Dysregulation at multiple points of the kynurenine pathway is a ubiquitous feature of renal cancer: implications for tumour immune evasion

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BACKGROUND: Indoleamine 2,3-dioxygenase (IDO), the first step in the kynurenine pathway (KP), is upregulated in some cancers and represents an attractive therapeutic target given its role in tumour immune evasion. However, the recent failure of an IDO inhibitor in a late phase trial raises questions about this strategy.

METHODS: Matched renal cell carcinoma (RCC) and normal kidney tissues were subject to proteomic profiling. Tissue immunohistochemistry and gene expression data were used to validate findings. Phenotypic effects of loss/gain of expression were examined in vitro.

RESULTS: Quinolinate phosphoribosyltransferase (QPRT), the final and rate-limiting enzyme in the KP, was identified as being downregulated in RCC. Loss of QPRT expression led to increased potential for anchorage-independent growth. Gene expression, mass spectrometry (clear cell and chromophobe RCC) and tissue immunohistochemistry (clear cell, papillary and chromophobe), confirmed loss or decreased expression of QPRT and showed downregulation of other KP enzymes, including kynurenine 3-monooxygenase (KMO) and 3-hydroxyanthranilate-3,4-dioxygenase (HAAO), with a concomitant maintenance or upregulation of nicotinamide phosphoribosyltransferase (NAMPT), the key enzyme in the NAD+ salvage pathway.

CONCLUSIONS: Widespread dysregulation of the KP is common in RCC and is likely to contribute to tumour immune evasion, carrying implications for effective therapeutic targeting of this critical pathway.

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BACKGROUND
Renal cancer is one of the ten most common adult cancers, accounting for over 100,000 deaths worldwide each year.1 It is also a cancer with one of the highest projected increases in incidence over the next two decades.2 Almost 90% of these cancers arise within the renal parenchyma and are termed renal cell carcinomas (RCCs). The most common (75%) histological subtype is clear cell RCC (conventional) (ccRCC), which are characterised by loss of the VHL tumour-suppressor gene, followed by papillary (10–15%) and chromophobe (5%) RCC. Each is considered to arise from distinct parts of the human nephron, are genetically distinct3 and vary considerably in their clinical behaviour.

Treatment options for patients with RCC have burgeoned in recent years, but despite this, it is a cancer that remains incurable for most patients with advanced disease. As in a number of other tumour types, immunotherapy, in the form of checkpoint inhibitors (CPIs), has come to the forefront of patient treatment, both in the first- and second-line settings. However, while some patients are observed to have deep and durable responses to these agents, many patients fail to respond.4 It appears therefore that, alone, CPIs are not always sufficient to overcome immune evasion and immune tolerance by tumours. Hence, increasing attention is being focussed on combining these drugs with other immune-modifying targeted agents.5

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Alterations in cellular metabolism are a hallmark of cancer, most notably perhaps the ‘Warburg effect’, that describes the increased rate of glycolysis with reduced oxidative phosphorylation characteristic of tumours. We were among the first to demonstrate this comprehensively in renal cancers, using a proteomic approach, showing an upregulation in the majority of proteins in the glycolytic pathway and a parallel downregulation of mitochondrial enzymes in comparison to normal renal tissues, highlighting novel opportunities for therapeutic targeting.

More recently, alteration in the metabolism of the essential amino acid tryptophan in cancer, through the kynurenine pathway, has come to wide attention as a mechanism by which tumours may escape immune control and promote disease progression. The enzymes indoleamine 2,3-dioxygenase (IDO1, IDO2) and tryptophan 2,3-dioxygenase (TDO) initiate the first steps in the kynurenine pathway, converting tryptophan to kynurenine, with the TDO-dependent pathway in the liver normally accounting for the majority of tryptophan metabolism and IDO-mediated metabolism predominantly occurring secondary to inflammation and cytokine-induced upregulation. In normal physiology, IDO plays an important role in tolerance to non-self-antigens, for example foetal antigens, where such immune nonresponsiveness may be important. Upregulation of IDO leads to tryptophan depletion and kynurenine accumulation, which appear to work in concert to mediate immunosuppression, via T cell anergy and apoptosis and suppressed T cell differentiation. The harnessing of this phenomenon by tumours has led to the development of inhibitors of IDO1 that have progressed to clinical trials in combination with CPI. Despite much promise, initial results have, however, been disappointing and the future of these agents currently remains uncertain.

