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Transitional cell carcinomas and nonurothelial carcinomas of the urinary bladder differ in the promoter methylation status of the caveolin-1, hDAB2IP and p53 genes, but not in the global methylation of Alu elements

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Abstract. Tumor suppressor genes play a prominent role in the modification and progression of urinary bladder carcinogenesis as a result of classic genetic alterations. Little is known about the potential significance of epigenetic events, mediated by DNA hypermethylation. This prompted our investigation to explore the global Alu methylation and the promoter methylation of the novel putative tumor suppressor genes caveolin-1 and hDAB2IP, and of p53 in transitional cell carcinomas (TCC), squamous cell carcinomas and undifferentiated small cell carcinomas of the urinary bladder. Quantitative GeneScan analysis revealed that the various histopathological tumor entities showed considerable interindividual variations in the global methylation, but the overall rate did not significantly differ between the various cancer subtypes. With methylation-specific PCR, a high frequency of methylation of the promoter region of the caveolin-1 gene was detected in undifferentiated small cell carcinomas (50%) and in squamous cell carcinomas (25.9%), while TCC were found not to be methylated. By immunohistochemistry, all squamous cell carcinomas showed a strong diffuse overexpression of caveolin-1, whereas undifferentiated small cell cancers lacked any expression. High-grade, high-stage TCC disclosed a higher incidence (60%) and a substantially stronger expression than low-grade, low-stage TCC (42.9%). Our findings suggest that hypermethylation of the caveolin-1 gene and an abnormal protein expression play a crucial role in cell differentiation, and in the phenotypical conversion of TCC into nonurothelial carcinomas. Promoter methylation of the hDAB2IP gene occurred more frequently in advanced muscle invasive (72.7%) than in superficial noninvasive (50%) TCC. DNA hypermethylation of p53 was detected in a quarter of the low-grade, low-stage TCC and undifferentiated small cell carcinomas, but only sporadically in squamous cell carcinomas, and was absent in high-grade, high-stage TCC. In conclusion, aberrant methylation and abnormal protein expression of the caveolin-1-gene is involved in the formation of nonurothelial carcinomas of the urinary bladder and promoter methylation of the hDAB2IP gene in the progression of TCC from a low to a high malignant potential.

Introduction

In Europe and North America approximately 93% of carcinomas occurring in the urinary bladder are transitional cell carcinomas (TCC), developing either by a multistep process from preneoplastic hyperplasias or, less frequently, from an *in situ* carcinoma of the urothelium. Their biological potential differs considerably, superficial low-grade, low-stage TCC showing a substantially better clinical outcome than muscle invasive high-grade, high-stage carcinomas. Rarely, nonurothelial carcinomas occur, such as squamous cell carcinomas, adenocarcinomas and undifferentiated small cell carcinomas which histogenetically originate either directly from the lining of transitional cell epithelium or, more commonly, secondarily from pre-existent TCC by a metaplastic process (squamous cell carcinomas and adenocarcinomas) or by a dedifferentiation (undifferentiated small cell carcinomas) of urothelial cells (1-4). Nonurothelial vesical carcinomas show an inherent aggressive clinical behaviour, a propensity for metastatic disease and a poor prognosis.

Although intensive efforts have been undertaken to unveil the molecular-genetic signaling pathways responsible for the differing clinical behaviour of bladder cancers and the conversion from a low- to a high-grade of malignancy, the underlying mechanisms are still not clear. However, there is strong evidence that functional reduction or inactivation of tumor suppressor (5-8) and DNA repair genes (9-14) are

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implicated in the modification, progression and metastasis of urinary bladder carcinogenesis. Repression of normal tumor suppressor gene function can be caused by classic genetic alterations (point mutations, deletions, amplifications) or may be alternatively mediated by an epigenetic mechanism independent of changes of the genome sequences. It has become increasingly apparent that methylation of normally unmethylated CpG islands in the promoter regions of tumor suppressor genes may result in transcriptional silencing with loss of gene function and potentially play a critical role in epigenetic regulation of tumorigenesis (15-19). For example, aberrant methylation of the tumor suppressor genes p14ARF, p15INK4b and p16INK4a, particulary involved in bladder carcinogenesis (5-8,20), was reported to be correlated with progression and a poorer clinical outcome of several cancer types (21-23) including carcinomas of the urinary bladder (24-28).

To obtain insights into the significance of the methylation machinery for the pathogenesis and biological behaviour of bladder cancers, we explored the role of global DNA methylation and the promoter methylation of the novel putative tumor suppressor genes caveolin-1 and hDAB2IP, and of p53 in low malignant superficial and advanced muscle invasive transitional cell carcinomas, and, for comperative purposes, in squamous cell and undifferentiated small cell carcinomas. To date, the functional importance of cytosine methylation at the promoter sites of the caveolin-1 and hDAB2IP genes for oncogenesis has been studied for the most part in tumor cell lines, yielding a growing body of evidence that methylation-mediated silencing at a transcriptional level abrogate the normal, potentially tumor suppressor function of both genes (15-19). Only a few investigations have aimed at revealing the clinicopathological relevance of DNA hypermethylation of the caveolin-1, hDBA2IP and p53 genes in human primary cancers, including prostate, breast, cervical and lung carcinomas (29-34).

