultados também suportam a hipótese de que as cistites crônicas e o urotélio normal de pacientes com carcinoma de bexiga podem conter anormalidades cromossômicas importantes, que podem predizer risco para progressão e recorrência tumoral. Assim, a técnica de FISH pode ser uma ferramenta útil para o diagnóstico precoce, em pacientes com risco de desenvolver a doença e acompanhamento em casos de recorrência e metástases.

Palavras-chave: neoplasias da bexiga; carcinoma; cistite; aneuploidias; fluorescência em hibridização in situ; cromossomos.

INTRODUCTION

Bladder carcinoma is a relevant worldwide public health problem. The predominant histological type of bladder cancer is transitional cell carcinoma (TCC) of urothelial origin. Several etiologic factors have been related as cause of bladder cancer, such as smoking and occupational exposure to certain aromatic amines.¹ The tumoral stage and grade are important parameters for prognosis. The acquisition of genetic alterations along the tumoral progression is an important event increasing the malignant potential of cancer cells.² Partial deletions of chromosomes 9 and 17p and gain of 1q, 5p, 7p, 11q and 17q were defined as the most important alterations in TCC.^{3,4} These abnormalities are related with molecular changes in tumor suppressor genes (CDKN2, ARF, RB and TP53), oncogenes (BCL-2 and c-ERBB-2), and other genes such as EGF and EGF-R.⁵

FISH is a sensitivity technique for the detection of urothelial carcinoma, superior to cytology. This technique has the potential to monitor patients with bladder cancer for tu-

mor recurrence.⁶

The purpose of this study was to evaluate the occurrence of aneuploidy involving chromosomes 7, 9 and 17 by FISH technique in interphase nuclei of bladder carcinoma, chronic inflammation (cystitis) and macroscopically normal urothelium biopsies from patients with TCC. FISH technique may be a useful tool for early diagnosis and follow up the patients with risk to recurrence and metastasis.

MATERIAL AND METHODS

A total of 19 specimens were obtained immediately after surgical resection from 16 patients (11 men and 5 women) with bladder cancer. Mean age of these patients was 68 years (ranging from 52 to 83 years). Tumor stage and grade were defined according to WHO system.⁷ The specimens were diagnosed as follows: 13 transitional cell carcinoma (TCC), 1 undifferentiated anaplastic carcinoma (UAC), 2 unspecific chronic cystitis (UCC) and 3 macroscopically normal urothelium (MNU). All specimens were obtained prior to radiation- and chemotherapy. From healthy donors,

we have obtained 5 specimens of urine exfoliated cells (donors' range age: 15 to 27 years) and 5 of peripheral blood lymphocytes (donors' range age: 28 to 81 years). All patients and healthy controls gave written informed consent for the study. This work was approved by the Ethics in Research Committee of UNESP, São José do Rio Preto - SP Campus and National Committee of Ethics in Research (CONEP) (process number: 409/99).

The FISH technique was performed in interphase nuclei with the following probes: alpha-satellite centromeric DNA probes for chromosomes 7 and 17 (Oncor) labeled with biotin and classical satellite DNA probe for chromosome 9, directly labeled with Spectrum Red-dUTP (Vysis). The bladder tissues collected in saline solution were mechanically disaggregated, treated with hypotonic sodium citrate solution (1%) for 30 min and fixed in 3:1 methanol/acetic acid overnight. The remaining cell fragments were disaggregated through incubation in 60% acetic acid for 4 h and the resulting cell suspension was dropped onto microscope slides, which were stored at -70°C up to the FISH assay.

Frozen slides were thawed at room temperature, dehydrated for 2 min each in 70%, 85% and 100% ethanol series, and air-dried. Each slide was treated with acetic acid 60% and pepsin 0.008% in 0.001M HCl for 1-5 min. Slides were rinsed in 1×PBS and dehydrated in ethanol. Next, 10µ of hybridization mixture containing 20ng of DNA probe, 50% formamide, 10% dextran sulfate, and 2×SSC was applied to each slide. A coverslip was added and sealed with rubber cement. Probe and target DNAs were denatured simultaneously at 84°C for 8 min and hybridization was carried out overnight in a moist chamber at 37°C. Post-hybridization washes included three washes in 50% formamide/2×SSC for 5 min at 37°C and three washes in 2×SSC (pH 7.0) for 5 min each at 37°C. The slides hybridized with biotin-labeled probes (7 and 17) were detected with avidin-FITC and the signal was amplified using biotinylated anti-avidin antibody, followed by another layer of avidin-FITC (Vector). After three buffer washes (2 min each), the nuclei were counterstained by adding propidium iodide (1µg/ml) or DAPI (2µg/ml) in an antifade solution.

