Original Study

Pazopanib-Induced Liver Toxicity in Patients With Metastatic Renal Cell Carcinoma: Effect of *UGT1A1* Polymorphism on Pazopanib Dose Reduction, Safety, and Patient Outcomes

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Abstract

Treatment of patients with metastatic renal cell carcinoma with pazopanib has been associated with an increased probability of developing liver toxicity in the presence of polymorphisms in the gene *UGT1A1* (uridine diphosphate glucuronosyltransferase 1A1), leading to treatment interruptions and permanent discontinuation. We have demonstrated that *UGT1A1*-guided dosing can manage pazopanib-induced liver toxicity and also that *UGT1A1* polymorphisms are associated with improved outcomes, despite pazopanib interruption and substantial dose reductions.

Background: Pazopanib can induce liver toxicity in patients with metastatic renal cell carcinoma (mRCC). We assessed the effect of a TA repeat polymorphism in the *UGT1A1* (uridine diphosphate glucuronosyltransferase 1A1) gene encoding uridine diphosphate glucuronosyltransferase 1A1 on liver toxicity, dose reductions, and patient outcomes. **Patients and Methods:** Patients with mRCC treated with first-line pazopanib developing liver toxicity underwent genotyping for the *UGT1A1* polymorphism. Liver toxicity was assessed using the Common Terminology Criteria for Adverse Events, version 4.0. Progression-free survival and overall survival were assessed using the Kaplan-Meier and log-rank methods. **Results:** Of 261 patients, 34 (13%) had developed liver toxicity after a median of 29 days (range, 5-155 days). Grade 4, 3, and 2 alanine aminotransferase or bilirubin had increased in 2 (6%), 17 (50%), and 8 (24%) patients, respectively. The *UGT1A1* assessment demonstrated that 18 patients (53%) had TA6/TA7, 7 (21%) had TA7/TA7, and 9 (26%) had wild-type TA6/TA6. The *UGT1A1* polymorphism was associated with improved median progression-free survival (TA6/TA6, 5.5 months; TA6/TA7, 34.2 months; TA7/TA7, 22.3 months; unknown *UGT1A1* status, 9.2 months; *UGT1A1* polymorphisms combined vs. unknown status, $P = .021$). *UGT1A1* polymorphism was associated with improved median overall survival (TA6/TA6, 8.1 months, TA6/TA7 or TA7/TA7 not reached, unknown *UGT1A1* status, 16.6 months; *UGT1A1* polymorphisms combined vs. unknown status, $P = .033$). Patients with *UGT1A1* polymorphism safely resumed pazopanib at ultra-low doses determined by the degree of liver toxicity and *UGT1A1* polymorphism. **Conclusions:** *UGT1A1* polymorphisms were associated with improved outcomes, despite pazopanib interruption and dose reductions. *UGT1A1* assessment could improve the management of pazopanib-induced liver toxicity in patients with mRCC.

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Introduction

The management of metastatic renal cell carcinoma (mRCC) has evolved considerably within the past decade.[1] The first-line treatment options include tyrosine kinase inhibitors inhibiting vascular endothelial growth factor and checkpoint immunotherapy (CPI).[2]

Compared with other vascular endothelial growth factor inhibitors for the treatment of mRCC, pazopanib has resulted in a greater incidence of aspartate aminotransferase and/or alanine aminotransferase (ALT) elevation.[3] Data from phase II and III trials of pazopanib showed elevated ALT and bilirubin levels in 41.4% and 24.8% of patients, respectively, with elevated ALT levels >3 times the upper limit of normal (ULN); ie, development of grade 2 toxicity using the Common Terminology Criteria for Adverse Events in one fifth of patients.[4] The median time to the onset of adverse events was 42 days.[5] These findings have prompted close observation of liver enzyme levels after the initiation of pazopanib, with treatment interruptions if elevated and, in many cases, permanent discontinuation, because hepatotoxicity can be severe or, even, fatal.[6]