This is the first study analysing the role of promoter methylation of the candidate tumor suppressor genes caveolin-1 and hDAB2IP in the development of carcinomas of the urinary bladder with special reference to the various histopathological cancer types. We detected a frequent DNA hypermethylation of caveolin-1 in squamous cell and undifferentiated small cell carcinomas, while TCC were not methylated. The rate of aberrant methylation of hDAB2IP proved to be high in the various cancer types and appeared to be associated with progression of TCC from a low to a high malignant potential.

Materials and methods

Specimens. Fourteen superficial low-grade (grade 1), low-stage (pTa) papillary and 11 muscle invasive nonpapillary (solid) high-grade (grade 3), high-stage (pT2b) transitional cell carcinomas (TCC) of the urinary bladder were analysed. Additionally, 27 muscle invasive squamous cell carcinomas (grades 1-3) and 8 muscle invasive undifferentiated small cell carcinomas were included in the study. The tumor samples were obtained from patients, who had undergone transurethral resection or radical cystectomy. The specimens were fixed in formalin and embedded in paraffin. For histopathological diagnosis, sections of 4 μm thickness were prepared and routinely stained with hematoxylin and eosin. The carcinomas were classified using the classification of the World Health Organisation (35) and staged according to the guidelines of the International Union against Cancer (36).

Immunohistochemistry. For immunohistochemical evaluation of the caveolin-1 protein, sections of 2 μm thickness were prepared from the formalin-fixed paraffin-embedded specimens, mounted on silane-coated slides, dewaxed in xylene, rehydrated in graded ethanol series and washed with distilled water. The sections were then incubated in citrate buffer (pH 6.0) at a temperature of approximately 90˚C over hot water vapor in a chamber for 45 min. Thereafter, the sections were cooled for 20 min, rinsed in distilled water and Tris-buffered saline (TBS pH 7.4; 0.05 M) containing Tween (0.03%). The primary antibody (caveolin-1 mouse IgG1 monoclonal; clone 2297; BD Biosciences, Heidelberg, Germany) was applied at a dilution 1:250 for 30 min at room temperature. After being washed with TBS, the sections were subjected to the biotinylated secondary antibody (Chem Mate™ detection kit alkaline phosphatase, code No. 5005; Dako Diagnostika, Hamburg, Germany) for 20 min. Following washing in TBS, the samples were treated with streptavidin alkaline phosphatase (Chem Mate detection kit, Code No. K5005; Dako Diagnostika) for 20 min. After rinsing in TBS, fast red was used as a chromogen (Chem Mate detection kit, Code No. K5005; Dako Diagnostika) to visualise the sites of immunoprecipitation. The sections were counterstained with Mayer's haemalum. Negative control staining was obtained by omission of the primary antibody. Immunohistochemical reactions were performed for all carcinomas except for a single case of a high-grade, high-stage TCC, and in normal, non-neoplastic urothelium (11 cases). Cytoplasmic immunoreactivity was semi-quantitatively stratified into the following categories: no detectable staining, focal weak to moderate deposits (between 10 and 50% of tumor cells), focal strong immunoprecipitations (between 10 and 50%), diffuse weak to moderate positivity, and diffuse strong immunoreactivity. Endothelial cells of blood vessels and muscle cells served as internal control and their staining intensity as a standard for comparison. hDAB2IP immunostaining could not be performed because an antibody suitable for paraffin-embedded tissue is not commercially available.

Molecular-genetic assays. Histological sections of 5 μm thickness were prepared from the paraffin-embedded specimens. Following identification of the tumor tissue in a section stained with hematoxylin and eosin, unstained tumor material in the consecutive sections was mechanically separated from nonneoplastic tissues and scraped from the slides.

DNA extraction and ß-globin PCR. DNA isolation was performed applying the QIAamp tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Integrity of the DNA was checked with ß-globin PCR. The ß-globin primer sequences yielding a 125 bp product were: sense 5'-CTT CTG ACA CAA CTG TGT TCA CT-3' and antisense 5'-TCA CCA CCA ACT TCA TCC ACG T-3'. For amplification, 2 μl of DNA solution were supplemented of 10x PCR-buffer, 1 μl of dNTP's (Roche Molecular icals, Mannheim, Germany) at a concentration of 10 mM each, 20 pM of each primer solutions, 1 unit of thermostable HotStar Taq DNA polymerase (Qiagen) and water to a final volume of 50 μl. Following an initial denaturing step at 95˚C for 15 min, DNA was amplified by 40 cycles. In each cycle a melting step at 95˚C for 30 sec was followed by an annealing step for 1 min at 60˚C, and an extension step for 1 min at 72˚C. The final PCR cycle consisted of an extension step for 7 min at 72˚C using a Primus-25 DNA thermal cycler (MWG, Ebersberg, Germany).

