Tumour necrosis factor receptor-associated factor-1 (TRAF-1) expression is increased in renal cell carcinoma patient serum but decreased in cancer tissue compared with normal: potential biomarker significance

R. Rajandram1,2,3, N. Y. Yap1, J. Pailoor4,5, A. H. A. Razack1,5, K. L. Ng1,3,5, T. A. Ong1,5, C. Morais3 and G. C. Obe3

1Department of Surgery, Faculty of Medicine, University Malaya, Kuala Lumpur, and 2University Malaya Cancer Research Institute (UMCRI), Kuala Lumpur, Malaysia; 3Centre for Kidney Disease Research, School of Medicine, The University of Queenslands, Translational Research Institute, Brisbane, Qld, Australia; 4Department of Pathology, Faculty of Medicine, University Malaya, Kuala Lumpur, Malaysia; 5University Malaya Medical Centre, Kuala Lumpur, Malaysia

Summary
Renal cell carcinoma (RCC) generally has a poor prognosis because of late diagnosis and metastasis. We have previously described decreased tumour necrosis factor receptor-associated factor-1 (TRAF-1) in RCC compared with paired normal kidney in a patient cohort in Australia. In the present study, TRAF-1 expression in clear cell RCC (ccRCC) and normal kidney was again compared, but in a cohort from University Malaya Medical Centre. Serum TRAF-1 was also evaluated in RCC and normal samples. Immunohistochemistry with automated batch staining and Aperio ImageScope morphometry was used to compare TRAF-1 in 61 ccRCC with paired normal kidney tissue. Serum from 15 newly diagnosed and untreated ccRCC and 15 healthy people was tested for TRAF-1 using ELISA. In this cohort, TRAF-1 was highly expressed in proximal tubular epithelium of normal kidney, and significantly decreased in ccRCC tissue (p < 0.001). Conversely, TRAF-1 in serum from ccRCC patients was significantly increased over control serum (132 ± 30 versus 54 ± 14 pg/mL, respectively; p = 0.013). Decreased TRAF-1 in RCC tissue, reported previously, was confirmed. This, along with significantly increased serum TRAF-1 may indicate the protein is actively secreted during development and progression of ccRCC. Therefore, the increased serum TRAF-1 may be a useful non-invasive indicator of RCC development.

Key words: Apoptosis, kidney cancer, renal cell carcinoma, TRAF, tumour necrosis factor receptor-associated factor.

Received 23 January, revised 1 May, accepted 5 May 2014

INTRODUCTION
Renal cell carcinoma (RCC) is the most common of the kidney cancers, accounting for 90–95% of neoplasms of the kidney. It is the third most common genitourinary cancer, behind prostate and bladder cancer, and accounts for around 3% of all human malignancies.1,2 The most common of the RCCs, at approximately 85%, is clear cell RCC (ccRCC).1–3 RCCs are traditionally thought to arise from the epithelial cells of the renal tubule, most commonly the proximal tubular epithelium, especially for ccRCC.4–6 Patients usually have no symptoms during early phases of renal cancer growth and, in many cases, the diagnosis of RCC is made incidentally while scanning the kidneys for other diseases, or made late with patients presenting with RCC metastases. The average survival following metastatic RCC is about 4 months, and only 10% of patients survive 1 year.5 Thus, the identification of effective therapeutic biomarkers for early diagnosis and for disease progression is imperative. This requires identification of tissue-specific molecules that are implicated in the development and progression of RCC.

