We investigated the use of genotype-interpreted measurements of the tumor marker Ca 19-9 in the urine of bladder cancer patients as a marker of the extent of urothelial disease. Ca 19-9 in urine (sialyl-Lea/creatinine ratio) was measured in 81 bladder cancer patients and correlated to T-category, histologic grade, and presence of urothelial dysplasia. As reference group, Ca 19-9 ratio was measured in urine from 21 apparently healthy individuals. The amount of sialyl-Lea expressed is influenced by the Lewis genotype and secretor status. Accordingly, secretor status was determined in urine by a novel ELISA method, and the Lewis genotypes of all of the individuals were determined by PCR cleavage methods. Ca 19-9 concentrations in urine were higher \( (P < 0.01) \) in bladder cancer patients than in healthy individuals and significantly \( (P = 0.02) \) higher in cancer patients with concomitant urothelial dysplasia than in those with normal urothelium. For individuals Lewis-genotyped as homozygous wild-type, Ca 19-9 concentrations in urine were higher, both in cancer patients \( (P = 0.06) \) and in healthy individuals \( (P = 0.004) \), than in the heterozygous individuals. Furthermore, nonsecretor cancer patients had higher \( (P < 0.01) \) Ca 19-9 concentrations in urine. Attention is drawn to the possibility of a general genotype interpretation of a result in clinical chemistry.

The disease course of treated bladder carcinoma patients is characterized by multiple recurrences in approximately one-half of the patients, and among these 10–15% will progress to invasive cancer disease [1]. Much work has been done to find methods to detect bladder cancer at an early stage and identify variables that can predict the disease course in individual patients. Urothelial dysplasia concomitant with a bladder tumor is an important prognostic factor for future new occurrences [2]. The workload of diagnostic and follow-up cystoscopies and subsequent analysis of tumor biopsies and selected site biopsies is a considerable burden to departments of urology and pathology. Consequently, there is a demand for reliable noninvasive methods for diagnosis, disease course prediction, and follow-up of bladder cancer patients.

The definition of cutoff values for biological markers above which disease or relapse after treatment is to be suspected is difficult. This is partly because of an interindividual variation, related to obvious causes like sex, age, hormonal status, and others but also to individual differences in genetic makeup. Very few studies have investigated the possible effect of different genotypes on the concentration of biomarkers encoded for by genes that have a high frequency in the population for heterozygosity for nonfunctional alleles. Previously, the concentration of Ca 19-9 has been shown to be increased in bladder cancer patients [3, 4]. The intention of this study has been to evaluate the influence of Lewis allelotype and secretor phenotype on the concentration of Ca 19-9 in urine from healthy individuals and bladder cancer patients.

The tumor marker Ca 19-9 recognizes the carbohydrate structure sialyl-Lewis a (sialyl-Lea) [5], which is synthesized by a sialyltransferase and the Lewis transferase (Fig. 1). The latter is encoded by the Lewis gene \( (FUT3) \) and is an \( \alpha(1,3/1,4)-l\)-fucosyltransferase responsible for the synthesis of both Le\(^a\) and Le\(^b\). Because of the competition for enzyme acceptors, the concentrations of Ca 19-9 in serum is low among secretors (who make Le\(^b\)) and high among nonsecretors [6] (Fig. 1).

Several missense mutations have been found in the Lewis gene, and when these mutations present in the homozygous form, they can cause inactivation of the enzyme [7–11]. Furthermore, a gene-dosage-like effect has been shown to exist for healthy individuals heterozygously mutated in the Lewis gene, leading to a lower concentration of the tumor marker Ca 19-9 in serum compared with homozygous wild-type individuals [12].
Consequently, we have proposed that cutoff values for Ca 19-9 measurements be redefined on the basis of secretor and Lewis genotyping [12].

The aim of this study was to compare the urinary concentrations of genotype-interpreted carbohydrate tumor marker Ca 19-9 in bladder cancer patients and healthy individuals and, subsequently, to compare the Ca 19-9 concentrations in the cancer patients with T-category, histologic grade, and presence of concomitant urothelial atypia. To investigate the possibility of using Ca 19-9 measurements in urine as a biomarker for bladder cancer, we undertook this study.

Materials and Methods

Patient Material

Urine for Ca 19-9 and creatinine measurements and peripheral blood leukocytes for DNA extraction were obtained from 86 consecutive patients with histologically confirmed transitional cell carcinoma of the bladder. Five patients were excluded from the study because of treatment with radiotherapy. There were 62 men with a mean age of 70 (range, 45–89) years, and 19 women with a mean age of 67 (42–83) years. No difference was observed between men and women (data not shown).

