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Contrasting roles of the ABCG2 Q141K variant in prostate cancer

Kathryn M. Sobek^a, Jessica L. Cummings^{a,1}, Dean J. Bacich^{a,2}, and Denise S. O'Keefe^{a,2}

^aDepartment of Urology, University of Pittsburgh School of Medicine Pittsburgh, Pennsylvania, U.S.A

¹Department of Critical Care Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, U.S.A

²Department of Urology, UT Health Science Center, San Antonio, Texas, U.S.A

Abstract

ABCG2 is a membrane transport protein that effluxes growth-promoting molecules, such as folates and dihydrotestosterone, as well as chemotherapeutic agents. Therefore it is important to determine how variants of ABCG2 affect the transporter function in order to determine whether modified treatment regimens may be necessary for patients harboring ABCG2 variants. Previous studies have demonstrated an association between the ABCG2 Q141K variant and overall survival after a prostate cancer diagnosis. We report here that in patients with recurrent prostate cancer, those who carry the ABCG2 Q141K variant had a significantly shorter time to PSA recurrence post-prostatectomy than patients homozygous for wild-type ABCG2 ($P = 0.01$). Transport studies showed that wild-type ABCG2 was able to efflux more folic acid than the Q141K variant ($P < 0.002$), suggesting that retained tumoral folate contributes to the decreased time to PSA recurrence in the Q141K variant patients. In a seemingly conflicting study, it was previously reported that docetaxel-treated Q141K variant prostate cancer patients have a longer survival time. We found this may be due to less efficient docetaxel efflux in cells with the Q141K variant versus wild-type ABCG2. In human prostate cancer tissues, confocal microscopy revealed that all genotypes had a mixture of cytoplasmic and plasma membrane staining, with noticeably less staining in the two homozygous KK patients. In conclusion, the Q141K variant plays contrasting roles in prostate cancer: 1) by decreasing folate efflux, increased intracellular folate levels result in enhanced tumor cell proliferation and therefore time to recurrence decreases; and 2) in patients treated with docetaxel, by decreasing its efflux, intratumoral docetaxel levels and tumor cell drug sensitivity increase and therefore patient survival time increases. Taken together, these data suggest that a patient's ABCG2 genotype may be important when determining a personalized treatment plan.

Corresponding Author: Dr. Denise S. O'Keefe. Department of Urology, School of Medicine, UT Health Science Center. 7703 Floyd Curl Drive, Mail cod 7845, San Antonio, TX 78229. Phone: (210) 562-4098. Fax: (210) 567-6868. OKeefeD@uthscsa.edu.

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Conflict of Interest

The authors declare no conflicts of interest.

Keywords

ABCG2; Docetaxel; Folate; Prostate Cancer; Q141K variant

Introduction

Prostate cancer is the most frequently diagnosed cancer and the second-leading cause of cancer-related deaths in American men.¹ Radical prostatectomy offers excellent long-term cancer control; however approximately one third of patients experience a prostate-specific antigen (PSA) recurrence within a decade.^{2, 3} Freedland et al.⁴ noted the median time from PSA recurrence to prostate cancer death is 16 years. Also, patients in the current era are younger and have longer life expectancies.⁵ On the other hand, some patients experience faster cancer progression and death. The variability of prostate cancer outcomes may not be completely explained by prostate cancer aggressiveness such as Gleason score. One interesting mechanism could be the presence of efflux pumps to adjust intracellular levels of needed materials.

