

Relationship of CD95 and COX-2 in renal cell carcinomas with survival and other prognostic parameters: A tissue microarray study

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Abstract

Objective: To investigate the expression of cyclooxygenase-2 and cluster of differentiation 95 in renal cell carcinomas having different clinico-pathological characteristics.

Methods: The study entailed histopathological diagnoses carried out on paraffin blocks at the Department of Pathology of the Medical Hospital of Duzce University, Turkey, between 2005 and 2011. Immunohistochemical staining for cyclooxygenase-2 and cluster of differentiation 95 was performed on tissue microarray using standard procedures. Each patient's age and gender as well as the tumour's grade, stage, diameter, ureteral surgical margins, vascular invasion, capsule invasion and subtype were assessed. In order to determine if the cases were still alive, relatives were telephoned and identity registration records were checked. SPSS 18 was used for statistical analysis.

Results: There were 49 paraffin blocks in the study. Significant correlations were found between cyclooxygenase-2 and tumour subtype ($p=0.044$) as well as between cyclooxygenase-2 and tumour diameter ($p=0.026$). There was a significant correlation between cluster of differentiation 95 and the Fuhrman grade ($p=0.050$).

Conclusion: Expression of cluster of differentiation 95 and cyclooxygenase-2 may be correlated with prognostic parameters in renal cell carcinoma and may also be associated with tumour progression.

Keywords: CD95, Cyclooxygenase-2, COX-2, Prognosis, Renal cell carcinomas. (JPMA 65: 597; 2015)

Introduction

Renal cell carcinoma (RCC) is the most common cancer of the kidney, with approximately 209,000 new cases per year worldwide, and causing 102,000 deaths.¹

RCC is characterised as a complex neoplasm which consists of several different tumour subtypes having distinct clinical and genetic features. New cytogenetic findings and molecular markers that assist in prognosis, diagnosis and therapy have led to several major revisions in the histological classification of RCC.²

RCC is clinico-pathologically defined as a heterogeneous disease and classified as a clear-cell, chromophobe, papillary and collecting duct carcinoma.³ Clear cell RCC (CC-RCC) is the most common histological subtype, and comprises approximately 75% of all RCCs. Papillary RCC (P-RCC) is the second most common subtype. It is sub-

classified into types I and II, and makes up approximately 10% of all RCCs. Sarcomatoid RCC (S-RCC) is a high-grade histological variation that can arise from all types of RCC, but it is not a separate entity. Chromophobe RCC (CH-RCC) is the third most common subtype, comprising approximately 5% of all RCCs.²

Cluster of differentiation 95 (CD95), also known as Fas, is a major apoptotic regulator, and Fas-mediated apoptosis has been observed in certain kinds of malignancies.⁴⁻⁶ However, in RCC, the Fas/Fas ligand (FasL) system has not been well-characterised. Apoptosis of cancer cells resulting from immunotherapy has been closely linked to Fas-mediated apoptosis.⁶

Cyclooxygenase-2 (COX-2) is transiently induced by pro-inflammatory cytokines and growth factors, and is involved in inflammation and mitogenesis.⁷ In one non-small cell lung cancer cell line study, COX-2 was shown to decrease the degradation of survival, leading to its stabilisation and the subsequent inhibition of apoptosis.^{8,9}

The progression of pathogenesis in CC-RCC was found to be associated with many pathways while the pathways associated with the pathogenesis of non-clear cell RCCs (NCC-RCC) have so far not been identified.⁹

The aim of this study was to investigate the expressions of

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COX-2 and CD95 in RCCs (CC and NCC) showing different clinico-pathological characteristics, and also to determine if a relation existed between COX-2 and CD95 expressions.

Materials and Methods

The study entailed histopathological diagnoses carried out on paraffin blocks at the Department of Pathology of the Medical Hospital of Duzce University, Turkey, between 2005 and 2011, after approval of the institutional ethics committee.

