The *UGT1* locus is a determinant of prostate cancer recurrence after prostatectomy

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Abstract

The prognostic significance of common deletions in uridine diphospho-glucuronosyltransferase 2B (*UGT2B*) genes encoding sex steroid metabolic enzymes has been recently recognized in localized prostate cancer (PCa) after radical prostatectomy (RP). However, the role of germline variations at the *UGT1* locus, encoding half of all human UGTs and primarily involved in estrogen metabolism, remains unexplored. We investigated whether variants of *UGT1* are potential prognostic markers. We studied 526 Caucasian men who underwent RP for clinically localized PCa. Genotypes of patients for 34 haplotype-tagged single-nucleotide polymorphisms (htSNPs) and 11 additional SNPs across the *UGT1* locus previously reported to mark common variants including functional polymorphisms were determined. The risk of biochemical recurrence (BCR) was estimated using adjusted Cox proportional hazards regression and Kaplan–Meier analysis. We further investigated whether variants are associated with plasma hormone levels by mass spectrometry. In multivariable models, seven htSNPs were found to be significantly associated with BCR. A greater risk was revealed for four *UGT1* intronic variants with hazard ratios (HRs) of 1.59–1.88 (*P* < 0.002) for htSNPs in *UGT1A10*, *UGT1A9*, and *UGT1A6*. Conversely, decreased BCR was associated with three htSNPs in introns of *UGT1A10* and *UGT1A9* (HR = 0.56–0.58; *P* ≤ 0.01). An unfavorable *UGT1* haplotype comprising all risk alleles, with a frequency of 14%, had a HR of 1.68 (95% CI = 1.13–2.50; *P* = 0.011). Significant alteration in circulating androsterone levels was associated with this haplotype, consistent with changes in hormonal exposure. This study provides the first evidence, to our knowledge, that germline polymorphisms of *UGT1* are potential predictors of recurrence of PCa after prostatectomy.

Key Words

- biochemical recurrence
- germline polymorphisms
- glucuronidation
- prostate cancer
- UGT

Introduction

Sex steroids play a central role in the development and progression of prostate cancer (PCa) (Mohler et al. 2012, Ryan et al. 2013). Recent evidence supports the notion that patient’s genetic background influences the outcome (Audet-Walsh et al. 2011, 2012, Nadeau et al. 2011, Boyd et al. 2012, Reese et al. 2012, Levesque et al. 2013, 2014a, b, Wyatt et al. 2013). In particular, common deletions of the uridine diphospho-glucuronosyltransferase (UGT) genes *UGT2B17* and *UGT2B28* involved in the catabolism of androgenic sex steroids have been recognized as
Prognostic markers for localized PCa after radical prostatectomy (RP) (Nadeau et al. 2011). The UGT-mediated metabolic pathways, governed by the two gene families UGT1 and UGT2, are among key regulators of sex steroid exposure responsible for their inactivation via glucuronic acid conjugation, which prevents binding to their respective nuclear receptors and facilitates their excretion. In support of the idea that inherited genetic variations can alter the hormonal environment to which cancer cells are exposed, common gene deletions of UGT2B17 and UGT2B28 have also been associated with altered levels of circulating androgen steroids (Nadeau et al. 2011).

Half of the human UGTs are encoded by the UGT1 gene located on chromosome 2q37, which encodes nine functional enzymes. In contrast to UGT2 enzymes, UGT1As are mainly regulators of tissue exposure to estrogens, because most of them are involved in the glucuronidation of parental estrogen (estradiol (E2) and estrone (E1)), and inactivation of their hydroxyl moieties at position 2 or 4 as well as methoxy catechol estrogens (Gall et al. 1999, Lepine et al. 2004, Murai et al. 2006, Starlard-Davenport et al. 2007, Zhou et al. 2010, Sneitz et al. 2013). Conversely, there is little evidence to support them having a role in the inactivation of androgens, e.g., the potent androgen, dihydrotestosterone (DHT; Gall et al. 1999, Zhou et al. 2010). UGT1A1, UGT1A3, UGT1A8, UGT1A9, and UGT1A10 conjugate E1 and E2 most efficiently, whereas UGT1A4 is less efficient. (Lepine et al. 2004, Starlard-Davenport et al. 2007, Sneitz et al. 2013). Several groups have reported that estrogens and their metabolites contribute to both the development and progression of PCa (Cavaliere & Rogan 2011, Neltes et al. 2011, Hu et al. 2012). Genetic polymorphisms in factors that govern estrogen biosynthetic and metabolic pathways have been linked to altered exposure to sex steroid hormones and risk of PCa (Gu et al. 2014, Kanda et al. 2015). It has been reported that low-activity promoter alleles of the E2-conjugating enzyme UGT1A1 (rs3064744; (TA)ₙ repeat) are associated with a risk of low-grade PCa and a slight elevation of estrogen levels but have no gene-dosage effect (Tang et al. 2011).

