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Original article

Chromosomal imbalances in successive moments of human bladder urothelial carcinoma☆

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Abstract

Objective: To understand developmental characteristics of urinary bladder carcinomas (UBC) by evaluating genomic alterations and p53 protein expression in primary tumors, their recurrences, and in the morphologically normal urothelium of UBC patients.

Methods: Tumors and their respective recurrences, six low-grade and five high-grade cases, provided 19 samples that were submitted to laser microdissection capture followed by high resolution comparative genomic hybridization (HR-CGH). HR-CGH profiles went through two different analyses—all tumors combined or classified according to their respective histologic grades. In a supplementary analysis, 124 primary urothelial tumors, their recurrences, and normal urothelium biopsied during the period between tumor surgical resection and recurrence, were submitted to immunohistochemical analyses of the p53 protein. During the follow-up of at least 21 patients, urinary bladder washes citologically negative for neoplastic cells were submitted to fluorescence in situ hybridization (FISH) to detect copy number alterations in centromeres 7, 17, and 9p21 region.

Results and Conclusions: HR-CGH indicated high frequencies (80%) of gains in 11p12 and losses in 16p12, in line with suggestions that these chromosome regions contain genes critical for urinary bladder carcinogenesis. Within a same patient, tumors and their respective recurrences showed common genomic losses and gains, which implies that the genomic profile acquired by primary tumors was relatively stable. There were exclusive genomic alterations in low and in high grade tumors. Genes mapped in these regions should be investigated on their involvement in the urinary bladder carcinogenesis. Successive tumors from same patient did not present similar levels of protein p53 expression; however, when cases were grouped according to tumor histologic grades, p53 expression was directly proportional to tumor grades. Biopsies taken during the follow-up of patients with history of previously resected UBC revealed that 5/15 patients with no histologic alterations had more than 25% of urothelial cells expressing the p53 protein, suggesting that the apparently normal urothelium was genomically unstable. No numerical alterations of the chromosomes 7, 17, and 9p21 region were found by FISH during the periods "free-of-neoplasia." Our data are informative for further studies to better understand urinary bladder urothelial carcinogenesis.

Keywords: Urinary bladder cancer; HR-CGH; FISH; p53

1. Introduction

Transitional cell urinary bladder cancer (UBC) accounts for up to 5% of all new human neoplasia. Accordingly, this tumor is the fifth most common cancer in the Western world. More than 60% of them recur at least once and may progress to less differentiated and or invasive neoplasm in 10%–15% of all cases [1].

UBCs are characterized by chromosomal and genetic alterations [2] but during periods between the resection of

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the initially diagnosed tumor and its late recurrences, there are not undisputable histologic alterations in the mucosa. However, during these periods, from hereon indicated as "free-of-neoplasia" intervals, the urothelium contains transformed cells distributed throughout the mucosa, which probably are responsible for urothelial instability and disease progression [3]. A previous study from this laboratory recorded significantly increased number of chromosomal breaks in otherwise morphologically normal urothelial cells obtained by bladder washing during the "free-of-neoplasia" period [4].

Many tumor characteristics have been studied in an attempt to predict the variable UBC behavior, such as histopathologic and cytologic features [5], and also some putative molecular markers [6]. UBCs are characterized by a number of chromosomal and genetic alterations of which cytogenetic loss of chromosome 9 is particularly frequent, occurring in more than 50% of the cases [7]. Activating point mutations in the *FGFR3* gene are found in Ta stage, but rarely in invasive tumors [2]. The hitherto best known molecular event predictive of malignant progression is the loss of the p53 protein function, which is found in the majority of advanced UBC [8,9]. The current World Health Organization (WHO) classification tries to integrate tumor morphology with its biological behavior and molecular characteristics and considers a strong argument for the presence of genetically unstable neoplasia, those tumors that show high p53 protein immunoexpression [6].