This may be because of patient selection and lack of suitable profiling of immuno-regulating metabolism, emphasising the need for a deeper understanding of these pathways.

Here, using a proteomic-based approach, we show that the kynurenine pathway is more broadly disrupted than has been previously considered, extending beyond IDO1, that this is a common event in RCC and is not just restricted to the clear cell histological subtype and suggests possible redundancy in the pathway within the tumour setting. Our findings are of significance in terms of highlighting various aspects of this pathway for potential therapeutic targeting, patient stratification and may have implications for other cancers.

METHODS
Reagents
Reagents were purchased as follows: general chemicals (Sigma, Poole, UK and VWR, Poole, UK), goat serum and human serum albumin, mouse monoclonal anti-β-actin antibody clone AC15 (Sigma), Hybond™ super NC membrane, Pharmalyte pH 3–10, IPG strips, dry strip cover fluid, bromophenol blue and PlusOne Silver Stain (GE Healthcare, Little Chalfont, UK), CHAPS (Calbiochem, San Diego, USA), LMP agarose, Minimum Essential Medium (MEM)- α medium, L-glutamine, trypsin with EDTA, G418, HBSS and diego, USA), LMP agarose, Minimum Essential Medium (MEM)- α medium supplemented with 10% v/v FCS and 1% v/v L-glutamine, as previously described. All cell lines were screened for mycoplasma contamination.

Quinolate phosphoribosyltransferase (QPRT) expression and QPRT knockdown stable transfectants
QPRT gene expression constructs containing empty vector (pFB-HYG) or QPRT (pFB-HYG-QPRT) were created. The QPRT insert was made by PCR of human cDNA (forward primer: GCCTCGACGACTGTTTGTTGGGACTTGGAGC) followed by digestion with SalI and Xho1 and ligation into the multiple cloning site of pFB. Correct sequence was confirmed by sequencing. These constructs were amplified in XL1 Blue competent Escherichia coli (Stratagene), then introduced into 293-PhoenixA cells using SiPORT transfection agent (Ambion). Supernatant containing viral particles was harvested on days 3 and 4, and polybrene was added to 8 μg/ml. Four ml of medium was used to infect each T75 flask of a VHL-defective 786-0 cell line lacking endogenous VHL followed by selection with hygromycin (0.5 mg/ml) and were designated 786-pFB and 786-QPRT, respectively. Cells were maintained in MEM-α medium supplemented with 10% FCS, 1% v/v L-glutamine, G418 (1 mg/ml) and hygromycin selection (0.5 mg/ml).

QPRT short hairpin RNA (shRNA) constructs containing a non-specific shRNA (pRetroSuper-shRNA-scramble) or shRNA targeting QPRT (pRetroSuper-shRNA-QPRT) were created using the following oligonucleotides (capitals indicate nucleotides corresponding to QPRT sequence or control, lower case indicates loop and linker sequences):

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shQPR: Forward gatccccGGCTTGTATTTCTCCCTCAatccagagagTCAGGGGA GAAATCAGGGCTtttttgaaa Reverse agcttttccaaagacGCTGGATTCTCTCCCTCActcctgagatgAGG GAGAAATCAGGGCGggg
Scramble: Forward gatccccCTTCCAGCGTACCTGCTGcctcaagagaCGAGGTAAACGCT GAGAACTTCCGGggg
Reverse gttttctggagaatCTTACGCCGTTACCTCGGTcctcaagagacGGACGCTAAC GCTGTAAGggg
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Oligonucleotide pairs were annealed by heating to 100 °C for 2 min and cooling slowly to room temperature and ligated into HindIII/BglII-digested pRetroSuper-puro (a gift from Darren Tomlinson). Constructs were amplified in XL1 Blue competent E. coli (Stratagene), then introduced into 293-PhoenixA cells using SiPORT transfection agent (Ambion). Supernatant containing viral particles was harvested on days 3 and 4, and polybrene was added to 8 μg/ml. Four ml of the medium was used to infect each T75 flask of 786-1-VHL cells, followed by selection with puromycin (2 μg/ml). Cell lines were maintained in MEM-α medium supple-
mented with 10% FCS, 1% v/v L-glutamine, G418 (1 mg/ml) and puromycin (2 µg/ml).

