

2016

ABCB5-Targeted chemoresistance reversal inhibits merkel cell carcinoma growth

Sonja Kleffel

Nayoung Lee

Cecilia Lezcano

Brian Wilson

Kristine Sobolewski

See next page for additional authors

Follow this and additional works at: <https://ro.ecu.edu.au/ecuworkspost2013>



Part of the [Oncology Commons](#)

[10.1016/j.jid.2015.12.038](https://ro.ecu.edu.au/ecuworkspost2013/2041)

Kleffel, S., Lee, N., Lezcano, C., Wilson, B. J., Sobolewski, K., Saab, K. R., ... & DoRosario, A. (2016). ABCB5-Targeted Chemoresistance Reversal Inhibits Merkel Cell Carcinoma Growth. *Journal of Investigative Dermatology*, 136(4), 838-846. Available [here](#)

This Journal Article is posted at Research Online.

<https://ro.ecu.edu.au/ecuworkspost2013/2041>

Authors

Sonja Kleffel, Nayoung Lee, Cecilia Lezcano, Brian Wilson, Kristine Sobolewski, Karim Saab, Hansgeorg Mueller, Qian Zhan, Christian Posch, Christopher Elco, Andrew DoRosario, Sarah Garcia, Manisha Thakuria, Yaoyu Wang, Linda Wang, George Murphy, Markus Frank, and Tobias Schatton

ABCB5-Targeted Chemoresistance Reversal Inhibits Merkel Cell Carcinoma Growth



JID Open

Sonja Kleffel¹, Nayoung Lee¹, Cecilia Lezcano², Brian J. Wilson³, Kristine Sobolewski¹, Karim R. Saab³, Hansgeorg Mueller¹, Qian Zhan², Christian Posch¹, Christopher P. Elco^{1,2}, Andrew DoRosario⁴, Sarah S. Garcia^{1,4}, Manisha Thakuria^{1,4}, Yaoyu E. Wang⁵, Linda C. Wang^{1,4}, George F. Murphy², Markus H. Frank^{1,3,6} and Tobias Schatton^{1,3}

Merkel cell carcinoma (MCC) is a highly aggressive neuroendocrine skin cancer with profound but poorly understood resistance to chemotherapy, which poses a significant barrier to clinical MCC treatment. Here we show that ATP-binding cassette member B5 (ABCB5) confers resistance to standard-of-care MCC chemotherapeutic agents and provide proof-of-principle that ABCB5 blockade can inhibit human MCC tumor growth through sensitization to drug-induced cell cytotoxicity. ABCB5 expression was detected in both established MCC lines and clinical MCC specimens at levels significantly higher than those in normal skin. Carboplatin- and etoposide-resistant MCC cell lines exhibited increased expression of ABCB5, along with enhanced ABCB1 and ABCC3 transcript expression. ABCB5-expressing MCC cells in heterogeneous cancers preferentially survived treatment with carboplatin and etoposide *in vitro* and in human MCC xenograft-bearing mice *in vivo*. Moreover, patients with MCC also exhibited enhanced ABCB5 positivity after carboplatin- and etoposide-based chemotherapy, pointing to clinical significance of this chemoresistance mechanism. Importantly, ABCB5 blockade reversed MCC drug resistance and impaired tumor growth in xenotransplantation models *in vivo*. Our results establish ABCB5 as a chemoresistance mechanism in MCC and suggest utility of this molecular target for improved MCC therapy.

Journal of Investigative Dermatology (2016) 136, 838–846; doi:10.1016/j.jid.2015.12.038

INTRODUCTION

Merkel cell carcinoma (MCC) is a highly aggressive neuroendocrine cancer of the skin that, on a case-by-case basis, is more deadly than melanoma (Poulsen, 2004). Although MCC is relatively rare, its incidence has tripled over the past two decades and continues to rise by 8% annually (Hodgson, 2005). Current treatment modalities are local excision surgery of primary lesions and chemo- and radiation therapy of metastatic disease (Eng et al., 2007). Although combination therapy with carboplatin and etoposide, the first-line chemotherapeutic agents utilized in advanced-stage MCC, yields initial response rates of up to 60%, most patients experience

disease relapse, usually with fatal outcomes (Tai et al., 2000). Because of the limited availability of human MCC cell lines and patient samples from this rare form of cancer (Becker, 2010), mechanisms of MCC chemotherapy resistance are largely understudied, and treatment of patients with advanced disease poses a significant challenge. Thus, elucidating the mechanisms underlying MCC therapeutic resistance is of critical importance for improving patient survival.

We have previously cloned and characterized ATP-binding cassette member B5 (ABCB5) (Frank et al., 2003, 2005, 2011; Ksander et al., 2014; Lin et al., 2013; Schatton et al., 2008, 2010, 2015; Wilson et al., 2011, 2014), which has been shown to serve as a clinically relevant multidrug resistance mediator in human malignant melanoma (Chartrain et al., 2012; Frank et al., 2005; Wilson et al., 2014), colorectal cancer (Wilson et al., 2011), and hepatocellular carcinoma (Cheung et al., 2011). Moreover, ABCB5 expression correlates with tumor virulence and clinical cancer progression in these malignancies (Cheung et al., 2011; Gazzaniga et al., 2010; Schatton et al., 2008; Setia et al., 2012; Sharma et al., 2010; Wilson et al., 2011). Given its role in multidrug resistance of multiple malignancies, we hypothesized that ABCB5 might also identify therapy-refractory tumor populations in MCC.

The herein reported results establish ABCB5 expression in MCC and show that ABCB5 marks therapy-refractory tumor subpopulations after standard-of-care carboplatin- and etoposide-based combination chemotherapy in patients with MCC. Similarly, in MCC xenotransplantation models, ABCB5⁺ tumor cells also preferentially survive carboplatin- and etoposide-induced cytotoxicity. Moreover, antibody-mediated

¹Harvard Skin Disease Research Center, Department of Dermatology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA; ²Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA; ³Department of Medicine, Boston Children's Hospital, Harvard Medical School, Boston, Massachusetts, USA; ⁴Dana Farber/Brigham and Women's Cancer Center, Harvard Medical School, Boston, Massachusetts, USA; ⁵Center for Cancer Computational Biology, Dana Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts, USA; and ⁶School of Medical Sciences, Edith Cowan University, Joondalup, WA, Australia

Correspondence: Tobias Schatton, Department of Dermatology, Brigham and Women's Hospital, Harvard Institutes of Medicine, Rm. 673C, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115, USA. E-mail: tschatton@bwh.harvard.edu or Markus H. Frank, Boston Children's Hospital, Enders Research Building, Rm. 507, 300 Longwood Avenue, Boston, Massachusetts 02115, USA. E-mail: markus.frank@childrens.harvard.edu

Abbreviations: ABCB5, ATP-binding cassette member B5; MCC, Merkel cell carcinoma; NSG, non-obese diabetic/severely combined immunodeficiency (NOD/SCID) IL-2Rγ^{-/-}

Received 23 April 2015; revised 9 December 2015; accepted 28 December 2015; accepted manuscript published online 29 January 2016

ABCB5 blockade sensitizes MCC cells to carboplatin- and etoposide-mediated cell killing concomitant with significantly enhanced inhibition of MCC xenograft growth.