ATP-binding cassette sub-family G member 2 (ABCG2) is one such efflux pump that has been implicated in the multidrug resistance phenotype due to its ability to efflux chemotherapeutics, such as doxorubicin and mitoxantrone.⁶ ABCG2 is a 72 kDa protein that is part of the ATP-binding cassette protein family. It is a half-transporter that functions as a homodimer to efflux many molecules including folates^{7, 8} and dihydrotestosterone.⁹ A nonsynonymous single nucleotide polymorphism, C421A, that substitutes a glutamine for lysine at amino acid position 141 (Q141K), decreases the function of ABCG2, thereby increasing the sensitivity of the cells to chemotherapeutics compared to wild-type ABCG2.^{10–14} The Q141K variant has been reported in up to 14% of the Caucasian population and as high as 35% in Chinese and Japanese populations.^{15, 16} The expression of ABCG2 appears to indicate more aggressive prostate cancer that is potentially resistance to the current standard-of-care chemotherapeutic agents.

ABCG2 variants may be related to survival mechanisms in men with PSA recurrent prostate cancer after radical prostatectomy. The decreased function of the efflux of folate and dihydrotestosterone may provide a selective advantage for cancer cells. Tumor cells divide directly in response to available folate, and it was recently observed that the prostate has a high requirement for folate due to polyamine biosynthesis.¹⁷ Moreover, we previously reported that increased fasting serum folate levels in prostate cancer patients correlates with increased Ki67 index,¹⁸ an indicator of cellular proliferation. Therefore, we hypothesize that increased intracellular folate levels in tumors carrying the Q141K variant play a significant role in driving the proliferation of the malignant cells, thereby decreasing patient survival.

Therefore, the goal of our study was to determine the potential dual roles of the Q141K variant in prostate cancer. To do this, we tested the hypothesis that folate and docetaxel are differentially effluxed by the wild-type ABCG2 and the Q141K variant proteins using both *in vitro* methods and patient samples.

Materials and Methods

ABCG2 Genotyping

DNA was extracted from lymphocytes (obtained from the University of Pittsburgh Health Sciences Tissue Bank under the University of Pittsburgh Institutional Review Board Protocol #970480) with the QIAamp® DNA Micro Kit (Qiagen, Germantown, MD). The Q141K coding site was amplified with the primers Forward (5' - CCATCATTATGTCTCATTAATAATGC) and Reverse (5' - CCTGAATGACCCTGATAATCCG). The PCR was performed with Titanium buffer and Taq (Clontech, Mountain View, CA), 25 µM Forward and Reverse primer, and 10 mM dNTPs. PCR products were digested with *MseI* (NEB, Ipswich, MA) overnight and visualized on a 10% TBE polyacrylamide gel. The CC genotype resulted in 2 bands (34bp and 124bp), the CA genotype resulted in 4 bands (34bp, 44bp, 80bp, and 124bp), and the AA genotype resulted in 3 bands (34bp, 44bp, and 80bp). Forty-one patient samples were analyzed. However, one patient who recurred one month after surgery and thus likely already had disseminated disease, was excluded from analysis.

Tissue Culture

LNCaP and PC3 (obtained from American Type Culture Collection) were maintained in RPMI 1640 medium supplemented with 10% or 5% FBS, respectively, L-glutamine, and penicillin/streptomycin. HEK293 (gift of Dr. Zhou Wang) was maintained in DMEM medium supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Lentiviral Transduction and Site-Directed Mutagenesis

ABCG2 cDNA was excised with *XhoI* and *EcoRI*, from the *pCMV6-AC* plasmid (Origene, Rockville, MD) and sub-cloned with *BamHI* and *EcoRI* into the lentiviral vector, *pLVX-puro* (Clontech, Mountain View, CA). The University of Pittsburgh Cancer Institute Lentiviral Core generated active viral particles that were transduced into the LNCaP, PC3, and HEK293 cell lines. Cells transduced with a GFP lentiviral vector (*pLKO.1-puro-CMV-TurboGFP*) were used as a control. Transduced LNCaP, PC3, and HEK293 cells were selected with puromycin (2 µg/mL, 1 µg/mL, and 3 µg/mL, respectively). The Quikchange XL site directed mutagenesis kit (Stratagene, Agilent Technologies, Santa Clara, CA) was used according to the manufacturer's instructions to generate the Q141K variant. The primers Forward (5' -TGACGGTGAGAGAAAACCTTAAAGTTCTCAGCAGCT) and Reverse (5' -AGCTGCTGAGAACTTTAAGTTTTCTCTCACCGTCAG) were used. The plasmid was sequence-verified. After generation of the selected transduced cell lines, STR PCR technology was used to validate the cell lines.¹⁹ All experiments used mixed clones to avoid effects attributable to specific integration of the transgene.