In order to document developmental characteristics of urinary bladder tumors, primary UBC and their recurrences were cytogenetically evaluated for their genomic expression by high resolution comparative genomic hybridization (HR-CGH). Besides, in an independent cohort, the immunoexpression of the p53 protein was evaluated in primary tumors and their recurrences. Finally, during the "free-of neoplasia" periods, negative urinary bladder washes were submitted to fluorescent in situ hybridization (FISH) in order to detect possible biomarkers for early detections of UBCs. For this purpose, probes to three regions frequently altered in urothelial tumor cells, centromeres 7, 17, and 9p21 [10,11], were used.

2. Materials and methods

2.1. Patients and tumor specimens

This study was approved by the Committee for Ethics in Research of the Botucatu Medical School (FMBo), UNESP, SP, Brazil (Protocol no. 399/2005). UBCs patients were diagnosed, treated, and followed up at the Urology Service of the FMBo/UNESP General Hospital; tissue samples from the primary tumors and from their recurrences were paraffin blocked and archived at the Department of Pathology, FMBo, UNESP.

2.2. High resolution-comparative genomic hybridization (HR-CGH)

Patients selected to have their tumors analyzed by HR-CGH included 10 men (71.7 ± 10 years old) and 1 woman (83 years old). They had not been submitted to any neoadjuvant therapy previously. Risk factors for UBC such as smoking were not explored for this study. Primary tumors and their recurrences from 3 patients with low-grade (LG) (1 with 2 successive recurrences; therefore, 7 samples) and 4 with high-grade (HG) papillary carcinomas (8 samples) were selected for HR-CGH analyses according to the tumor grade, totalizing 15 samples. Single primary tumor samples of 3 patients with LG and 1 with HG were added to these patients, totalizing 19 samples (i.e., 11 primary tumors and 8 recurrences).

Samples of neoplastic tissues were obtained by laser capture microdissection (LCM) (Pix Cell II Laser Capture Microdissection; Arcturus Inc., Life Technologies, Carlsbad, CA). The DNA was extracted by Nucleon for Hard Tissue kit (GE Healthcare, Buckinghamshire, England) and stored at –80°C. Tumoral DNA and references were amplified and labeled by PCR-based protocols single cell comparative genomic hybridization (SCOMP) as described by Stoecklein et al. [12], with modifications. Phytohemaglutinin-stimulated normal lymphocytes were prepared as targets for HR-CGH experiments using a standard protocol [13]. The hybridization and washes were performed as described by Kallioniemi et al. [14]. Briefly, target DNA and normal reference DNA were labeled with biotin-14-dATP (Invitrogen, Carlsbad, CA) and digoxigenin-11-dUTP (Roche, Mannhein, Germany), respectively. Tumor and Cot1 DNA (Invitrogen, Carlsbard, CA), both labeled, were co-hybridized to normal chromosomes metaphases of lymphocytes from healthy donors. Slides were hybridized for 72 h, washed, and counterstained with 4,6-diamidino-2-phenylindole (Vector, Burlingame, CA). HR-CGH images were captured using an Olympus AX61 fluorescence microscope (Olympus Optical, Hamburg, Germany) equipped with a CCD camera (Photometrics CH 250; Huntington Beach, CA). The software CGH View 3.0 (Applied Spectral Imaging, Beith Haemek, Israel) was used for image analysis. In each case, 15–30 metaphases were analyzed. Chromosomal imbalances were detected by standard reference intervals (SRI), as described in Kirchhoff et al. [15]. Superposed chromosomes and heterogeneous hybridization patterns were excluded from the analyses. A library with differentially labeled normal samples was constructed to select the upper and lower limits for chromosomal gains and losses. The 99.5% confidence interval of each mean ratio profile value was compared with a corresponding 99.5% standard reference interval (SRI) based on an average of 15 normal DNA samples obtained from volunteers without cancer (140 lymphocyte cells). The SRI was scaled automatically to fit the individual test case.

The databank of NCBI Map Viewer website [16] was inquired regarding the genes localized in the chromosomes