Quantification of global DNA methylation. Global DNA methylation of repetitive Alu elements was performed according to the method described by Yang and coworkers (37) with the following modifications. PCR reactions were carried out in a total volume of 50 μl using a Primus-25 DNA thermal cycler. Each reaction mixture contained 5 μl DNA, 1 unit HotStar Taq DNA polymerase (Qiagen), 2 μl 10x reaction buffer [Tris-HCl (pH 8.7), KCl, $(NH4)_2SO_4$, 15 mM $MgCl_2$], dNTPs (Roche Molecular Biochemicals) each at a concentration of 200 μM, 5 pmol sense and 5 pmol anti-sense primer. Thermocycling consisted of an initial 15-min step at 95˚C to denaturate the DNA and to activate the HotStar Taq DNA polymerase. The amplification conditions were 40 x at 96˚C for 90 sec, 43˚C for 60 sec, 72˚C for 120 sec. The reaction was completed with a final extension step at 72˚C for 7 min. Following amplification, agarose gel (3% agarose with 5 μl/40 ml ethidium bromide) electrophoresis revealed PCR products of the expected sizes (155 bp), indicating high specificity of the PCR. Primers used were: Alu sense 5'-GAT CTT TTT ATT AAA AAT ATA AAA ATT AGT-3' and anti-sense 5'-GAT CCC AAA CTA AAA TAC AAT AA-3'. The 5'-end of the sense primer was labelled with the fluorescent dye FAM. The PCR fragments were isolated from the PCR mixture using the QIAquick PCR purification kit as recommended by the manufacturer (Qiagen) and digested by 10 units of the restriction enzyme MboI (Fermentas, Lietua) at 37˚C according to the manufacturer's instructions. The enzyme cuts only methylated Alu repetitive elements. The DNA of each sample was analysed in two separate PCR runs and cleavage reactions, respectively. For fluorescence-based quantification of the DNA amount, 5 μl of each cleavage mixture were loaded onto the genetic DNA-analyser 310 (Applied Biosystems, Darmstadt, Germany). For internal size standard, 400 HD [ROX] (Applied Biosystems) was added to the solution and two parallel reactions were carried out for each sample. The sizes of the peak areas of the electropherograms were determined by GeneScan software. The peak area of the 155 bp uncut non-methylated Alu fragments was divided by the peak area of the 124 bp cleaved methylated Alu fragments obtained in both reactions and the mean value calculated, yielding the global methylation rate.

Methylation-specific PCR of the caveolin-1, hDAB2IP and p53 genes. DNA (1 μg tumor) was treated with sodium bisulphite using the CpGenome DNA modification kit (Intergen Company, Oxford, UK) according to the manufacturer's instructions. Modified DNA (2 μl, 1/5 volume) were used for PCR amplification. PCR reactions were performed at a total volume of 50 μl using the Primus-25 DNA thermal cycler. Each reaction mixture contained 5 μl DNA, 1 unit HotStar Taq DNA polymerase (Qiagen), 2 μl 10x reaction buffer [Tris-HCl (pH 8.7), KCI, $(NH4)_2SO_4$, 15 mM $MgCl_2$], dNTPs (Roche Molecular Biochemicals) each at a concentration of 200 μM, 5 pmol sense and 5 pmol anti-sense primer. The thermocycling conditions consisted of an initial 15-min step at 95˚C to denaturate the cDNA and to activate the HotStar Taq DNA polymerase. The amplification conditions for the various genes were as follows: methylated and non-methylated p53: 94˚C for 30 sec, 55˚C for 60 sec, 72˚C for 60 sec; methylated caveolin-1: 94˚C for 30 sec, 55˚C for 60 sec, 72˚C for 60 sec, non-methylated caveolin-1: 94˚C for 30 sec, 58˚C for 60 sec, 72˚C for 60 sec; methylated hDAB2IP: 94˚C for 20 sec, 61˚C for 30 sec, 72˚C for 30 sec, non-methylated hDAB2IP: 94˚C for 20 sec, 50˚C for 30 sec, 72˚C for 30 sec. Following amplification, agarose gel electrophoresis revealed PCR products of the expected sizes indicating high specificity of the PCR. The reaction was completed with a final extension step at 72˚C for 7 min. Caveolin-1 PCR primers were those used by Chan and coworkers (30): sense 5'-GGT ATT TTT GTA GGC GCG TC-3' and antisense 5'-CTA ACA ACA AAA AAC GAA AAA CG-3' for methylated sequences (product size, 174 bp), sense 5'-GTT TAT ATT GGG TAT TTT TGT AGG TGT GT-3' and antisense 5'-TCC CCA AAA TTC TAA CAA CAA AAA ACA AAA AAC-3' for non-methylated sequences (product size, 174 bp). hDAB2IP PCR primers were those applied by Dote and coworkers (33): sense 5'-GAG GTG AGC GGG GCG GTC-3' and anti-sense 5'-CGC TAT TAC CTT AAC GAC GCC GA-3' for methylated sequences (product size, 163 bp), sense 5'-GAG GTG AGT GGG GTG GTT-3' and anti-sense 5'-CAC TAT TAC CTT AAC AAC ACC AA-3' for non-methylated sequences (product size, 163 bp). The p53 primers were those described by Yeh and coworkers (38): sense 5'-ATT TAC GGT ATT AGG TCG GC-3' and anti-sense 5'-ACA CGC TCC CAA CCC GAA CG-3' for methylated sequences (product size, 310 bp), sense 5'-TTT AAA ATG TTA GTA TTT ATG GTA TTA GGT TGG T-3' and anti-sense 5'-CAT CAT AAA AAA CAC ACT CCC AAC CCA AAC A-3' for non-methylated sequences (product size, 310 bp).

Statistical analyses. For statistical analysis between the differences in the global methylation rates of the caveolin-1, hDAB2IP and p53 genes for the various histopathological types of carcinomas, the non-parametric two-sided Kruskal-Wallis test was used; a p-value <0.05 was considered to be significant. The Pearson chi-square test was used to statistically analyse the differences between the frequencies of promoter methylation of the 3 genes for the different cancer types; to correct for multiplicity, Bonferroni adjustment was applied (a p-value of 0.008 was taken to be statistically significant). The non-parametric two-sided Wilcoxon-Mann-Whitney test for paired group comparisons was employed to assess a potential correlation between the rate of global methylation and the frequency of promoter methylation separately for the various phenotypical tumor entities (a p-value of 0.05 was regarded to be statistically significant). The analyses were performed with Statistica version 7.0 (StatSoft Incorporation, Tulsa, OK, USA).

