

# Increased BTB-Kelch type substrate adaptor protein immunoreactivity associates with advanced stage and poor differentiation in renal cell carcinoma

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Received November 6, 2008; Accepted December 22, 2008

DOI: 10.3892/or\_00000383

**Abstract.** Renal cell carcinoma (RCC) is a malignancy with increasing incidence. Despite the well-known prognostic factors - the stage, grade and histological subtype - the clinical course of RCC can seem quite random. The aim of this study was to evaluate markers of the oxidative system as candidate prognostic factors for RCC. Our study population consisted of 152 patients who underwent operation for RCC between 1990 and 1999. The tumours were examined with three immunohistochemical markers of the oxidative system, thioredoxin (Trx), NF-E2-related factor (Nfr2) and BTB-Kelch type substrate adaptor protein (Keap1). Cytoplasmic Keap1 staining was related to poorer prognosis in renal cancer-specific survival. The difference was statistically significant ( $P=0.02$ ). Keap1 staining was associated with a more advanced stage and a higher nuclear grade. Cytoplasmic Trx staining was associated with a trend of better prognosis in renal cancer-specific survival. Nfr2 staining was not a prognostic factor in renal cancer-specific survival. In RCC, Keap1 is associated with a more advanced disease, a higher grade and a poorer prognosis.

## Introduction

Renal cell carcinoma (RCC) is a malignancy with an increasing incidence and mortality rate (1). At the moment of diagnosis as many as one-third of the patients may have a metastasised disease. After curative treatment about half of the patients will

have a recurrence (2). The stage, Fuhrman grade, histologic subtype and many histological features such as microscopic venous invasion, necrosis and sarcomatoid features are well-known prognostic factors for RCC (3,4). Many markers associated with angiogenesis, apoptosis and proliferation have been studied as prognostic factors for RCC but no immunohisto-chemical prognostic markers for RCC are in routine use as yet (5). In the era of new treatment possibilities even for metastasised disease there is a definite need for new prognostic factors for RCC so that the treatment and follow-up of the individual patient can be planned better.

Oxidative stress can cause genomic damage and induce carcinogenesis (6). Reactive oxygen species (ROS) are oxygen molecules which have at least one unpaired electron on their outer orbital (7). ROS are involved in a variety of different cellular processes such as apoptosis, cell proliferation and carcinogenesis (6). The potential role of ROS in tumour progression includes the activation of growth factors, hypoxia inducible factor and matrix metalloproteinases and the regulation of apoptosis. There is increased oxidative stress in tumours (8). In many malignancies there is a hypoxic environment which causes alterations in the reduction/oxidation reaction (redox) state of the cells, increases mitochondrial generation of ROS and induces the stabilisation of the hypoxia inducible factor. Antioxidants and antioxidant enzymes may be important both in normal tissue and cells and malignancies but their importance is not yet well understood (9).

NF-E2-related factor (Nfr2) is a transcription factor which is redox-regulated. Nfr2 is normally repressed through its cytoplasmic location by binding to the cytoskeleton-associated protein Keap1 (BTB-Kelch type substrate adaptor protein). It has been shown that stresses that activate Nfr2 induce the dissociation of Nfr2 from Keap1. Nfr2 binds to the antioxidant response element (ARE) of genes and controls the expression of an array of detoxification and antioxidant proteins. Keap1-Nfr2 complex is thought to serve as a cytoplasmic sensor of oxidative stress (10). Keap1 is suggested to be a negative regulator of Nfr2 activity (11).

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**Key words:** renal cell carcinoma, BTB-Kelch type substrate adaptor protein, thioredoxin, oxidative system, prognosis

Thiol-containing enzyme proteins such as thioredoxins (Trx) are important in maintaining the cellular redox state in cells (12). Thioredoxin is a cysteine containing protein and an antioxidant enzyme which has been shown to be overexpressed in several tumour types and which has prognostic significance (13). It has antioxidative, anti-apoptotic and proliferative capacity (14). It has been shown that RCCs which express Trx have a low apoptic index (15).

The aim of this study was to investigate these markers of the oxidative system as candidate prognostic factors for RCC by the use of immunohistochemistry.

## Materials and methods

**Patients.** Our study population consisted of 152 patients who underwent operation for RCC between 1990 and 1999 at Oulu University Hospital. Seven patients were operated by resection, 145 by radical nephrectomy. The follow-up details were collected from patient records. The follow-up was complete in all cases. The research plan was approved by the local ethics board. The stage of the tumours was assigned by use of the TNM staging of renal cell carcinoma (16).