RNA isolation, DNA isolation and Quantitative PCR

RNA was extracted from cell lines using the Trizol reagent (Invitrogen, Carlsbad, CA). One microgram of RNA was reverse transcribed. DNA was extracted with the Blood and Tissue Micro Kit (Qiagen, Germantown, MD). ABCG2 expression was measured in a PCR reaction

containing iQTM Supermix (Bio-Rad, Hercules, CA), 500 nM of the forward primer (5'-CATTGCATCTTGGCTGTCAT), 500 nM of the reverse primer (5'-GTCCTGGGCAGAAAGTTTTGT), 500 nM of the probe ([FAM]-CCACGATATGGATTTACGGCTTTGC-[BHQ1]), and 2 µL of cDNA. GusB was used as a reference gene. Each GusB PCR reaction contained iQTM Supermix (BioRad), 500 nM of the forward primer (5'-CTCATTTGGAATTTTGCCGATT), 500 nM of the reverse primer (5'-CCGAGTGAAGATCCCCCTTTT), 500 nM of the probe ([FAM]-TGAACAGTCACCGACGAGAGTGCT-[BHQ1]), and 2 µL of cDNA. Each primer and probe set was optimized to be efficient (ranged from 93.1% to 100.9%). Samples were run in duplicate on an iQ5 thermocycler (Bio-Rad, Hercules, CA) and analyzed using the Ct method.

Western blotting

Protein was extracted with RIPA buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris, pH 8.0, 0.1% SDS, 1 mM EDTA-free protease inhibitor). Lysates were electrophoresed on a 10% polyacrylamide Tris gel, transferred to a PDVF membrane and probed with the ABCG2 antibody BXP-21 (Millipore, Billerica, MA) or β-actin (Sigma-Aldrich, St. Louis, MO).

miRNA expression

A modified protocol for stem-loop RT-qPCR for miRNA analysis from Current Protocols in Molecular Biology ²⁰ and Chen et al. ²¹ was used. Briefly, 100 µM of reverse transcription primers (primers from Li et al. ²²: miR-519c RT: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACATCCTC, miR-520h RT: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACACTCTA, and U74: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACAATTGT) were stem-looped. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). One microgram of RNA was reverse transcribed (RT). A reverse transcriptase negative control was used for each primer and RNA sample. qPCR reactions were performed containing 1X iQTM SYBR® Green Supermix (Bio-Rad, Hercules, CA), 1.5 µM of the specific miRNA forward primer (miR-519c: 5'-GGCGGGAAAGTGCATCTTTT, miR-520h: 5'-GGCGACAAAGTGCTTCCCTT, and U74: 5'-CCTGTGGAGTTGATCCTAGTCTGGGTG), 0.7 µM of the universal reverse primer (5'-GTGCAGGGTCCGAGGT), and 0.67 µL of RT product.

Tritiated Folic Acid Transport Assay

The transport assay protocol was modified from Hamid et al. ²³ Cells were grown to approximately 80% confluency, counted by hemocytometer, and aliquoted in triplicate or quadruplicate at 1×10^6 cells/tube. Cells were incubated in incubation solution pH 5.5 (10 mM HEPES, 10 mM Tris, 80 mM mannitol, 100 mM MES) for 30 m. Cells were incubated for 30 m in ³H-folic acid (Moravak Biochemicals, Brea, CA) at 37°C and 5% CO₂. The reaction was stopped with ice-cold stop solution pH 7.4 (280 mM Mannitol, 20 mM Tris, 20 mM HEPES) and rinsed four times with cold PBS. Cells were added to scintillation fluid and retained tritiated folic acid was measured on a Wallac 1409 DSA Liquid Scintillation Counter (Perkin-Elmer, Waltham, MA).