Areas showing histopathological features of RCC were selected on archival Haematoxylin and Eosin (H&E) slides and then representative areas of the tumour were marked on the corresponding paraffin block for tissue microarray (TMA) construction. The 5mm cylindrical tissue samples were taken from the selected regions of the donor paraffin block and transferred to the receiver paraffin block using a tissue-arraying tool. Multiple sections, 5µm in thickness, were then cut. The slides were stained with the usual H&E. All the diagnoses were made by two pathologists, following the pathology of World Health Organisation (WHO Classification of Tumours).¹⁰

Immunohistochemical staining was performed on the 5µm sections of formalin-fixed, paraffin-embedded material. The slides were de-paraffinised with xylene, rehydrated with ethanol, and then non-enzymatic antigen retrieval was applied to each slide. Thereafter, they were washed with 10mM phosphate-buffered saline (PBS), pH 7.5. Immunohistochemical staining was performed manually using the standard avidin-biotin peroxidase complex technique (Neomarkers, Thermo Fisher Scientific, Fremont, CA, USA). Briefly, the slides were incubated at 37°C for 60min with the following primary antibodies (all from Neomarkers, Thermo Fisher Scientific): CD95 (Ab-3 Clone GM30, 1:30 dilution), anti-COX-2 (RB-9072-PO, 1:40 dilution). The slides were then washed twice for 5 min with 10mM PBS and incubated with biotinylated rabbit anti-goat immunoglobulin (Ig) G (1:200 dilution; Dako, Carpinteria, CA, USA) for 1h at room temperature. After a final washing, the colour reaction was developed using 0.5% diaminobenzidine and 0.01% hydrogen peroxide. The sections were counterstained with H&E before being mounted. As a negative control, the primary antibody was removed from the samples.

Evaluation of immunohistochemical staining was performed independently by two pathologists. Each stained slide was evaluated and scored according to the brown cytoplasmic staining intensity: 0 (negative), 1 (weak), 2 (medium), and 3 (strong). The following score results were obtained for the extent of staining: 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). For both

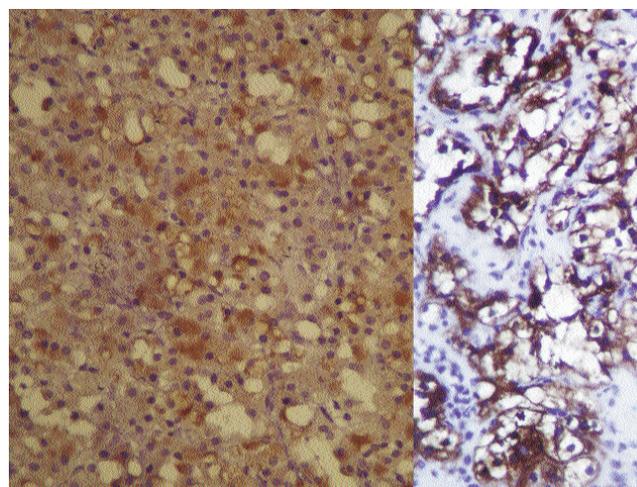
COX-2 and CD95 the total for the intensity and extent scores was used as the final staining score (0-7). A final staining score of ≥ 4 for a tumour was considered to be positive (1) and tumours scoring ≤ 3 were considered to be negative (0).¹⁰

The slides were then examined with a Nikon i80 microscope. Archival records of the patients and all preparations (prepared slides and paraffin blocks) were revised and evaluated. For immunohistochemical examination, the most appropriate paraffin blocks of tumours were selected. Each patient's age and gender as well as the tumour's grade, stage, diameter, ureteral surgical margins, lymphovascular invasion (LVI), capsular invasion (CI), and subtype were assessed. In order to determine if the cases (patients) were still alive, relatives were telephoned and identity registration records were checked.

SPSS 18 and PASW were used for statistical analysis. Descriptive statistics of variables were computed as frequency and percentage, median (minimum, maximum). The relationships between groups and categorical or ordinal variables were investigated by using either the Chi-Square test or Kruskal-Wallis test. P values less than 0.05 were accepted as statistically significant.

Results

The 49 paraffin blocks in the study related to 12(24.5%) women and 37(75.5%) men, with an overall median age of 62.5 years (range: 32-96 years). The observed subtypes were 21(42.9%) CC-RCC, 9(18.4%) CH-RCC, 15 (30.6%) P-



COX-2: Cyclooxygenase-2
CD95: Cluster of differentiation 95

Figure: CD95 expression in tumour tissue (CD95, $\times 400$) and COX-2 expression in tumour tissue (COX-2, $\times 400$, right).

