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ScienceDirect
Experimental and Molecular Pathology 82 (2007) 292–297
Experimental and Molecular Pathology
www.elsevier.com/locate/yexmp

In situ detection of global DNA hypomethylation in exfoliative urine cytology of patients with suspected bladder cancer

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Received 20 May 2006, and in revised form 4 August 2006
Available online 5 October 2006

Abstract

Global DNA hypomethylation is a common phenomenon in bladder cancer. Therefore we investigated whether it is possible to detect and assess global DNA hypomethylation in bladder cancer using a specific monoclonal antibody for 5-methyl-cytosine. Cytospins from exfoliative urine cytology specimens of patients with bladder cancer or a history of bladder cancer, control patients with benign urological diseases and of young healthy volunteers were analyzed.

Urothelial carcinoma (UC) cells showed various degrees of nuclear destaining indicating global DNA hypomethylation whereas all specimens from healthy volunteers showed granular nuclear staining indicating regular methylation of repeated DNA sequences. Lowest 5-methylcytosine immunostaining scores were observed in carcinoma cells and a statistically significant difference was observed between urothelial cells of healthy controls or patients with benign disease compared to bladder cancer patients ($p<0.01$, $p<0.05$, respectively). In UC cases even morphologically normal urothelial cells often displayed evident hypomethylation. Likewise, in patients with a history of UC, but no cystoscopic evidence of recurrence, morphologically non-malignant urothelial cells presented with some degree of demethylation.

Our results strongly support the hypothesis of early global demethylation in bladder cancer. Immunocytochemical staining with the 5-methylcytosine antibody allows simultaneous individual assessment of nuclear morphology and methylation status of a given sample.

Keywords: Hypomethylation; Bladder cancer; Cytological detection; 5-methyl-cytosine

Introduction

Epigenetic changes contribute to carcinogenesis and have therefore been studied intensively in many human cancers. Alterations of cytosine methylation at CpG dinucleotides in DNA are particularly well characterized. Changes in DNA methylation comprise aberrant hypermethylation of specific promoter regions and global hypomethylation affecting primarily repeated DNA sequences (Jones and Baylin, 2002; Ehrlich, 2002). Hypermethylation is usually associated with transcriptional silencing of specific genes, while global hypomethylation appears to contribute to chromosomal instability (Hoffmann and Schulz, 2005). Both types of alterations have also been described in urothelial carcinoma (UC) of the bladder (Chan et al., 2003; Markl et al., 2001; Salem et al., 2000; Maruyama et al., 2001; Jürgens et al., 1999; Florl et al., 1999). DNA hypermethylation in bladder cancer appears to gradually increase with tumor progression (Salem et al., 2000; Maruyama et al., 2001; Catto et al., 2005) and affects several genes in a variable pattern. In contrast, DNA hypomethylation is highly prevalent throughout all stages of UC and may represent an early step in the development of this cancer type. In normal cells, methylcytosine is concentrated in regions of the genome with a high content of repetitive DNA such as pericentromeric regions, the inactive X-chromosome and facultative heterochromatin. As a consequence of global hypomethylation, this distribution can change in cancer cells. The use of antibodies

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0014-4800/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexmp.2006.08.002

specific to 5-methylcytosine allows to visualize DNA methylation patterns at the cytological level, as demonstrated in lung cancer (Piyathilake et al., 2003) and cervical cancer (De Capoa et al., 2003). Thus, hypomethylation in UC ought to result in a detectable change in nuclear staining by an anti-methylcytosine antibody. In lung and cervical cancers, detection of cells with hypomethylated genomes has been suggested to be helpful for cancer detection and diagnosis. Detection of cancer cells by cytological investigation of urine samples is one of the most important procedures for initial diagnosis and monitoring of UC, too, but is hampered by limited sensitivity. We therefore explored whether methylcytosine staining of urine cytological specimens of patients with bladder cancer detects global hypomethylation and could improve diagnosis of urothelial carcinoma.

Materials and methods

Samples and controls

Urine specimens from 79 patients were collected at the Department of Urology, Heinrich-Heine-University, Duesseldorf between August 2003 and April 2004. On arrival at the laboratory, all samples were primarily processed for routine diagnostic purposes. They were centrifuged at 670×*g* for 5 min. The pellet was fixed with Saccomanno fixative (50% ethanol, 2% polyethylene glycol). A part of the cell pellet was used to prepare conventional smears stained according to Papanicolaou. Out of these urine samples, specimens from patients with histologically confirmed UC (21 males, 14 females; median age, 72 years, range 54–94 years), with a history of UC but without evidence for tumor recurrence (5 females; median age, 74 years; range, 56–90 years) or with benign disease of the urinary tract (6 males, 5 females; median age, 61 years; range, 34–78 years; 6 benign prostate hyperplasia, 3 urge incontinence, 2 urocystitis) were randomly selected. Information regarding clinical diagnosis and clinical staging according to the UICC TNM Classification of Malignant Tumours (Sobin and Wittekind, 1997) was obtained from patients chart review. In addition, urine samples from 14 healthy volunteers (12 males, 2 females; median age, 28 years; range, 23–43 years) were collected.