RESULTS AND DISCUSSION

In healthy human skin, ABCB5 is expressed only on rare subsets of cells (Frank et al., 2003; Schatton et al., 2015). Similarly, in human malignant melanoma, ABCB5 expression is confined to relatively rare tumorigenic minority populations (Schatton et al., 2008). Clinical human MCC specimens obtained at various stages of disease progression (n = 85), on the other hand, demonstrated marked ABCB5 membrane expression by cytokeratin 20-positive MCC cells (Figure 1a). Although cell-cell membrane apposition made it difficult to enumerate numbers of positive cells

and specimens displayed heterogeneity for ABCB5 expression, ABCB5 immunoreactivity was typically observed in the majority of tumor cells. Supplementary Table S1 online summarizes the clinical parameters for all MCC biopsy specimens analyzed. Aggregate quantitative reverse transcription polymerase chain reaction (RT-PCR)-based analysis of all tissue specimens showed significantly higher ($P < 0.001$) ABCB5 mRNA expression in biopsies from patients with MCC (n = 85) compared with normal human skin (n = 10) (Figure 1b). Based on the previously described correlation of ABCB5 frequency with chemotherapeutic refractoriness in cancer patients (Chartrain et al., 2012; Cheung et al., 2011; Wilson et al., 2011), we next examined ABCB5 expression levels in MCC samples obtained before and after first-line chemotherapy from three patients afflicted by this

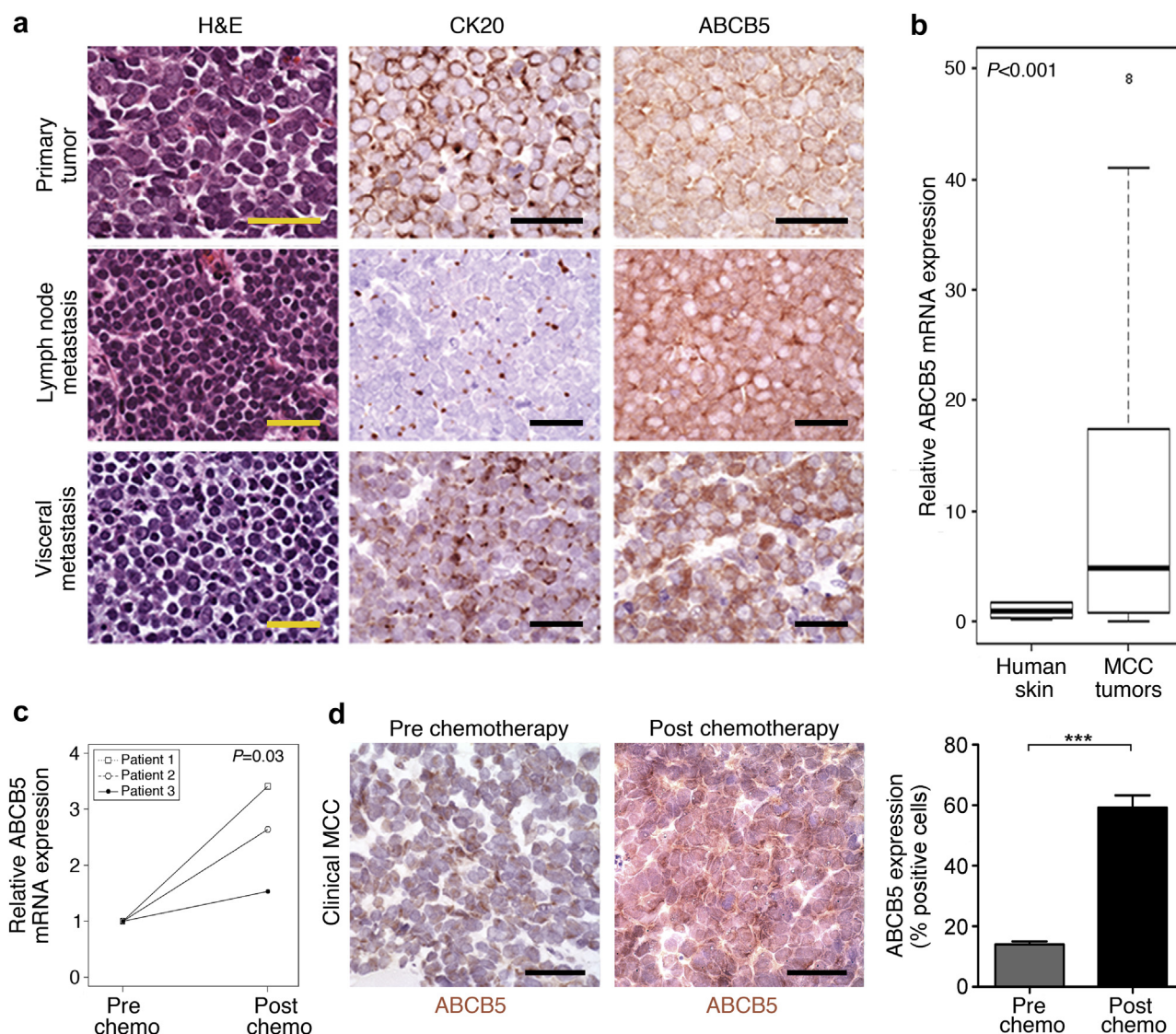


Figure 1. ABCB5 expression in patients with MCC before and after first-line chemotherapy. (a) Representative H&E, CK20, and ABCB5 immunohistochemistry of a primary MCC tumor, a lymph node and visceral metastasis. (b) Relative ABCB5 mRNA expression in normal human skin (n = 10) versus clinical MCC specimens (n = 85), as determined by quantitative RT-PCR. (c) Relative ABCB5 mRNA expression and (d) immunohistochemically determined ABCB5 protein expression (mean \pm SEM) by patient-matched pre- and post-chemotherapy MCC biospecimens (n = 3, respectively). Size bars = 50 μ m. (NS: not significant, $***P < 0.001$, as determined by the unpaired (b) or paired (c, d) Student's *t*-test). ABCB5, ATP-binding cassette member B5; CK20, cytokeratin 20; H&E, hematoxylin and eosin; MCC, Merkel cell carcinoma; RT-PCR, reverse transcription polymerase chain reaction; SEM, standard error of the mean.