Results

Molecular-genetic findings

Global methylation. The entire collective of carcinomas analysed contained on average 9.4-times more non-methylated repetitive than methylated Alu sequences, indicating that 10.6% of the 1.5 million Alu sequences covering the genome of each tumor cell were methylated. The quantitative data of global methylation determined in the various histopathological cancer types are listed in Tables I-IV. There were no substantial differences between the various carcinomas, which all showed considerable interindividual variations of the methylation rate. The ratios ranged between 2.1 and 15.8 in low-grade, low-stage TCC; between 6.0 and 13.9 in high-grade, high-stage TCC; between 5.0 and 23.8 in squamous cell carcinomas; and between 5.4 and 12.3 in undifferentiated small cell cancers. The lowest mean and median values were observed in squamous cell carcinomas. An example of the results obtained in two squamous cell caracinomas applying GeneScan software is presented in Fig. 1.

Promoter methylation of the caveolin-1 gene. None of the superficial low-grade, low-stage and advanced high-grade, high-stage TCC revealed a methylation at the promoter site of the caveolin-1 gene (Tables I and II). Squamous cell carcinomas were hypermethylated in 25.9% and undifferentiated small cell carcinomas in 50% of the cases (Tables III and IV). A statistically significant relationship between the rate of global methylation and DNA hypermethylation did not exist, and the frequencies of promoter methylation between the two cancer types was not statistically different.

Promoter methylation of the hDAB2IP gene. The rate of methylation at the promoter region of the hDAB2IP gene was high in all carcinomas examined (Tables I-IV), the highest frequency being observed in advanced high-grade, high-stage TCC (72.7%) which, however, proved not to be significantly different from the rate of the other carcinoma types. The promoter methylation was statistically not correlated with the pattern of global methylation in any of the various histopathological cancer types.

Promoter methylation of the p53 gene. Promoter methylation of the p53 gene (Tables I-IV) was detected in a quarter of the low-grade, low-stages TCC (28.6%) and undifferentiated small cell carcinomas (25.0%), lacking a significant correlation with the global methylation rate. Squamous cell carcinomas showed a DNA hypermethylation in only single cases, while none of the high-grade, high-stage TCC were hypermethylated. A statistically significant interrelationship between the promoter methylation of the caveolin-1, hDAB2IP and p53 genes could not be observed.

Immunohistochemical findings. Positive immunostaining of caveolin-1 protein was evidenced by red-coloured granular deposits within the cytoplasm of the carcinoma cells. The intensity of the immunoprecipitations was assessed by comparison with those observed in the endothelium of blood vessels and of muscle cells which showed a strong positivity. The normal, nonneoplastic urothelium revealed no immunoreactivity or, rarely, a minimal immunoreactivity. The results obtained in the various histopathological cancer types are summarised in Table V. More than half of the low-grade, low-stage TCC (57.1%) lacked an expression of the caveolin-1 protein (Fig. 2A), 42.9% showed a focal or a diffuse weak to moderate expression (Fig. 2B). Of the high-grade, high-stage TCC, 40% did not express the protein, the others (60%) displayed a strong focal or diffuse immunoreactivity, similar to that of the endothelium (Fig. 2C). All squamous cell carcinomas exhibited a strong diffuse immunostaining. In less differentiated squamous cell carcinomas (grade 3) all cells throughout the tumor stained positively (Fig. 2D), in well-differentiated carcinomas (grade 1) mainly the peripherally located cells of the tumor complexes were positive, while the tumor cells in the center were immunonegative (Fig. 2E). Undifferentiated small cell carcinomas failed to express the caveolin-1 protein in all cases (Fig. 2F).

Table I. Global methylation (ratio peak areas of 155 bp non-methylated Alu fragments/124 bp-methylated Alu fragments) and promoter methylation of low-grade (grade 1) and low-stage (pTa) papillary transitional cell carcinomas of the urinary bladder (+ promoter methylation present, - promoter methylation absent).

		Promoter methylation		
Case	Global methylation	CAV-1	hDAB 2IP	p53
1	11.75	-	-	-
2	11.21	-	-	-
3	11.15	-	+	-
4	11.39	-	-	-
5	8.31	-	+	-
6	7.88	-	+	-
7	14.61	-	+	+
8	9.63	-	+	-
9	15.79	-	-	-
10	8.37	-	+	+
11	8.02	-	+	-
12	13.65	-	-	-
13	3.09	-	-	+
14	2.14	-	-	+
		Incidence		
	Mean value, 9.79±3.91	0/14	7/14	4/14
	Median value, 10.39	0%	50%	(28.6%)

Discussion

Emerging evidence indicates that methylation of cytosine in CpG dinucleotides at the 5' promoter region of tumor suppressor genes (15-19) and of the mismatch repair genes hM2H1 and hMSH2 (40-46) plays a crucial role during tumorigenesis based on an epigenetic mechanism. Although a wealth of data exists obtained from *in vitro* experiments using