One proposed mechanism of liver toxicity is the inability to metabolize bilirubin owing to genetic polymorphism in the gene *UGT1A1* (uridine diphosphate glucuronosyltransferase 1A1). The enzyme encoded by this gene is the primary contributor to conjugation of bilirubin, and reduced levels will cause isolated, elevated levels of unconjugated bilirubin. One genetic variant (*UGT1A1**28, also known as TA7) causes Gilbert syndrome, a hereditary disorder predisposing patients to benign, elevated levels of unconjugated bilirubin associated with mild jaundice.[7] In patients with mRCC treated with pazopanib, polymorphism of either 1 or both alleles of the gene *UGT1A1* (TA6/TA7 or TA7/TA7) has been associated with a greater probability of hyperbilirubinemia compared with patients with the wild-type TA6/TA6 genotype, with an odds ratio of 13 when homozygous for TA7.[7,8]

The *UGT1A1* enzyme is inhibited in the presence of pazopanib.[9] Also, the risk of hyperbilirubinemia with a TA7 allele is greater with the use of pazopanib.[8] Thus, we validated and implemented genetic testing for *UGT1A1* polymorphisms as standard practice. We subsequently used the findings to guide the management of severe pazopanib-induced liver toxicity. The aim of the present study of patients with mRCC treated with first-line pazopanib was to analyze *UGT1A1* polymorphisms compared with the patient outcomes and treatment management.

Patients and Methods

Study Design and Study Population

We performed a retrospective, longitudinal cohort study of prospectively collected data from consecutive patients with mRCC who had been treated with first-line pazopanib at the Department of Oncology, Aarhus University Hospital. The starting dose of pazopanib was at the discretion of the treating physician, with consideration of real-world patients, frail patients, elderly patients, and patients with comorbidities. Blood samples from those patients who had developed grade ≥2 elevated liver enzymes using the Common Terminology Criteria for Adverse Events, version 4.0, were tested for the polymorphic *UGT1A1* gene. Pazopanib was interrupted until the liver enzyme levels had normalized and was resumed at lower doses according to the degree of liver toxicity and the *UGT1A1* genotyping results. The largest dose reductions were for those patients with TA7/TA7. The baseline patient characteristics and data on biochemical markers, overall survival (OS), and progression-free survival (PFS) were retrieved from the medical records. At baseline, no patient had had a known history of chronic liver disease or iron overload. The Danish Data Protection Agency (journal no. 1-16-02-428-13) and the Danish Health Authority (journal no. 3-3013-542/1) approved the present study.

UGT1A1 *Analysis*

Genetic testing was established in 2014 as the standard of care. Genomic DNA was isolated from EDTA (ethylenediaminetetraacetic acid)-stabilized blood samples. The human *UGT1A1* gene (NG_033238.1) served as a template for the design of the primers, and the nucleotide sequence was obtained from the National Center for Biotechnology Information platform (available at: https://www.ncbi.nlm.nih.gov/). Standard polymerase chain reaction (PCR) amplification was performed using forward primer 5′-FAM-GCTCC-ACCTTCTTTATCTCTGAAAGTGAACT-3′ and reverse primer 5′-CTAACAAAAGACTCTTTCACATCCTCCCTTTGGA-3′. After PCR amplification, fluorescein amidite labeling allowed for the detection of 448- to 450-bp fragments, which corresponded to 6 and 7 TA repeats, respectively. This analysis was performed using the Applied Biosystems 3500 Genetic Analyzer mounted with a 50-cm capillary (Applied Biosystems, ThermoFisher Scientific, Waltham, MA) using POP7 polymer and the GeneScan 500 ROX dye size standard (35-500 bp; Applied Biosystems) according to the manufacturer's instructions. Data analysis was performed using GeneMapper software, version 4.1 (Applied Biosystems). Details of the method are presented in Supplemental Methods and Supplemental Table 1 (available in the online version).

The laboratory participates in external proficiency testing, and the *UGT1A1* TA polymorphism genotyping method we have described allows for the proper identification of the external quality controls organized and distributed by the Reference Institute for Bioanalytics (Bonn, Germany).