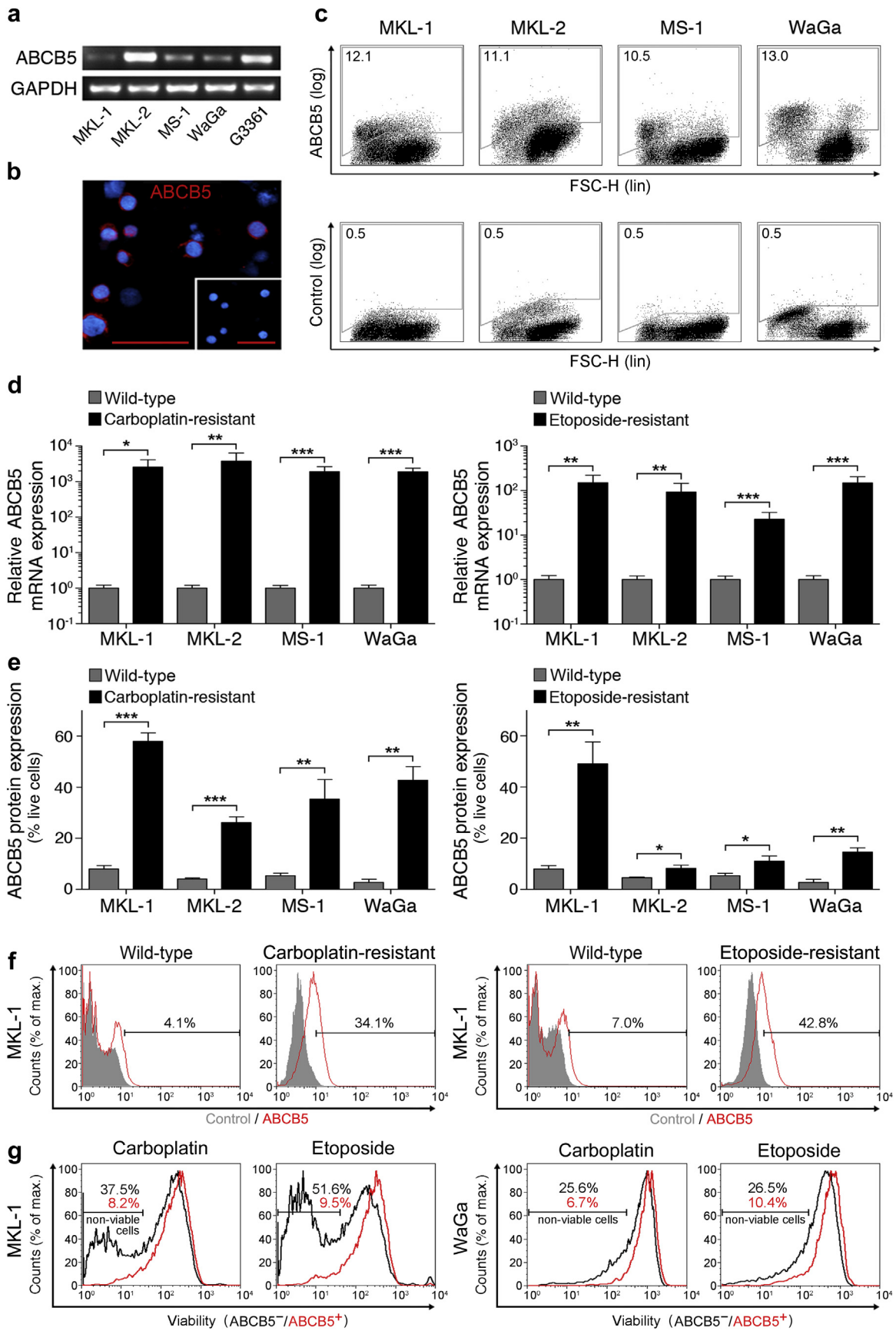


Figure 2. ABCB5 expression in response to in vitro carboplatin and etoposide treatment. (a) ABCB5 mRNA expression by MKL-1, MKL-2, MS-1, and WaGa MCC cell lines, as determined by RT-PCR (positive control: G3361 melanoma cells). (b) Representative ABCB5 immunofluorescence staining (red) of a cytopsin preparation of WaGa cells (inset: isotype control antibody staining). Nuclei are counterstained with 4',6-diamidino-2-phenylindole (blue). Size bars = 50 μ m.

extraordinarily rare orphan disease with availability of this unique biopsy material. Analysis of patient-matched pre- and postchemotherapy MCC specimens revealed significantly increased ABCB5 mRNA expression in postchemotherapy local recurrences compared with prechemotherapy biopsy specimens, both at the mRNA (Figure 1c) and immunoreactive protein levels (cell frequency $59.2 \pm 4.1\%$ vs. $14.0 \pm 1.0\%$, mean \pm standard error of the mean, respectively, $P < 0.001$) (Figure 1d). These findings in patient specimens were consistent with the possibility that ABCB5⁺ MCC cells are preferentially resistant to treatment with the first-line chemotherapeutic agents, carboplatin and etoposide.

On the basis of previous studies implicating ABCB5 expression in conferring chemotherapeutic resistance in several human cancers (Chartrain et al., 2012; Cheung et al., 2011; Frank et al., 2005; Wilson et al., 2011, 2014), we next examined the potential functional contribution of ABCB5 to carboplatin and/or etoposide resistance in MCC. We first demonstrated ABCB5 mRNA expression in the established human MCC cell lines, MKL-1, MKL-2, MS-1, and WaGa (Guastafierro et al., 2013; Houben et al., 2010; Rodig et al., 2012; Rosen et al., 1987), by RT-PCR amplification and sequencing (Figure 2a). All four MCC lines also showed ABCB5 surface protein expression, as determined by immunofluorescence staining (Figure 2b) and by flow cytometric analysis, with ABCB5⁺ cell frequencies (mean \pm standard error of the mean) averaging $10.0 \pm 1.8\%$ for MKL-1, $9.1 \pm 2.4\%$ for MKL-2, $8.3 \pm 1.5\%$ for MS-1, and $16.9 \pm 5.4\%$ for WaGa cells (Figure 2c). To explore the potential role of ABCB5 in MCC refractoriness to first-line chemotherapy, we next investigated ABCB5 expression in control (wild-type) versus MCC lines rendered drug resistant via continuous exposure to carboplatin or etoposide over a 2-month period. First, we confirmed preferential survival of carboplatin- and etoposide-resistant compared with wild-type MCC cells for the respective drugs (Supplementary Figure S1a online). Subsequent quantitative RT-PCR analyses revealed markedly increased ABCB5 mRNA expression levels in both carboplatin- and etoposide-resistant MKL-1, MKL-2, MS-1, and WaGa lines compared with the respective wild-type cell lines (Figure 2d). At the protein level, exposure to cytotoxic levels of carboplatin or etoposide resulted in significantly increased ABCB5 expression among viable MKL-1, MKL-2, MS-1, and WaGa cells compared with vehicle-treated controls, respectively (Figure 2e and f). Although the percentage of ABCB5⁺ cells was markedly enhanced in chemorefractory MCC cell lines, we also noted that a significant proportion of carboplatin- and etoposide-resistant cells did not display ABCB5 expression.

To directly demonstrate that ABCB5⁺ tumor cell subsets preferentially survive carboplatin- and etoposide-induced

cytotoxicity, we compared the viability of ABCB5⁺ versus ABCB5⁻ MKL-1 and WaGa cells grown in the presence of cytotoxic carboplatin or etoposide levels. We found that ABCB5⁺ cells cultured under these conditions demonstrated increased viability compared with ABCB5⁻ MCC populations (Figure 2g), indicating that ABCB5⁺ MCC subsets preferentially survive drug-induced cell killing. However, we cannot entirely exclude the possibility of induction of ABCB5 expression, as opposed to preferential survival. Because other ABC transporters, including ABCB1, ABCC3, and ABCG2, are known mediators of carboplatin and etoposide resistance in other cancers (Dean et al., 2001), we examined whether drug-resistant MCC lines also expressed high levels of these ABC transporters, in addition to ABCB5. With the exception of etoposide-resistant MKL-1 cells, all drug-resistant MCC cell lines examined showed a significant increase in ABCB1 and ABCC3, but not ABCG2 transcript expression compared with the respective wild-type cell lines (Supplementary Figure S1b), raising the possibility that several ABC transporters, in addition to ABCB5, might contribute to chemoresistance in drug-induced MCC cell lines. Together, these results suggested a direct relationship between therapeutic resistance to both carboplatin and etoposide treatment and ABCB5 expression in the MCC lines evaluated, and further suggest the potential contribution of additional ABC transporters (ABCB1 and ABCC3) to MCC chemoresistance.