Germline variations in genes involved in the biosynthesis and metabolism of androgens and estrogens have been associated with progression of PCa (Audet-Walsh et al. 2011, 2012, Nadeau et al. 2011, Levesque et al. 2013, 2014a,b). The relevance of studying steroidogenic pathways in the context of progression of PCa arises from observations that PCa tumor cells can use multiple biotransforming pathways to de novo synthesize more potent hormones (Montgomery et al. 2008, Mitsiades et al. 2012). In support of these results, we have recently reported that single-nucleotide polymorphisms (SNPs) in genes associated with E2-related metabolic pathways, either individually or in combination, are predictors of progression of PCa. These variants are located in genes encoding cytochrome P450 1B1, which is involved in the production of 4-hydroxy metabolites of E2 and E1 (4-OH), catechol-O-methyltransferase, which forms 2-methoxy catechol estrogens, sulfotransferase 2B1, which catalyzes the formation of less-reactive sulfate metabolites, and 17β-hydroxysteroid dehydrogenase type 2, which is involved in the conversion of E2 to E1 (Levesque et al. 2014a). However, it remains unclear whether the outcome of PCa is influenced by common germline polymorphisms in the estrogen catabolic pathway mediated by UGT1 gene products.

The objective of this study was to determine whether common variations across the UGT1 locus, which encodes multiple estrogen-metabolizing UGT1A enzymes, are associated with a modified risk of biochemical recurrence (BCR) after prostatectomy in patients with localized PCa and to ascertain their relationships with levels of endogenous circulating hormones. After adjustments for known clinico-pathological variables, seven UGT1 SNPs were found to be significantly associated with time to BCR, three SNPs were protective and four were associated with an increased risk. Additional analyses identified an unfavorable UGT1 haplotype, characterized by the latter four risk markers, that is also linked with changes in exposure to circulating hormones.

Materials and methods

Clinical data and outcome collections

The cohort included 526 Caucasian patients with localized PCa. All patients underwent RP at l’Hôpital-Dieu de Québec Hospital (QC, Canada) between February 1999 and December 2002 (Audet-Walsh et al. 2011, Nadeau et al. 2011). All participants provided written informed consent for genetic analysis, and the Local Research Ethics Committee of the CHU de Québec approved the research protocol. The clinical characteristics of the studied cohort are given in Table 1.

Genetic analysis and steroid measures

Polymorphisms of the UGT1 locus were assessed by PCR amplification by Sequenom iPLEX matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. With the objective of covering most of the haplotype
dose, polymorphisms in UGT1 were chosen on the basis of results from our previous study of Caucasian volunteers recruited in the same geographical region (Menard et al. 2009) and as described by the genetics of the CEU (Utah residents with Northern and Western European ancestry) HapMap population. A region covering all exons, introns, and 5 kb upstream and downstream of each gene was screened using a haplotype-tagging SNP (htSNP) strategy (Audet-Walsh et al. 2011, 2012). Overall, we genotyped 34 htSNPs (Fig. 1) with an additional 11 associated SNPs across the UGT1A locus previously reported to mark common variants in regulatory domains, exons, and intron–exon boundaries, including some functional polymorphisms (Supplementary Table 1, see section on supplementary data given at the end of this article). This included genotyping of the UGT1A1 promoter variant (rs3064744; c.-54/-53insTA) genotyped by direct sequencing of PCR-amplified products, as described previously (Girard et al. 2008). Negative controls were included for every run of analysis, and quality controls consisting of random samples were successfully performed on 5% of the study cohort. SNPs that had 5% missing genotypes were excluded from the analysis. None of the studied SNPs deviated from the Hardy–Weinberg equilibrium. A total of 495 plasma samples were available and collected the morning of the surgical procedure. Levels of circulating sex steroids for this cohort of patients have been reported (Nadeau et al. 2011). Data are expressed as the mean ± the s.e.m. To ensure the validity of each analysis, quality controls that included known steroid concentrations with deuterated isotopes were included in each run of analysis.