Although there is now evidence for a significant role for the tumour necrosis factor (TNF) receptor-associated factor (TRAF) family of proteins in the pathogenesis of some cancers like lymphomas and myelomas,6–11 little is known about their role in the development and progression of RCC. The TRAF family consists of six members (TRAF-1 to TRAF-6) that have tumour necrosis factor (TNF) receptor-associated factor (TRAF) family of proteins in the pathogenesis of some cancers like lymphomas and myelomas,6–11 little is known about their role in the development and progression of RCC. The TRAF family consists of six members (TRAF-1 to TRAF-6) that have conserved C-terminal domain required for binding signal-transduction adaptor proteins to the TNF receptor.12–14 Two additional functional domains located at the N terminus, the zinc finger and the RING finger domains, may be essential for the activation of specific downstream signalling components and transcription factors, such as nuclear factor kappaB (NF-kB).13,14 TRAF-1 is an adaptor protein that involved in the regulation of cell survival, proliferation, differentiation and stress responses.11,12,15 Recent published data from our laboratory demonstrated that RCC cells, treated in vitro with an immunotherapy (interferon-gamma) with and without radiation to induce apoptosis, had significantly increased expression of TRAF-1 DNA and protein concurrent with increased apoptosis.16 Use of silencing RNA to TRAF-1 in RCC cell culture experiments decreased therapy-induced apoptosis. In a later publication on TRAF-1 expression in human RCC and normal kidney samples at Princess Alexandra Hospital in Brisbane, Australia, high TRAF-1 was found in the proximal tubular epithelium of normal kidney, with a significant decrease in ccRCC from our Australia patient samples.17 The decrease in the RCC samples may help explain, in part, the negligible amount of apoptosis in RCC, and the resistance of RCC to therapies.18,19 No serum was available for TRAF-1 measurements from this patient cohort.
The aim of the present study was to investigate TRAF-1 expression levels in RCC and paired normal kidney from a cohort of patients from a Malaysian hospital, to verify or otherwise the results of our work with the Australian cohort of patients. Additional investigations to our previous publication involved analysis of levels of TRAF-1 in serum from newly diagnosed, untreated ccRCC patients prior to nephrectomy, compared with age and gender-matched healthy controls to investigate any association between serum and tissue levels.

MATERIALS AND METHODS

Ethics approval for use of the human kidney tissue samples and serum was obtained from the University Malaya Medical Centre (UMMC) Ethics Committee. Written consent was obtained from all patients. The present study examined 61 cases of formalin fixed and blocked ccRCC tissue with paired normal kidney. The kidney cancer samples were taken from the periphery of the cancer, away from any necrotic core. In addition, serum was used from 15 newly diagnosed and untreated ccRCC patients prior to nephrectomy, and 15 age and gender matched healthy volunteers. Clinical data for all patients were available.

Immunohistochemistry and quantitative analysis

The present study assessed complete sections and blocks of ccRCC or normal kidney, compared with our previous study where small cores of kidney were used in multi-sample tissue microarrays.17 Entire histology sections were used to ensure a more comprehensive comparison of TRAF-1 expression in ccRCC versus paired healthy kidney. We did this because a common criticism of tissue microarray is that the small cores of sampled cancer may not be representative of the whole tumour,25 particularly in heterogeneous cancers such as RCC.12 New histology sections for each specimen were stained with haematoxylin and eosin (H&E). Other sections were cut onto Superfrost Plus histology slides (Thermo Scientific, USA) for immunohistochemistry (IHC). Primary antibodies for TRAF-1 (1:1000; IHC World, US Biological, USA) were used for IHC. Positive tissue samples (human small intestine) were used for TRAF-1 to verify its activity in human tissue, and negative controls without primary antibody were prepared for each batch stain. Non-specific binding of peroxidase or antibody was blocked with 0.1% sodium azide in 0.3% hydrogen peroxide (H2O2) in Tris-buffered saline (TBS; 10 min), followed by 5% non-fat milk powder in TBS containing 0.05% Tween-20 (Blotto; 20 min), then a 1:100 dilution of normal serum in 1% bovine serum albumin (BSA) in TBS (5 min). Antibodies were diluted in 1% BSA in TBS. The IHC procedure was performed using a Bond-Max automated immunostainer (Vision BioSystems, Australia) in the Histology Unit, Queensland Institute for Medical Research, Brisbane, Australia by Histology Manager, Mr Clay Winterford. The kit used for IHC was a Bond Polymer Refine Detection kit (Vision Biosystems, Cat no: DS9800). The chromogen was diaminobenzidine hydrochloride (DAB). Sections were lightly counterstained with haematoxylin and then dehydrated in a series of ethanol, cleared in xylene and mounted with glass coverslips in Depex. Thus the slides were stained in a batch in a constant environment, making comparisons in expression patterns among samples as controlled as possible. After IHC, sections were scanned in an Aperio ScanScope XT slide scanning system (Aperio Technologies, USA) at ×20 magnification. Digital images of the sections were captured using ScanScope. Three random fields of the same size were selected per RCC and paired normal kidney section (20 pairs allowed this comparison). A quantitative scoring system of overall expression, intensity and subcellular localisation of TRAF-1 was performed, using the positive pixel staining algorithms of Aperio Imagescope.22 For quality assurance, slides were further evaluated under low power routine microscopy to provide a semi-quantitative estimate of staining intensity. Morphological evidence of apoptosis and proliferation was sought in each scanned view.21