To explore the potential role of ABCB5 as a carboplatin and/or etoposide resistance mediator in MCC, we evaluated cell viability in MCC cultures exposed to increasing concentrations of carboplatin or etoposide in the presence of an anti-ABCB5 blocking monoclonal antibody (mAb) (Frank et al., 2003, 2005; Ksander et al., 2014; Schatton et al., 2008; Wilson et al., 2014) versus isotype control mAb. ABCB5 blockade reversed carboplatin and etoposide resistance of both MKL-1 and WaGa cells (Figure 3a), resulting in significantly enhanced cell killing at all carboplatin concentrations greater than 1 μM versus controls and at etoposide concentrations as low as 10 nM in MKL-1 cells, and reductions of the LD₅₀ in both MKL-1 (LD₅₀ [carboplatin] 2.3 μM vs. 5.9 μM and LD₅₀ [etoposide] 25.0 nM vs. 62.4 nM, respectively) and WaGa cells (LD₅₀ [carboplatin] 3.1 μM vs. 7.6 μM and LD₅₀ [etoposide] 98.4 nM vs. 130.4 nM, respectively) (Figure 3a). Drug-induced selection for ABCB5⁺ MCC cells (Figure 2d–g) might hereby explain why the ABCB5 blocking mAb mediates carboplatin- and etoposide-induced cytotoxicity in proportions of MCC cells that exceeded those frequencies observed in native cell lines (Figure 2c), especially at higher drug concentrations. Treatment with the anti-ABCB5 mAb alone had no significant effect on in vitro cell survival (Figure 3b), but, in line with

(c) Representative flow cytometry of ABCB5 protein expression by MKL-1, MKL-2, MS-1, and WaGa cells (n = 6 independent experiments). (d) Relative ABCB5 mRNA expression (mean \pm SEM) by MCC cells resistant to 50–150 μM carboplatin or 1–3 μM etoposide compared with that in wild-type MCC cells, as determined by quantitative RT-PCR (n = 6–9 each, representative of 2–3 independent experiments). (e) Flow cytometrically determined ABCB5 protein expression (% live cells, mean \pm SEM) in wild-type versus carboplatin- or etoposide-resistant MCC cells (n = 3–8 independent experiments, respectively). (f) Representative flow cytometry of ABCB5 protein expression (MKL-1) in experimental groups as in (e) and of (g) cell viability (calcein AM positivity) of wild-type ABCB5⁺ versus ABCB5⁻ MCC cells cultured in the presence of cytotoxic carboplatin (250 μM) or etoposide (5 μM) concentrations (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as determined by the unpaired Student's *t*-test). ABCB5, ATP-binding cassette member B5; DAPI, 4',6-diamidino-2-phenylindole; MCC, Merkel cell carcinoma; RT-PCR, reverse transcription polymerase chain reaction; SEM, standard error of the mean.

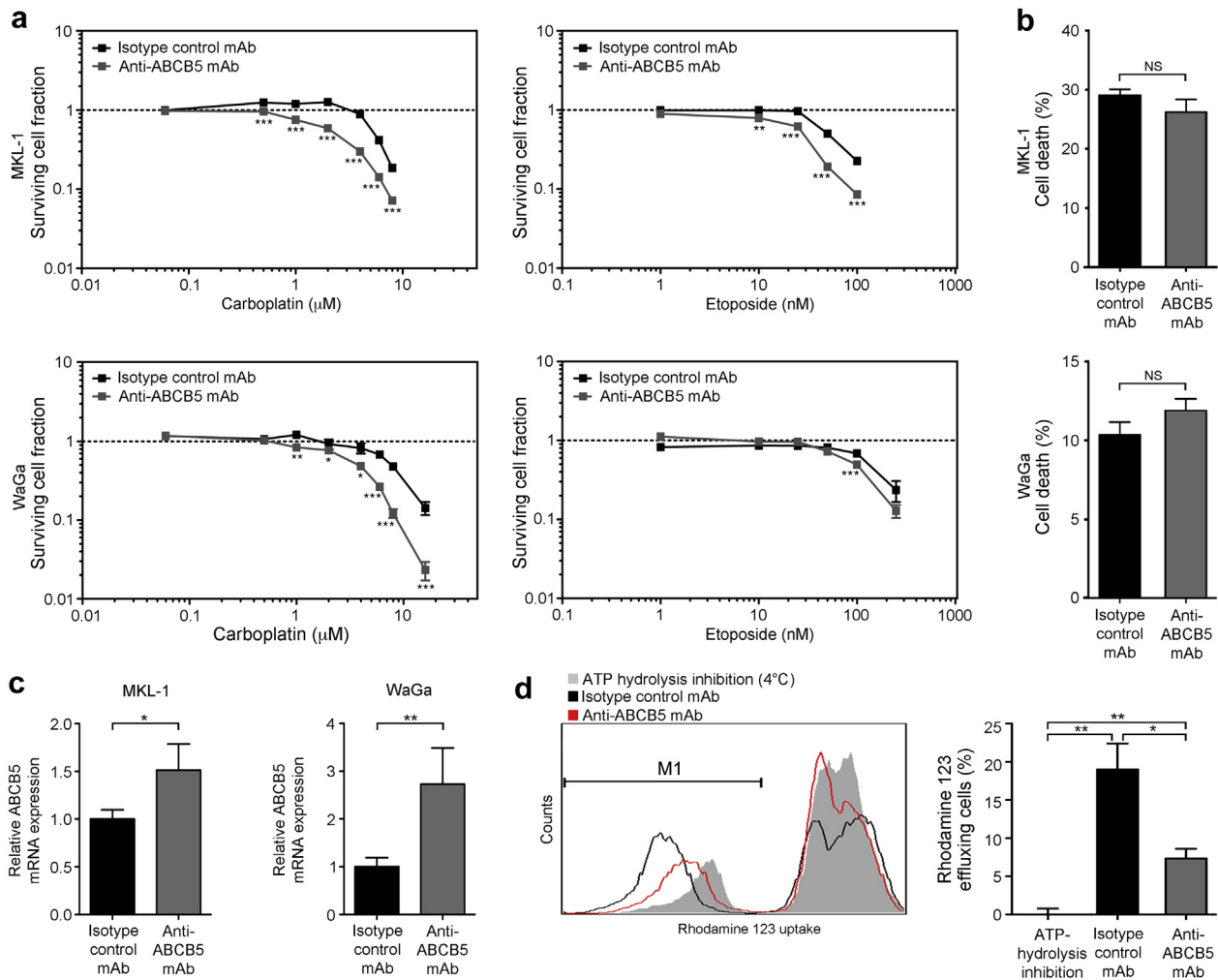


Figure 3. Chemoresistance reversal of human MCC cells by ABCB5 inhibition. (a) Effects of anti-ABCB5 versus isotype control antibody on carboplatin- or etoposide-induced MKL-1 and WaGa cell killing, determined by the MTT assay. Surviving cell fractions are plotted against drug concentration (n = 6 each, representative of n = 3 independent experiments). (b) Flow cytometric assessment of cell death (percent Annexin-V⁺/7AAD⁺ cells, mean ± SEM, n = 3) and (c) relative ABCB5 mRNA expression (mean ± SEM) in isotype control- versus anti-ABCB5 antibody-treated MCC cultures (n = 3–9, respectively). (d) Representative histogram plot and percent rhodamine 123-effluxing wild-type MKL-1 cells (mean ± SEM) under conditions of isotype control or ABCB5-blocking antibody treatment (37 °C), or with incubation at 4 °C to block ATP-dependent efflux (n = 3 independent experiments) (NS: not significant, *P < 0.05, **P < 0.01, ***P < 0.001, as determined by the unpaired Student's *t*-test). ABCB5, ATP-binding cassette member B5; MCC, Merkel cell carcinoma; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SEM, standard error of the mean.