At the time of primary transurethral treatment and (or) after recurrences, biopsy specimens were taken from apparently normal urothelium at preselected sites as described previously [13]. All urine samples were screened for leukocytes and nitrite, and positive samples were excluded from the investigation.

T-classification according to UICC [14] was based on biopsy specimens obtained at transurethral resection and findings at bimanual palpation under general anesthesia. The tumors were graded according to the cellular criteria of Bergquist et al. [15]. Urothelial dysplasia of selected site biopsies were graded similarly.

 Apparently healthy volunteers (8 men and 13 women) with no known bladder diseases were included as a reference group. All 21 were blood-typed as Lewis-positiv e secretors (Leb) by routine blood bank procedures. Nine (43%) were homozygous wild type, and 12 (57%) were heterozygous mutated in the Lewis gene.

All procedures were approved by the local scientific ethical committee.

Lewis Genotyping

DNA was extracted from leukocytes by using Puregene DNA Isolation kit (Gentra Systems, Inc., Los Angeles, CA) according to the manufacturer’s protocol. Lewis genotyping for the mutations T59G and C314T was done by using PCR-cleavage assays as described previously [12]. For the performance of PCR-cleavage reactions for the mutations T59G and C314T, the sequences of the sense and antisense oligonucleotide primers, the annealing temperature of the PCR reactions, the sizes of the products obtained, and the restriction enzymes used for the cleavage of the PCR products, respectively, were as follows: T59G, VE1 mms/EL3as [7] (5’-ccatgccccgccgtgtggccgccc-3’/5’-gggagtggt-gtcctgcgggaggacccact-3’), 65 °C, 140, MspI; and C314T, EL3 s [7]/VE4as (5’-agttggctcctcagagacaccc acctc-3’/5’-ggtgcagctgatccctcagggtgcacagcgt-3’), 63 °C, 204, NlaIII. PCR reactions were performed with 25 pmol of each primer, 1.25 mmol/L deoxynucleotide triphosphates, and 0.5 unit of Taq polymerase in 25 μL of buffer (50 mmol/L KCl, 10 mmol/L Tris base, pH 8.3, 2 mmol/L MgCl2). Negative PCR controls (amplification without template DNA) were included in all experiments. For T59G, the expected fragments sizes after cleavage with MspI of the mutated allele were 24 and 116 bp. For C314T, the expected fragment sizes for the mutated allele were 105, 34, and 65 bp. The digested products were separated by 4% and 3% agarose gel electrophoresis, respectively (Metaphor® agarose, FMC; Fig. 2).

Secretor Typing of Urine

All incubations were at room temperature. Urine samples were centrifuged, and the supernatants were used for the assay. MaxiSorp plates (Nunc) were coated overnight with capture lectin wheat germ agglutinin (Sigma Chem-
ical Co.) 5 mg/L in coating buffer. The coated wells were emptied, washed with washing buffer (phosphate-buffered saline, pH 7.2, plus 1 mL/L Tween) three times, and then blocked for 30 min with 100 μL of coating buffer containing bovine serum albumin, 20 g/L. After blocking and a wash, urine samples were diluted in washing buffer to a creatinine content of 1.5 mmol/L and added to the wells. After incubation for 1 h, the wells were washed with washing buffer and incubated for 1 h with the detecting antibodies anti-Lea (BioClone; Ortho Diagnostic Systems) and anti-Leb (Seraclone; Biotest AG). After washing, peroxidase-conjugated rabbit anti-human IgM (DAKO), diluted 2000-fold in washing buffer, was added, and the plates were incubated for 1 h. The plates were developed with o-phenylenediamine (DAKO), 7 g/L in 0.1 mol/L sodium citrate, pH 5.0, containing 0.3 mL/L H2O2. The reaction was stopped after 2–3 min by the addition of 1 mol/L sulfuric acid. Absorbance at each well was read at 492 nm, and the results were calculated after subtraction of the blank values obtained in the absence of the detecting antibodies anti-Lea and anti-Leb. All assays were performed in duplicate. Positive controls for the detecting antibodies consisted of urine obtained from apparently healthy adults, typed as secretors and nonsecretors, respectively, on fresh blood samples, by standard agglutination methods performed in a specialized blood grouping laboratory at Aarhus University Hospital (Skejby, Denmark).