**Tumour samples.** Tissue microarrays of formalin-fixed, paraffin-embedded tumours were prepared and used for further analysis. All tumours were reclassified and graded according to the WHO classification by two pathologists (17).

**Immunostaining procedure.** Paraffin-embedded multi-tissue blocks were cut to 3- $\mu$ m thick sections and mounted on pre-coated slides. The sections were first deparaffinised in xylene and rehydrated in descending ethanol series. In order to enhance immunoreactivity, the sections were incubated in a citrate buffer (pH 6.0) and boiled. Endogenous peroxidase activity was eliminated by incubation in hydrogen peroxide and absolute methanol. The antibodies used in the study were: polyclonal goat IgG antibody to Keap1 (Keap-T E-20 SC-15246 Santa Cruz Biotechnology, Inc., dilution 1:100), goat anti-human IgG antibody to Trx (populi No 705 American Diagnostica, Inc., dilution 1:200) and rabbit polyclonal antibody to Nfr2 (Nfr2 C-20: sc-722 Santa Cruz Biotechnology, Inc., dilution 1:100). AEC (aminoethyl-carbazole) was used as a chromogen.

**Immunohistochemical evaluation of the oxidative system markers.** Immunohistochemical staining was evaluated simultaneously by four observers (P.H., S.K., Y.S. and H.R.) and a consensus was reached. Immunostaining for cytoplasmic Keap1 was classified as follows: negative, <10% positive, 10-40% positive and >40% positive. Immunostaining for nuclear and cytoplasmic Trx was classified negative, <25% positive, 25-75% positive and >75% positive. In further analysis for cytoplasmic Keap1 and Trx, positive subgroups were combined and cytoplasmic immunostaining for Keap1 and Trx was considered positive when staining was detected. Immunostaining for nuclear Nfr2 was classified negative, <20% positive, 20-40% positive and >40% positive. Immunostaining for cytoplasmic Nfr2 was classified negative, <20% positive, 20-50% positive and >50% positive.

Table I. Distribution of clinical and tumor stages (TNM classification), histological evaluation and immunostaining for Keap1, Trx and Nfr2.

Clinical stage	
I	70 (46%)
II	12 (8%)
III	51 (34%)
IV	19 (12%)
Tumour stage	
T1	75 (49%)
T2	12 (8%)
T3	59 (39%)
T4	6 (4%)
Histological subtype	
Clear cell	134 (88.2%)
Papillary	11 (7.2%)
Chromophobic	5 (3.3%)
Unclassified	2 (1.3%)
Fuhrman grading	
Grade I	5 (3%)
Grade II	83 (55%)
Grade III	40 (27%)
Grade IV	22 (15%)
Keap1	
Negative	65 (45%)
Positive	79 (55%)
<10%	44 (31%)
10-40%	24 (17%)
>40%	11 (8%)
Cytoplasmic Trx	
Negative	20 (14%)
Positive	123 (86%)
<25%	14 (10%)
25-75%	38 (27%)
>75%	71 (50%)
Nuclear Trx	
Negative	32 (22%)
Positive	111 (78%)
<25%	29 (20%)
25-75%	46 (32%)
>75%	36 (25%)
Cytoplasmic Nfr2	
Negative	4 (3%)
Positive	135 (97%)
<20%	50 (36%)
20-50%	52 (37%)
>50%	33 (24%)
Nuclear Nfr2	
Negative	26 (19%)
Positive	113 (81%)
<20%	61 (44%)
20-40%	38 (27%)
>40%	14 (10%)

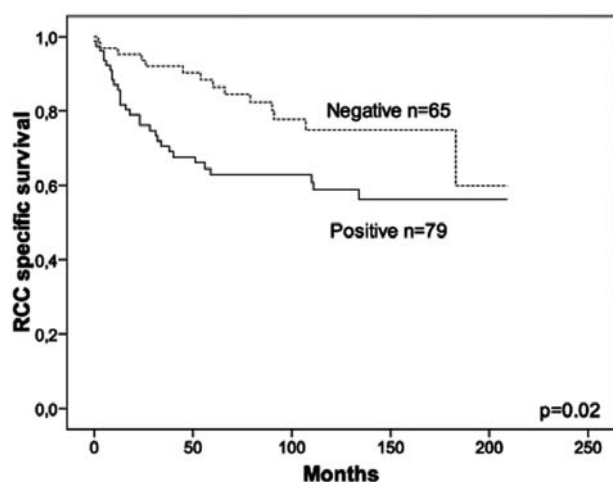


Figure 1. Keap1 as a prognostic factor in RCC-specific survival. Kaplan-Meier curve for survival of 144 patients.