blocking specificity, induced compensatory increases in ABCB5 mRNA expression compared with isotype control mAb treatment (Figure 3c). To more rigorously demonstrate that the anti-ABCB5 mAb used in our study blocks ABCB5 function in MCC, we next examined whether it blocked cellular efflux of the green fluorescent dye and known ABCB5 substrate (Frank et al., 2003; Lin et al., 2013), rhodamine 123 (Rh123) in MCC cells. Compared with isotype control mAb-treated MCC cells, of which a subpopulation of 19.0 ± 2.8% (mean ± standard error of the mean, n = 3) effluxed Rh123 over a 120-minute incubation period at 37 °C, mAb-mediated ABCB5 blockade significantly (P < 0.05) inhibited Rh123 efflux by >60% (Figure 3d). The effect of ABCB5 blockade on Rh123 efflux was assessed compared with incubation of MCC cells for 120 minutes at 4 °C (Figure 3d), which blocks ATP hydrolysis and hence ABC transport function (Frank et al., 2003; Lin et al., 2013). Together, these results established that ABCB5 is expressed as a functional

xenobiotic efflux transporter in MCC, and that ABCB5 blockade reverses carboplatin and etoposide resistance in this malignancy.

To investigate the relationship between ABCB5 and first-line chemotherapy resistance in MCC in vivo, we also examined ABCB5 expression and carboplatin or etoposide treatment response of ABCB5⁺ cancer cell populations in an established model system (Lezcano et al., 2014) utilizing non-obese diabetic/severely combined immunodeficiency IL-2Rγ^{-/-} (NSG) mice bearing MKL-1 or WaGa xenografts. MKL-1 and WaGa tumors grew at similar rates (Supplementary Figure S2a online) and exhibited cytokeratin 20 and ABCB5 expression profiles similar to those found in patient MCC samples (Supplementary Figure S2b). We found that the carboplatin and etoposide doses (Fichtner et al., 2008) administered to NSG mice for six consecutive days resulted in significant volume reduction of preestablished MKL-1 and Wa xenograft tumors, whereas vehicle-treated controls showed continued tumor growth

(Figure 4a). Quantitative RT-PCR analysis revealed greater than eightfold elevation of ABCB5 mRNA expression by tumor xenograft tissue harvested from carboplatin- and etoposide-treated versus vehicle control-treated MKL-1 ($P < 0.001$) or WaGa xenograft-bearing mice ($P < 0.05$), respectively (Figure 4b). Compared with tumor xenografts of vehicle control-treated mice, residual MKL-1 and WaGa specimens resected from NSG mice treated with carboplatin or etoposide also showed 2.0- to 4.3-fold enhanced ($P < 0.001$, respectively) ABCB5 protein expression by viable MCC cells (Figure 4c), consistent with our in vitro results (Figure 2). In line with our in vitro findings, we also found that ABCB1 and ABCC3 transcript levels tended to be elevated in both MKL-1 and WaGa tumors resected from NSG mice treated with carboplatin or etoposide compared with those treated with vehicle control (Supplementary Figure S3 online).

To determine if ABCB5 blockade in the context of carboplatin- or etoposide-based monotherapy has an additive inhibitory effect on MCC growth in vivo, we administered submaximal doses of carboplatin (30 mg/kg) or etoposide (5 mg/kg) in combination with anti-ABCB5 mAb (Frank et al.,

2003, 2005; Ksander et al., 2014; Lin et al., 2013; Schatton et al., 2008, 2015; Wilson et al., 2014) or isotype control mAb to MKL-1 or WaGa tumor xenograft-bearing NSG mice. Compared with the respective vehicle control groups, low-dose carboplatin or etoposide treatment resulted in significantly ($P < 0.01$) attenuated growth of both MKL-1 and WaGa tumor xenografts (Figure 5a). Importantly, combination therapies involving anti-ABCB5 mAb plus carboplatin or anti-ABCB5 mAb plus etoposide resulted in significantly greater inhibition of tumor growth compared with treatment with either chemotherapeutic alone ($P < 0.05$, respectively) (Figure 5a). Immunohistochemical analysis of serial sections of resected MKL-1 and WaGa specimens revealed binding of in vivo-administered mAb to tumor target tissue, which coincided with regions of ABCB5 positivity, in anti-ABCB5 mAb-co-treated, but not isotype control mAb-co-treated carboplatin or etoposide treatment groups (Figure 5b), supporting the notion of a direct anti-ABCB5 mAb effect on ABCB5⁺ MCC target cells. Although MKL-1 and WaGa xenografts of NSG mice treated with either carboplatin or etoposide plus isotype control mAb showed only