Dual-color FISH assays performed in the samples TCC13, IAC14 and MNU01 using centromeric probes of the chromosomes 7 (biotin-labeled) and 9 (Spectrum Red), or 9 (Spectrum Red) and 17 (biotin-labeled).

For each probe, two independent observers, according criteria described by Eastmond et al.⁸ evaluated signals from about 300 nuclei. In brief, only intact and non-overlapping nuclei were counted, and only completely separated signals were scored individually, bilobed signals were counted as one. Cutoff levels were based on the upper limit mean +4SD of the exfoliated urothelial cells samples. The cutoff values for monosomy 7, 9, and 17 were respectively set at 26.8%, 22.8%, and 24.1%; for trisomy 7, 9 and 17 were respectively set at 5.0%, 7.5%, and 6.2%, and for tetrasomy 7, 9 and 17 were set at 2.0%. Statistical analysis was performed by chi-squared test. A P value of <0.05 was considered significant.

RESULTS

Significant differences in the number of copies of chromosomes 7 ($X^2=15.5$, $DF=2$, $P<0.05$) and 9 ($X^2=18.3$, $DF=2$, $P<0.05$) per cell were found between the lymphocytes and the bladder exfoliated cells. Thus we used the exfoliated urothelial cell samples as controls because these represent the same cellular type of the tumoral tissue.

The chromosomal aberrations are summarized in Table 1. Numerical aberrations were detected in 15 of 19 specimens (79%), the most common being gain of chromosomes 7 and 17 which was observed in 8 of 19 (42%) cases. Monosomy 9 was the less frequent change, observed in 32% of the cases (4 TCC and 2 cystitis) whereas, trisomy and tetrasomy of chromosome 9 were present in 6 other samples (5 TCC and 1 normal urothelium). Figure 1 (A-F) illustrates some chromosome aberrations.

Two carcinomas, an invasive grade IV TCC (TCC13) and an invasive primary UAC (UAC14), exhibited trisomy and tetrasomy for chromosomes 7, 9 and 17 suggesting that the cells were polyploidy. Dual-color FISH assays performed in the samples TCC13, UAC14 and MNU01 using centromeric probes of the chromosomes 7 and 9, or 9 and 17 detected 8 fluorescent spots in more than 50% of the ana-

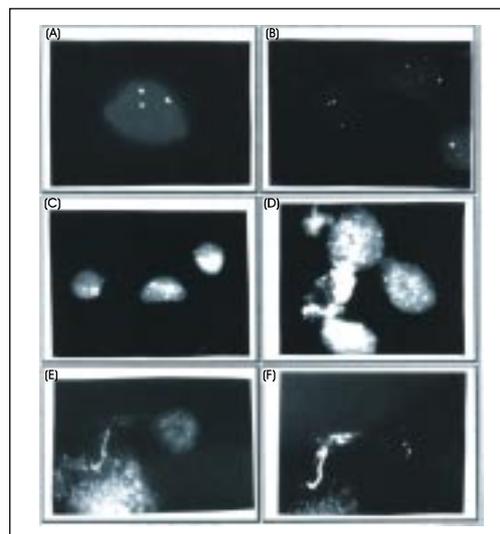
lyzed nuclei, reinforcing the occurrence of tetraploidy. The normal control MNU01, taken from a macroscopically normal bladder site of patient TCC13, showed the same numerical alterations as the tumoral tissue. In additional one normal urothelium sample (MNU02) biopsied from patient TCC07, presented trisomy 7, while in other normal sample (MNU03) biopsied from patient TCC13, numerical aberrations were not observed.

Table 1. Distribution of numerical aberrations in bladder carcinoma, cystitis, and normal bladder tissue samples.