Two-dimensional (2D) gel electrophoresis
For global protein profiling of 786-0 cells +/- VHL, protein extracts (80 µg protein for analytical gels and 1 mg for preparative gels) were analysed by 2D polyacrylamide gel electrophoresis (2D-PAGE) over a pH range of 4–7 using a combined IPGPhor and Multiphor approach.28 Protein samples were loaded onto IPG strips by overnight in-gel rehydration and focussing carried out for a total of 65 kVh. Strips were equilibrated in running buffer, placed onto polyacrylamide gels (10% resolving gel with 4% stacking gel) and electrophoresed overnight (12.5 °C, 18 mA/gel). Gels were stained using OWL silver stain and scanned using a Personal PAGE system. Gels were stripped by overnight in-gel rehydration and focussing carried out for 2 min, air dried, washed briefly in TBS-T, endogenous peroxidase blocked using 0.6% v/v hydrogen peroxide in methanol for 5 min and washed again. After overnight incubation at 4 °C in rabbit antiserum to QPRT diluted 1:20,000 in TBS/0.1% w/v HSA with y in TBS-T, endogenous peroxidase blocked using 0.6% v/v hydrogen peroxide in methanol for 5 min and washed again. After overnight incubation at 4 °C in rabbit antiserum to QPRT diluted 1:20,000 in TBS/0.1% w/v HSA with 0.1% w/v sodium azide, slides were washed in TBS-T and labelled using the rabbit EnVision+ detection system with DAB substrate according to the manufacturer’s instructions. Slides were counterstained with Mayer’s haematoxylin and mounted using DePeX mounting medium. Negative control sections were probed with an irrelevant antibody. Immunohistochemistry on sections of frozen tumour and normal tissue (5 µm OCT-embedded) was similarly performed.22

Western blotting of protein lysates of cell lines or matched tumour/normal tissue was performed as previously described24 with samples separated by 10% sodium dodecyl sulfate (SDS)-PAGE and transferred to Hybrid-C Super NC membrane in Towbin’s buffer. After blocking with TBS-T/10% w/v dried skimmed milk, blots were probed with antibodies to QPRT (1:20,000), VHL (1 µg/ml) and β-actin (5 ng/ml; protein-loading control). After washing, blots were incubated with anti-rabbit or anti-mouse HRP-conjugated Envision+ reagent, then washed again and exposed to film. In all cases, western blots were normalised using densitometric scanning of parallel Coomassie blue-stained gels for total protein load, given the limitations of housekeeping genes.25 Additional blotting against beta actin was variably employed.

Measurement of quinolinic acid (QUIN) in renal tissue samples
Sections from matched pairs of frozen tumour/normal tissue were cut into 1 M HCL and stored at −80 °C. QUIN levels were measured in Schwarz laboratory by gas chromatography MS, performed as previously described.26

Effects of QPRT on cell proliferation
786-pFB and 786-iQPRT cells in an exponential phase of growth were harvested and plated in 96-well plates (1 × 104 cells/well) and cultured for 24, 48 and 72 h at 37 °C with 5% CO2/95% air. Viable cells were quantified using WST-1 reagent according to the manufacturer’s protocol, and absorbance was measured at 450 and 650 nm. Wells were seeded in triplicate, and three independent experiments were run.

Effects of QPRT on anchorage-independent colony-formation assay
To investigate anchorage-independent growth of 786-0 cell lines stably transduced with constructs of interest, WT VHL (786+ VHL)+shRNA scramble control, WT VHL (786+VHL)+shRNA QPRT, VHL negative+empty vector (786+pFB) and VHL negative+QPRT (786+iQPRT) were cultured at 4 × 104 cells/well as previously described.27 Viable colonies were stained with 8 mM p-iodonitrotetrazolium violet, and colonies with a diameter of >1 mm were then counted within 10 random fields of view using an eyepiece graticule (1 cm2 area, made up of 10 × 10 mm squares) on a bright field microscope. The mean number of colonies per 10 cm2 from four independent experiments was determined. Statistical significance was assessed by Student’s t test. The NIH3T3 cell lines containing either a control or H1047R vector were employed as negative and positive controls for the assay, respectively.