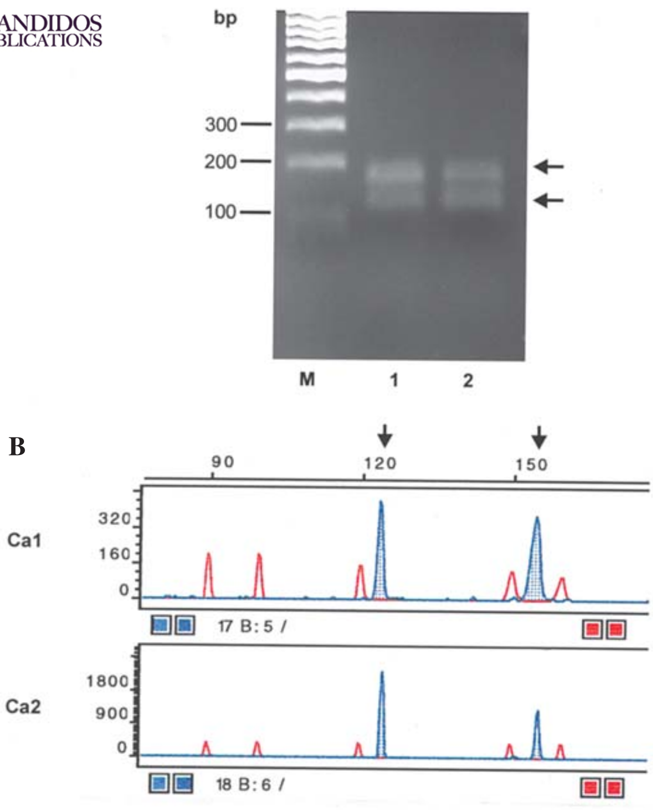

Figure 1. Fluorescence-based quantitative densitometric analysis of global Alu metylation performed on the Genetic DNA-analyser 310. (A) Bisulfite-treated and MboI-cleaved Alu PCR fragments amplified from two squamous cell carcinomas (lanes 1 and 2) were separated by electrophoresis on 3% agarose gel (lane M molecular weight standard). The upper band documents the non-cleaved (non-methylated) 155 bp, and the lower band the cleaved (methylated) 124 bp Alu fragments (arrows). (B) Quantities of the PCR fragments (determined by GeneScan software) of the two carcinomas (Ca 1 and Ca 2) shown in part A. Blue peak areas contain the 124 bp and 155 bp Alu fragments, red peak areas represent the internal size standard. Data demonstrate differing amounts of MBoI-cut and uncut Alu elements in both tumors, exemplifying the basic finding of considerable interindividual variations of global methylation of bladder cancers.

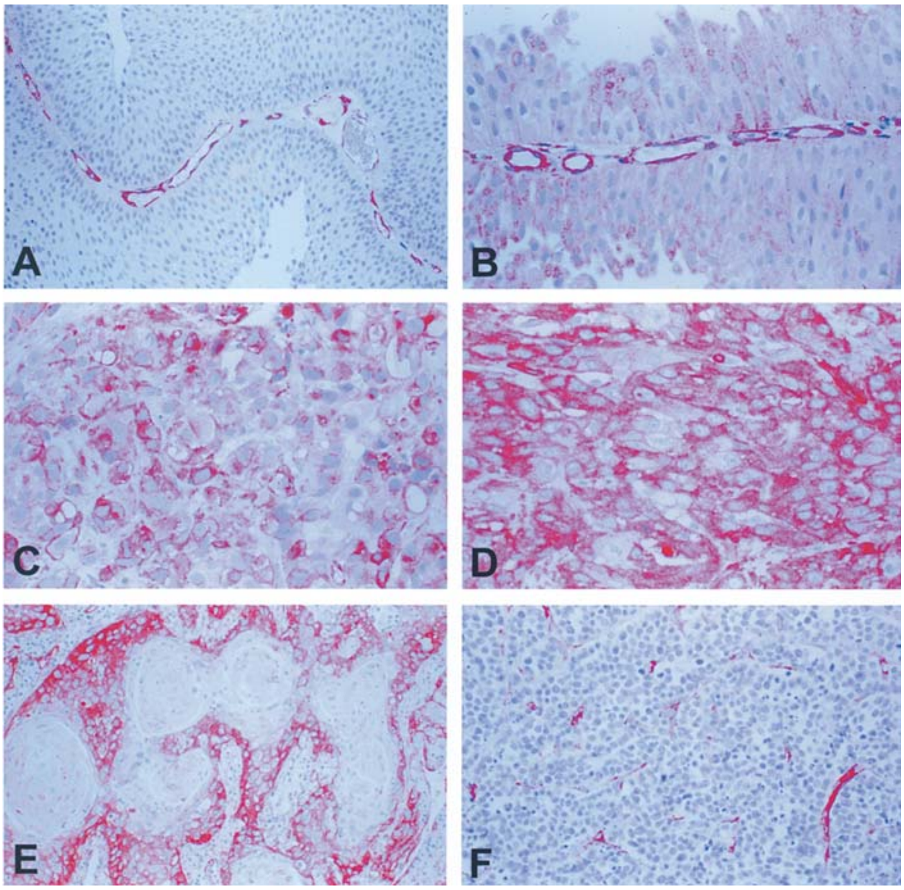

Figure 2. Immunohistochemical expression of caveolin-1 protein in various histopathological types of bladder carcinomas. (A) Low-grade, low-stage papillary transitional cell carcinoma with negative immunoreactivity; endothelial cells of the small blood vessels of the stromal stalk showing a strong positive red-coloured staining. (B) Papilla of a low-grade, low-stage papillary transitional cell carcinoma revealing a moderate positivity compared to the endothelium of the centrally located blood vessels. (C) High-grade, high-stage solid transitional cell carcinoma with a strong intracytoplasmic staining. (D) Less differentiated squamous cell carcinoma (grade 3) with a strong diffuse expression. (E) Well-differentiated squamous cell carcinoma (grade 1) displaying deposits only in the peripherilly located cells of the tumor complexes. (F) Undifferentiated small cell carcinoma lacking expression; interspersed blood vessles with strong immunoprecipitation.