Statistical Analysis

Using *UGT1A1* status, the patients were analyzed in groups: TA6/TA6, TA6/TA7, TA7/TA7, and unknown *UGT1A1* status. Descriptive statistics were calculated using frequencies and proportions for the categorical variables. The Memorial Sloan Kettering Cancer Center and International mRCC Database Consortium (IMDC) prognostic groups were used for risk stratification at the initiation of first-line therapy.[10] OS was defined as the interval between systemic therapy initiation and the date of death or last follow-up visit. PFS was defined as the interval between treatment initiation and disease progression using the RECIST (response evaluation criteria in solid tumors), version 1.1,[11] death, or last follow-up visit. For the PFS and OS analyses, the median and 95% confidence intervals (CIs) were estimated using Kaplan-Meier analysis and log-rank statistics. Computed tomography assessments of the chest, abdomen, and pelvic were performed at baseline and every third month until disease progression or death. The investigator-assessed best objective response was evaluated using the RECIST, version 1.1. Statistical analyses were performed using

SPSS, version 25 (IBM Corp, Armonk, NY). All *P* values were 2-sided.

Results

Demographic Data and Baseline Characteristics

A total of 261 patients had received first-line treatment with pazopanib (Table 1). The median age was 67 years, and 71% of the patients were men. All the patients were white, and 74% had undergone nephrectomy. Risk allocation using the IMDC criteria rated 17% as favorable, 44% as intermediate, and 29% as poor risk. Of the 261 patients, 38% were alive at a median follow-up of 28.2 months (range, 2.3-62.2 months). The median PFS and OS for the entire cohort was 9.5 months (95% CI, 7.7-11.2) and 17.7 months (95% CI, 14.0-21.3), respectively.

The baseline patient characteristics are presented in Table 1. Of the 261 patients, 34 (13%) had developed liver toxicity after a median of 29 days (range, 5-155 days) and had undergone *UGT1A1* analysis. The average starting pazopanib dose for these patients was 488 mg/d. Grade 4, 3, and 2 ALT elevation had occurred in 1, 16, and 8 patients, respectively. Grade 4 or 3 bilirubin elevation was noted in 1 patient each.

UGT1A1 *Polymorphism Associated With Outcome*

The *UGT1A1* assessment showed that 18 patients (53%) had TA6/TA7, 7 (21%) had TA7/TA7, and 9 (26%) had wild-type TA6/TA6. *UGT1A1* polymorphism was associated with improved median PFS. The patients with wild-type TA6/TA6 had a median PFS of 5.5 months (95% CI, 5.3-5.7). In contrast, patients carrying 1 or 2 TA7 alleles (TA6/TA7 and TA7/TA7) had a median PFS of 34.2 months (95% CI, 6.8-61.6) and 22.3 months (95% CI, not estimable), respectively. Finally, patients with unknown *UGT1A1* status had a median PFS of 9.2 months (95% CI, 7.2-11.1 months; Figure 1A). The PFS for patients carrying 1 or 2 TA7 alleles combined (TA6/TA7 and TA7/TA7) was significantly increased compared with that for patients with TA6/TA6 ($P = .012$), TA6/TA6 or unknown status ($P = .018$), and unknown status ($P = .021$).

UGT1A1 polymorphism was also associated with improved median OS. The median OS was not reached for either TA6/TA7 or TA7/TA7. In contrast, TA6/TA6 patients had a median OS of 8.1 months (95% CI, 7.5-8.7 months), and patients with unknown *UGT1A1* status had a median OS of 16.6 months (95% CI, 13.0-20.2 months; Figure 1B). The OS for patients carrying 1 or 2 TA7 alleles combined was significantly increased compared with that for patients with wild-type TA6/TA6 or unknown *UGT1A1* status ($P = .03$), TA6/TA6 ($P = .054$), and unknown status ($P = .033$). No association with the best objective response was noted.