Interrogation of LC-MS/MS RCC proteomic data sets for kynurenine pathway changes
As part of a large proteogenomic study of RCC (manuscript in preparation), LC-MS/MS proteomic data sets were created containing 13 matched tumour/normal pairs of ccRCC and 7 matched tumour/normal pairs of chromophobe RCC following expert pathological review of the selected blocks. We were able to interrogate this data for evidence of protein expression of enzymes from the kynurenine pathway. For each sample, 30-µm sections equivalent to 3 cm2 surface area of tissue were lysed in excess lysis solution (250 µl of 3% SDS in 50 mM Tris-HCl, pH 7.6), and DNA was sheared with brief sonication. Samples were then heated at 95 °C for 10 min, centrifuged at 13,000 × g for 8 min, supernatant removed, and protein concentration was measured by BCA assay. Dithiothreitol was added to samples at a final concentration of 30 mM, and samples were heated at 95 °C for 5 min. Seventy µg of protein was processed by the iTRap protocol as previously described.28 Label-free MS and data analysis were conducted essentially as previously described29 but using an
Among QPRT-positive cell lines, only 10% of colonies were estimated (Fig. 2f). Colony size was also affected by QPRT expression status. Knockdown of QPRT in 786-0 cells increases cellular anchorage-dependent proliferation, whereas all 786-0 cells, irrespective of whether +/− VHL, formed small colonies (diameter of >1 mm), 786−/− VHL cells, we identified QPRT as being undetectable in −/− VHL cells and expressed at relatively high levels following re-introduction of WT VHL (Fig. 1a and Supplementary Table 2). This was confirmed using immunocytochemistry and western blotting of the 786-0 −/− VHL cell lines (Fig. 1b, c). However, this apparent VHL-dependent expression of QPRT was not observed in UMRC2 and RCC4+/− VHL cell line pairs (Fig. 1d), which all retained expression. Western blotting of frozen tissue lysates from ten ccRCC/normal kidney matched pairs with tumour VHL mutation status defined in all but one pair (Fig. 1e) showed significant loss or downregulation of QPRT expression in ccRCC tissues compared to their normal counterparts in nine cases, although no relationship with VHL mutation status or mutation type was apparent. The loss of QPRT in clear cell tumours was confirmed in 12/13 patients by IHC (Fig. 1f).

Taken together, these results indicate that loss of QPRT is a common event in ccRCC although regulation by VHL in vitro is cell line dependent. QPRT catalyses the conversion of QUIN, produced within the kynurenine pathway, to nicotinamide adenine dinucleotide (NAD+). The reaction catalysed by QPRT is a rate-limiting step in this pathway, therefore loss of QPRT may lead to an increase in the level of QUIN. In support of this hypothesis, we found increased levels of QUIN in ccRCC tissues relative to patient-matched normal kidney cortex (Fig. 1g).

IHC analysis of the kynurenine pathway TMA FFPE sections (4 μm) were mounted onto Plus Frost slides, and dewaxing and epitope recovery was carried out by heating in a Cookworks pressure cooker for 5 min on high followed by 25 min on low temperature in Access Revelation solution. Endogenous peroxidase activity was blocked in Bloxall and 2.5% v/v normal horse serum used as a protein block. Antibodies were optimally diluted in Antibody Diluent and detected using rabbit HRP-conjugated secondary antibodies followed by Impact DAB substrate for 5 min at room temperature, then counterstained with Mayer’s haematoxylin for 30 s dehydrated, cleared in xylene and mounted in DPX.

RESULTS

QPRT is downregulated in ccRCC

From our 2D-PAGE-based comparison of whole-cell lysates of 786-0 −/− VHL cells, we identified QPRT as being undetectable in −/− VHL cells and expressed at relatively high levels following re-introduction of WT VHL (Fig. 1a and Supplementary Table 2). This was confirmed using immunocytochemistry and western blotting of the 786-0 −/− VHL cell lines (Fig. 1b, c). However, this apparent VHL-dependent expression of QPRT was not observed in UMRC2 and RCC4+/− VHL cell line pairs (Fig. 1d), which all retained expression. Western blotting of frozen tissue lysates from ten ccRCC/normal kidney matched pairs with tumour VHL mutation status defined in all but one pair (Fig. 1e) showed significant loss or downregulation of QPRT expression in ccRCC tissues compared to their normal counterparts in nine cases, although no relationship with VHL mutation status or mutation type was apparent. The loss of QPRT in clear cell tumours was confirmed in 12/13 patients by IHC (Fig. 1f).

