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Differential urinary specific gravity as a molecular phenotype of the bladder cancer genetic association in the urea transporter gene, *SLC14A1*

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Abstract

Genome-wide association studies (GWAS) identified associations between markers within the solute carrier family 14 (urea transporter), member 1 (*SLC14A1)* gene and risk of bladder cancer. SLC14A1 defines the Kidd blood groups in erythrocytes and is also involved in concentration of the urine in the kidney. We evaluated the association between a representative genetic variant (rs10775480) of *SLC14A1* and urine concentration, as measured by urinary specific gravity (USG), in a subset of 275 population-based controls enrolled in the New England Bladder Cancer Study. Overnight urine samples were collected and USG was measured using refractometry. Analysis of covariance was used to estimate adjusted least square means for USG in relation to rs10775480. We also examined the mRNA expression of both urea transporters, *SLC14A1* and *SLC14A2*, in a panel of human tissues. USG was decreased with each copy of the rs10775480 risk T allele (p-trend= 0.011) with a significant difference observed for CC vs. TT genotypes ($p\text{-value}_{tukey}$=0.024). RNA-sequencing in the bladder tissue showed high expression of *SLC14A1* and the absence of *SLC14A2*, while both transporters were expressed in the kidney. We suggest that the molecular phenotype of this GWAS finding is the genotype-specific biological activity of SLC14A1 in the bladder tissue. Our data suggest that SLC14A1 could be a unique urea transporter in the bladder that has the ability to influence urine concentration and that this mechanism might explain the increased bladder cancer susceptibility associated with rs10775480.

Keywords

Bladder Cancer; Urinary Specific Gravity; Genome-wide association study; Epidemiology

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INTRODUCTION

Recent results from genome-wide association studies (GWAS) have identified the urea transporter gene, solute carrier family 14, member 1 (*SLC14A1)* located on chromosome 18 as a new bladder cancer susceptibility locus.[1, 2] The *SLC14A1* gene encodes the type B urea transporter (UT-B) which functions to regulate urine concentration in the kidney and as determinant of the Kidd (JK) blood group antigen in erythrocytes.[3] The GWAS variants tag the common blood group alleles Jka and JKb as well as the more recently discovered $JK^{a\text{-weak}}$ allele.[1] The most associated GWAS variants are intronic single nucleotide polymorphisms (SNPs) rs7238033, rs10775480 and rs10853535 which capture the separation of the Jk^a vs JK^b and $JK^{a\text{-weak}}$ blood group antigens, defined by the two functional variants, rs1058396 (Asp280Asn) and rs2298720 (Glu44Lys).[1] Information about the functional consequence of the observed phenotypes with respect to renal function is minimal. In experimental models using UT-B knockout mouse strains, UT-B null mice had higher urine volume and lower urine concentration compared to wild-type mice.[4] In humans, carriers of a very rare blood group, JK-null, characterized by the absence of both JK^a and JK^b antigens also have impaired urinary concentrating capacity.[5] Based on these observations we hypothesized that bladder cancer risk might be related to a differential ability of SLC14A1 protein isoforms to transport urea and concentrate the urine.

To examine this hypothesis, we evaluated urine concentration, as measured by specific gravity,[6] in a subset of population-based controls enrolled in the New England Bladder Cancer Study (NEBCS), which is a part of a bladder cancer GWAS.[1;7] Specifically, we evaluated the relationship between specific gravity and a representative common SNP, rs10775480 of *SLC14A1*, which is associated with bladder cancer risk.[1] To further explore and interpret our observations, we performed RNA-sequencing analysis of both human urea transporter genes, *SLC14A1* and *SLC14A2*, in a panel of human tissues, including kidney and bladder.

MATERIALS AND METHODS

Study Population and Sample Collection

Details of the NEBCS have been described previously.[8] Briefly, the NEBCS is a large population-based case-control study of 1,193 histologically confirmed cases of urinary bladder carcinoma (including carcinoma *in situ*) and 1,418 controls aged 30–79 years living in Maine, Vermont, and New Hampshire. Subjects were enrolled between September 1, 2001 and October 31, 2004 and were interviewed at home by trained personnel using a detailed computer-assisted personal interview (CAPI). A standardized, structured questionnaire elicited demographic data, including current height and weight, as well as information on major known or suspected risk factors for bladder cancer including information on smoking, urination frequency at various times of the day and night, history of urinary tract infection, and usual intake of specific fluids (water, tea, soda, juice, milk, coffee, alcohol). Approximately 20% of selected subjects provided an overnight urine sample around the time of interview. Of the 278 controls who provided written informed consented for specimen collection, an overnight urine sample was collected from 275 (participation rate=98.9%). All collection materials were provided, including a cooler and ice pack to cool the sample until pick-up by the interviewer at a pre-determined date. Subjects were asked to begin collecting urine after their evening meal up until and including the first morning void the next day. A questionnaire to document food and fluid consumption, smoking status, vitamin intake, and medication use for the two days preceding the overnight urine collection was also included for completion. Exfoliated buccal cells for DNA extraction were collected from mouthwash samples at the time of the CAPI.