Serum Folate Assay

Serum folate levels were determined using the microbiological folate assay, as described in Tomaszewski et al.¹⁸

Cytotoxicity Assay

To determine the IC₅₀ value of docetaxel, an MTT assay was performed. Briefly, 5 000 cells/well were plated in a 96 well dish 24 h prior to the addition of docetaxel. Docetaxel (Sigma-Aldrich, St. Louis, MO) ranging from 0–100 nM was added to the cells and grown at 37°C and 5% CO₂. To determine the role that ABCG2 plays in docetaxel efflux, 1 µM Ko143 (Ko143 concentrations over 1 µM do not specifically inhibit ABCG2²⁴) was added to the ABCG2 expressing cell lines for 15 m before the docetaxel was added. After 96 h, fresh media and 10 µL of 5mg/mL of MTT reagent A (BioExpress, Kaysville, UT) were added. After four hours the media was removed and 100 µL of DMSO was added. Plates were read on a SpectraMax M2^e plate reader at a wavelength of 540 nm (Molecular Devices, Sunnyvale, CA).

Immunofluorescence

Fresh frozen Gleason 7 prostate cancer tumors were embedded in O.C.T. compound (Tissue-Tek, Sakura Finetek, Torrance, CA) and eight micron sections were placed on glass slides. 293-GFP, 293-ABCG2, and 293-Q141K were grown in chamber slides to confluency. Tissues were rehydrated with PBS or the cells were washed with PBS before fixation with 2% paraformaldehyde and permeabilization with 0.1% Triton X-100. Samples were blocked in 2% BSA before a two hour incubation in the ABCG2 antibody (Millipore, Billerica, MA) (clone BXP-21, 1:100 dilution) and a one hour incubation in the Alexa Fluor 488 secondary antibody. Nuclei were stained with 0.5 µg/mL (cells) or 0.75 µg/mL (tissues) of Hoechst 33342 (Sigma-Aldrich, St. Louis, MO). Slides were coverslipped with Fluorescent Mounting Medium (Dako, Santa Clara, CA) and imaged on an Olympus FV1000 Confocal Microscope (Shinjuku, Tokyo, Japan).

Statistics

SigmaPlot Version 12 (Systat Software Inc., San Jose, CA) was used for statistical analyses. PSA recurrence was analyzed by a Kaplan-Meier survival analysis. The tritiated folic acid transport and docetaxel cytotoxicity was analyzed by one-way ANOVAs for repeated measures, and adjusted for pair-wise multiple-comparisons using the Holm-Sidak method. A t-test was used to analyze the serum folate concentration and genotype experiment.

Results

The Q141K variant decreases time to PSA recurrence in prostate cancer patients

A previous study showed that androgen independent prostate cancer patients with the Q141K variant have a shorter overall survival time than patients with wild-type ABCG2. We took a slightly different approach and examined the effect wild-type and variant ABCG2 had on time to PSA recurrence in prostate cancer patients. Forty-one prostate cancer patients were genotyped at the ABCG2 C421A locus. This cohort of prostate cancer patients had

biochemical recurrence after a prostatectomy but received no drug therapy. The allelic frequencies were comparable to that expected in the Caucasian population^{14, 15, 25, 26} (i.e. 17% of patients were heterozygous/encoding the QK variant protein and 80% of patients were wild-type/encoding the QQ protein). Patients with the Q141K variant had a significantly shorter time to PSA recurrence post-prostatectomy than wild-type patients (mean time to PSA recurrence: 15.5 months vs. 51 months, $P=0.01$) (Fig. 1). This finding suggests an important biological role for ABCG2 in tumors that have the capacity to cause recurrent disease.