Safety

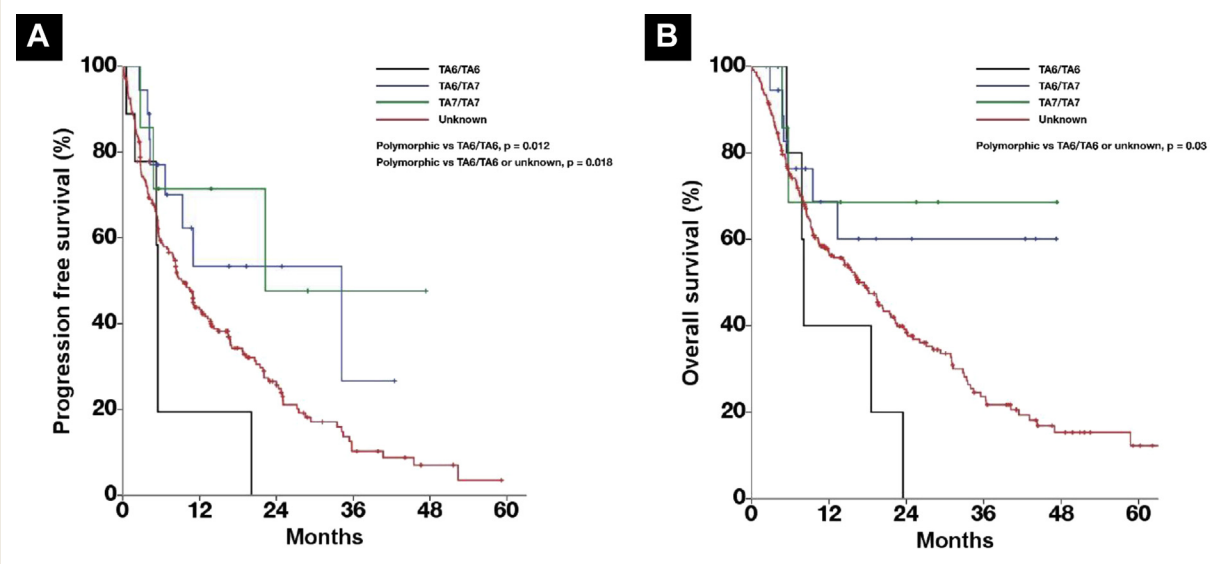
Liver toxicity grade ≥2 resulted in pazopanib interruption until normalization of liver enzymes. The interruption was a median of 23 days (range, 7-320 days). The longest interruption was a median of 75 days (range, 13-320 days) for the TA7/TA7 patients compared with a median of 22 days (range, 7-215 days) for the TA6/TA7 patients and 28 days (range, 14-57 days) for the TA6/TA6 patients (Table 2). Patients with *UGT1A1* polymorphism safely resumed pazopanib at very low doses determined by the

Table 1 Clinical and Histopathologic Baseline Patient Characteristics (n = 261)

Characteristic	*UGT1A1* Status, n (%)			
	TA6/TA6 (n = 9)	TA6/TA7 (n = 18)	TA7/TA7 (n = 7)	Unknown (n = 227)
Gender				
Female	4 (44)	8 (44)	4 (57)	58 (26)
Male	5 (56)	10 (56)	3 (43)	169 (74)
Age, y				
Median	73	71	65	67
Range	65-79	56-87	48-82	40-96
IMDC risk				
Favorable	0 (0)	5 (28)	2 (29)	36 (16)
Intermediate	8 (89)	9 (50)	5 (71)	93 (41)
Poor	1 (11)	4 (22)	0 (0)	71 (31)
Missing	0 (0)	0 (0)	0 (0)	27 (12)
Previous nephrectomy	4 (44)	13 (72)	6 (86)	170 (75)
Clear cell histologic type	8 (89)	16 (89)	6 (86)	205 (90)
Metastatic location				
Brain	0 (0)	0 (0)	2 (29)	10 (4)
Liver	2 (22)	4 (22)	1 (14)	30 (13)
Lung/pleura	4 (44)	6 (33)	4 (57)	135 (60)
Bone	3 (33)	8 (44)	1 (14)	88 (39)
Lymph node	6 (67)	9 (50)	4 (57)	112 (49)

Abbreviations: IMDC = International Metastatic Renal Cell Carcinoma Database Consortium; *UGT1A1* = uridine diphosphate glucuronosyltransferase 1A1.