Table II. Global methylation (ratio of peak areas of 155 bp non-methylated Alu fragments/124 bp-methylated Alu fragments) and promoter methylation of high-grade (grade 3) and high-stage (pT2b) non-papillary (solid) transitional cell carcinomas of the urinary bladder (+ promoter methylation present, - promoter methylation absent).

		Promoter methylation		
Case	Global methylation	CAV-1	hDAB 2IP	p53
1	13.86	-	+	-
2	13.66	-	-	-
3	10.44	-	+	-
4	6.68	-	+	-
5	9.19	-	+	-
6	7.11	-	+	-
7	6.03	-	+	-
8	5.96	-	+	-
9	14.59	-	+	-
10	10.12	-	-	-
11	9.63	-	-	-
		Incidence		
	Mean value, 9.75±3.18	0/11	8/11	0/11
	Median value, 9.63	(0%)	(72.7%)	(0%)

Table III. Global methylation (ratio of peak areas of 155 bp non-methylated Alu fragments/124 bp-methylated Alu fragments) and promoter methylation of squamous cell carcinomas of the urinary bladder (+ promoter methylation present, - promoter methylation absent).

		Promoter methylation		
Case	Global methylation	CAV-1	hDAB 2IP	p53
1	10.29	-	-	-
2	6.64	-	-	-
3	23.84	+	+	-
4	8.36	+	-	-
5	7.43	+	+	-
6	7.25	+	+	-
7	9.46	-	+	-
8	7.84	-	+	-
9	4.95	-	-	-
10	9.06	-	+	-
11	9.54	-	-	-
12	8.80	-	-	-
13	8.93	-	+	-
14	5.69	-	-	-
15	8.13	-	+	-
16	9.81	-	-	-
17	5.56	-	-	-
18	9.62	-	-	-
19	10.9	-	+	-
20	5.32	-	-	-
21	11.1	+	-	-
22	7.68	+	-	-
23	8.16	-	+	+
24	9.71	+	-	-
25	8.30	-	+	-
26	6.61	-	+	-
27	7.79	-	+	+
		Incidence		
	Mean value, 8.77±3.44	7/27	13/27	2/27
	Median value, 8.30	(25.9%)	(48.1%)	(7.4%)

different tumor cell lines, the functional significance and clinical relevance of aberrant DNA methylation for primary human cancers remains obscure. This prompted our investigation to analyse the methylation status of the caveolin-1, hDAB2IP and p53 genes in transitional cell carcinomas of various grades and stages, and, for comparison, in squamous cell carcinomas and undifferentiated small cell carcinomas of the urinary bladder. Based on the hypothesis that caveolin-1 and hDAB2IP might function as tumor suppressors, we originally expected that progression of TCC from a low to a high malignant potential would be associated with increasing rates of promoter methylation, and that high malignant squamous cell and undifferentiated small cell carcinomas would show a higher methylation frequency than TCC.

Methylation status and protein expression of the caveolin-1 gene. The caveolin-1 gene maps to chromosome 7q 31.1 and codes for a 21- to 24-kDa protein which is a major integral and structural component of caveolae, which are vesicular invaginations of the plasma membrane (47). Caveolin scaffolding proteins contain glycosphingolipid-rich microdomains, identified to mediate molecular transport, to regulate a variety of signaling pathways including Src family tyrosine kinases, epidermal growth factor receptor, transforming growth factor, protein kinase, H-Ras, c-erbB-2, nitric oxide synthase and cyclin D1 pathways, and to be implicated in cellular cholesterol homeostasis (47). Caveolae were found in many cell types particularly in adipocytes, endothelial cells, muscle cells and pneumocytes of type 1. It has become evident that the caveolin-1 gene is a target in oncogenesis and that promoter methylation causes transcriptional silencing, abrogating a possible tumor suppressor function of the gene by epigenetic control.

Analysing the methylation status of caveolin-1 in urinary bladder carcinomas, we were unable to detect a promoter methylation either in superficial low-grade, low-stage or in advanced muscle invasive high-grade, high-stage TCC. This finding indicates that DNA hypermethylation of the caveolin-1 gene does not play a role during development and progression of TCC from a low- to a high-grade of malignancy. In contrast,

. Global methylation (ratio peak areas of 155 bp ylated Alu fragments/124 bp-methylated Alu fragments) and promoter methylation of undifferentiated small cell carcinomas of the urinary bladder (+ promoter methylation present, - promoter methylation absent).

Case	Global methylation	Promoter methylation		
		CAV-1	hDAB 2IP	p53
1	9.26	-	-	+
2	9.40	+	-	-
3	5.44	+	+	-
4	12.3	+	+	-
5	7.20	+	-	+
6	9.14	-	-	-
7	11.45	-	+	-
8	10.85	-	+	-
		Incidence		
	Mean value, 9.38±2.25	4/8	4/8	2/8
	Median value, 9.33	(50%)	(50%)	(25%)

half of the undifferentiated small cell and a quarter of the squamous cell carcinomas were identified to be methylated at the promoter region. Formation of hypermethylated DNA exclusively in nonurothelial carcinomas, but not present in TCC, suggests methylation-mediated alteration of the caveolin-1 gene function to be associated with changes in cell differentiation and the phenotypic conversion of urothelial into nonurothelial carcinomas. Studies analysing the methylation status of the caveolin-1 gene in other cancers are scarce. An increased promoter methylation was demonstrated in prostate cancer compared to normal prostate epithelium (29). Similarly, a significantly higher frequency of methylated CpG island of caveolin-1 was recently determined in breast carcinomas (25.5%) than in normal breast parenchyma (7.3%), but an association with tumor grades and stages could not be observed (31). Cervical carcinomas showed a methylation in only 6% (30) and colorectal cancers in 3.8% of the cases (32) regardless of the tumor grades and lymph node metastases.