Expression of exogenous ABCG2 in prostate cancer cell lines causes a selective disadvantage

Prostate cancer cell line models were generated to determine whether the shorter time to PSA recurrence in the Q141K variant patients was due to an increased retained folic acid. ABCG2 protein expression in the prostate cancer cell lines, LNCaP and PC3 was undetectable by western blot. Therefore we transduced the LNCaP and PC3 cell lines with lentivirus expressing either ABCG2 (*pLVX-ABCG2*) or a control (*pLVX-GFP*). Despite selection, expression of the ABCG2 protein in the transduced prostate cancer cell lines was undetectable (Fig. 2a). The LNCaP-ABCG2 cell line grew poorly, and further analysis on this cell line could not be conducted.

Because the prostate cancer cell lines expressing ABCG2 were important for our studies, we investigated why the ABCG2 protein was undetectable in the transduced cell lines. Expression of *ABCG2* mRNA in the PC3-ABCG2 cell line was approximately 280× greater than in the PC3-GFP cell line (Fig. 2b). This confirmed that the *ABCG2* cDNA in the lentiviral vector was being transcribed but that a pre- or post-translational mechanism was repressing the ABCG2 protein.

The ABCG2 cDNA used for this study contained part of the 3'-UTR. A literature search revealed that two known miRNA (miR) recognition sites in the 3'-UTR repress ABCG2 (miR-519c in the MCF-7 breast cancer cell line²² and miR-520h in the PANC-1 pancreatic cancer cell line²⁷). Stem loop RT-qPCR revealed that the expression of miR-519c was increased in the PC3-ABCG2 cell lines relative to the PC3-GFP cell line, but there was no significant expression of miR-520h (Fig. 2c and 2d). After deletion of the miR recognition sites from the 3'-UTR region of the plasmid, the LNCaP and PC3 cell lines were transduced again and named LNCaP-delABCG2 and PC3-delABCG2, respectively. ABCG2 protein expression was detected, albeit at levels much lower than in the 293-ABCG2 cell line (HEK293 cells transduced with the *pLVX-ABCG2* vector to be used as a positive control for ABCG2 expression) (Fig. 2e). This data demonstrated for the first time that ABCG2 in prostate cancer cell lines may also be regulated by miR-519c and/or -520h.

After several passages of the PC3-delABCG2 and LNCaP-delABCG2 cell lines, ABCG2 protein expression decreased in PC3-delABCG2 and was undetectable in LNCaP-delABCG2, despite continued selection for the lentiviral vector (Supplemental Fig. 1). Unfortunately, after a rigorous effort to express ABCG2 in these prostate cancer cells, further analysis could not be conducted on these cell lines. This data suggests that ABCG2

confers a selective disadvantage for prostate cancer cell lines, regardless of androgen receptor status (the PC3 cell line is AR negative, and the LNCaP cell line is AR positive).

Exogenous wild-type and Q141K variant ABCG2 proteins in HEK293 cells are similarly expressed and functional

Despite the selective disadvantage of expressing ABCG2 in the prostate cancer cell lines, we were still very interested in determining whether the Q141K variant differentially effluxes folic acid and docetaxel compared to wild-type ABCG2. Therefore, we utilized HEK293, a cell line that readily expresses exogenous proteins. We transduced the HEK293 cell line (endogenous ABCG2 protein is undetectable) with either the lentiviral vector for wild-type ABCG2, the Q141K variant, or the control (293-ABCG2, 293-Q141K, and 293-GFP, respectively). We did not detect a significant difference between the mRNA expression in the wild-type and Q141K variant by quantitative real-time PCR (Fig. 3a). The wild-type and Q141K variant proteins were expressed at similar levels (Fig. 3b). The functionality of the exogenous ABCG2 protein was confirmed by a Hoechst 33342 efflux assay (Supplemental Fig. 2).