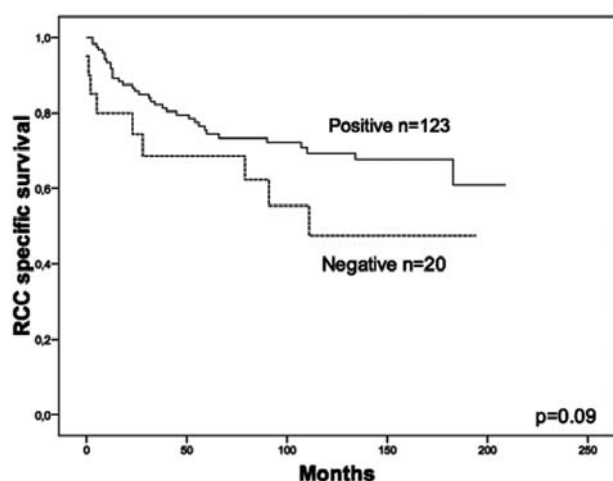


Figure 2. Trx as a prognostic factor in RCC-specific survival. Kaplan-Meier curve for survival of 143 patients.

Table II. Association between Keap1 immunostaining and stage and grade.

	Keap1	
	Negative	Positive
Stage		
I	38 (58%)	27 (42%)
II	7 (70%)	3 (30%)
III	18 (36%)	32 (64%)
IV	2 (10%)	17 (90%)
Grade		
I	3 (60%)	2 (40%)
II	42 (54%)	36 (46%)
III	13 (34%)	25 (66%)
IV	6 (29%)	15 (71%)

Table III. Association between Trx immunostaining and stage and grade.

	Cytoplasmic Trx	
	Negative	Positive
Stage		
I	8 (12%)	57 (88%)
II	2 (20%)	8 (80%)
III	8 (16%)	42 (84%)
IV	2 (11%)	16 (89%)
Grade		
I	0 (0%)	4 (100%)
II	7 (9%)	71 (91%)
III	5 (13%)	33 (87%)
IV	7 (33%)	14 (67%)

**Statistical analyses.** SPSS for Windows 15 (Chicago, IL) was used for statistical analysis. Survival was analysed with the Kaplan-Meier curve and significance of associations with log-rank test. The Cox regression multivariate model was used as in multivariate analysis.

## Results

The median age of the patients was 63 years at the time of diagnosis (range 29-86 years). Seventy-seven (51%) patients were women, 75 (49%) men. The median follow-up time was 90 months (range 0-209 months). During follow-up, 44 (29%) patients died of RCC, 40 (26%) died of other causes and 68 (45%) patients were still alive. The distribution of clinical stages and T-classes (TNM classification), the histological evaluation of tumours and the immunostaining for Keap1, Trx and Nfr2 are described in Table I. Sixty-five tumours (45%) were negative in cytoplasm immunostaining for Keap1, 79 (55%) were positive. In cytoplasmic immunostaining for Trx, 20 tumours (14%) were negative and 123 (86%) positive.

Keap1 staining was related to poorer prognosis in renal cancer-specific survival (Fig. 1). The difference was statistically significant ( $P=0.02$ ). Cytoplasmic Trx staining was associated with a trend of better prognosis in renal cancer-specific survival but the difference was not statistically significant ( $P=0.09$ ) (Fig. 2). Nuclear Trx staining was not a statistically significant prognostic factor in renal cancer-specific survival. Nfr2 staining in neither cytoplasm nor nucleus was a prognostic factor in renal cancer-specific survival. Keap1 staining was associated with a more advanced stage and a higher nuclear grade (Table II). In multivariate analysis with stage and grade, Keap1 staining lost its significance as an independent prognostic factor. Cytoplasmic Trx positivity was associated with lower Fuhrman grade (Table III).

In this material, the stage and grade were both statistically significant prognostic factors in renal cancer-specific survival. In multivariate analysis, the stage seemed to be a statistically significant prognostic factor in renal cancer-specific survival, but Fuhrman grade lost its power as an independent,

statistically significant prognostic factor. Fuhrman grade was associated with a more advanced stage.