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IHC TMA analysis of the kynurenine pathway by TMA

In normal renal cortex, weak-to-moderate granular staining of proximal tubules (predominantly cytoplasmic) was observed for IDO1 and moderate/strong expression of KYNU, QPRT and KMO, with absent-to-moderate staining for NAMPT. In the case of IDO1, staining was noticeably localised to basolateral aspect of the tubules. Glomerular reactivity was also seen for NAMPT and KYNU. In the medulla, tubules were largely negative, and two cases showed weak expression of IDO1, NAMPT and QPRT, although one of these showed distinct populations of tubules with moderate QPRT staining (Fig. 6).

In ccRCC cases, expression of IDO1, KYNU, QPRT and KMO was greatly reduced compared with normal kidney cortex and, in some cases, was absent (Fig. 6, Supplementary Table 4). IDO1 was not detected in tumour cells but only in occasional inflammatory cells or in endothelial cells, and KMO1 was also seen in some endothelial cells. For QPRT, five cases were completely negative, four cases showed only focal positivity and two weak-to-moderate staining. Conversely, NAMPT expression was absent from clear cell tumour cells in two cases but positive in five (from weak to strong)
with focal positivity seen in a further four cases. Positive staining was also seen with occasional inflammatory cells and endothelial cells. Of note, the strongest staining for any of the enzymes amongst the ccRCC cases was observed in the rhabdoid cells contained in tumour 4728. A similar pattern of generally reduced staining compared with normal kidney was also seen with papillary and chromophobe cases and generally weak-to-moderate NAMPT. The findings were consistent with our MS results and demonstrate that disruption of the kynurenine pathway is a feature of RCC across histological subtypes.

DISCUSSION

This study provides the first comprehensive demonstration of a wide and coordinate dysregulation of the kynurenine pathway in RCC and that this is a common, unifying event, highlighting its importance in the pathogenesis of these cancers and potentially providing insights of relevance to therapeutic targeting.

The kynurenine pathway is the main route for degradation of the essential amino acid tryptophan and de novo synthesis of NAD⁺, generating numerous other active intermediate metabolites including kynurenine, kynurenic acid, anthranilic acid,
Fig. 2 Loss of QPRT expression increases anchorage-independent growth. 

a Western blot showing QPRT expression in control and QPRT-transfected VHL negative 786-0 cell lines. 

b QPRT expression in control and anti-QPRT shRNA transfected, VHL-expressing 786-0 cell lines. A single band at the expected molecular weight was observed. 

c, d Relative cell number of 786-0 cell lines plus or minus QPRT, as measured using WST1 assay. 

e, f Impact of QPRT transfection or knockdown on relative colony number in soft agar colony-formation assay and representative images. Colony number per 10 cm² are provided as a mean of the sum over four independent experiments and then standardised as a percentage to either 786-pFB or 786-VHL controls. Significant differences are indicated (asterisk (*); 786-+QPRT versus 786-+pFB, \( p = 0.017 \), 786-+VHL+shQPRT versus either control, \( p = 0.001 \)).
picolinic acid and QUIN. Dysregulation of the pathway was initially highlighted in neuropsychiatric disorders but has now been implicated more widely and increasingly in tumorigenesis and immune evasion. Although in the liver, where the majority of tryptophan degradation occurs, constitutively expressed TDO2 is the initial rate-limiting enzyme in the pathway, in most other tissues IDO1 is the first and rate-limiting enzyme. Inducible by inflammatory cytokines, IDO/IDO1 has been reported to be expressed in many cancers and hypothesised to play a role in tryptophan degradation and accumulation of active metabolites in the kynurenine pathway, both of which result in T cell/immune suppression leading to the idea of IDO1 inhibitors as useful anticancer therapeutics to overcome immune resistance, for example in combination with vaccine strategies. However, dysregulation at multiple points of the kynurenine pathway is...
IDO1 expression is highly dependent on tumour type, and many cancers, including renal, melanoma and thyroid, have absent or low expression of IDO1 in tumour cells in most cases. This has been confirmed subsequently with the demonstration of IDO1 expression in endothelial cells and macrophages in RCC tissues and a recent extensive study across many cancers where, although 80% of renal carcinomas were positive, IDO1 was absent from tumour cells and present predominantly in endothelial cells or in some lymphocyte-rich stroma. This in agreement with our IHC results and with the relatively low expression being undetectable by MS.