Cytospins

Saccomanno fixed cells were centrifuged onto polylysine coated glass slides at 1000 rpm for 10 min using a cytotunnel (Thermo Electron, Germany) with a diameter of 6 mm.

Treatment of cytospinned cells

Control and pathological slides were stained according to Papanicolaou to visually check cell morphology. The cells were refixed in cold absolute methanol–glacial acetic acid (3:1) for 15 min, dried and incubated at 50°C for 48 h. Slides were placed cell side up in a Petri dish, covered with a layer of PBS (phosphate-buffered saline: 20 g NaCl, 85 ml 0.25 M Na_2HPO_4, 15 ml 0.25 M KH_2PO_4 in 2.4 ml distilled water pH 7.4) and exposed to UV-light (254 nm) for 10 h. Immunostaining with a monoclonal antibody directed against 5-methylcytosine (Ab-1, Oncogene, USA) was performed according to vendor's instructions using the ABC immunostaining kit (Vector Laboratories, USA). The cells were counterstained with hematoxylin and the antigen-antibody complex was visualized with the substrate diaminobenzidine tetrahydrochloride.

Assessment of immunostaining

Two observers scored the nuclear immunostaining by grading its intensity on a scale of 0 (no staining) to 4 (intense staining). The staining intensity of each cell was evaluated and recorded separately. The resulting scores of individual cells were cumulated and divided by the number of cells investigated, to obtain a weighted average immunostaining score. To adjust for technical variations in staining intensity, a ratio of average immunostaining score of analysis cells and staining intensity of reference cells (neutrophils or squamous cells) was calculated. The final score reported is the average of the two observers. A low 5-methylcytosine immunostaining score (5-mc immunostaining score) thus indicates global hypomethylation of DNA. The observers were blinded as to the clinical diagnosis. The staining intensity of at least 100 urothelial (normal or tumor) cells was estimated. In hypocellular preparations, all urothelial and tumor cells present on a slide were evaluated. For the 5-mc assessment in the urothelial cancer cell lines T24 and HT1376, more than 300 cells each were analyzed. Since no normal reference cells were available in the tumor cell lines, their staining scores are not directly comparable to those of the urine samples.

Southern blot analysis of LINE-1 hypomethylation

Southern blot analysis of LINE-1 hypomethylation has been described previously in detail (Florl et al., 1999). In short, 1 μg DNA each was extensively digested with either the methylation-sensitive restriction enzyme *Hpa*II or its non-methylation-sensitive isoschizomer *Msp*I, separated on agarose gels, blotted and hybridized with a ^{32}P-labeled specific LINE-1 probe. After *Hpa*II digestion, decreased methylation of LINE-1 sequences results in the appearance of new bands in the 1.0 to 4.0 kb range on Southern blots whose intensities relative to the MspI signals, after correction for unequal loading of the two lanes, can be used as a quantitative measure of genome-wide hypomethylation.

Statistical analysis

Descriptive statistics such as median, range, mean, standard deviation and 95%-confidence interval were calculated for the 5-mc immunostaining score for each group of individuals and each cell type investigated. Comparison between two groups was performed by the Mann–Whitney *U*-test. Associations between variables were estimated by the Spearman's rank correlation. All statistical tests are 2-sided. The level of significance was set to $p<0.05$.

Results

Comparison of Southern blot analysis and 5-mc staining of T24 and HT1376

Southern blot analysis (Fig. 1) demonstrated almost complete methylation of LINE-1 sequences in normal tissues, but significant hypomethylation in T24 (23%) and even stronger hypomethylation in HT1376 (56%). Corresponding assessment of 5-mc immunostaining for T24 and HT1376 showed comparable differences of the methylation status with a higher 5-mc staining score of 2.89 for T24 compared to 2.14 for HT1376 (Fig. 2).

5-mc staining of urine samples

A 5-mc staining score was only determined in those samples in which a sufficient number of homogeneously stained reference cells was available. This was often not the case, especially in samples from healthy persons that had a low cell count overall. Therefore, out of 79 urine samples only 31 specimens were used for the qualitative analysis.

The reference cells (neutrophils or squamous cells) always showed a strong staining of the nucleus (Fig. 3). All cells