Table-1: Correlation between COX-2 and subtype.

| | COX-2 | | Total | P |
|---------|-------|----|-------|-------|
| | 0 | 1 | | |
| NCC-RCC | 15 | 13 | 28 | 0.044 |
| CC-RCC | 17 | 4 | 21 | |
| Total | 32 | 17 | 49 | |

COX-2: Cyclooxygenase-2

NCC-RCC: Non-clear cell renal cell carcinoma

CC-RCC: Clear cell renal cell carcinoma.

Table-2: Relationships between diameter and COX-2 and Fuhrman grade.

| | | Diameter ≤ 7 cm | | Diameter > 7 cm | | P |
|---------|---|-----------------|---------|-----------------|---------|---------|
| | | Count | Percent | Count | Percent | |
| Fuhrman | 1 | 21 | 56% | 2 | 16% | p=0.041 |
| | 2 | 13 | 35% | 7 | 58% | |
| | 3 | 3 | 8% | 3 | 25% | |
| COX-2 | 0 | 21 | 56% | 16 | 43% | p=0.026 |
| | 1 | 11 | 91% | 1 | 9% | |

COX-2: Cyclooxygenase-2

The COX-2 staining ratio was significantly higher for the NCC-RCC subtype (46.4%) than for the CC-RCC subtype (8.9%). Tumours less than 7cm in diameter had significantly higher COX-2 staining intensity (43.2%).

RCC, 2(4.1%) multilocular cystic carcinoma (MLC-RCC), and 2(4.1%) S-RCC, making up a total of 21(43%) CC-RCCs and 28(57%) NCC-RCCs.

The tumour stage (pT) distribution was 37 (77%) pT1 with tumours 7cm or less in diameter, and 12(23%) pT2 with tumours more than 7cm. LVI was positive in 6(12.2%)

cases and CI was positive in 4(8.2%).

RCC grades based on Fuhrman nuclear grading was grade 1, 23(46.9%), grade 2,20(40.8%), and grade 3,6(12.2 %). In terms of survival, 44(89.8%) were still alive, 2(4.1%) survived for more than 6 months, and 3(6.1%) survived for less than 6 months.

There were significant correlations between COX-2 and subtype ($p < 0.044$) and COX-2 and diameter ($p = 0.026$). The COX-2 staining ratio was significantly higher for the NCC-RCC subtype 13/28(46.4%) than for the CC-RCC subtype 4/21(8.9%) ($p < 0.044$). Tumours less than 7cm in diameter had significantly higher COX-2 staining intensity ($p < 0.026$).

Significant correlations were found between LVI and diameter ($p = 0.026$), LVI and Fuhrman grade ($p = 0.033$), Fuhrman grade and diameter ($p = 0.041$), and Fuhrman grade and CI ($p = 0.027$) (Tables-1-2). There was significant correlation between CD95 and Fuhrman grade ($p = 0.050$) (Table-3).

Survival was not evaluated since 44(89.8%) of the patients were still alive. A high Fuhrman grade was statistically significant in predicting poorer survival (LVI and CI; $p = 0.033$ and $p = 0.027$, respectively) (Table-4).

Discussion

RCCs account for 90% of all primary malignant renal tumours in adults.¹¹ Currently, staging and nuclear grading for RCC are considered to be the most important predictors of survival.¹²⁻¹⁴ Several studies have identified additional prognostic factors, such as tumour size, cell

Table-3: Relationships between Fuhrman grade and lymphovascular invasion, capsular invasion, and CD95.

| | | Fuhrman (1) | | Fuhrman (2) | | Fuhrman (3) | | Fuhrman (4) | | p |
|------|---|-------------|---------|-------------|---------|-------------|---------|-------------|---------|---------|
| | | Count | Percent | Count | Percent | Count | Percent | Count | Percent | |
| LVI | 0 | 23 | 100 % | 16 | 80 % | 4 | 66 % | 0 | 0 % | p=0.033 |
| | 1 | 0 | 0 % | 4 | 20 % | 2 | 33 % | 0 | 0 % | |
| CI | 0 | 23 | 100 % | 18 | 90 % | 4 | 66 % | 0 | 0 % | p=0.027 |
| | 1 | 0 | 0 % | 2 | 10 % | 2 | 33 % | 0 | 0 % | |
| CD95 | 0 | 18 | 81 % | 17 | 85 % | 3 | 42 % | 0 | 0 % | p=0.05 |
| | 1 | 4 | 18 % | 3 | 15 % | 4 | 58 % | 0 | 0 % | |

LVI: Lymphovascular invasion. CI: Capsular invasion. CD95: Cluster of differentiation 95.