Figure 1 *UGT1A1* (Uridine Diphosphate Glucuronosyltransferase 1A1) Polymorphisms Are Associated With Improved Outcomes: (A) Progression-Free Survival and (B) Overall Survival



degree of liver toxicity and *UGT1A1* polymorphism. The largest dose reductions were required for patients with TA7/TA7 to a mean daily dose of 167 mg compared with 217 mg for TA6/TA7 patients and 329 mg for TA6/TA6 patients (Table 2). None of the patients died of liver toxicity.

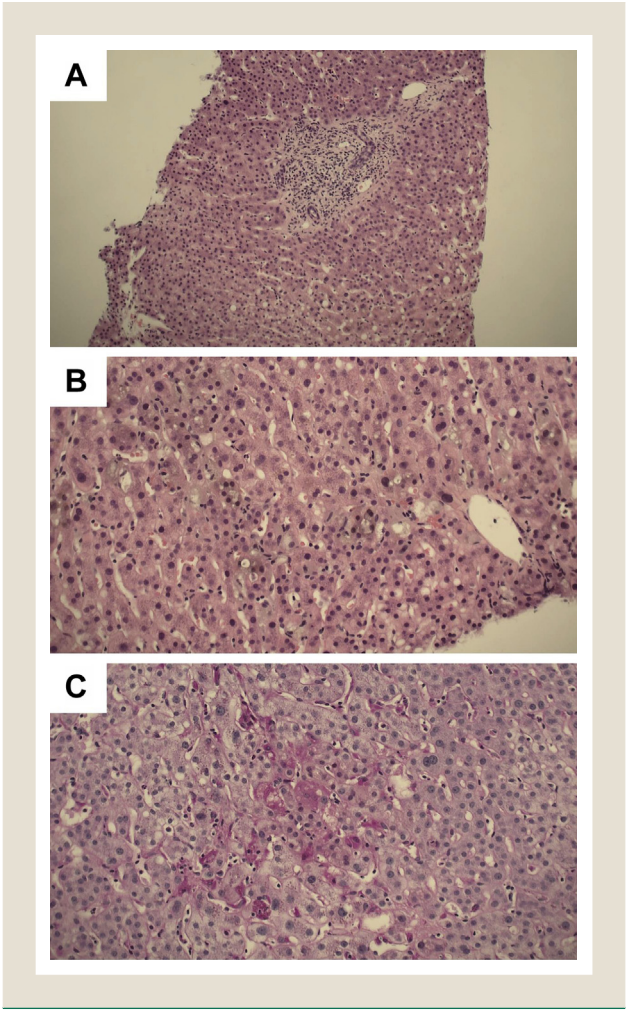
Of the 34 patients, 4 (12%) had been admitted to the hospital because of liver toxicity and 3 (9%) had been treated with prednisolone (median dose, 50 mg; range, 25-100 mg). One patient with grade 4 elevated bilirubin levels had undergone a liver biopsy (Figure 2), which demonstrated cholestatic hepatitis. The patient

Table 2 Liver Toxicity and Pazopanib Dose Stratified by *UGT1A1* Status

Characteristic	*UGT1A1* Status, n (%)		
	TA6/TA6 (n = 9)	TA6/TA7 (n = 18)	TA7/TA7 (n = 7)
Interval to elevated liver enzymes, d			
Median	30	28	29
Range	7-155	20-84	5-57
Maximum ALT			
Grade 1	2 (22)	1 (6)	2 (29)
Grade 2	1 (11)	5 (28)	2 (29)
Grade 3	3 (33)	11 (61)	2 (29)
Grade 4	1 (11)	0 (0)	0 (0)
Maximum bilirubin			
Grade 1	0 (0)	0 (0)	2 (29)
Grade 2	0 (0)	1 (6)	0 (0)
Grade 3	0 (0)	1 (6)	0 (0)
Grade 4	0 (0)	1 (6)	0 (0)
Length of pazopanib interruption, d			
Median	28	22	75
Range	14-57	7-215	13-320
Mean pazopanib dose before interruption, mg	511	467	514
Mean pazopanib dose after interruption, mg	329	217	167

Abbreviations: ALT = alanine aminotransferase; *UGT1A1* = uridine diphosphate glucuronosyltransferase 1A1.