The Q141K variant effluxes folates less efficiently than wild-type ABCG2 in vitro and in vivo

To determine whether the Q141K variant affects the efflux of folic acid, tritiated folic acid transport assays were performed. As expected the 293-ABCG2 cells retained 34% less folic acid than the 293-GFP cells (Fig. 4a, $P < 0.001$), while the 293-Q141K cells retained 18% less tritiated folic acid compared to the 293-GFP control cells ($P < 0.001$). Addition of Ko143 abolished the effect in both cell lines. While it was already known that ABCG2 is able to efflux folic acid, we observed a statistically significant difference in efflux between the wild-type and Q141K variant-expressing cells ($P = 0.002$), demonstrating for the first time that the Q141K variant decreases folic acid efflux.

Our laboratory previously reported that the serum folate levels in prostate cancer patients were positively correlated with tumor cell proliferation.¹⁸ ABCG2 is expressed in the liver, particularly the bile canalicular membrane. Given this, we hypothesized that the ABCG2 genotype at nucleotide 421 may be correlated with serum folate levels based on our *in vitro* observations (Fig. 4a). We observed significantly lower serum folate levels in the Q141K variant patients than in wild-type patients (Fig. 4b, $P = 0.029$). This finding suggests that the Q141K variant patients efflux folate less efficiently into the bile for distribution via the enterohepatic cycle, which is known to contribute to 40–60% of serum folate.²⁸ This observation indicates that ABCG2 may play an important role in regulating folate homeostasis. Taken together, we observed that the Q141K variant decreased folate efflux both *in vitro* and *in vivo*.

Expression of wild-type ABCG2 confers docetaxel resistance and the Q141K variant confers partial resistance

A cytotoxicity assay was performed to test whether ABCG2 effluxes docetaxel and to determine if expression of the Q141K variant would affect the efflux of docetaxel. As shown in Figure 5, wild-type ABCG2 cells showed enhanced resistance to docetaxel relative to the

control cells ($IC_{50} = 9.96$ nM vs. 4.50 nM, $P = 0.001$). Inhibition of ABCG2 by Ko143 abrogated the growth advantage in docetaxel ($IC_{50} = 9.96$ nM vs. 3.69 nM; $P = 0.007$). Compared to the control cells, the Q141K variant was significantly more resistant to docetaxel ($IC_{50} = 7.51$ nM, $P = 0.011$), but was less resistant than the wild-type cells. Taken together, we concluded that wild-type ABCG2 can efflux docetaxel leading to a resistant phenotype. The Q141K variant can efflux docetaxel, which conferred a partial resistance to docetaxel.

The Q141K variant and wild-type ABCG2 are differentially localized in vitro and in vivo

We hypothesized that the wild-type ABCG2 effluxed folate and docetaxel more efficiently than the Q141K variant because the two proteins were differentially localized. It has been reported that wild-type ABCG2 protein is predominantly localized at the plasma membrane, and the Q141K variant is localized intracellularly.^{10, 12, 13, 29, 30} We determined that the wild-type protein was predominantly at the plasma membrane, whereas the Q141K variant protein was dispersed throughout the intracellular compartment as well as on the plasma membrane (Fig. 6a). These findings were consistent with previous reports and confirmed our hypothesis.

We also wanted to determine if the wild-type ABCG2 protein and the Q141K variant were differentially localized in prostate cancer patient tissue. In a cohort of fifty-five Gleason 7 prostate cancer patients, 25% expressed the variant allele (CA or AA). Two of these patients were homozygous (AA). All genotypes had a mixture of cytoplasmic and plasma membrane staining (Fig. 6b). There were no observable differences in localization or staining frequency and intensity between the wild-type homozygotes (CC) and heterozygotes (CA). However, ABCG2 staining intensity and frequency in the two homozygous variant (AA) patient samples was noticeably decreased at the same exposure time. Of note, we detected nuclear localization of ABCG2 for all genotypes (Fig. 6b, arrows), which has been reported in glioblastoma multiforme cells³¹ but not in normal or cancerous prostate cells.