Table-4: Distribution of prognostic parameters.

| Subtype | Gender | pT | LVI | CI | Fuhrman grade |
|---------|--------|-----|----------|----------|---------------|
| CC-RCC | F | pT1 | positive | positive | 1 |
| NCC-RCC | M | pT2 | negative | negative | 2 |
| | | | | | 3 |
| | | | | | 6 |

CC-RCC: Clear cell renal cell carcinoma. NCC-RCC: Non-clear cell renal cell carcinoma. pT: Tumour stage. LVI: Lymphovascular invasion. CI: Capsular invasion.

type, histological subtype, deoxyribonucleic acid (DNA) ploidy, proliferation markers, angiogenesis, nuclear morphometry, and a combination of these parameters.¹²⁻¹⁴ The Fuhrman grading system is the one most commonly used for classifying RCC.¹³⁻¹⁵ A study reported that Fuhrman grade should be the standard grading system for P-RCC.¹⁵ This study found that the Fuhrman scheme correlated with tumour diameter.

Several studies attempting to determine potential molecular prognostic factors for RCC have produced inconclusive results.¹⁵⁻²¹ The present study showed that COX-2 may correlate with tumour subtypes CC-RCC and NCC-RCC. COX-2 staining may be significant in NCC-RCC than in CC-RCC, but the results did not show conclusive relationship between COX-2 and tumour diameter.

Previously, there have been only a few studies dealing with the relationship between COX-2 expression and RCC. Some studies have shown that COX-2 expression is associated with the ability to metastasise and the tumour stage, size and grade, but a study found that COX-2 did not seem to be an independent prognostic indicator in predicting outcomes for patients with RCC.¹⁶ Increased COX-2 expression has been seen in several types of human cancers. Carcinogenesis and poor prognostic outcome have been attributed to over expression of COX-2.¹⁵⁻²¹

Expression of COX-2 is significantly associated with various clinico-pathological variables, including the T stage, N stage, M stage, and tumour grade. The COX-2 expression was not analysed as an independent prognostic factor for cancer-specific survival because of claims that the COX-2 is not a significant prognostic factor in RCC,¹⁹⁻²³ but it was analysed as an independent risk factor for large tumour size (>7cm), although one study did not find an association with tumour stage or grade in RCC.²¹ Other findings showed that COX-2 expression was linked with tumour size, but was not an independent prognostic factor for cancer-specific survival.¹⁹ It has recently been proposed that COX-2 is a contributing factor to the inhibition of apoptosis.¹⁵⁻²⁰

The process of the COX-2 inhibition of apoptosis is possibly connected with the removal of the substrate arachidonic acid by COX-2 catalytic activity or the generation of prostaglandin products.¹⁵⁻²⁰

The present study found that COX-2 correlated with prognostic parameters (subtype and diameter). COX-2 expression was found to be higher in pT1 than in pT2 stage. This may be attributed to the fact that in the majority of cases (37), the tumours were 7cm in diameter or less. In the present study, COX-2 was found in the

tumour subtype NCC-RCC, and grade 1 staining (COX-2) was found in pT1 tumours. The reason may be small number of patients and it may not be high staining.

Some studies have reported that younger patients included a higher proportion of NCC-RCC histological subtypes.²² In this study, significant relationship between age and subtype was not found.

It has been proposed that the alteration of the down-regulated expression of CD95 may be a contributing factor in the carcinogenesis of RCC. In RCC, tumour size has been implicated as an independent prognostic parameter, while tumour volume itself is believed to reflect the invasive nature of RCC.^{4-6,23-25} In these studies, the extent of apoptosis observed in neoplastic tissue was correlated with the rate of cell proliferation and patient survival.^{4-6,23-25} Furthermore, data showed that Fas expression could contribute to the generation and progression of breast cancer, as the levels of expression of CD95 were in correlation with metastasis of tumours into the axillary lymph nodes.^{4-6,23-25} In the present study, it was found that CD95 may correlate with the Fuhrman grade ($p=0.05$).

Conclusion

There may be an association between COX-2 expression and between CD95 expression and the Fuhrman grade. More extensive research in this field could be of great value in future RCC treatment methods.

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