Specific Gravity Assessment

Urine was stored in a cooler until it was picked up by study personnel and transferred to the repository (SeraCare Life Sciences, Inc.). Immediately upon arrival the volume of the urine was recorded and pH was determined, then aliquots were prepared and samples were transferred to −80°C for storage. For specific gravity measurements, 1.8 ml aliquots were thawed and urinary specific gravity was measured by refractometer (also at SeraCare Life Sciences, Inc.) for all 275 samples.

Genotyping

DNA was extracted from exfoliated buccal cells using standard phenol-chloroform extraction methods. The samples were genotyped by the Cancer Genetics Research Laboratory of the Division of Cancer Epidemiology and Genetics of the National Cancer Institute as a part of a bladder cancer GWAS.[1, 7] Three GWAS variants, rs7238033, rs10775480 and rs10853535, provide similar association with bladder cancer and capture the separation of Jk^a vs JK^b and $JK^{a\text{-weak}}$ blood group antigens, defined by the two functional variants, rs1058396 (Asp280Asn) and rs2298720 (Glu44Lys). SNP rs10775480 was used for this study as a representative variant to derive information on Jk blood antigens, which otherwise could be obtained by genotyping of rs1058396 (Asp280Asn) and rs2298720 (Glu44Lys).[1]

RNA-sequencing

RNA-sequencing (RNAseq) of seven tumor and five adjacent normal bladder tissue samples has been previously described.[9] Briefly, 1 μg of each total RNA sample was used for polyA mRNA selection and fragmentation, followed by ligation to 65-bp paired-end adaptors. Each library was enriched by 12 cycles of PCR and run at a concentration of 4.5 pM on HiSeq2000 Sequencing Systems (Illumina Inc.) using one lane per sample. On average, 52 million paired 107-bp reads were generated for each sample. All sequencing reads were processed and aligned to the UCSC Homo sapiens reference genome (hg19) by TopHat v1.2.0,[10] allowing a maximum of two mismatches with the reference sequence. RNA-sequencing data for all other human samples are a part of Illumina's Human BodyMap 2.0 project (NCBI GEO GSE30611). All RNA-seq results were visualized using Integrative Genomics Viewer.[11]

Statistical Analysis

Univariate linear regression analyses were used to identify predictors of overnight specific gravity, including all relevant available variables in the CAPI and the overnight urine collection questionnaire. Important predictors included: age, gender, urination frequency after dinner (as assessed on CAPI), current body weight (kg), volume of the overnight urine (mL), and amount of total fluids consumed (as assessed on CAPI). Appropriate transformations of variables were performed and Spearman correlation coefficients were used to assess correlations between variables. Analysis of covariance (ANCOVA) was used to estimate adjusted least square means for specific gravity by rs10775480 genotype. Variables identified as important predictors of specific gravity were then included as possible covariates in ANCOVA models and the most parsimonious model was selected. The final model covariates included age, gender, current weight, and overnight urine volume. All appropriate regression diagnostics were performed to assess model fit. Comparisons between adjusted means were performed using unadjusted and multiple comparison adjustment methods (Tukey). Stratified analyses were also conducted for all predictive variables. All statistical analyses were performed using SAS software version 9.2 (SAS Institute, Inc., Cary, NC).

RESULTS

Controls selected for overnight urine collection were representative of the total control population of the NEBCS (Table 1); they tended to be between 65–74 years of age, male, white, and former smokers. All of the overnight specific gravity measurements were within the biologically plausible range. The minor allele frequency of rs10775480 (risk T allele) in this set was 0.46 and similar to that observed in the whole GWAS set of controls (0.43).[1]

Adjusted mean values of overnight specific gravity among NEBCS controls in relation to rs10775480 genotype are presented in Table 2. Individuals with the CC genotype had the highest adjusted mean levels of urinary specific gravity (1.016, standard error = 0.00056). Carriage of one or two of the rs10775480 risk alleles (T) resulted in decreased urinary specific gravity, 1.0154 and 1.0139 respectively (p-trend= 0.011), with a significant difference observed in adjusted specific gravity means for CC vs. TT genotypes ($p\text{-value}_{tukey}$=0.024). Stratified analyses consistently showed the lowest adjusted mean specific gravity levels among those with the homozygous variant genotype, however, none of the interactions were statistically significant (data not shown).

