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Decreased apoptosis repressor with caspase recruitment domain confers resistance to sunitinib in renal cell carcinoma through alternate angiogenesis pathways

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ABSTRACT

Apoptosis repressor with caspase recruitment domain (ARC), an endogenous inhibitor of apoptosis, is upregulated in a number of human cancers, thereby conferring drug resistance and giving a rationale for the inhibition of ARC to overcome drug resistance. Our hypothesis was that ARC would be similarly upregulated and targetable for therapy in renal cell carcinoma (RCC). Expression of ARC was assessed in 85 human RCC samples and paired non-neoplastic kidney by qPCR and immunohistochemistry, as well as in four RCC cell lines by qPCR, Western immunoblot and confocal microscopy. Contrary to expectations, ARC was significantly decreased in the majority of clear cell RCC and in three (ACHN, Caki-1 and 786-0) of the four RCC cell lines compared with the HK-2 non-cancerous human proximal tubular epithelial cell line. Inhibition of ARC with shRNA in the RCC cell line (SN12K1) that had shown increased ARC expression conferred resistance to Sunitinib, and upregulated interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF). We therefore propose that decreased ARC, particularly in clear cell RCC, confers resistance to targeted therapy through restoration of tyrosine kinase-independent alternate angiogenesis pathways. Although the results are contrary to expectations from other cancer studies, they were confirmed here with multiple analytical methods. We believe the highly heterogeneous nature of cancers like RCC predicate that expression patterns of molecules must be interpreted in relation to respective matched non-neoplastic regions. In the current study, this procedure indicated that ARC is decreased in RCC.

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1. Introduction

Renal cell carcinoma (RCC) is a highly metastatic, heterogeneous, and treatment-resistant cancer with over 50 pathological entities [1,2]. The most common subtype is clear cell RCC (ccRCC, 70–80%), followed by papillary RCC (pRCC, 10–15%), chromophobe RCC (chRCC, 5%) and collecting duct RCC (<1%) [3]. Despite the introduction of many targeted therapies in clinical practice [4] metastatic RCC remains an incurable terminal disease. About 30% of patients do not respond to targeted therapy, and the remaining 70%, who initially respond, will develop resistance between 6 and 11 months [5,6].

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Resistance to treatment, although multifactorial, is largely driven by defective apoptotic pathways. There are two well-known apoptotic pathways: extrinsic and intrinsic [7]. The extrinsic pathway is mostly initiated by death ligands, which form a death-inducing signalling complex (DISC). The intrinsic pathway is largely initiated by drugs, especially chemotherapeutics, which alter the mitochondrial membrane potential through the translocation of the cytoplasmic pro-apoptotic molecule Bax to the mitochondrial membrane transition pores [8]. Both extrinsic and intrinsic pathways converge at caspase-3, and its cleavage executes apoptosis. These processes are tightly-regulated by many endogenous apoptosis inhibitors, of which apoptosis repressor with caspase recruitment domain (ARC) is one. Most of the endogenous apoptosis inhibitors either inhibit the intrinsic or the extrinsic pathways, but ARC is unique in that it inhibits both pathways [8].

In 2008, Heikus and colleagues [9], using 47 RCC and adjacent non-neoplastic regions of the kidney, reported increased ARC mRNA in RCC when compared with adjacent non-neoplastic areas of the kidneys. In 2014, Razorenova et al. [10] also showed that ARC was over-expressed in RCC samples. These authors also reported that ARC was expressed in the RCC cell line Caki-1 and that inhibition of ARC using shRNA sensitised these cells to cisplatin treatment. These results offered a rationale for further exploration of the molecular mechanisms of drug resistance in RCC secondary to ARC up-regulation.

The primary aim of the present investigation was to study the molecular mechanisms of ARC-mediated resistance of RCC to targeted therapies. As a first step, it was decided to investigate the expression of ARC in 48 human RCC samples along with paired non-neoplastic kidney samples by immunohistochemistry. Contrary to expectations, we found ARC protein was significantly decreased in the majority of RCC samples, particularly clear cell RCC, compared with paired normal kidney. This report therefore details our further analyses: ARC mRNA expression in a different paired set of 37 RCC samples; mRNA and protein in 4 RCC cell lines with and without Sunitinib therapy compared with the HK-2 human proximal tubular epithelial cell line; confocal microscopy to investigate protein localization in the cell lines; and inhibition of ARC with shRNA in an ARC-overexpressing cell line.