The performance of the assay was tested by typing urine from 31 healthy subjects (24 secretors and 7 nonsecretors) and 33 bladder cancer patients (25 secretors and 8 nonsecretors) and comparing the results with convenient secretor typing on erythrocytes. No overlap between secretors and nonsecretors was observed in healthy individuals and in bladder cancer patients (Table 1). Intraassay variation (CV) for Lea and Leb ELISA readings was determined on duplicate analyses on a series of clinical specimens as 12.9% (0.0–35.4%) and 2.2% (0.1–4.2%) for secretor-positive healthy subjects (n = 10), 3.8% (0.4–6.4%) and 8.1% (0.0–20.2%) for nonsecretor healthy subjects (n = 6), 12.5% (1.2–21.1%) and 1.3% (0.0–4.5%) for secretor-positive bladder cancer patients (n = 10), and 2.1% (0.6–8.0%) and 10.8% (1.8–35.5%) for nonsecretor bladder cancer patients (n = 10). All subjects were Lewis positive.

### Table 1. Lea\textsuperscript{a}/Leb\textsuperscript{b} ratios in healthy subjects and bladder cancer patients, blood grouped by secretor status.

<table>
<thead>
<tr>
<th>Blood group\textsuperscript{*}</th>
<th>Individuals</th>
<th>N</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secretors: Healthy subjects</td>
<td>24</td>
<td>0.014 (0.002–0.303)</td>
<td></td>
</tr>
<tr>
<td>Le(a–b+)</td>
<td>25</td>
<td>0.163 (0.047–0.427)</td>
<td></td>
</tr>
<tr>
<td>Nonsecretors: Healthy subjects</td>
<td>7</td>
<td>19.7 (3.1–72.7)</td>
<td></td>
</tr>
<tr>
<td>Le(a+b–)</td>
<td>8</td>
<td>13.8 (5.5–22.2)</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{*} On the basis of erythrocyte phenotyping.

**Determination of CA 19-9 and Creatinine**

Urine samples were centrifuged, and the supernatants were assayed for concentrations of tumor-associated antigen Ca 19-9 by using a solid-phase, two-site chemiluminescence enzyme immunometric assay for use with the Immulite Automated Analyzer (Diagnostic Products Corp.) according to the manufacturer’s instructions. Intraassay variation (CV) was determined on duplicate analyses on a series of clinical specimens as 5.9% in the range of <20 kU/L (n = 16), 4.8% in the range of 20–200 kU/L (n = 12), and 6.7% in the range of >200 kU/L (n = 8). Interassay reproducibility for urine was tested by two successive assays (double determinations) of a series of concentrations (nine high, >200 kU/L, and nine low, <20 kU/L) during 1 week. CVs were 4.7% (range, 0.5–10.6%) and 5.0% (range, 0.0–10.1%) for high and low concentrations, respectively. Intraassay and interassay CVs for urine samples were comparable with those reported for serum pools. Urinary content of creatinine was determined by using a routine alkaline picrate method. To allow for different concentrations of the urinary samples, Ca 19-9 concentrations were expressed as Ca 19-9/creatinine ratios. Ca 19-9 was expressed as kU/L and creatinine as mmol/L.

**Statistical Analysis**

Distributions were compared by using the Mann–Whitney U-test. Unless otherwise stated, P < 0.05 was considered statistically significant.

**Results**

Eighty-one patients with histologically confirmed transitional cell carcinoma of the bladder were included in this investigation. Seven of them had invasive tumors (T1–T4), and 74 had noninfiltrating tumors (T1).

Secretor typing of the urine by a novel lectino-immunoassay assay showed that 60 patients (79%) were secretors, whereas 16 (21%) were nonsecretors (Fig. 3). Secretor typing on urine could not be performed in the five Lewis-negative patients.

As a result of the Lewis genotyping for the two mutations that are representative of the mutated Lewis alleles present in the Danish population, bladder cancer patients and reference individuals could be classified as either homozygous wild-type, heterozygous mutated, or homozygous mutated in the Lewis gene [12]. Five of 81 patients (6%) were genotyped as homozygous mutated, i.e., Lewis negative. Fifty-one (63%) were homozygous wild-type, and 25 (31%) were heterozygous mutated in the Lewis gene. The distribution of genotypes agrees with the Hardy–Weinberg theorem on the assumption that the frequency of genuine Lewis negatives is ~7%. The five Lewis-negative individuals had no detectable Ca 19-9 in urine and were excluded from data processing.

As shown in Fig. 4, Ca 19-9 concentrations were significantly higher (P = 0.004) in reference subjects who were homozygous wild-type in the Lewis gene compared
with those who were heterozygous mutated in the Lewis gene (Mann–Whitney U-test). All reference subjects were secretors.