Serum analysis by ELISA

The serum analysis included patients with newly diagnosed ccRCC who were nephrectomised at the UMMC, Kuala Lumpur, between August 2011 and June 2012. Patients with a history of other cancers or who were already on treatment were excluded. A total of 15 ccRCC, and 15 healthy control serum samples from separate volunteers with no medical history of cancer, were analysed. RCC patients and controls were matched for age (range for RCC patients 43–75 years, mean 62.13 ± 9.14 years; range for controls 41–74 years, mean 61.20 ± 9.93 years; p > 0.05) and gender (RCC 9 males; controls 8 males) as closely as possible. Blood samples (6 mL whole blood) were collected in BD Vacutainer SST tubes (BD, USA). Approximately 30 min after blood collection, serum was separated by centrifugation at 1000 × g and aliquoted into cryovials for storage at –80°C before analysis. The serum samples were assayed in triplicate using the TRAF-1 enzyme-linked immunosorbent assay (ELISA) kit (Casabio, China), according to the manufacturer’s guideline. Briefly, 100 μL of standards and serum samples were added and incubated for 2 h at 37°C in microtitre plates coated with TRAF-1 antibody. The plates were washed to remove unbound TRAF-1 protein before the detection antibody and substrate solution were added. Approximately 15 min was allowed for colour to develop. The colorimetric density of each well was measured at 450 nm using a microplate reader when standard dilutions were set as 1000, 500, 250, 125, 62.5, 31.3, 15.6 and 0 pg/mL. The standard curve was prepared and the values of the samples were calculated. Quantitative comparisons were then made from the triplicate analyses of each patient or control.

Statistical analyses

Data were analysed using Student’s t-test for paired groups and p < 0.05 was considered to be significant.

RESULTS

TRAF-1 had decreased expression in clear cell RCC compared with normal kidney

Figure 1 shows some examples of the differences in paired ccRCC and normal kidney samples for TRAF-1 protein expression. TRAF-1 localised strongly to the proximal tubular epithelium in normal kidney (Fig. 1A,E), generally accepted to be the site of origin of ccRCC. Qualitative analysis showed a lower overall cell expression intensity of TRAF-1 in ccRCC (Fig. 1B,D,F) compared with normal kidney. The TRAF-1 expression characteristic was routinely homogeneous in the whole of the cancer tissue. Quantitative estimation of the expression intensity using Aperio ScanScope system showed that TRAF-1 expression was significantly lower in ccRCC samples (p < 0.001) compared with paired normal kidney (Fig. 2). In data not demonstrated, all Fuhrman grades of RCC had decreased TRAF-1, and there was no significant difference amongst the grades.

As was recorded for our previous study,17 there was negligible apoptosis or active cell proliferation detected in any of the RCC or normal kidney sections. One novel characteristic of note in the ccRCC sections was strong TRAF-1 in inflammat-ory cells in and near the RCC (arrowed in Fig. 1F).

Serum TRAF-1 was increased in RCC patient samples compared with healthy controls

Serum TRAF-1 was assessed using a quantitative sandwich ELISA technique. Figure 3 demonstrates the raw data plot of RCC versus controls, with one outlier measurement for ccRCC patients (132 ± 30 pg/mL) removed, and one zero value for the controls removed to keep group numbers standard. TRAF-1 in serum from ccRCC patients was significantly increased over normal serum (132 ± 30 for ccRCC patients versus 54 ± 14 pg/mL for healthy controls; p = 0.013). If excluded values were included, the group means were 201 ± 74 for ccRCC patients and 50 ± 13 pg/mL for controls (p < 0.028). As far as we can ascertain, there have been no other publications of serum TRAF-1 levels in healthy people. The size of the RCC tumour may impact on the level of serum TRAF-1, but the significance of the difference should take into account patient variability in...
tumour size, if this does have an effect on serum TRAF-1 levels.