### Statistical analysis

To analyze associations between polymorphisms and BCR, each htSNP was first categorized according to homozygosity/heterozygosity (i.e. major allele homozygote, heterozygote, and minor allele homozygote), because the functions of most of these htSNPs are unknown. Rare homozygotes (frequency <2%) were combined with heterozygotes. Cox regression analysis was performed for each SNP with adjustment for clinico-pathological variables. Multivariable models included prostate-specific antigen (PSA) at diagnosis, Gleason score, pathological T stage, age at diagnosis, surgical margins, nodal invasion status, smoking status, neoadjuvant therapy, adjuvant therapy. All covariates were treated as categorical, and ≤4% of all covariates had missing values. The censoring variable was BCR, as reported previously (Nadeau et al. 2011). Haplotypes were inferred using Phase version 2.1.1 (Stephens et al. 2001, Stephens & Donnelly 2003). Pairwise linkage disequilibrium was determined with HAPLOVIEW 3.32 (www.broad.mit.edu/mpg/haploview). Kaplan–Meier analyses (log-rank) and univariate Cox regression analyses were performed for each SNP. As we tested multiple polymorphisms (n = 45), false-discovery rates (q values) were calculated to determine the degree to which the tests were prone to false positives using the R QVALUE package (http://genomics.princeton.edu/storeylab/qvalue/).

Analysis of covariance was used to compare means of each natural log-transformed hormonal variable between haplotype groups adjusted for age. Patients with missing genotype or hormone level data were excluded. For comparisons among haplotype groups, any significant
differences revealed by analysis of covariance were further investigated using Fisher’s least significant difference post-hoc pairwise comparison. Patients who had previously received androgen deprivation therapy were excluded from our analyses. Statistical analyses were performed using PASW Statistics version 17 (SPSS, Inc.).

Results

Overall, 24.7% of patients experienced recurrence after prostatectomy (median follow-up time, 7.4 years). None of the germline variations tested was associated with the main predictors of BCR (PSA at diagnosis, Gleason score, pathological T stage, and surgical margin status; Supplementary Table 2, see section on supplementary data given at the end of this article). After adjustments for known prognostic factors had been made, seven variants located in the intronic or regulatory regions of UGT1 were associated with an altered risk of BCR. The relative frequency of each hSNP and its corresponding hazard ratio (HR; 95% CI) are presented in Table 2. One variation in the UGT1A10 (rs1823803) and two variations in the UGT1A9 intronic region (rs2741048 and rs2602381) were associated with a significantly decreased risk of BCR with HR values between 0.56 and 0.58 (P<0.01). Conversely, hSNPs located in the UGT1A10 intronic region (rs17862847 and rs7608713) and in the UGT1A9 intronic region (rs4663871) were associated with an increased risk for BCR (HR=1.59–1.88; P<0.02). In addition, one variation rs6751673 in UGT1A6 was positively associated with BCR and displayed an increased risk of events (HR=1.68; P=0.008). The Kaplan–Meier curves showing the BCR-free survival for the UGT1 markers significantly associated with BCR and their log-rank P values are available in the supplementary material (Supplementary Fig. 1, see section on supplementary data given at the end of this article). No significant association was observed for any of the known functional UGT1 variants evaluated, e.g., the TATA box promoter variant UGT1A1*28 (rs34815109; c.-54/-53insTA), UGT1A3 V47A
Table 2 Significant associations of UGT1 polymorphisms with BCR

<table>
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Significant changes in androgen glucuronides, E2, and E1 could be detected (data not shown).