We performed RNA-sequencing analysis of the 600-Kb region covering *SLC14A1* and *SLC14A2* genes on chr18q12.3 in a panel of human tissues (Illumina's Human Body Map project) including normal bladder and tumor samples. This analysis showed that *SLC14A2* was mainly expressed in the kidney and breast, but not in the bladder. *SLC14A1* was found to be expressed in several tissues, including normal and human bladder, prostate and brain, and weaker expression was detected in the kidney (Figure 1).

DISCUSSION

In a subset of 275 controls with overnight urine collection enrolled in a population-based case-control study of bladder cancer, we observed differential urine concentrations, as measured by specific gravity, by bladder cancer susceptibility SNP rs10775480 within *SLC14A1*. Specifically, carriage of the bladder cancer risk allele resulted in a significant decrease in urinary specific gravity after controlling for all available predictors. These results suggest a link between urine concentration and susceptibility to bladder cancer.

In bladder carcinogenesis, the carcinogenic stimulus is believed to be presented to the urothelium through the urine; this concept is also known as the urogenous contact hypothesis.[12] Based on this hypothesis, active carcinogens dissolved in urine come in contact and transform cells of bladder epithelium. Thus, factors affecting urine volume and concentration, including urination frequency and fluid intake, have been evaluated as risk factors for the disease in epidemiologic studies. Increased fluid intake has been related to bladder cancer but with inconsistent results with some studies finding a decreased risk,[13–15] some a positive risk,[16] and others showing no effect.[17–20] The impact of urination frequency on bladder carcinogenesis has also been equivocal[17, 18, 21, 22] likely because of the complex interrelationship between urine concentration, fluid intake, and urination habits. No study, to our knowledge, has specifically evaluated urine concentration and risk of bladder cancer.

Based on the previous observations that individuals with the extremely rare JK-null blood group, defined by *SLC14A1*, are unable to efficiently concentrate the urine, we hypothesized that bladder cancer risk might be related to this impaired urine concentrating ability. To test this hypothesis, we examined urine specific gravity, a measure of urine concentration, in 275 controls with genotype data. We observed a significantly decreased specific gravity with every copy of the risk allele T of rs10775480, which was independent of urination frequency, fluid intake and could not be explained by urine volume. We also examined the expression of both *SLC14A1* and *SLC14A2* in a panel of human tissues, and

found *SLC14A2* expression predominantly in the kidney, while *SLC14A1* was found in several tissues, including the bladder, with similar expression pattern in normal and cancerous urothelial tumor tissue. These data suggest that *SLC14A1* is a unique urea transporter in the bladder that has the ability to influence urine concentration. The composition of urine in the bladder is thought to reflect that produced by the kidney however, our data suggests, and others have shown,[23, 24] that the urothelium has a significant transport function resulting in the ability to change urine composition.[25] This transport might also allow for the co-transfer of other carcinogenic compounds present in the urine directly to the bladder epithelium which may increase bladder cancer risk. The exact mechanism for bladder carcinogenicity is unclear as several mechanisms including direct carcinogenicity to the urothelium, the interaction of carcinogens with either urinary proteins or urothelial surface molecules, or the interaction of growth factors with their receptor have been postulated even for well-established bladder carcinogens.[26]

Although there is limited experimental evidence, alternative mechanisms have also been proposed. For example, there is some experimental evidence suggesting that increased urinary volume (which is coupled with a decrease in specific gravity) may be a risk factor for bladder carcinogenesis.[27] Increased volume of urine is thought to produce distention of the urothelium, which is coupled with an increase in the number of urothelial cells to compensate for the increased surface area of the bladder lumen and an increase in cellular proliferation, which may promote carcinogenesis.[26] Our results suggest lower urine concentration, independent of urine volume, is a molecular phenotype of the genetic association with bladder cancer risk in *SLC14A1.* This, and other potential mechanisms, needs further evaluation in future experimental studies.