Case	Histopatologic Diagnosis (Grade)	Sex/Age (years)	Other Information	Aneuploidies		
				7	9	17
Carcinomas						
CC 01	TCC (I)	M/82	Primary			
CC 02	TCC (I)	M/64	1 st rec			
CC 03	TCC (I)	M/62	4 th rec	Tris, Tetras		Tris
CC 04	TCC (I)	M/65	Primary		Monos	Tris
CC 05	TCC (I)	M/83	3 rd rec		Monos	Tris, Tetras
CC 06	TCC (II)	F/77	1 st rec, invasive	Tris		
CC 07	TCC (II)	F/52	Primary	Tris	Tris, Tetras	Tris
CC 08	TCC (III)	F/69	Primary, invasive		Monos	
CC 09	TCC (III)	M/69	4 th rec, invasive		Tris, Tetras	
CC 10	TCC (III)	F/51	1 st rec			
CC 11	TCC (III)	M/67	Primary, invasive	Tris, Tetras	Tris, Tetras	Tris
CC 12	TCC (IV)	M/75	Primary, invasive		Monos	
CC 13	TCC (IV)	M/62	Primary, invasive	Tris, Tetras	Tris, Tetras	Tris, Tetras
UAC 14	UAC	M/71	Primary, invasive	Tris, Tetras	Tris, Tetras	Tris, Tetras
Cystitis						
UCC 01	UCC	M/68	1 st rec		Monos	
UCC 02	UCC	F/62	4 th rec		Monos	
Normal bladder						
MNU 01		M/65		Tris, Tetras	Tris, Tetras	Tris, Tetras
MNU 02		F/52		Tris		
MNU 03		M/62				

TCC=transitional cell carcinoma; UAC=undifferentiated anaplastic carcinoma; UCC=unspecific chronic cystitis; MNU=macroscopically normal bladder; rec=recurrence; monos=monosomy; tris=trisomy; tetras=tetrasomy.

Figure 1. Interphase nuclei disaggregated from bladder carcinomas showing (A) trisomy of chromosome 7 (UAC14), (B) trisomy and tetrasomy of chromosome 17 (TCC13), (C) monosomy of chromosome 9 (TCC12), (D) tetrasomy of chromosome 9 (TCC13), (E) and (F) MNU01, respectively, showing tetrasomy 9 and 7 in the same nucleus. Arrows point the nuclei with aneuploidies.



DISCUSSION

Although peripheral blood lymphocytes constitute a good system for cytogenetic analyses, our study showed that bladder exfoliated cells obtained after urinary sedimentation from healthy young people constitute a simple and non-invasive system for FISH analysis. Fresh collected urine exfoliated cells represent an advantage over normal cells collected through invasive methodology as bladder irrigation⁹ or formalin-fixed, paraffin-embedded tissues.³

In the present study, nuclei isolated of fresh tissue of bladder carcinoma, cystitis and macroscopically normal urothelium showed mainly trisomies 7 and 17, and monosomy 9. Monosomy 9 was less frequent and occurred in 32% of the samples, mainly lower grade tumors (I and II) and cystitis. These results do not exclude partial chromosome 9 loss as a frequent event, since a pericentromeric probe was used. Linn et al.¹⁰ also detected low frequency of monosomy 9 in TCC. However, when gene-specific probes were used for chromosome loci (9q22 and 9p21), deletions on 9p and 9q were found in majority of urothelial carcinoma. Some early superficial papillary tumors showed deletion of chromosome 9p without deletion of 9q, suggesting 9p deletions as a very early event in the development of papillary urothelial carcinoma.¹¹ Loss of chromosome 9 in TCC patients was related with high risk of recurrence and possible progression. Thus, the detection of this alteration in early tumor stage may be used as marker of poor prognosis.³ Trisomy and tetrasomy 9 was also evident in 32% of samples, frequently observed together with trisomy/tetrasomy 7 and 17. These cases consisted mainly of high grade, invasive primary tumors, thus suggesting an association of these polysomies with high-grade bladder carcinomas. Eleuteri et al.¹² reported trisomy 9 in bladder TCC associated with high grade and hyperploidy evaluated by flow cytometry, while monosomy 9 was prevalent in diploid or near-diploid cases and had no relationship with histological grade.