Discussion

We provide evidence for the prognostic value of UGT1 for localized PCA. As has been observed UGT2B deletions, we found that several UGT1 htSNPs are associated with an increased risk of recurrence. The markers positively linked with a greater risk of BCR are located in intronic regions of exons 1A6, 1A9, and 1A10. This finding supports our hypothesis that genetic variants of UGT1 may modify the progression of PCA – probably by altering sex steroid metabolism – although direct biological evidence for this hypothesis is lacking.

We had previously reported a significant decrease in circulating androgen metabolites, namely ADT-G and (rs6431625; c.140T>C), or other non-synonymous common variations located in exons UGT1A3 and UGT1A4, and in the common exons 2–5 or the 3′ UTR.

Five haplotypes inferred using the seven positive UGT1 markers are significantly associated with the occurrence of BCR at an allelic frequency of ≥5% in our cohort. The occurrence rates of BCR were 23, 22, 33, 21, and 19%, respectively, for haplotypes 1–5 (H1–H5; Fig. 2A). After adjustments for other variables, we found that H3, characterized by the presence of the four unfavorable alleles and no protective allele, displayed a greater risk of BCR (Table 3). H3 carriers display a HR of 1.68 (P = 0.011) compared with the reference H1 that includes none of the increased risk and/or the protective alleles described earlier. We also observed that individuals who carry both H3 and two or more deleted copies of UGT2B17 and UGT2B28 tend to have decreased BCR-free survival as shown by the Kaplan–Meier curves for BCR. The BCR-free stratifications are 71 and 55% respectively, when H3 is sub-classified according to the number of UGT2B deletions (0–1 UGT2B deletion (n = 112) versus ≥2 UGT2B deletions (n = 33); Fig. 2B).

We also compared the mean levels of plasma unconjugated sex steroids and androgen glucuronides for H3 carriers with only zero or one UGT2B deletion, because individuals with more than two UGT2B deletions had been previously reported to present reduced circulating levels of DHT metabolites, namely androsterone glucuronide (ADT-G) and 3α-androstenediol-17G (Nadeau et al. 2011). Compared with non-carriers of UGT1 risk alleles (i.e. H1), carriers of H3 presented a 15% lower and 22%, 81%, and 81% lower mean levels of (rs6431625; c.140T>C), or other non-synonymous common variations located in exons UGT1A3 and UGT1A4, and in the common exons 2–5 or the 3′ UTR.

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with the exception of UGT1A3 and UGT1A4, which have weak activity toward DHT and its metabolite ADT compared with UGT2B enzymes (Gall et al. 1999, Zhou et al. 2010). This is consistent with our observation that no significant changes in circulating androgen glucuronides are observed for carriers of UGT1-related risk haplotypes. UGT1A enzymes are well known to be involved in the metabolism of estrogens, including the parental estrogens E₂ and E₁, and catechol estrogens and their methoxy derivatives; however, no significant changes in circulating levels of unconjugated E₁ and E₂ could be detected in our study. It remains to be ascertained if levels of estrogen metabolites, including their glucuronide derivatives, are modified by variants of UGT1. We found positive markers mostly in regions related to UGT1A9 and UGT1A10, with their gene products being mainly involved in the metabolism of 2-OH-E₂, 4-OH-E₂, and 2-methoxy-E₂ (Lepine et al. 2004). We are currently developing a mass spectrometry assay to address whether the identified UGT markers could be involved in recurrence of PCa by promoting unbalanced glucuronidating metabolism for these specific estrogen metabolites or by their relative abundance compared with that of androgens, thereby favoring progression of cancer. The carcinogenicity of catechol oxidative metabolites has been recognized for various cancers, including PCa (Bosland & Mahmoud 2011, Cavalieri & Rogan 2011). Results of a pilot study by Kosti et al. (2011) indicated that some catechol estrogens are present in greater concentrations in the urine of PCa patients compared with that of healthy men, indicating an association between estrogen metabolites and risk of PCa. However, based on a method developed by Xu et al. (2007), they did not directly measure the levels of estrogen glucuronide derivatives but reported total estrogen concentrations (glucuronidates + sulfates + unconjugated sex steroids following hydrolysis with sulfatase and β-glucuronidase).