2. Materials and methods

2.1. Ethics approval

Approvals for the use of human tissue samples were obtained from the Human Research Ethics Committee of the Princess Alexandra Hospital (PAH) and the Human Ethics Committee of University of Queensland, Brisbane, Australia. Written informed-consent was obtained from patients before the collection of samples (Ethics approval numbers 2006/189 and HREC/05/QPAH/95).

2.2. Tissue microarray and immunohistochemistry

Forty-eight formalin-fixed paraffin-embedded (FFPE) archival kidney cancer and matched morphologically normal regions of the kidneys collected from patients who underwent nephrectomy for kidney cancer between 1990 and 2011 at the PAH were used in this study. None of the patients received prior treatment before nephrectomy. Tumour grade and stage were determined by Fuhrman criteria and Tumour Node Metastasis (TNM) classification, respectively, by a qualified pathologist. Tissue cores (0.6 mm in diameter) were punched from selected areas of the paraffin blocks with core punch needles (Beecher Instruments, Inc. Sun Prairie, WI, USA) and tissue microarrays (TMA) were constructed using a Galileo TMA CK3000 Tissue Microarrayer (Fantoli, Milan, Italy) as previously described [11]. The TMAs were batch-immunostained for ARC with a validated antibody (abcam; ab118929) as previously described [11]. Digital images of the entire TMA were captured using the Aperio ScanScope XT Slide Scanner (Aperio Technologies, Vista, CA, USA) under 20 × objective magnification. A quantitative scoring of ARC expression was analysed using the positive pixel algorithm of Aperio Imagescope [11].

2.3. ARC mRNA expression studies

Thirty-seven RCC and morphologically normal regions of matched kidneys were collected from patients who underwent nephrectomy for kidney cancer between June 2013 and December 2014 at the PAH. None of the patients received prior treatment before nephrectomy. The samples were snap-frozen in liquid nitrogen and stored at −80 °C until analysis. RNA was isolated using RNeasy Fibrous Tissue Mini Kit, following the instructions of the supplier (Qiagen, Maryland, USA). cDNA was synthesised using a High Capacity cDNA Reverse Transcription Kit (Life Technologies). Fully validated TaqMan Gene Expression Assay for ARC (Hs00608346_m1) was used with the SensiFAST™ Probe No-ROX Kit (Bioline, London, UK) in a LightCycler 480 (Roche Applied Science, Penzberg, Germany) to determine relative gene expression by the comparative Ct method. The TATA box binding protein (TBP Hs00427620_m1; Life Technologies) was used as internal control.

2.4. Cell culture

Metastatic human RCC cell lines ACHN, Caki-1 and 786-0, and the immortalised normal human proximal tubular epithelial cell line HK-2, were obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA. Another human metastatic RCC cell line, SN12K1, was obtained from Professor D Nicol, formerly at PAH, Brisbane, Australia, through his collaborations with Dr IJ Fidler, Cancer Research Institute, MD Anderson Cancer Center, Orlando, FL, USA. The cell lines were cultured in DMEM/F12 supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco, Invitrogen,CA, USA) at 37 °C in an atmosphere of 95% air and 5% carbon dioxide.

2.5. qPCR and western blotting of cell lines

Cells were grown to approximately 90% confluence in 10 cm petri dishes. The culture medium was removed and the cells were washed in 1× ice-cold phosphate buffered saline (PBS). RNA was isolated using RNeasy Mini Kit, following the instructions of the supplier (Qiagen, Maryland, USA). The remaining qPCR methods were as for tissue, described previously. For protein, whole cell lysates were prepared by lysing the cells in 300 μl of radio immunoprecipitation assay (RIPA) buffer (Sigma–Aldrich, Missouri, USA), followed by centrifugation at 13,000 rpm for 15 min at 4 °C. The supernatants were collected and the protein contents measured using bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL, USA). The lysates were aliquoted and stored at − 80 °C until further use. The proteins (50 μg) were resolved in 10% Tris–HCl gel and electro-transferred into polyvinylidene fluoride membranes (Millipore Corporation, MA, USA). Equal loading of proteins was confirmed by staining the membrane with Ponceau S solution. Standard Western blotting procedures were followed. The primary antibody was used at a dilution of 1:1000 and the secondary antibody at a dilution of 1:5000. The signals were detected by Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). The differences in intensities of the signals were analysed by ImageJ software (National Institutes of Health, Bethesda, MD). The results are expressed as the ratio of house-

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