Urine samples from secretor-positive bladder cancer patients showed a significantly \( (P < 0.01) \) higher concentration of Ca 19-9 than did the secretor-positive healthy individuals (Fig. 5). On the basis of a comparison of Ca 19-9 in nonsecretor and secretor patients, ignoring non-significant differences in tumor stage and grade between the two groups, an examination of Ca 19-9 ratios showed a considerably higher concentration \( (P < 0.01) \) in urine from nonsecretors (Fig. 5).

Secretor-positive cancer patients were then grouped according to the Lewis genotypes, heterozygous mutated and homozygous wild-type. Statistical analyses indicated a higher Ca 19-9 ratio in homozygous wild-type patients than in heterozygous patients \( (P = 0.06; \text{Fig. 6}) \).

From all patients, selected site biopsies were taken from eight predefined sites in the bladder when the bladder tumor was removed. However, to compare Ca
19-9 concentrations in urine from patients with and without concomitant dysplasia, we included only the 52 patients who were both Lewis positive and secretors, and who had their urine samples taken the same day they underwent surgery. From the pathology examination of the eight bladder biopsies, we judged that 30 patients (58%) had concomitant urothelial dysplasia, 7 of whom (23%) had carcinoma in situ, and 22 patients (42%) had normal urothelium. Secretor-positive bladder cancer patients with concomitant epithelial dysplasia showed significantly ($P < 0.02$) higher Ca 19-9 in urine than did cancer patients with normal epithelium (Fig. 7). When the patients were divided the second time into four groups according to Lewis genotype, a pattern of increasing Ca 19-9 concentrations appeared (Fig. 8).

Ca 19-9 ratios were not correlated to tumor grade. The patients with invasive tumors were too few in number for an evaluation of the correlation between Ca 19-9 concentrations and tumor stage.

We conclude that genotype evaluation of Ca 19-9 measurements in urine is a prerequisite for the interpretation of the concentration of this tumor marker. Furthermore, an increased concentration of Ca 19-9 ratio in urine seems to reflect both the presence of concomitant urothelial dysplasia and the presence of a tumor. On this basis, Ca 19-9 ratio in urine may serve as a biomarker and help identify the patients with field defect of the bladder, i.e., patients who need to be followed closely.

**Discussion**

We have analyzed the creatinine-adjusted Ca 19-9 concentrations in the urine from bladder cancer patients and healthy individuals. Bladder cancer patients and reference individuals were grouped according to secretor type and Lewis genotype by means of an ELISA method on urine and PCR cleavage assays, respectively. Cancer patients, furthermore, were classified according to T-category, histologic grade, and presence or absence of concomitant epithelial dysplasia.

We have shown that Ca 19-9 ratio in urine is increased in patients with bladder cancer, and that the highest concentrations are found in urine from patients whose selected site biopsies from apparently normal epithelium showed concomitant urothelial dysplasia. The Ca 19-9 concentrations in urine from healthy individuals as well as from bladder cancer patients depend on the individual Lewis genotype and secretor type and can be interpreted correctly only when the types are known.

The amount of Ca 19-9 in urine seems to reflect not only the presence of tumor(s) but also the existence of urothelial dysplasia or carcinoma in situ in biopsies from the bladder mucosa. We do not know the origin of urinary Ca 19-9 at present. Although it is found in ducts of the prostate gland and in seminal fluid [16, 17], it more probably originates from the urothelium or the kidney.

In the human bladder, marked tumor-associated
changes in blood group antigen expression have been demonstrated [18–20]. ABH and Le^a^ antigens are expressed in normal urothelium but are often replaced by Le^b^ and Ley in high-grade invasive tumors [21, 22]. Thus, one would not expect a high amount of sialyl-Le^a^ from these tumors, which agrees with our present data. In normal urothelium, sialyl-Le^a^ is present only in the luminal umbrella cell layer. However, in carcinoma in situ and in papillomas, the sialyl-Le^a^ structure is expressed in all cell layers of the urothelium [21]. This increased expression in carcinoma in situ alterations could explain the increased Ca 19-9 in urine from patients who, in addition to the tumor, have concomitant dysplasia or carcinoma in situ of a fraction of the urothelial mucosa. At present, these cases of field disease are diagnosed on biopsies taken at eight preselected sites in the bladder mucosa. Often, there is more than one dysplastic biopsy, indicating that a relatively large area of the mucosa is diseased. It is, however, difficult to get a quantitative impression of the extent of the dysplastic field on the basis of these eight biopsies. The genotype-interpreted Ca 19-9 measurement might be a marker that could supply such quantitative or semiquantitative information on the extent of the field change and its progression or regression during the urothelial disease. The presence of some overlap between the two groups, however, requires that additional studies are performed to determine the clinical applicability of the tumor marker. Other markers are presently approved by the Food and Drug Administration for bladder cancer testing. Future studies may show whether one marker is sufficient or whether combinations of markers will have better clinical performance.