The most common chromosomes abnormalities in our samples were trisomy and/or

tetrasomy 7 and 17, occurring in 42% of cases. Trisomy 7 jointly with monosomy 9 are considered early chromosome changes in bladder cancer.^{3,4,13} Pycha et al.⁴ described high frequency of trisomy 7 in bladder cancer related with advanced clinical stages, corroborating other studies that associated this alteration with progression of lower to higher grade lesions, and with tumoral recurrence.³ Chromosome 7 harbors the gene encoding the EGF-R related with regulation of cell growth. In TCC, the overexpression of this gene has been associated with an increased rate and short time recurrence.⁵ However, trisomy 7 as the sole clonal chromosomal aberration has been reported in a wide variety of tumors,¹⁴ as well as in macroscopically normal tissue adjacent to solid tumors¹⁵ and non-neoplastic lesions.¹⁶ Thus, the importance of a solitary trisomy 7 as a neoplasia-associated change has been questioned for a long time.

Trisomy of chromosome 17 is a frequent event in bladder tumors and also was observed in 42% of the samples in our study, jointly with aneuploidy of the chromosomes 7 and 9, indicating its later participation in bladder carcinogenesis. Some studies related this abnormality with an increased risk of tumoral recurrence and progression,^{15,17} which associated polysomy 17 with invasive tumors and advanced stage. Molecular studies have indicated gain in 17q11-q25, maybe related with the oncogene *c-ERBB-2* (*Her-2/neu*) mapped at 17q12-21. This oncogene codes for a receptor protein p185, with partial homology to the EGF-R protein. In bladder cancer, both *Her-2/neu* gene amplification and protein overexpression are observed in higher stages and nodal metastasis, which highlight this gene as a potential tumor marker.^{18,19}

Halling et al.⁶ evaluated urine specimens of patients with a history of urothelial carcinoma by multi-color FISH with centromeric probes to chromosomes 3, 7 and 17, and 9p21 locus. FISH assay demonstrated high sensitivity and specificity for the detection of urothelial carcinoma by presence of polisomic cells.

In addition, we detected anomalies in chromosomes 7, 9, and 17 in macroscopically normal bladder tissue biopsied from the patients with bladder carcinomas. One of three

samples (MNU01) of the normal bladder presented the same chromosomal changes observed in the primary TCC (TCC13). The patient had resected the entire bladder, device tumoral multicentricity. Frequent recurrences and a tendency for multicentricity are characteristic features of bladder tumors, suggesting that bladder cancer is a field disease involving the entire urothelium. Anomalies of the surrounding urothelium may therefore be highly relevant to patient prognosis. Hartmann et al.¹⁵ observed genetic changes in urothelial hyperplasias and normal urothelium in patients with papillary bladder cancer. Therefore, genetic investigations of normal urothelium biopsies in bladder cancer patients could provide insights into the primary alterations of bladder carcinogenesis. Our findings also demonstrated that apparently normal urothelium tissue of patients with tumor history do not constitute adequate negative control samples in genetic studies.

High grade TCC samples (III and IV) had higher frequency of trisomy and tetrasomy of chromosomes 7, 9, and 17 in comparison with low grade (I and II), evidencing chromosome instability. The samples TCC13 and UAC14 showed increased frequencies of polysomies 7, 9, and 17, suggesting tetraploidy (4n) that was corroborated by the dual-color FISH assays. These abnormalities are consistent with the tumor progression model, in which the polyploidy occurs as a late event in carcinogenesis.

Chromosomal evaluations in cystitis are rare. The present study found aneuploidy of chromosome 9 in the unspecific chronic cystitis samples from patients with prior history of bladder TCC. Ghaleb et al.²⁰ observed numerical aberrations of chromosomes 9 and 17 in urothelium of patients with bilharzial cystitis, therefore monosomy 9 may be an early chromosomal change and a predictor of incipient carcinoma in patients with this condition.

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CONCLUSIONS

This study strengthens a role for chromosomes 7, 9 and 17 in urothelial carcinogenesis. In conjunction with the literature, it is possible to postulate that anomalies in chromosomes 7 and 9 are related to the initiation process and in chromosome 17 with tumoral progression and recurrence. Thus, interphase FISH using probes targeting these chromosomes may be indicated for early diagnosis in patients at risk of disease and for follow-up in cases of recurrence and metastase.

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