## Discussion

Our study showed that Keap1 staining is related to a poorer prognosis of the RCC patient. Increased Keap1 immunoreactivity was associated with an advanced stage and a higher grade of RCCs, which can largely explain the poorer prognosis of these patients. The Keap1-Nrf2 interaction is thought to be an important regulatory nodal point in the overall response to oxidative or xenobiotic stress. It is assumed that the Keap1-Nrf2 complex serves as a cytoplasmic sensor of oxidative stress which is based on the presence of highly reactive sulfhydryl groups in both these proteins (10). It is believed that Keap1 is a negative regulator of Nrf2 activity and either too much or too little Nrf2 activity can result in physiological impairment (11). Cell culture experiments have demonstrated that stresses which activate Nrf2 induce the dissociation of Nrf2 from Keap1. When the cell is exposed to inducers of phase II enzymes, the Keap1-Nrf2 complex disrupts and Nrf2 is allowed to translocate into the nucleus. In the nucleus, Nrf2 binds to the ARE and activates transcription of various detoxification and antioxidant proteins (10). It has recently been demonstrated that loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth (18). In non-small cell lung cancer biallelic inactivation of Keap1 is a frequent genetic alteration which leads to loss of Keap1 function and constitutive activation of Nrf2-mediated gene expression (19). In breast cancer, a mutation of Keap1 has been found to impair its ability to repress Nrf2 activity (20). Increased expression of Nrf2 has been demonstrated in head and neck squamous cell carcinomas (21).

Despite the association of Keap1 immunoreactivity with a poorer prognosis of the RCC patient, we could not find the same correlation with Nrf2, whose counterpart Keap1 is thought to be. The molecular basis for this observation is unclear. One explanation could be that immunohistochemical quantification of Nrf2 may not correlate with functionally active Nrf2. Recently it has been demonstrated that cysteine residues have a critical role in maintaining Keap1 function. Modification of cysteine residues by ROS-induced stress allows the dissociation of Nrf2 from Keap1 (22). There are, however, other regulatory mechanisms such as phosphorylation and proteosomal degradation which are also important in regulating Nrf2 activity (23,24). The significance of these mechanisms could not be assessed in this study, although they might explain some of the discrepancy between the detected immunohistochemical staining of Keap1 and Nrf2.

In this study there was a tendency for Trx to be present in tumours of patients with a better prognosis. Trx promotes cell proliferation and it is capable of increasing the growth rate and colony formation of cells (25). Trx can also increase oxidant and drug resistance of various cells (26). A variety of stress stimuli such as hypoxia and photochemical or ultra violet radiation can activate Trx (13). The expression of Trx has been shown in various tumours. In gastric carcinomas Trx has been shown to associate with increased proliferation and decreased apoptosis (27). On the other hand, in hepatocellular carcinoma, high Trx expression is related to decreased cell

proliferation (28). In small-cell lung carcinoma, Trx is highly expressed and takes part in activation of transcriptional factors and regulates apoptosis. In high grade tumours, Trx expression is diminished, suggesting loss of redox regulation (29). Trx expression has also been described in astrocytic brain tumours where it may influence the biological behaviour of the tumour, possibly modulating cell proliferation and necrosis of the tumour (30). In earlier studies it has been shown that Trx positivity associates with lower apoptotic index in RCC (15). It would be reasonable to suppose that these patients would have a better prognosis. In this study, Trx was related to a better prognosis of the RCC patient. The difference was not, however, statistically significant. We suppose that our study population is too small to show a statistically significant difference in survival with Trx staining but it is still possible that the correlation exists.

The advantages of our study were a long follow-up time and detailed follow-up information. Our study population was large enough to prove the effect of the traditional prognostic factors - the stage and Fuhrman grade. Histological subtype, however, seemed not to be a statistically significant prognostic factor, presumably because of the small number of patients with histology other than clear cell carcinoma.

The oxidative system is involved in RCC and it may be that in the future there will be markers of oxidative stress or the antioxidative system which can help us assess the prognosis of RCC patients. In RCC, Keap1 is related to a more advanced disease, a higher grade and a poorer prognosis.

## Acknowledgements

We would like to thank Manu Tuovinen and Riitta Vuento for their skillfull technical assistance, Pasi Ohtonen, M.Sc., for assistance with statistical analyses and Oulu University, Oulu University Hospital and Finnish Urological Association for financial support.

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