Thus far, the carbohydrate tumor marker Ca 19-9 (or GI-MA) has been used mainly for serological diagnosis and follow-up of gastrointestinal and pancreatic malignancies [23]. A few studies have also investigated the use of this tumor marker in the serum and urine of patients with bladder cancer [3, 4, 24]. These data need to be reinterpreted in light of the secretor and Lewis genotypes of these patients. Very high serum or urine concentrations are, of course, abnormal, and in a follow-up situation the patient serves as his own reference. However, declining values of Ca 19-9 may present a problem when the concentration close to the normal reference interval is reached. The cutoff amount defining pathologically increased values is quite different among secretors and nonsecretors and for the groups internally among Lewis heterozygous and Lewis homozygous individuals. The recent cloning of the secretor gene (FUT2) and the identification of enzyme-inactivating mutations and gene fusion [25–30] may even make it possible to classify the secretors as homozygous wild-type and heterozygous at the FUT2 locus, once the geographical distribution of inactivating mutations has been documented. Finally, the sialyltransferase adding the sialic acid to the carbohydrate chain could also be involved in a gene-dosage dependent effect. This is, however, not very likely, as several sialyltransferases are present and may have overlapping functions.

The antigenic determinant for Ca 19-9, sialyl-Le^a^, serves as a ligand for the endothelial leukocyte homing receptor E-selectin [31, 32], and it is suggested that E-selectin-mediated binding of tumor cells to the endothelium is involved in the hematogenous metastasis of tumor cells [33]. Whether Ca 19-9 in urine indicates tumor cells with a high density of sialyl-Le^a^ on the cell membrane is unknown. However, if it is true, these tumor cells might have an ideal surface for binding to activated endothelial cells, and on the basis of this mechanism, might be prone to make hematogenous metastasis.

The synthesis of Ca 19-9 is complex because there are three genes involved: the secretor gene, the gene encoding the sialyltransferase, and the Lewis gene. The gene products are glycosyltransferases, which in turn will add monosaccharides to the growing chain of carbohydrates on glycoproteins or glycolipids. We have shown previously that the Lewis glycosylation of circulating glycoproteins is gene-dosage dependent because individuals with two intact alleles synthesize markedly more than individuals with one allele. In this report, we extend this observation to Lewis glycosylation of glycoproteins in urine. We state the hypothesis that the same difference between
homozygous and homozygous wild-type individuals would be expected, when Ca 19-9 concentrations are increased because of cancer disease. In favor of this hypothesis is the recent finding that the accumulation of Ca 19-9 in colorectal tumors is mainly caused by the activation of α-2,3-sialyltransferase and not by the Lewis enzyme [34]. Furthermore, Lewis enzyme activity in malignant bladder tissue does not differ from the enzyme activity in normal bladder tissue [35]. α-2,3-Sialyltransferase activity has not been investigated in bladder cancer. The increased concentration of Ca 19-9 in urine might, on the basis of the data presented above, reflect a shift in glycosylation pathway. This might favor the synthesis of sialyl-Lea as terminal structure or the increased synthesis of the glycoproteins that carry sialyl-Lea as side chains, or both.

Further investigations are needed to evaluate whether high concentrations of creatinine-adjusted Ca 19-9 are specific for bladder cancer and concomitant urothelial dysplasia, or whether this is merely a diffuse marker for an abnormal urothelial mucosa.

Prospective comparative studies of the ability of Ca 19-9 and routine procedures to signal bladder cancer and (or) predict clinical course are also needed to define the exact role of Ca 19-9 in diagnosis and follow-up of bladder cancer. Such investigations may also elucidate whether biochemical alterations in the form of raised Ca 19-9 concentrations may be detectable before abnormal pathology.

In conclusion, the findings in this report draw attention to the possibility of a general genotype interpretation of results in clinical chemistry. The determination of biomarker concentrations in body fluids could be influenced by the individual genotype for the biomarker, because heterozygous individuals may produce less biomarker than homozygous individuals in normal as well as in diseased cells. Perhaps the future will show not only age- and sex-corrected reference intervals but also genotype-corrected reference intervals. In short, we believe we are in an era where individual biological variation is being explored by genetic tools, in hopes that it may lead to a more precise interpretation of clinical chemistry results in individual patients.

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