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Original Research

Suppression of the ATP-binding cassette transporter ABCC4 impairs neuroblastoma tumour growth and sensitises to irinotecan *in vivo*

Jayne Murray [a,1], Emanuele Valli [a,b,1], Denise M.T. Yu [a,b,1], Alan M. Truong [a,c], Andrew J. Gifford [a,b,d], Georgina L. Eden [a], Laura D. Gamble [a], Kimberley M. Hanssen [a,c], Claudia L. Flemming [a], Alvin Tan [a], Amanda Tivnan [a], Sophie Allan [a], Federica Saletta [e], Leanna Cheung [a], Michelle Ruhle [a], John D. Schuetz [f], Michelle J. Henderson [a,b], Jennifer A. Byrne [e,g], Murray D. Norris [a,h,2], Michelle Haber [a,2], Jamie I. Fletcher [a,b,*,2]

[a] *Children's Cancer Institute Australia, Lowy Cancer Research Centre, UNSW Australia, NSW 2052, Australia*
[b] *School of Women's and Children's Health, UNSW Australia, NSW 2052, Australia*
[c] *School of Medical Sciences, UNSW Australia, NSW 2052, Australia*
[d] *Department of Anatomical Pathology (SEALS), Prince of Wales Hospital, Randwick, NSW 2031, Australia*
[e] *Children's Cancer Research Unit, Kids Research Institute, The Children's Hospital at Westmead, Westmead, NSW 2145, Australia*
[f] *Department of Pharmaceutical Sciences, St. Jude Children's Research Hospital, Memphis, TN, USA*
[g] *University of Sydney Discipline of Child and Adolescent Health, The Children's Hospital at Westmead, Westmead, NSW 2145, Australia*
[h] *University of New South Wales Centre for Childhood Cancer Research, UNSW Australia, NSW 2052, Australia*

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Abstract The ATP-binding cassette transporter ABCC4 (multidrug resistance protein 4, MRP4) mRNA level is a strong predictor of poor clinical outcome in neuroblastoma which may relate to its export of endogenous signalling molecules and chemotherapeutic agents. We sought to determine whether ABCC4 contributes to development, growth and drug response in neuroblastoma *in vivo*. In neuroblastoma patients, high ABCC4 protein levels were associated with reduced overall survival. Inducible knockdown of ABCC4 strongly inhibited the growth of human neuroblastoma cells *in vitro* and impaired the growth of neuroblastoma

* *Corresponding author*: Children's Cancer Institute Australia, PO Box 81, Randwick, NSW 2031, Australia. Fax: +61 (2) 9662 6583.
E-mail address: JFletcher@ccia.unsw.edu.au (J.I. Fletcher).
[1] These authors are co-first authors. [2] These authors are co-senior authors.

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xenografts. Loss of *Abcc4* in the Th-*MYCN* transgenic neuroblastoma mouse model did not impact tumour formation; however, *Abcc4*-null neuroblastomas were strongly sensitised to the ABCC4 substrate drug irinotecan. Our findings demonstrate a role for ABCC4 in neuroblastoma cell proliferation and chemoresistance and provide rationale for a strategy where inhibition of ABCC4 should both attenuate the growth of neuroblastoma and sensitise tumours to ABCC4 chemotherapeutic substrates.

1. Introduction

Neuroblastoma is an extracranial solid tumour of infancy and early childhood arising from the sympatico-adrenal lineage of the neural crest [1]. It is a heterogenous disease with few recurrent somatic mutations [2], and the treatment for high-risk disease still relies heavily on conventional cytotoxic agents. *ABCC4*, encoding for the ATP-binding cassette transporter protein ABCC4 (multidrug resistance protein 4, MRP4), is transcriptionally regulated by MYCN [3], which is a driver of neuroblastoma tumourigenesis [4] and an established poor prognostic factor [5,6], and high *ABCC4* mRNA expression strongly predicts poor clinical outcome across multiple patient cohorts [7,8]. In cultured cells, ABCC4 confers resistance to several anti-cancer drugs, including the camptothecin irinotecan, a drug used in the treatment of neuroblastoma [9]; however, it is unknown whether ABCC4 protein expression has prognostic value or affects chemotherapy response in tumours. ABCC4 also exports endogenous signalling molecules that may influence tumour survival and proliferation, including cyclic nucleotides and eicosanoids [10,11], and we previously reported that transient RNAi-mediated ABCC4 knockdown reduced proliferation and colony-forming ability in two neuroblastoma cell lines [7] in the absence of chemotherapy. More rigorous assessment of ABCC4 as a therapeutic target in neuroblastoma is clearly warranted.

Here we demonstrate that the suppression of ABCC4 inhibits the growth of multiple neuroblastoma cell lines *in vitro* and established human neuroblastoma xenografts in immune-deficient mice, suggesting that blocking ABCC4 function might be beneficial in established tumours, even without chemotherapy. In a neuroblastoma-prone transgenic mouse model, constitutive absence of *Abcc4* did not affect neuroblastoma formation suggesting that ABCC4 function does not contribute to the genesis of this tumour. Nonetheless, the murine neuroblastomas derived from the *Abcc4*-deficient animals were sensitised to irinotecan in an allograft model. Our findings demonstrate ABCC4 inhibition as an approach to chemosensitisation of neuroblastomas.

2. Materials and methods

2.1. Tissue microarray

Tissue microarray (TMA) sections with clinical annotation, from the Children's Hospital at Westmead Tumour Bank, were stained with haematoxylin and eosin (H&E) or for ABCC4 (rat monoclonal anti-MRP4 M4I-10, Abcam ab15602, 1:50 dilution, 3 μg/mL). Cores from 98 patients diagnosed between 1979 and 2013 were scored for ABCC4 staining by a paediatric pathologist blinded to clinical parameters [12]. Staining was scored for intensity (0, absent; 1, weak; 1.5 weak–moderate; 2, moderate, 2.5 moderate–strong; and 3, strong) and percentage of positive staining (0, 0%; 1, 1–10%; 2, 11–50% and 3, 51–100%) and overall score (0–9) was determined by multiplying staining intensity and percentage scores, with duplicate cores averaged. Photos were from an Olympus BX53 light microscope and DP-73 camera with cellSens software.

2.2. Cell culture

Cells lines verified by short tandem repeat profiling (CellBank Australia, Westmead Australia) were cultured at 37 °C, 5% CO_2 in Dulbecco's Modified Eagles Medium with 10% foetal bovine serum (ThermoTrace, Nobel Park, Australia) for BE(2)-C, or Roswell Park Memorial Institute medium with 10% foetal bovine serum (CHP-134 and NB69). Transfections used Lipofectamine RNAiMAX (Life Technologies, Mulgrave Australia) and 20 nM siRNA (Supplementary Table 1). Stable cell lines expressing doxycycline-inducible ABCC4 shRNA were generated by lentiviral transduction with the pFH1UTG vector [13] (Supplementary Table 1) packaged using the psPAX2 and pMD2.G plasmids (gift from Didier Trono, Lausanne, Switzerland). Doxycycline treatment was 1 μg/mL (72 h treatment, media change to replenish doxycycline every 48 h).

2.3. Western blot

Western blotting antibodies were: ABCC4 (rat monoclonal M4I-10; Enzo Life Sciences, Waterloo, NSW;