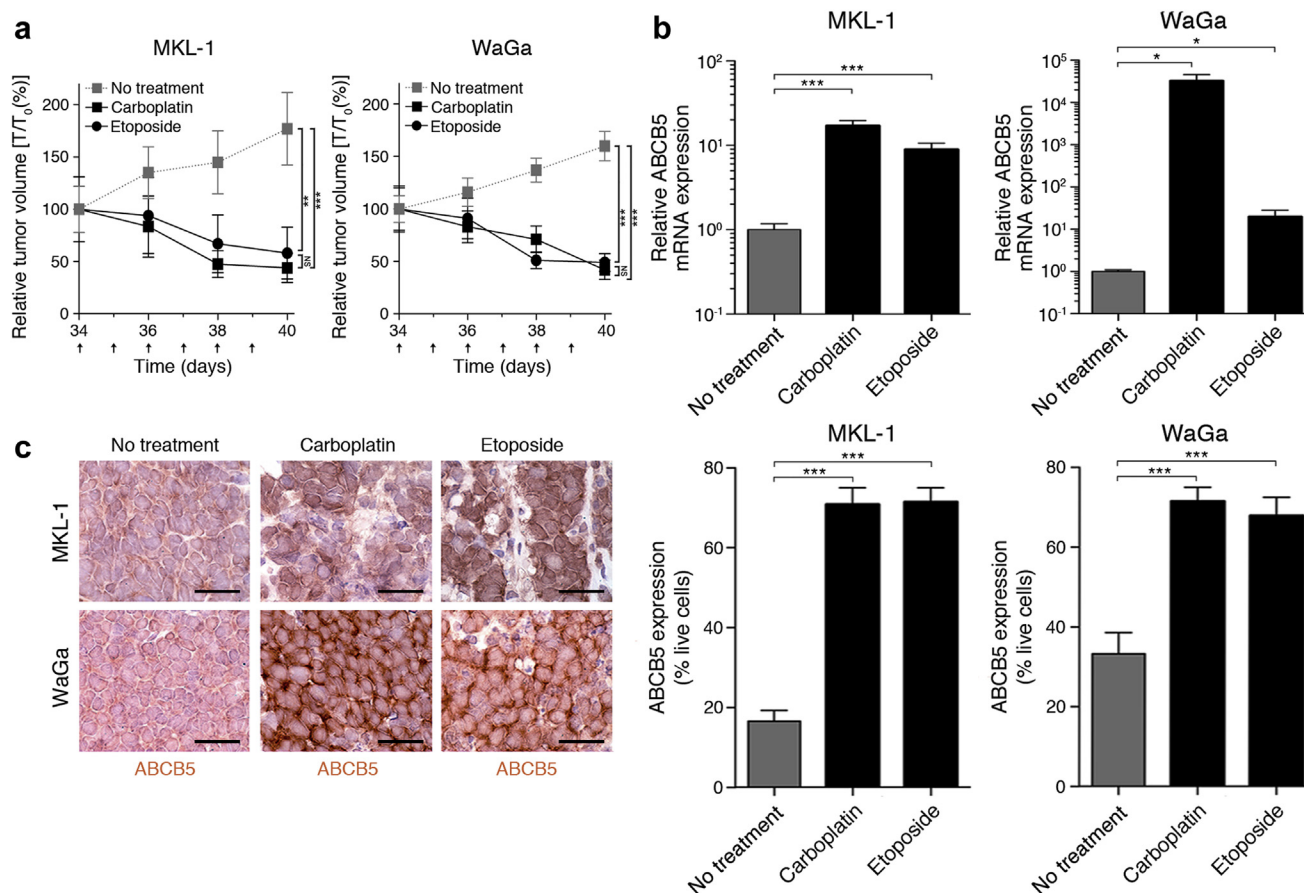


Figure 4. Response of ABCB5 expression to carboplatin and etoposide treatment in MCC tumor xenografts. (a) Tumor growth kinetics (mean \pm SD) of vehicle control-, carboplatin- (75 mg/kg/d) or etoposide-treated (10 mg/kg/d) MKL-1 and WaGa xenografts in NSG mice ($n = 6-8$, respectively). Arrows indicate days of carboplatin or etoposide administration. (b) Relative ABCB5 mRNA expression (mean \pm SEM) determined by quantitative RT-PCR and (c) immunohistochemically determined ABCB5 protein expression (mean \pm SEM) by MCC xenografts ($n = 6-8$, respectively) harvested 40 days after inoculation to NSG mice, in experimental groups as in (a). Representative images of ABCB5 immunohistochemistry are shown on the left. Size bars = 50 μ m (NS: not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, as determined by two-way ANOVA followed by the Bonferroni correction (a) or by the unpaired Student's t -test (b, c)). ABCB5, ATP-binding cassette member B5; ANOVA, analysis of variance; MCC, Merkel cell carcinoma; NSG, non-obese diabetic/severely combined immunodeficiency IL-2R $\gamma^{-/-}$; RT-PCR, reverse transcription polymerase chain reaction; SD, standard deviation; SEM, standard error of the mean.

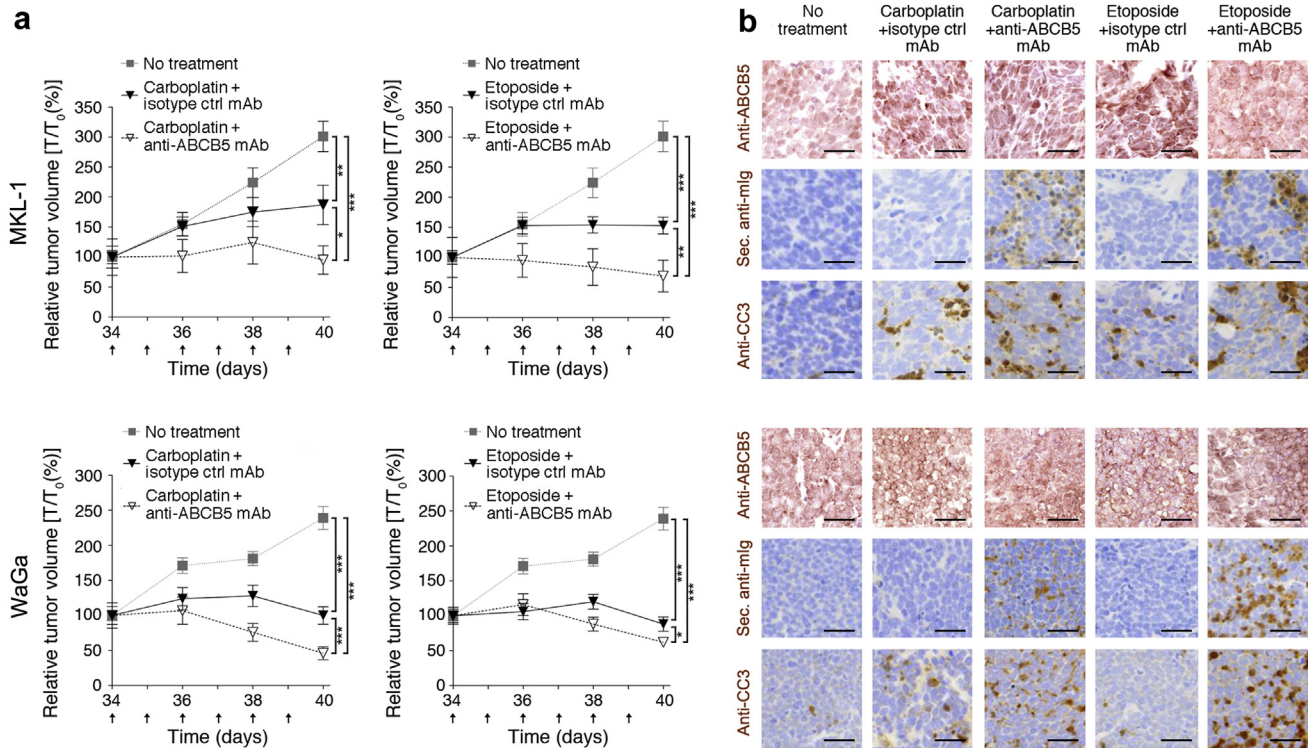


Figure 5. Effect of ABCB5 blockade on MCC tumor xenograft growth and chemotherapy-induced cytotoxicity. (a) Tumor growth kinetics (mean ± SD) and (b) ABCB5, secondary antibody (sec. anti-mIg staining, recognizing in vivo-administered anti-ABCB5 antibody) and cleaved caspase 3 (CC3) immunohistochemical staining of MKL-1 (top, n = 6–8) and WaGa (bottom, n = 8–9) xenografts dissected from vehicle control-, carboplatin- (30 mg/kg/d), or etoposide-treated (5 mg/kg/d) NSG mice also treated with anti-human ABCB5- or isotype control antibody (500 µg/d), respectively. Arrows in panel (a) indicate days of carboplatin or etoposide and isotype control or anti-ABCB5 antibody administration. Size bars = 50 µm (*P < 0.05, **P < 0.01, ***P < 0.001, as determined by two-way ANOVA followed by the Bonferroni correction). ABCB5, ATP-binding cassette member B5; ANOVA, analysis of variance; MCC, Merkel cell carcinoma; NSG, non-obese diabetic/severely combined immunodeficiency IL-2Rγ^{-/-}; SD, standard deviation.

moderate apoptotic cell death compared with vehicle controls (determined by cleaved caspase 3 immunostaining, Figure 5b), the combination therapies involving carboplatin or etoposide plus anti-ABCB5 mAb resulted in enhanced MCC apoptosis, in tumor areas that also showed binding of anti-ABCB5 mAb to MCC cells (Figure 5b). Without concurrent administration of carboplatin or etoposide, anti-ABCB5 mAb administration to MKL-1 or WaGa tumor xenograft-bearing NSG mice at the equivalent observation endpoint did neither result in significant differences in tumor growth (Supplementary Figure S4a online) nor in changes in apoptotic tumor target cell death, compared with isotype control mAb treatment alone (Supplementary Figure S4b).