Figure 2 Pazopanib-Induced Cholestatic Hepatitis. (A) Liver Needle Biopsy Showing Expanded Portal Tract With Mild Inflammation and Lymphocytes, Together With Scattered Neutrophil and Eosinophil Granulocytes. Parenchyma Shows Mild Regenerative Changes With Hepatocyte Disarray (Hematoxylin and Eosin Stain, Original Magnification ×300). (B) Higher Power View Showing a Central Vein to the Right. The Surrounding Hepatic Parenchyma Have Marked Cholestasis With Only Slight Accompanying Lobular Inflammation and a Lively Macrophage Reaction (Hematoxylin and Eosin Stain, Original Magnification ×500). (C) Central Areas Showing Cholestasis With a Marked Macrophage Reaction—Evidence of Previous Hepatitis With Healing (Periodic Acid-Schiff Diastase Stain, Original Magnification ×500)



responded to prednisolone 100 mg daily, had required pazopanib interruption for 215 days until the liver test results had normalized, and had resumed pazopanib at an ultra-low dose of 200 mg every third day for 3 months. The pazopanib dose was increased to 200 mg every second day for the subsequent 8 months, until the patient had declined further therapy. Repeated CT assessments of this patient had shown stable disease. Of the 9 patients with elevated liver enzymes and wild-type genotype TA6/TA6, the diagnostic evaluation demonstrated progressive disease in 7 patients (78%).

Discussion

To the best of our knowledge, the present study is the first to demonstrate *UGT1A1*-guided dosing to manage pazopanib-induced liver toxicity in patients with mRCC. In addition, our study is the first showing improved outcomes when stratified by *UGT1A1* genotyping. Liver toxicity affects a significant proportion of patients treated with pazopanib and most often results in drug interruption and permanent discontinuation. Our results have shown that it is feasible to implement assessment of *UGT1A1* status in daily clinical practice, allowing patients to safely resume pazopanib therapy. Also, improved survival of patients carrying 1 or 2 TA7 alleles was achieved despite the significant dose reductions, with the largest reductions required for TA7/TA7 patients. We were not blinded to the patients' *UGT1A1* status, and some outcome improvement could have resulted from the very low dosing of pazopanib rather than the genetic polymorphism itself. Thus, patients with hepatotoxicity and *UGT1A1* polymorphism should undergo rechallenge with pazopanib.

The PFS for the overall population (9.5 months) was comparable to that in the COMPARZ (pazopanib versus sunitinib in the treatment of locally advanced and/or metastatic renal cell carcinoma) study (sunitinib, 9.5 months; pazopanib, 8.4 months),[12] although many patients had begun treatment at <800 mg/d. Thus, some patients, and patients with *UGT1A1* polymorphism in particular, will benefit from very low dosing and rechallenge with reduced doses of pazopanib when liver toxicity has resolved.

With the genetic status included, this approach expands the Goldilocks dosing principle for patients with mRCC[13] (ie, providing an individual dose for the individual patient to ensure optimal patient outcomes with limited toxicity). Our data suggest that largescale dose reductions are possible, and most likely advisable, for patients with polymorphic *UGT1A1*, an addendum to normal dose reduction practice. The correct resumption dose for patients carrying 1 or 2 TA7 alleles with liver toxicity can be as low as 200 mg every third day. This approach might be applicable to other cancer treatments. Antineoplastic drugs known to be associated with an increased risk of toxicity in the presence of *UGT1A1* polymorphism include irinotecan, nilotinib, sorafenib, and belinostat.[14-17] Thus, further research is warranted.

In contrast to an earlier study,[8] we found only low levels of elevated bilirubin. The bilirubin levels in the patients heterozygous or homozygous for the TA7 allele were similar to those in the patients with wild-type TA6/TA6. Also, the interval to the development of elevated liver enzymes was slightly shorter than previously reported (29 vs. 42 days).[5] This could be explained, in part, by a lower starting dose and differing timing of the biochemical tests, reflecting the real-world setting. We found elevated ALT to be a more frequent sign of liver toxicity than was elevated bilirubin, with no differences between the groups. Therefore, other genes than *UGT1A1* might be involved in pazopanib-induced liver toxicity.