Our study has several strengths. We were able to account for important determinants of specific gravity using detailed collection of urination frequency and fluid intake information from questionnaires. In addition, information on medical history, medication use, body mass index, bladder cancer risk factors, and a range of other demographic and lifestyle variables were available for exhaustive confounding characterization and adjustment. In this study, we measured specific gravity, which is readily used to evaluate the kidney's ability to dilute or concentrate urine in order to maintain homeostasis. An alternative measure, urine osmolality, is also used to measure the concentrating ability of the kidneys;[6] however, we did not measure osmolality in the NEBCS. Previous studies have shown osmolality to correlate well with specific gravity measured by refractometry (R^2= 0.89–0.96),[28, 29] thus we expect these two measures to be very similar in this population of healthy controls. We also must consider that the observed association could be due to chance since this is, to our knowledge, the first study to test this phenotype in relation to *SLC14A1.* Replication of these findings is warranted. Our study provides the first indirect evidence that these functional differences exist, but specifically designed *in-vitro* biochemical experiments using allele-specific protein isoforms of SLC14A1 corresponding to all Jk antigens and measuring minute and transient changes in extracellular and intracellular urea concentration are needed.

In conclusion, we observed that carriage of the bladder cancer risk variant T allele of rs10775480 in the urea transporter *SLC14A1* is associated with a significant decrease in urine concentration (i.e. urinary specific gravity). Previous data have linked impaired urinary concentrating capacity to those lacking UT-B protein which is coded by *SLC14A1.* Here we suggest that common variation in this region also impacts urinary concentrating capacity as well as bladder cancer risk. More work is needed to understand the specifics of the mechanism due to the complex interrelationship between several determinants of urine concentration.

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Novelty and impact: In this study we report a novel association between urine concentration, as measured by urinary specific gravity, and the bladder cancer susceptibility locus rs10775480 in *SLC14A1*. Our data suggest that SLC14A1 could be a unique urea transporter in the bladder that has the ability to influence urine concentration and ultimately bladder cancer susceptibility.