Discussion

The current investigation verified in a Malaysian RCC patient cohort that TRAF-1 is present in the proximal tubular epithelium of normal kidney and at significantly reduced levels in ccRCC, thereby confirming our previous work in an Australian cohort of patients. However, serum levels were the inverse of the tissue expression profile: serum TRAF-1 was higher in RCC patients than age and gender-matched controls. The role of TRAF proteins in RCC has not been clearly defined. One possibility for decreased TRAF-1 in RCC is that the low expression is relative to the low or negligible apoptosis levels seen in RCC histology. Our previous results in cell culture indicated that TRAF-1 expression was increased in RCC cells in culture when high levels of apoptosis were induced. The increased TRAF-1 in serum may reflect active secretion of the protein from the tissue as the RCC develops and, therefore,
There was also negligible apoptosis or cell proliferation seen in this new RCC patient cohort. This method confirmed the patients were used for as representative a result as possible in inflammatory TNF in TRAF-1 deficient mice. From our regulator of TNF signalling as demonstrated by enhanced pro-apoptotic TNF in TRAF-1 deficient mice. From our studies and those of others, we believe TRAF-1 expression or activation does impact on levels of apoptosis in RCC, but in a pro-apoptotic manner and the low levels of TRAF-1 in ccRCC may help explain why RCCs do not respond well to most therapies. This analysis is very complex, because there are many molecular controls of apoptosis. Gobe and colleagues found that, in RCC with increased expression of anti-apoptotic Bcl-2 and/or Bcl-XL, levels of apoptosis were minimal, and this might have assisted with progression of the cancers and resistance to treatments. However, in this same report a positive link between low expression of Bcl-2 and/or Bcl-XL in normal tissue and low expression of these proteins in RCC was seen in some patients. Enhanced resistance of RCC to anti-apoptotic Bcl-2 and/or Bcl-XL, levels of apoptosis were described, although Wu and colleagues found that regulatory molecules for apoptosis in RCC are not functioning normally or are missing. Similar principles may also apply to TRAF-1 and its presence and role in RCC apoptosis. The disparate outcome of decreased tissue versus increased serum TRAF-1 in ccRCC versus controls indicates a complex pathophysiology of TRAF-1 in RCC development and progression that needs further dissection.

TRAF-1 is also involved in the regulation of inflammatory pathways, often in association with NF-κB. TRAF-1, along with TRAF-2, are downstream intermediaries of the TNF receptor signal transduction pathways, regulating TNF-α activation of c-Jun N-terminal kinase and NF-κB signaling pathways. Additionally, expression of TRAF-1 is up-regulated as a result of lymphocyte activation, and in B-cells TRAF-1 acts together with TRAF-2 to enhance CD40-mediated activation signals. RCC is considered an immunogenic tumour, with tumour infiltrating lymphocytes and intratumoural neutrophils. Although there was lower intensity of TRAF-1 in the tumour tissue, we found increased TRAF-1 expression in inflammatory cells detected in the cancers. The distribution of inflammatory cells was considered insufficient to explain the overall increased serum TRAF-1 in RCC patients, but these cells may contribute to serum TRAF-1, as it is known that cytokines, chemokines and their receptors are sometimes secreted by the tumour. For example, the levels of IL-6, IL-1β and TNF-α were higher in the serum of RCC patients compared to healthy controls, and RCC cells produced TNF-α. Thus, in the current investigation, we have suggested that high serum TRAF-1 may be due to active secretion away from the RCC cells, but increased TRAF-1 in serum may also be associated with secretion from the highly-expressing tumour-related inflammatory cells.

In conclusion, the TRAFs have disparate roles in normal tissues and multiple cancer types, and they have been proposed as targets for therapeutic intervention in some cancers. TRAF-1 has diverse roles in cancer cell survival, apoptosis, and inflammation, but its role in RCC development and progression remains unclear. Specifically, from the results reported by us and others, TRAF-1 has decreased expression in RCC compared with normal kidney. We report here that TRAF-1 in serum has increased levels in RCC patients compared with healthy controls. These data require further investigation to determine why serum TRAF-1 increases in RCC patients in association with decreased TRAF-1 in RCC tissue. There is some evidence that reduced TRAF-1 may have a modulatory role on apoptosis. However, the data do indicate a potential use of TRAF-1 in diagnostic techniques using serum that may help indicate an actively growing and/or inflamed RCC.

Conflicts of interest and sources of funding: The authors acknowledge funding received from the University Malaya Research Grant (UMRG) number RG524/13 and the Fundamental Research Grant Scheme Malaysia number FRGS FP012-2013B. The authors state that there are no conflicts of interest to disclose.

Address for correspondence: Associate Professor Dr G. Gobe, Centre for Kidney Disease Research, School of Medicine, Translational Research Institute, Kent Street, Woolloongabba, Brisbane, QLD 4012, Australia. E-mail: g.gobe@uq.edu.au

References


