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Contrasting roles of the ABCG2 Q141K variant in prostate cancer

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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.

1. 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

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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.

2. Materials and methods

2.1. 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′-CCATCATTATGTCTCATT-AAAATGC) 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 (34 bp and 124 bp), the CA genotype resulted in 4 bands (34 bp, 44 bp, 80 bp, and 124 bp), and the AA genotype resulted in 3 bands (34 bp, 44 bp, and 80 bp). 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.

2.2. 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% CO_2.

2.3. 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′- TGACGGTGAGAGAAAACTTAAAGTTCTCAGCAGCT) and Reverse (5′- AGCTGCTGAGAACTTTAAGTTTTCTCTCACC-GTCAG) 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.

2.4. 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 iQ™ Supermix (Bio-Rad, Hercules, CA), 500 nM of the forward primer (5′-CATTGCATCTTGGCTGTCAT), 500 nM of the reverse primer (5′-GTCCTGGGCAGAAGTTTTGT), 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 iQ™ Supermix (BioRad), 500 nM of the forward primer (5'-CTCATTTGGAATTTTGCCGATT), 500 nM of the reverse primer (5′-CCGAGTGAAGATCCCCTTTTT), 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 ΔΔCt method.

2.5. 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).

2.6. 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′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGC-ACTGGATACGACATCCTC, miR-520h RT: 5′-GTCGTATCCAGTGCAG-GGTCCGAGGTATTCGCACTGGATACGACACTCTA, and U74: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAATT-GT) 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 iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA), 1.5 μM of the specific miRNA forward primer (miR-519c: 5′-GGCGGGAAAGTGCATCTTTTT, miR-520h: 5′-GGCGACAAAGTGCTTCCCTT, and U74: 5′-CCTGTGGAGTTGATC-CTAGTCTGGGTG), 0.7 μM of the universal reverse primer (5′-GTGCAGGGTCCGAGGT), and 0.67 μL of RT product.

2.7. 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 ^{3}H-folic acid (Moravek Biochemicals, Brea, CA) at 37 °C and 5% CO_2. 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).

2.8. Serum folate assay

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