Discussion

The presence of the Q141K variant appears to have a contradictory role in prostate cancer outcomes. We hypothesized that cells expressing the Q141K variant would retain more folic acid, which would lead high rates of proliferation and shorter survival times. We also hypothesized that cells expressing the Q141K variant would retain more docetaxel, which would lead to increased tumoral drug concentrations and longer survival times.

Our data support the hypothesis that patients carrying the Q141K variant have a shorter time to disease-specific recurrence. This is likely, at least in part, due to the decreased ability of the Q141K variant to efflux folates. It is well established that the proliferation rate of tumor cells in culture is dependent on the availability of folate as it is necessary for DNA synthesis. We previously observed that tumors proliferated on average six times faster in patients with Gleason 7 prostate tumors that have high fasting serum folate levels than those patients with low serum folate levels.¹⁸ In this study, we observed that patients in this cohort carrying the Q141K variant had lower serum folate levels, again providing evidence that the Q141K variant decreased the efflux function of ABCG2. As we have shown here for the first time,

expression of the Q141K variant results in decreased efflux of folic acid and serum folate compared to the wild-type ABCG2. Therefore, a patient with the variant ABCG2 protein may have increased intratumoral folate levels, allowing cancer cells to proliferate faster than cells effluxing folate. As a result of the increased proliferation, the time to PSA recurrence could be shortened in prostate cancer patients expressing the Q141K variant protein.

This study also supports the hypothesis that wild-type cells are significantly more resistant to docetaxel than control cells. This effect is specific to ABCG2 because an ABCG2 inhibitor abolished it. Furthermore, the presence of the Q141K variant appears to increase the sensitivity of the cell to docetaxel. Thus, expression of the Q141K variant would affect the clinical response to docetaxel. Since inhibitors to ABCG2 are currently being designed to prevent efflux of chemotherapeutics out of cancer cells, it is important to know that patients carrying the Q141K variant will not efflux these drugs as efficiently. Therefore, knowledge of a patient's ABCG2 genotype at this locus would be important, as patient's harboring the variant may require modified therapeutic treatment regimens.

The mechanism responsible for the differential efflux of substrates by ABCG2 and the Q141K variant is unclear.^{10, 12–14, 29, 30, 32–37} Furukawa et al.¹⁰ observed that the Q141K variant is more susceptible to ubiquitin-mediated proteasomal degradation and therefore would not be trafficked to the cell membrane. Likewise, we observed that the Q141K variant protein localized to the cytoplasm more often than the wild type protein in both *in vitro* models, which offers one explanation for a decrease in function in the Q141K variant. Others have demonstrated that the function of the ABCG2 Q141K variant is decreased due to disruption at the nucleotide-binding domain.³⁰ The Q141 position contributes to a critical domain-domain interface between the cytosolic nucleotide-binding domain and the transmembrane domain. The Q141K variant leads to instability in the nucleotide-binding domain thus causing decreased steady-state protein levels and functionality. Therefore, expression, localization, and/or structure of the ABCG2 Q141K variant protein may contribute to the inability of the protein to efflux substrates as efficiently as the wild-type protein.

In this study, we sought to decipher the roles that ABCG2 and the Q141K variant play in prostate cancer after the publication of two studies that at first glance, appear to be contradictory: one study showing that Q141K variant prostate cancer patients have a shorter overall survival time than wild-type patients, and the second demonstrating that docetaxel-treated prostate cancer patients who were heterozygous for the Q141K variant had a longer survival time than wild-type patients. Our study supports both findings, but also provides evidence that Q141K tumors likely have a shorter time to PSA recurrence due to this variant effluxing folic acid less efficiently than wild-type ABCG2. Moreover, in mechanistic support of the second study, we demonstrate that the Q141K variant does not efflux docetaxel as efficiently as wild-type ABCG2. Taken together, these findings support a dual role for the Q141K ABCG2 variant in prostate cancer: 1) the Q141K variant increased time to PSA recurrence by retaining folate, and 2) the Q141K variant may increase survival times in docetaxel-treated prostate cancer patients. Modulation of ABCG2 expression and/or function at different stages of prostate cancer, especially with regard to patient genotype, warrants further investigation as the clinic moves towards personalized therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlight

- The presence of ABCG2 Q141K variant decreases time to PSA recurrence.
- Cells expressing the Q141K variant retain more folic acid than wild type.
- Cells expressing the Q141K variant are more sensitive to docetaxel.
- ABCG2 protein is repressed miR-519c and/or miR-520h in prostate cancer cell lines.