In this study, on the basis of previously established roles of ABCB5 as a clinically relevant chemoresistance mechanism in cutaneous melanoma (Chartrain et al., 2012; Frank et al., 2005; Wilson et al., 2014) and other cancers (Cheung et al., 2011; Wilson et al., 2011), we analyzed ABCB5 expression and function in MCC, through the study of MCC tumor biospecimens (n = 85) obtained from 66 patients, healthy human skin specimens from 10 individuals, and 4 established MCC cell lines. Our results provide evidence that ABCB5 is highly expressed in MCC and that its expression levels markedly surpass those in healthy human skin. Importantly, we show that ABCB5 identifies carboplatin- and etoposide-resistant MCC subsets in vitro and in tumor xenografts and clinical specimens in vivo. Additional ABC

transporters, namely ABCB1 and ABCC3, were also overexpressed in carboplatin- and etoposide-resistant compared to wild-type MCC cell lines, raising the possibility of multiple ABC transporter involvement in MCC chemoresistance. Moreover, we cannot exclude the possibility that more pronounced cellular quiescence in chemorefractory MCC cells may have contributed to resistance to the agents used. Although ABCB5 may function as an MCC efflux transporter, as evidenced by our findings of ABCB5 mAb-mediated MCC cell retention of the ATP transporter substrate rhodamine 123, the question of whether ABCB5 directly effluxes the standard-of-care clinical MCC therapeutic agents, carboplatin and etoposide, as a mechanism of resistance, or if alternative, efflux-independent ABCB5 functions (Ksander et al., 2014; Wilson et al., 2014) might be primarily responsible for MCC chemoresistance, requires further investigation.

The finding that ABCB5 serves as a chemoresistance mediator in MCC is of potential high clinical significance, because the emergence of resistance to first-line chemotherapy is a major impediment to successful MCC treatment (Eng et al., 2007; Tai et al., 2000). Indeed, because the addition of an ABCB5 blocking mAb to carboplatin or etoposide treatment resulted in enhanced tumor cell apoptosis and significant inhibition of tumor xenograft growth, our results establish initial proof-of-principle that ABCB5 can be targeted in MCC to attenuate resistance to clinically relevant chemotherapeutic agents. In summary,

our findings provide a clear rationale to translate ABCB5-targeted chemoresistance reversal strategies to the clinic to enhance the efficacy of currently available systemic MCC therapies.

MATERIALS AND METHODS

Clinical specimens, MCC cell lines, culture methods, and generation of drug-resistant MCC lines

MCC biospecimens and healthy human skin were obtained from patients and healthy volunteers in accordance with protocols approved by the institutional review boards of Partners Health Care Research Management and the Dana-Farber Cancer Institute and assurances filed with and approved by the US Department of Health and Human Services. Written informed consent was obtained from all subjects. Authenticated human MCC cell lines were obtained from Dr. James DeCaprio of the Dana-Farber Cancer Institute, Boston, MA (Rodig et al., 2012) and were cultured fewer than 6 months in Roswell Park Memorial Institute 1640 medium supplemented with 20% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin (Gibco, Life Technologies, Grand Island, NY). MCC cell lines were rendered drug resistant to carboplatin or etoposide by incubation in increasing doses of up to 150 μ M carboplatin or of up to 3 μ M etoposide over the course of 2 months, respectively.

RNA extraction, RT-PCR, and real-time quantitative PCR

Human ABCB5 was amplified and sequenced after reverse transcription of total RNA using ABCB5-specific primer pairs. Relative ABCB5, ABCB1, ABCC3, and ABCG2 transcript levels were determined by real-time quantitative RT-PCR and calculated using the $2^{-\Delta\Delta C_t}$ method (Schatton et al., 2008, 2010). See [Supplementary Materials](#) online for primer sequences.

Immunohistochemistry and immunofluorescence staining

Immunofluorescence labeling for ABCB5 in cytospin preparations of MCC cell lines, conventional histology, and immunohistochemical analysis of ABCB5, cytokeratin 20, and/or cleaved caspase 3 expression by tumor biospecimens obtained from patients with MCC or human MCC tumor xenografts, and of in vivo anti-ABCB5 mAb binding to MCC tumor xenograft tissue were carried out as described (Schatton et al., 2008, 2010). ABCB5 immunoreactivity was quantified using ImageJ software analysis, as described previously (Wilson et al., 2011).

Flow cytometry and cell viability measurements

ABCB5 surface protein expression by established wild-type, drug-exposed, or drug-resistant MCC lines with or without concurrent counterstaining with the viability dye, calcein acetoxymethyl ester, was analyzed by single- or dual-color flow cytometry (Schatton et al., 2008, 2010). Assessment of cell viability in anti-ABCB5 versus isotype control mAb-treated MCC cells was carried out by annexin V/7-amino-actinomycin D staining and subsequent flow cytometric analysis, as described (Schatton et al., 2010).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assays

To confirm preferential chemosensitivity of wild-type versus drug-resistant MCC lines and determine the effect of ABCB5 mAb blockade on carboplatin- or etoposide-induced cell killing, MCC cells were exposed to a range of concentrations of carboplatin or etoposide in the presence or absence of anti-ABCB5 mAb or isotype control mAb over a course of 7 days. Subsequently, in vitro growth kinetics of cells were assayed using the TACS 3-(4,5-dimethylthiazol-

2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay kit (Trevigen, Gaithersburg, MD).

Rhodamine 123 efflux transport assays

Efflux transport capacity for the green fluorescent dye, rhodamine 123, was assessed by flow cytometry for MCC cells incubated for 120 minutes at 37 °C with the 3C2-1D12 anti-ABCB5 blocking mAb (Frank et al., 2003, 2005; Ksander et al., 2014; Lin et al., 2013; Schatton et al., 2008; Wilson et al., 2011) versus isotype control mAb, as described previously (Frank et al., 2003; Lin et al., 2013).

Human MCC xenotransplantation, in vivo carboplatin and etoposide treatment, and anti-ABCB5 mAb targeting

NSG mice were maintained and experiments performed in accordance with IACUC-approved experimental protocols. For tumorigenicity studies, MCC cells were injected subcutaneously (1×10^7 cells/inoculum) into the flanks of recipient NSG mice (Schatton et al., 2008). At day 34 after tumor cell inoculation, mice were randomized to carboplatin, etoposide or vehicle control treatment groups and carboplatin (75 mg/kg) or etoposide (10 mg/kg) were administered daily by intraperitoneal injection for 6 consecutive days, as previously described (Fichtner et al., 2008). Control animals were given vehicle at equal volumes. For in vivo ABCB5 targeting experiments, human MCC cells were grafted, mice randomized to experimental treatment groups, and animals were injected intraperitoneally with anti-ABCB5 mAb or control mAb daily (500 μ g per injection, respectively) for 9 consecutive days with or without concurrent administration of carboplatin (30 mg/kg) or etoposide (5 mg/kg) for 6 consecutive days starting 72 hours after initial antibody treatment, respectively. Tumor volumes were measured daily for the duration of the treatment and tumor volumes were calculated as described (Schatton et al., 2008).

Statistics

Analyses were performed using the PRISM software (version 5 for Macintosh, GraphPad Software, La Jolla, CA) and R version 3.02. Statistical hypotheses were tested using the two-tailed Student's *t*-test, the nonparametric Mann-Whitney test, or two-way analysis of variance (ANOVA) followed by the Bonferroni correction. Data were tested for normal distribution using the D'Agostino and Pearson omnibus normality test. A two-sided value of $P < 0.05$ was considered statistically significant.