We initially observed that in a VHL−/− cell line pair, expression of QPRT increased following the introduction of VHL. QPRT is the final enzyme in the kynurenine pathway, converting QUIN to NAD. However, it is apparent that this is not mainly responsible for increased QPRT expression in endothelial cells or in some lymphocyte-rich stroma. The underlying biology leading to such dysregulation, and whether common or divergent mechanisms are responsible, remains uncertain, but its consistency across more than one type of RCC suggests that it is a key and potentially early event.

As predicted, we found that that loss of QPRT was associated with increased QUIN in tumour tissue, and metabolomic studies of urine and tissue samples from RCC patients reported higher concentrations of quinolinol compared with healthy controls or normal tissue. QUIN has been reported to activate β-Catenin and increase proliferation in colon cancer cell lines, and in RCC cell lines, variable effects of quinolinate on cell viability or proliferation have been reported although very different quinolinate concentrations have been used across studies. We did not observe increased cell proliferation in an in vitro QPRT knockdown model. However, we did see a pronounced increase in anchorage-independent growth in response to loss of QPRT. It is possible that this is mediated by QUIN accumulation and exerting this effect through a potential autocrine loop involving N-methyl-d-aspartate receptors (NMDARs), since this receptor–ligand binding is important for its role in neurological disease and expression of NMDAR subunits has been demonstrated in the normal kidney cortex and medulla and across multiple cancer types, with receptor blockade reducing cancer cell proliferation and invasive-ness in numerous cancers in vitro. QUIN is also known to be an immune modulator. For example, treatment with QUIN induced selective apoptosis in vitro of murine thymocytes and of T helper type 1 (Th1) but not of Th2 cells, and mice treated with QUIN had significantly reduced levels of immature thymocytes in the thymus. In a microenvironment deficient in tryptophan, QUIN was found to inhibit proliferation of both lymphocytes and natural killer (NK) cells. Interestingly, accumulation of QUIN has been reported to occur in human gliomas but accompanied by increased QPRT expression and supporting NAD generation through this pathway rather than the NAMPT-mediated pathway. QPRT was shown to be induced by oxidative stress,
Temozolomide and irradiation and to be associated with poorer prognosis in recurrent tumours after radiochemotherapy, potentially through increasing resistance. These results suggest that targeting QPRT itself may be a potential therapeutic option and indeed data from a cell line model have implicated upregulation of QPRT as conferring resistance to NAMPT inhibitors. Examination of a parallel existing LC-MS/MS proteomic data set generated by our group as part of an ongoing proteomic study in RCC not only confirmed loss of QPRT in RCC tissues but also showed loss of five other enzymes in the kynurenine pathway, namely HAAO, KMO, kynurenine formamidase and KYAT1 and 2. Remarkably little is known about these enzymes in cancer although upregulation of KMO has been described in hepatocellular carcinoma. This may be due at least in part to the lack of availability of good antibodies, at least until recently, which is why we generated our own QPRT antibody. Critically, one can expect that loss of KMO, alongside an increase in IDO expression, will lead to the accumulation in tumour tissue of kynurenine. In metabolomic studies of mouse RCC xenografts and human RCC tumours, significantly lower tryptophan and higher kynurenine levels and higher quinolinic and kynurenine levels, respectively, were seen in tumours compared with controls. A metabolomic study involving ccRCC along with chromophobe and papillary tissue samples also identified elevated kynurenine compared with controls but in ccRCC cases only. The immune-suppressive properties of kynurenine are well described, and it has been shown to inhibit T cell and NK cell proliferation and promote immune suppression via the aryl hydrocarbon receptor. Furthermore, it can promote cancer cell survival and motility. 3-Hydroxyanthranilic acid, the substrate for HAAO, has also been shown to have multiple roles in promoting tumour immune evasion, by promoting apoptosis of Th1 and NK cells, promoting differentiation of regulatory T cells and inhibiting T cell proliferation. This novel observation, that multiple enzymes in the kynurenine pathway downstream of IDO are all downregulated and in a manner likely to promote tumorigenesis and immune evasion, is striking. The loss of QPRT, and hence the blockade of de novo biosynthesis of

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**Fig. 5** Alterations to the kynurenine pathway in RCC. The various enzymes and intermediates generated through this de novo synthesis of NAD\(^+\) from tryptophan are shown together with the salvage pathway route for generation of NAD\(^+\) from nicotinamide, catalysed by NAMPT. Enzymes for which we have strong evidence of downregulation in RCC tissues are marked with a solid grey arrow. Enzymes or substrates observed to be upregulated are marked with a hatched arrow. Enzymes with evidence to suggest they may be down regulated are marked by an open grey arrow.