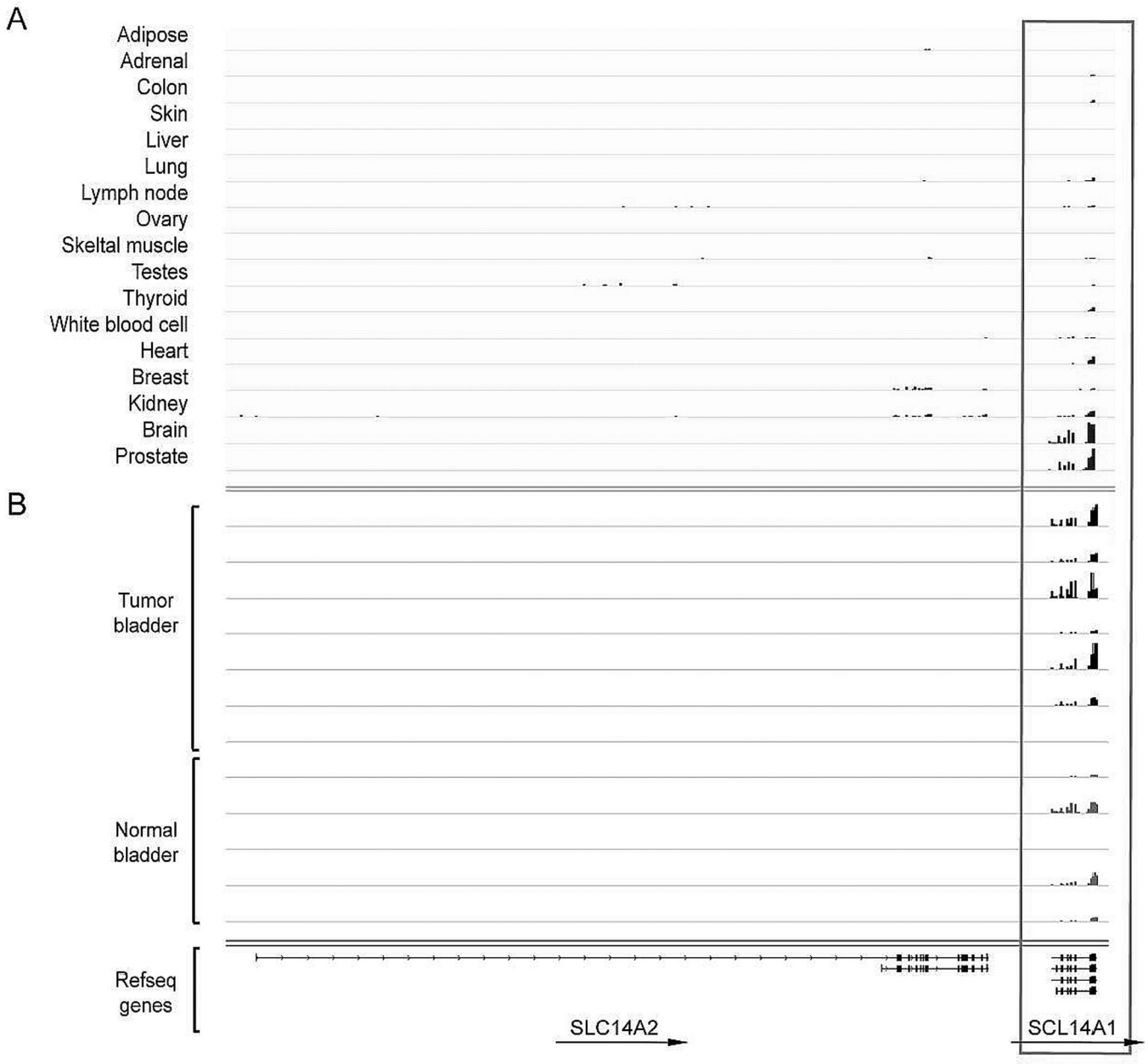

Figure 1.

RNA-sequencing results for the 600-Kb genomic region which includes *SLC14A2* and *SLC14A1* urea transporters on chr18q13.2 in a panel of human tissues from the Illumina's Human BodyMap 2.0 project and in normal and tumor bladder tissue samples. *SLC14A2* expression is detected mainly in breast and kidney tissue, but not in the bladder. *SLC14A1* is expressed in several tissues, including normal and human bladder, prostate and brain, and weaker expression was detected in kidney.

Table 1

Characteristic of controls in the New England Bladder Cancer Study

Characteristic	All Controls n=1,418 n (%)	Controls with Specific Gravity* Measurement n=275 n (%)
Age		
<55	255 (18.0)	45 (16.4)
55–64	336 (23.7)	61 (22.2)
65–74	544 (38.3)	122 (44.4)
75+	283 (20.0)	47 (17.1)
Gender		
Male	1039 (73.3)	195 (70.9)
Female	379 (26.7)	80 (29.1)
Race/Ethnicity		
White	1371 (96.7)	257 (93.5)
Other	47 (3.3)	18 (6.5)
State		
Maine	740 (52.2)	153 (55.6)
Vermont	252 (17.8)	45 (16.4)
New Hampshire	426 (30.0)	77 (28.0)
Smoking Status		
Occasional Smoker	40 (2.8)	6 (2.2)
Non-smoker	472 (33.3)	90 (32.7)
Former smoker	699 (49.3)	140 (50.9)
Current smoker	206 (14.5)	38 (13.8)
Unknown	1 (0.07)	1 (0.4)
Current Weight (kg)		
Median (IQR)	81.6 (71.2–93.4)	81.6 (70.3–95.3)
Specific Gravity *		
Median (IQR)	--	1.0150 (1.0109–1.0200)
Urine Volume (mL)		
Median (IQR)	--	811 (561–1311)
rs10775480		
CC	321 (22.6)	71 (25.8)
CT	549 (38.7)	116 (42.2)
TT	220 (15.5)	52 (18.9)
Missing	328 (23.1)	36 (13.1)
Minor allele frequency (T)	0.45	0.46

Percents may not sum to 100 due to rounding. Abbreviations: Inter-quartile range (IQR).

* Specific gravity of urine from overnight sample.

Table 2

Adjusted specific gravity means by *SLC14A1* rs10775480 genotype among controls in the New England Bladder Cancer Study

	N	Adjusted Specific Gravity Means*	Standard Error
rs10775480 genotype			
CC	71	1.0160†	0.00056
CT	116	1.0154†	0.00044
TT	52	1.0139†	0.00064
p-trend		**0.011**	

*Controlling for age, gender, current weight (kg), and overnight urine volume (mL)

†Difference between means:

CC vs. CT: $p\text{-value}_{unadjusted}$ = 0.366, $p\text{-value}_{tukey}$ 0.637
CC vs. TT: $p\text{-value}_{unadjusted}$ = 0.009, $p\text{-value}_{tukey}$ 0.024
CT vs. TT: $p\text{-value}_{unadjusted}$ = 0.040, $p\text{-value}_{tukey}$ 0.099