ORCID

Markus H. Frank: <http://orcid.org/0000-0002-1312-0488>

CONFLICT OF INTEREST

MHF is inventor or co-inventor of US and international patents assigned to Brigham and Women's Hospital and/or Boston Children's Hospital, Boston, MA, and licensed to Ticeba GmbH (Heidelberg, Germany) and Rheacell GmbH & Co. KG (Heidelberg, Germany). MHF serves as a scientific advisor to Ticeba GmbH and Rheacell GmbH & Co. KG and participates in corporate sponsored research collaborations with Rheacell GmbH & Co. KG.

ACKNOWLEDGMENTS

The authors thank Dr. James DeCaprio for providing Merkel cell carcinoma cell lines for our study. This work was supported by Brigham and Women's Hospital, Department of Dermatology funding for new investigators and a Fund to Sustain Research Excellence from the Brigham Research Institute (to TS), NIH/NCI grants 1R01CA113796 and 1R01CA138231 (to MHF), and 1R01CA158467 (to MHF and GFM). TS is the recipient of a Research Career Development award from the Dermatology Foundation. NL is the recipient of a Medical Student Grant from the American Skin Association.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <http://dx.doi.org/10.1016/j.jid.2015.12.038>.

REFERENCES

- Becker JC. Merkel cell carcinoma. *Ann Oncol* 2010;21(Suppl 7):vii81–5.
- Chartrain M, Riond J, Stennevin A, Vandenberghe I, Gomes B, Lamant L, et al. Melanoma chemotherapy leads to the selection of ABCB5-expressing cells. *PLoS One* 2012;7:e36762.
- Cheung ST, Cheung PF, Cheng CK, Wongg NC, Fan ST. Granulin-epithelin precursor and ATP-dependent binding cassette (ABCB5) regulate liver cancer cell chemoresistance. *Gastroenterology* 2011;140:344–55.
- Dean M, Rzhetsky A, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. *Genome Res* 2001;11:1156–66.
- Eng TY, Boersma MG, Fuller CD, Goytia V, Jonew WE, Joyner M, et al. A comprehensive review of the treatment of Merkel cell carcinoma. *Am J Clin Oncol* 2007;30:624–36.
- Fichtner I, Rolff J, Soong R, Hoffmann J, Hammer S, Sommer A, et al. Establishment of patient-derived non-small cell lung cancer xenografts as models for the identification of predictive biomarkers. *Clin Cancer Res* 2008;14:6456–68.
- Frank NY, Margaryan A, Huang Y, Schatton T, Waaga-Gasser AM, Gasser M, et al. ABCB5-mediated doxorubicin transport and chemoresistance in human malignant melanoma. *Cancer Res* 2005;65:4320–33.
- Frank NY, Pendse SS, Lapchak PH, Margaryan A, Shlain D, Doeing C, et al. Regulation of progenitor cell fusion by ABCB5 P-glycoprotein, a novel human ATP-binding cassette transporter. *J Biol Chem* 2003;278:47156–65.
- Frank NY, Schatton T, Kim S, Zhan Q, Wilson BJ, Ma J, et al. VEGFR-1 expressed by malignant melanoma-initiating cells is required for tumor growth. *Cancer Res* 2011;71:1474–85.
- Gazzaniga P, Cigna E, Panasi V, Devirgillis V, Bottoni U, Vincenzi B, et al. CD133 and ABCB5 as stem cell markers on sentinel lymph node from melanoma patients. *Eur J Surg Oncol* 2010;36:1211–4.
- Guastafierro A, Feng H, Thant M, Kirkwood JM, Chang Y, Moore PS, et al. Characterization of an early passage Merkel cell polyomavirus-positive Merkel cell carcinoma cell line, MS-1, and its growth in NOD scid gamma mice. *J Virol Methods* 2013;187:6–14.
- Hodgson NC. Merkel cell carcinoma: changing incidence trends. *J Surg Oncol* 2005;89:1–4.
- Houben R, Shuda M, Weinkam R, Schrama D, Feng H, Chang Y, et al. Merkel cell polyomavirus-infected Merkel cell carcinoma cells require expression of viral T antigens. *J Virol* 2010;84:7064–72.
- Ksander BR, Kolovou PE, Wilson BJ, Saab KR, Guo Q, Ma J, et al. ABCB5 is a limbal stem cell gene required for corneal development and repair. *Nature* 2014;511:353–7.
- Lezcano C, Kleffel S, Lee N, Larson AR, Zhan Q, DoRosario A, et al. Merkel cell carcinoma expresses vasculogenic mimicry: demonstration in patients and experimental manipulation in xenografts. *Lab Invest* 2014;94:1092–102.
- Lin JY, Zhang M, Schatton T, Wilson BJ, Alloo A, Ma J, et al. Genetically determined ABCB5 functionality correlates with pigmentation phenotype and melanoma risk. *Biochem Biophys Res Commun* 2013;436:536–42.
- Poulsen M. Merkel-cell carcinoma of the skin. *Lancet Oncol* 2004;5:593–9.
- Rodrig SJ, Cheng J, Wardzala J, DoRosario A, Scanlon JJ, Laga AC, et al. Improved detection suggests all Merkel cell carcinomas harbor Merkel polyomavirus. *J Clin Invest* 2012;122:4645–53.
- Rosen ST, Gould VE, Salwen HR, Herst CV, Le Beau MM, Lee I, et al. Establishment and characterization of a neuroendocrine skin carcinoma cell line. *Lab Invest* 1987;56:302–12.
- Schatton T, Murphy GF, Frank NY, Yamaura K, Waaga-Gasser AM, Gasser M, et al. Identification of cells initiating human melanomas. *Nature* 2008;451:345–9.
- Schatton T, Schutte U, Frank NY, Zhan Q, Hoerning A, Robles SC, et al. Modulation of T-cell activation by malignant melanoma initiating cells. *Cancer Res* 2010;70:697–708.
- Schatton T, Yang J, Kleffel S, Uehara M, Barthel SR, Schlapbach C, et al. ABCB5 identifies immunoregulatory dermal cells. *Cell Rep* 2015;12:1564–74.
- Setia N, Abbas O, Sousa Y, Garb JL, Mahalingam M. Profiling of ABC transporters ABCB5, ABCF2 and nestin-positive stem cells in nevi, in situ and invasive melanoma. *Mod Pathol* 2012;25:1169–75.
- Sharma BK, Manglik V, Elias EG. Immuno-expression of human melanoma stem cell markers in tissues at different stages of the disease. *J Surg Res* 2010;163:e11–5.
- Tai PT, Yu E, Winquist E, Hammond A, Stitt L, Tonita J, et al. Chemotherapy in neuroendocrine/Merkel cell carcinoma of the skin: case series and review of 204 cases. *J Clin Oncol* 2000;18:2493–9.
- Wilson BJ, Saab KR, Ma J, Schatton T, Putz P, Zhan Q, et al. ABCB5 maintains melanoma-initiating cells through a proinflammatory cytokine signaling circuit. *Cancer Res* 2014;74:4196–207.
- Wilson BJ, Schatton T, Zhan Q, Gasser M, Ma J, Saab KR, et al. ABCB5 identifies a therapy-refractory tumor cell population in colorectal cancer patients. *Cancer Res* 2011;71:5307–16.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>