Very few studies have documented the expression of UGT1 gene products in human prostate. Court et al. (2012) detected nine UGT1A transcripts in a pool of human prostates and detected all UGT1As but at significantly lower levels compared with those found in liver samples. During normal prostate differentiation, downregulation of UGT1A transcripts was observed in normal prostatic stem cells compared with more differentiated cells (Williamson et al. 2013). Furthermore, assessment of individual UGT1A transcripts revealed that the expression of UGT1A8 was elevated in progenitor cells, with no change in amounts of UGT2B expressed. UGT1A8 is the major UGT1A involved in inactivation of the carcinogenic metabolites.
4-OH-E1/E2 (Lepine et al. 2004). In support of a role for UGT1As in progression of PCa, transient repression of UGT1A expression in the PCa cell line LNCaP has been shown to contribute to activation of androgen receptors leading to upregulation of PSA along with increased cell survival in the standard steroid-containing medium (Williamson et al. 2013). This study also provided additional evidence that reduced expression of UGT1A is a feature of clinical PCa associated with a poorer prognosis and may be explained by the fact that upon depletion of UGT1A, a higher level of steroids is expected. However, Williamson and colleagues did not address whether levels of expression of UGT1A affect levels of steroids and whether estrogens rather than androgens are altered upon partial repression of UGT1A. In fact, their results obtained in vitro with LNCaP cells might be caused by the action of androgens and estrogens because both types of steroids are present in a complete medium and because androgens are converted to estrogens by the action of aromatase. A role for estrogens in this context is thus plausible because UGT1A more efficiently inactivates estrogens compared with androgens such as DHT, and it is known that estrogens are proficient in activating the mutated androgen receptor in the LNCaP cell line (Tan et al. 1997). In addition, expression of aromatase is upregulated by approximately 30-fold ($P<0.001$) in metastatic PCa tumors compared with primary tumors (Montgomery et al. 2008), indicating a greater production of estrogens during progression of PCa. Changes in UGT1A expression have also been reported in clinical specimens. In castration-resistant patients, low levels of expression of UGT1A have been found compared with that observed in early PCa (Varambally et al. 2005, Williamson et al. 2013), as well as decreased UGT1A immunostaining in aggressive disease (Williamson et al. 2013). Altogether, these findings provide evidence supporting the suggestion that reduced expression of UGT1A is linked to poor prognosis and may represent a mechanism for accumulation of sex steroids, probably estrogens, within PCa cells. In this study linking several genetic markers of the UGT1 gene to BCR, the decreased levels of the DHT metabolite ADT in carriers of the UGT1-risk haplotype may reflect enhanced production of estrogens from androgens or a modification of androgen metabolism, although additional investigations are required to validate this hypothesis.

In conclusion, our study provides the first evidence, to our knowledge, that germline polymorphisms in the UGT1 locus modify the likelihood of BCR after prostatectomy. Our findings also highlight the need to better
characterize the levels of E₂ and estrogen metabolites, the main known steroid hormone substrates of UGT1A enzymes, in PCa patients. Further studies are needed to define the underlying mechanisms through which UGT1As contribute to the biology of PCa and patient outcomes.

Acknowledgements
The authors thank Lyne Villeneuve for technical support. They acknowledge the employees of the genomics and the statistical service platforms of the Centre Hospitalier Universitaire de Québec, particularly Sylvie Levesque and C Guillemette.

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Author contribution statement
É Lévesque and C Guillemette conceived the study concept, and designed and supervised the study. L Lacombe and Y Fradet were responsible for patient recruitment and clinical data. I Laverdière, C Flageole, É Audet-Walsh and É Lévesque were involved in acquisition of data and statistical analysis. P Caron performed the experiments. I Laverdière, C Flageole, É Audet-Walsh, É Lévesque, and C Guillemette conceived the study concept, and designed and supervised the study. L Lacombe and Y Fradet were responsible for patient recruitment and clinical data. I Laverdière, C Flageole, É Audet-Walsh, É Lévesque, and C Guillemette wrote the manuscript. All authors contributed to the critical revision of the manuscript for important intellectual content. É Lévesque, C Guillemette, Y Fradet, and L Lacombe obtained funding for this work.

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Received in final form 27 November 2014
Accepted 1 December 2014
Made available online as an Accepted Preprint 1 December 2014