Figure 1. Q141K variant decreases time to PSA recurrence

Prostate cancer patients who had recurred after prostatectomy and received no drug treatment were evaluated for time to PSA. The genotype at the 421 locus that encodes for the Q141K variant was determined by PCR and restriction digest. Patients with the CA genotype (QK variant, $n = 7$) had a significantly shorter time to PSA recurrence than patients with the CC genotype (QQ wild-type, $n = 33$) (mean time to PSA recurrence: 15.5 months vs. 51 months, $P = 0.01$).

Figure 3. Expression and functional validation of wild-type ABCG2 and variant Q141K in HEK293 cells

A. ABCG2 mRNA expression was measured in 293-GFP, 293-ABCG2, and 293-Q141K. There is no significant difference in ABCG2 mRNA expression between the wild-type and variant cell lines. B. A western blot was performed to determine the expression of ABCG2 protein. Protein in 293-GFP was undetectable whereas ABCG2 expression in 293-ABCG2 and 293-Q141K was similar.

Figure 4. Folates are differentially effluxed by the wild-type and variant ABCG2 protein
 A. Radiolabeled folic acid retention was measured in the cell lines 293-GFP, 293-ABCG2, and 293-Q141K. 293-ABCG2 retained 33.7% less folic acid than 293-GFP ($P < 0.001$). 293-Q141K retained 17.8% less folic acid than 293-GFP ($P < 0.001$). 293-Q141K also retained significantly more folic acid than 293-ABCG2 ($P = 0.002$). There were 12 replicates for each cell line. When the ABCG2 specific inhibitor Ko143 was added, the 293-ABCG2 and 293-Q141K cell lines retained more folic acid (21% and 14%, respectively). B. Folate from Gleason 7 prostate cancer patients' serum was measured using a microbiological assay.

Patients with the variant ABCG2 (either QK or KK) have significantly lower serum folate concentrations compared to patients expressing wild-type ABCG2 (*, $P = 0.029$).

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Figure 5. ABCG2 expression increases resistance to docetaxel

An MTT assay was used to determine the IC₅₀ values of 293-GFP, 293-ABCG2, and 293-Q141K treated with docetaxel. 293-ABCG2 was 2.2× more resistant to docetaxel than 293-GFP ($P=0.001$). The wild-type was 1.3× more resistant than the Q141K variant, but this difference was not significant. However, the Q141K variant was 1.7× more resistant than the control ($P=0.011$). By adding Ko143, the resistance to docetaxel observed in the wild-type and variant cell lines was abolished ($P=0.007$ and $P=0.005$, respectively).

Figure 6. Localization of ABCG2 *in vitro* and *in vivo*

A. The 293-ABCG2 and 293-Q141K cell lines were stained with ABCG2 (green) and Hoechst (blue) and imaged by confocal microscopy. The localization of ABCG2 is predominately at the plasma membrane in the 293-ABCG2 cell line and both in the cytoplasm and plasma membrane in the 293-Q141K cell line. B. Prostate cancer tissue was stained with the ABCG2 antibody, BXP-21 and an Alexa Fluor 488 secondary antibody and the nuclei were stained with Hoechst 33342. Tissue from all three genotypes (QQ, QK, and KK) showed ABCG2 (green) staining at the plasma membrane, intracellular, and nuclear. Arrows represent nuclei that contain ABCG2 staining.