The immunohistochemical protein expression of caveolin-1 in carcinomas of the urinary bladder was observed to depend on the histopathological cancer types. All squamous cell carcinomas disclosed a strong diffuse immunoreactivity, but with a topographically differential pattern related to the grade of cellular differentiation. Less differentiated high-grade squamous cell carcinomas showed a positive immunostaining of all cells throughout the tumor, well-differentiated low-grade carcinomas mainly of the cells located in the periphery of the tumor complexes, whereas those in the centre were negative. TCC including all grades and stages displayed a positive immunoreactivity in half of the cases and in most of them the protein was expressed only focally. High-grade, high-stage TCC exhibited a higher incidence (60%) of a positive staining than low-grade, low-stage TCC (42.9%) and the precipitations proved to be considerably stronger in advanced compared to superficial carcinomas, indicating overexpression of caveolin-1 protein as a late event during urothelial carcinogenesis and to be correlated with tumor progression. Based on the fact that the normal transitional cell epithelium did not express the caveolin-1 protein, its appearance in TCC and squamous cell carcinomas is interpreted as a tumor-associated neo-expression. It is speculated that the protein produced is abnormal and potentially represents an oncoprotein, rather than functioning as a tumor suppressor. Since the promoter region of the caveolin-1 gene was not found to be methylated in TCC and in only a quarter of the squamous cell carcinomas, other

Table V. Immunohistochemical expression of caveolin-1 protein in transitional cell carcinomas (TCC) and nonurothelial carcinomas of the urinary bladder.

Type of carcinoma	Total No. of cases	Intensity of immunoreactivity					Incidence of positive cases (%)
		No staining	Focal weak to moderate	Focal strong	Diffuse weak to moderate	Diffuse strong	
Low-grade, low-stage TCC	14	8	5	-	1	-	42.9
High-grade, high-stage TCC	10	4	-	4	-	2	60.0
Squamous cell Carcinomas	27	-	-	-	-	27	100
Undifferentiated small cell carcinomas	8	8	-	-	-	-	0

causes than DNA hypermethylation may be responsible for the neo-formation of the protein, as for example genetic alterations. On the other hand, nearly all undifferentiated small cell carcinomas showed a promoter methylation in association with a lack of protein expression. Our combined molecular-genetic and immunohistochemical studies point to a differential functional role of the caveolin-1 gene in bladder carcinogenesis, particularly involved in the histogenetic formation of nonurothelial carcinomas. The only other immunohistochemical study concerned with caveolin-1 expression in bladder cancer available to date reported an increasing immunoreactivity with increasing tumor grades, TCC grade 3 being positive in 21% and grade 2 carcinomas in 3% of the cases, whereas well-differentiated TCC grade 1 were negative (48).

An overexpression of the caveolin-1 protein was also described in various other primary human cancers, such as in pancreatic ductal adenocarcinomas (49), renal cell carcinomas (50), prostate cancer (51), lung carcinomas (52,53), adenocarcinomas of the colon (54), esophageal squamous cell carcinomas (55) and papillary carcinomas of the thyroid (56), the higher expression levels in most of the cancers correlated with higher pathological stages, lymph node metastasis and a poorer prognosis, suggesting elevated caveolin-1 expression as a functional predictor and prognostic marker of survival time and metastatic disease. The role of caveolin-1 in cell differentiation and malignant transformation as documented in bladder carcinogenesis is supported by results obtained in certain other human primary cancers. Thus, undifferentiated (anaplastic) and follicular carcinomas and adenomas of the thyroid failed to express the caveolin-1 protein as opposed to papillary cancers which showed a positive immunoreactivity (56). Ovarian serous adenomas revealed a positive immunostaining, while mucinous adenomas as well as mucinous carcinomas were negative, and serous and endometrioid carcinomas exhibited substantially reduced expression levels, compared to normal ovarian surface epithelium (57). In colon carcinogenesis, most adenomas were negative for caveolin-1, but positive in adenocarcinomas compared to normal colonic epithelium.

There is a growing body of evidence that DNA hypermethylation of the caveolin-1 gene and an altered protein expression of the caveolin-1 gene are implicated in tumorigenesis. Data available do not allow us to definitely draw conclusions as to the functional role of the caveolin-1 gene during oncogenesis. The differential expression dependent on the organ involved and related to the morphological phenotype appears compatible with a role in cell differentiation and transformation, and possibly reflects modulation of differential signaling pathways by activation or inactivation of different domains of caveolin-1. Overexpression of the caveolin-1 protein in most cancers associated with a more aggressive clinical behaviour, an increased incidence of lymph node metastasis and an unfavourable prognosis lends support to the hypothesis that the protein produced may be abnormal and potentially implies an oncogenic activity.