Another proposed mechanism for liver toxicity is polymorphism in the hemochromatosis gene (*HFE* rs2858996 and rs707889 genotypes) or *HLA**B57:01. These observations were from genome

association studies with patients from 2 and 31 clinical trials, respectively, showing statistically significant associations between polymorphisms in the hemochromatosis gene and elevated ALT.[18,19] Additionally, ALT >3 × ULN and ALT >5 × ULN both occurred more often in carriers of the *HLA*-B*57:01 allele than in the noncarriers.[20]

The pazopanib concentration and exposure in plasma is crucial for patient outcomes in the adjuvant and metastatic settings.[21,22] However, plasma exposure has not been linked to the pazopanib prescribing dose, although influenced by other factors (ie, liver and gut enzyme processing).[21,22] Therefore, the assessment of *UGT1A1* polymorphism in patients experiencing liver toxicity is suggested. Moreover, therapeutic drug monitoring of pazopanib might prove even more effective for guiding therapy. A somewhat similar approach has been suggested for irinotecan, a drug also known to be associated with an increased risk of toxicity from *UGT1A1* polymorphism.[14,23] This should be examined further in clinical studies.

An intriguing finding was the high frequency of underlying progressive disease in patients with liver toxicity and the wild-type genetic variant TA6/TA6. We observed modest PFS and OS rates for these patients. Thus, elevated liver enzymes should prompt further clinical investigations to identify rapid tumor progression, allowing for treatment alterations in due time.

The treatment landscape for mRCC has changed in recent years. The approval of combined nivolumab plus ipilimumab for frontline treatment of patients with mRCC and intermediate or poor IMDC risk has led to changes in the recommended regimens.[2] Thus, most of the patients in the current study would have received different treatment today. However, pazopanib could still be used for patients with IMDC favorable risk, patients with autoimmune disease, patients requiring steroids, and those without access to CPI or after CPI failure. Therefore, the findings from the present study are important for mRCC management.

One of the strengths of the present study was the prospective data collection and complete data set for patients treated with first-line pazopanib. The study limitations included the small number of patients with genetic information, the retrospective analysis of the results, and that the prognostic IMDC factors were not evenly distributed among the genetic groups.

Conclusions

UGT1A1 polymorphisms were associated with improved outcomes, despite pazopanib interruption and substantial dose reductions. *UGT1A1* assessment could improve management of pazopanib-induced liver toxicity in patients with mRCC.

Clinical Practice Points

- Pazopanib can induce liver toxicity in patients with mRCC, leading to treatment interruptions and permanent discontinuation.
- Polymorphisms in the gene *UGT1A1* have been associated with a greater probability of liver toxicity.
- *UGT1A1*-guided dosing could be used to manage pazopanib-induced liver toxicity.
- *UGT1A1* polymorphisms were also associated with improved outcomes, despite pazopanib interruption and substantial dose reductions.
- Our study showed a feasible implementation of *UGT1A1* status assessment in daily clinical practice, allowing patients to safely resume treatment with pazopanib.
- To the best of our knowledge, using genetic status to guide treatment dosing has not previously been tested and could also be applicable for other anticancer drugs.

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Disclosure

F.D. has received funding from Pfizer, Novartis, and Ipsen (to the institution) outside the submitted work. The remaining authors declare that they have no competing interests.

Supplemental Data

Supplemental data and table accompanying this article can be found in the online version at https://doi.org/10.1016/j.clgc.2019.09.013.

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Supplemental Methods

UGT1A1 *Analysis*

Validation of the *UGT1A1* TA polymorphism analysis by fragment analysis was performed using 17 randomly chosen DNA samples from the Department of Clinical Biochemistry, Aarhus University Hospital.

DNA Extraction

Genomic DNA was isolated from EDTA-stabilized blood samples, using the Maxwell 16 instrument and the Maxwell 16 Blood DNA purification kit (Promega Biotech AB, Stockholm, Sweden) or the QIAsymhony SP using the DSP DNA Mini Kit (Qiagen AB, Sollentuna, Sweden), according to the manufacturer's instructions. After purification, the concentration of genomic DNA for the validation study was determined in a NanoDrop 2000C spectrophotometer (ThermoFisher Scientific, Waltham, MA).