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NAD+ may initially seem at odds with the requirement of cells, and especially cancer cells, for this molecule. However, NAD+ may also be made from nicotinamide via the salvage pathway of which the enzyme NAMPT is a key component, and this pathway is often preferred in cancer cells. Consistent with this, we observed upregulation of NAMPT occurring as a common event in RCC, confirming a recent IHC study. As such, NAMPT forms an attractive therapeutic target, and it is of note that KPT-9274, a NAMPT/PAK4 inhibitor, has recently been reported to have activity against renal cancer xenografts.

The kynurenine pathway forms a particularly attractive target for therapy, since it seems to regulate tolerance to non-self-antigens, thus reducing the risk of immune-related adverse events often seen with immune-checkpoint inhibition. Whether inhibition of IDO1 alone is sufficient to overcome pathway dysregulation is uncertain, as exemplified by the recent negative results seen with epacadostat in patients with melanoma.

Indeed, a planned Phase 3 trial of this agent in combination with pembrolizumab in patients with RCC has been halted based on these findings. While combined IDO1/TDO inhibitors are currently in early Phase trials (NCT03208959), our results suggest that, in RCC at least, the kynurenine pathway is much more widely dysregulated and may require further downstream modulation in addition to IDO inhibition.

Our study has its limitations and a number of questions remain unanswered that would need to be explored in future studies. Whether the phenotypic consequences of loss or gain of QPRT extend beyond effects on colony formation, for example, and whether such changes are consistently observed across RCC cell lines, remains uncertain. Furthermore, since the 786-0 cell line is known to contain a phosphatase and tensin homologue (PTEN) mutation (although rarely observed in ccRCC tissues), it would be of interest to examine how deficiency of PTEN and/or dysregulation of the phosphoinositide-3 kinase/AKT/mammalian target of rapamycin signalling pathway impacts QPRT expression. We have also not examined how dysregulation of the kynurenine pathway in RCC correlates with patient outcomes.

In conclusion, we have demonstrated a widespread and apparently coordinated dysregulation of the kynurenine pathway in RCC. These findings have implications for current strategies aimed at therapeutically targeting this critical pathway and highlight the potential for novel treatment strategies, such as inhibition of NAMPT.

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AUTHOR CONTRIBUTIONS
R.E.R., P.S.J. and N.S.V. supervised the study with A.H. and E.R.M. also contributing to study design; R.A.C. and R.E.B. conducted the initial 2D-PAGE work; J. Brown facilitated access to tissue specimens and clinical data; K.R.D., S.T., R.S. and A.Z. conducted the mass spectrometric studies; K.R.D., M.K. and J. Burns conducted and oversaw phenotypic studies; T.F. performed the assay for tissue QUIN concentrations; M.S. and H.S. performed the IHC; S.B. and M.M. provided expert pathological review; L.F., A.B., Y.R., M.L. and G.S. provided transcriptomic data sets; N.H. analysed the data and, together with R.A.C., N.S.V. and R.E.B., drafted the manuscript; all authors provided comments on the manuscript and had final approval of the submitted version.

ADDITIONAL INFORMATION
Ethics approval and consent to participate The study was approved by the Leeds East Research Ethics Committee (ethical approval 15/YH/0080) and performed in accordance with the Declaration of Helsinki. All samples were obtained with the patients’ informed consent.

Consent for publication No individually identifiable data are presented.

Data availability RNA-seq data, generated through our ICGC CAGEKID study, has been deposited in a public repository as described elsewhere: Scelo et al. Proteomic data sets are available on request.

Competing interests A.H. is Editor-in-Chief of British Journal of Cancer. R.S.’s current role is Publishing Editor for British Journal of Cancer.

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