Methylation status of the hDAB2IP and p53 genes. The novel candidate tumor suppressor gene hDAB2IP is located on chromosome 9q33.1-q33.3 and has been found to express a GTPase-activating protein that is involved in the modulation of Ras-mediated signal cascades. hDAB2IP forms a protein complex with DOC-2/DAB2 by interacting with the N-terminal domain of disabled-2. DOC-2/DAB2 contains a phosphotyrosine domain and multiple C-terminal proline-rich SH3-binding domains which interact with the Grb2 adapter protein (58-63). It is assumed that the hDAB2IP gene negatively regulates cell growth by down-regulation of Ras-controlled signal pathways. The potential role of hDAB2IP as a tumor suppressor is attributed to aberrant methylation of the CpG islands leading to transcriptional silencing by epigenetic regulation (64).

Our findings document a high frequency of promoter methylation of the hDAB2IP gene in bladder carcinomas ranging between 48.1 and 72.7%. The higher frequency of DNA hypermethylation in advanced muscle invasive high-grade, high-stage (72.7%) compared to superficial low-grade, low-stage (50%) TCC indicate a correlation of aberrant methylation of hDAB2IP with progression of TCC from a low to a high malignant potential. The relationship between the methylation status and the degree of malignancy existed only for TCC, while high malignant squamous cell and undifferentiated small cell carcinomas disclosed a rate of promoter methylation similar to that of low-grade, low-stage TCC. The significance of DNA hypermethylation of the hDAB2IP gene for tumor progression is also evident from results obtained in other types of cancers. Dote and coworkers (33) recently reported aberrant promoter methylation of hDAB2IP at the m2a region in 38% and in 33% at the m2b region of primary breast carcinomas, the methylation status being linked to an increased incidence of lymph node metastases. Primary lung cancers showed an aberrant methylation in 37% (m2a region) and 36% (m2b region) of the cases in association with an advanced disease stage (34). Down-regulation of the mRNA of disabled-2 in human prostate cancer cell lines referred to normal prostatic epithelia (64-67), a decreased protein expression or even a loss in ovarian carcinomas (68) and in choriocarcinomas (69) suggest methylation-mediated transcriptional silencing of the hDAB2IP gene and support the view that the gene functions as a tumor suppressor.

Regarding the importance of the tumor suppressor gene p53 for bladder carcinogenesis a wealth of data has been accumulated, documenting the prominent role of mutational alterations and protein overexpression for progression of TCC (70-73). The promoter methylation status of p53 has been explored in only a very few studies, as for example in oral cancer, yielding a low frequency of hypermethylation in 4.2% of the cases (38). We were able to detect a DNA hypermethylation in one-fourth of low-grade, low-stage TCC and undifferentiated small cell carcinomas, indicating that, in addition to genetic alterations, promoter methylation of p53 may be involved in the pathogenesis of these cancer types by epigenetic control, whereas p53 hypermethylation evidently does not play a role during formation of high-grade, high-stage TCC and squamous cell carcinomas.

Global methylation. Global hypomethylation of DNA reflecting a decreased methylation of CpG islands dispersed throughout the human genome is suspected to participate in cancer and progression (74,75). LINE-1 repetitive sequences ic DNA of several cancers including carcinomas of the urinary bladder revealed a higher level of hypomethylation than their normal tissue counterparts (76). Accordingly, urothelial carcinomas were recently reported to exhibit a high percentage of DNA hypomethylation in pericentrometric satellite regions 2 and 3 (41 and 44%, respectively), but rarely in non-cancerous tissue (7 and 0%, respectively), associated with and possibly inducing loss of heterozygosity on chromosome 9 (77). The DNA hypomethylation significantly correlated with the grade and stages of the TCC.

This is the first study to determine the degree of global DNA methylation in TCC and nonurothelial carcinomas of the urinary bladder applying quantitative GeneScan analysis of MboI-cut and uncut repetitive Alu sequences. This novel technical approach allows us to estimate global methylation with high accuracy and sensitivity. We observed marked inter-individual variations of the global methylation levels of the caveolin-1, hDAB2IP and p53 genes in the various histopathological tumor entities, but the overall rate of Alu methylation did not significantly differ between the different phenotypical cancer subtypes and proved not to be related to the grades and stages of the TCC. These data clearly indicate epigenetic stability of certain types of repetitive sequences during bladder carcinogenesis. Similarly, global and gene-specific epigenetic patterns were recognised, upon analysis of the p15, p16 and PAX6 genes, to be relatively stable over time in recurrent TCC and in cell lines derived thereof (78). The absence of an association between global Alu methylation and promoter hypermethylation in our study points to an independent role of DNA undermethylation in the development of bladder cancer, supporting the view of others (74). In summary, DNA undermethylation may be less important for the development of bladder cancer than has been suggested (75).

In conclusion, this is the first study to document a high frequency of promoter methylation of the caveolin-1 gene in undifferentiated small cell and squamous cell carcinomas of the urinary bladder, whereas TCC lacked DNA hypermethylation. Our findings provide evidence that aberrant methylation and an abnormal protein expression of caveolin-1 is involved in the metaplastic conversion and dedifferentiation, respectively, of transitional cell carcinomas into nonurothelial cancers. The rate of DNA hypermethylation of the hDAB2IP gene proved to be high in bladder cancers including all histopathological subtypes, but with a peak incidence in high-grade, high-stage TCC, suggesting promoter methylation to be implicated in the progression of TCC from a low to a high malignant potential. DNA hypermethylation of the p53 gene may play a role in the pathogenesis of low-grade, low-stage TCC and undifferentiated small cell carcinomas.

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