Primers for Fragment Analysis and Sequencing

Primers (Supplemental Table 1; available in the online) were designed to accommodate the requirements of the different polymerases for PCRs and sequencing using OligoAnalyzer, version 3.1, software available on the Integrated DNA Technologies platform (available at: https://eu.idtdna.com/calc/analyzer).

The human *UGT1A1* gene (NG_033238.1) served as the template, and the nucleotide sequence was obtained from National Center for Biotechnology Information platform (available at: https://www.ncbi.nlm.nih.gov/). Moreover, we assessed primer specificity using the BLAST (basic local alignment search tool) algorithm against the human genome. The forward primer for fragment analysis was labeled with fluorescein amidite (Eurofins MWG Operon, Ebersberg, Germany).

Fragment Analysis

PCR was performed in a 25-μL reaction mixture containing ~40 to 100 ng of genomic DNA, 0.3 μM of each primer, 0.2 mM of dNTPs, 1 × long PCR Enzyme Mix buffer, and 0.625 IU Long PCR Enzyme Mix (ThermoFisher Scientific) at the following temperatures: 94°C for 2 minutes, 34 cycles at 94°C for 20 seconds, 63°C for 30 seconds, and 68°C for 1 minute, with a final extension at 68°C for 1 minute in a Bio-Rad S- or C1000 thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA). After PCR amplification, the purity and yield were visualized and verified by electrophoresis of 2.5 μL of the PCR reaction in a 1.5% agarose gel.

Separation of the fluorescein amidite-labeled PCR fragments using the Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems) with POP7 polymer and mounted with a 50-cm capillary resulted in fragments of 448 to 450 bp, corresponding to 6 and 7 TA repeats, respectively.

A dilution series was performed to verify that diluting the PCR reactions 10 times in water produced robust signal intensities. Accordingly, 0.5 μL of the diluted PCR product was mixed with 14 μL HiDi formamide (Applied Biosystems) and 0.25 μL of the GeneScan 500 ROX dye size standard (35-500 bp; Applied Biosystems). The samples were denatured at 80°C for 2 minutes in a Bio-Rad S- or C1000 thermal cycler (Bio-Rad Laboratories) and placed on ice until analysis. Three positive controls (representing the TA6/TA6, TA6/TA7, and TA7/TA7 genotypes) and a no template control were included in the setup, along with validation or patient samples. Subsequent data analysis was performed using GeneMapper software, version 4.1 (Applied Biosystems). The laboratory participates in external proficiency testing organized by the Reference Institute for Bioanalytics. The *UGT1A1* TA polymorphism analysis was assessed by 2 samples twice yearly.

DNA Sequencing

To validate the fragment analysis protocol for detection of the *UGT1A1* TA polymorphism, we amplified and sequenced a 756- to 758-bp fragment of the promoter, harboring the TA polymorphism using genomic DNA from the 17 validation samples and 2 clinical samples.

The sequencing reactions were ethanol precipitated and diluted in HiDi formamide (Applied Biosystems) and separated using the Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems) with POP7 polymer and a 50-cm capillary.

Statistical Analysis

Statistical analysis was performed using SeqScape software, version 2.7 (Applied Biosystems), using NG_033238.1 as the reference sequence for alignment of the sequence traces.

Supplemental Table 1 **Primers for Assessment of TA Polymorphism in *UGT1A1* Promoter Region**

5′-3′ Sequence	Application	Ta (°C)	Size (bp)[a]
FAM-GCTCCACCTTCTT-TATCTCTGAAAGTGAACT	Fragment analysis	63	448-450
CTAACAAAAGACTCTTT-CACATCCTCCCTTTGGA			
GGTAACACTTGTTGGT-CTGTGGAAATACTA	PCR and sequencing	63	756-758
CATGACATCAAAGCTG-CTTTCTGCCA			
CTTTATCTCTGAAAGT-GAACT	Sequencing	NA	NA

Abbreviations: PCR = polymerase chain reaction; NA = not available; Ta = annealing temperature in PCR reactions.
[a]Sizes correspond to TA6 and TA7 alleles, respectively; however, the fragment analysis protocol will also detect the TA5 and TA8 alleles, if present.