Published in final edited form as:

Hum Pathol. 2012 September ; 43(9): 1394–1399. doi:10.1016/j.humpath.2011.10.014.

CD70 Expression Patterns in Renal Cell Carcinoma

Lucia B. Jilaveanu¹, Joshua Sznol¹, Saadia A. Aziz¹, Dylan Duchon¹, Harriet M. Kluger¹, and Robert L. Camp²

¹Department of Medicine, Section of Medical Oncology, Yale Cancer Center, Yale University School of Medicine, New Haven, Connecticut

²Department of Pathology, Yale University School of Medicine, New Haven, Connecticut

Abstract

CD70 is upregulated in several malignancies, where it induces cytotoxic effects on B and T lymphocytes leading to immune escape. Novel therapeutic agents targeting CD70 have entered clinical trials. We characterized expression of CD70 protein in RCC specimens of various histologic subtypes and assessed their prognostic value and association with clinical/pathological variables. We employed tissue microarrays containing 330 cases using a novel fluorescent immunohistochemistry-based method of Automated Quantitative Analysis (AQUA) of *in situ* protein expression. The mean expression of CD70 was almost twice as high in tumors relative to normal tissue ($p < 0.0001$). When broken into subsets, CD70 was higher in sarcomatoid and clear cell tumors ($p < 0.0001$), and variably elevated in oncocytomas and some papillary tumors. No association was found between CD70 expression and stage or grade. High CD70 expression was associated with decreased survival on univariate analysis in the clear cell subset of RCC, however, it did not retain significance on multi-variable analysis. Given the elevated expression of CD70 in clear cell, sarcomatoid, and some papillary tumors, our findings suggest that CD70 might be a good drug target in RCC. Additional studies are warranted to assess the association between expression of CD70 and response to therapy with CD70 targeting drugs in RCC.

Keywords

CD70; Renal Cell Carcinoma; therapeutic targets

INTRODUCTION

The incidence of renal cell carcinoma (RCC) in the United States is rising. In 2010, it is estimated that 58,240 new RCC cases (35,370 in men and 22,870 in women) were diagnosed and 13,040 deaths occurred (8,210 men and 4,830 women) (1). In recent years, a number of drugs targeting VEGF, VEGF receptors (VEGFR) or mTOR were approved for metastatic RCC based on an increase in progression free survival, when given alone or in conjunction with interferon α -2b (2–8). None of these agents, however, has been unequivocally shown to significantly improve overall survival, and they are not void of toxicity. A number of immunotherapy agents have activity in RCC, such as interleukin-2 (IL-2) and IFN- α , but

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Corresponding Author: Robert L. Camp, MD PhD, Department of Pathology, Yale University School of Medicine, 310 Cedar Street, New Haven, Connecticut, 06520; Robert.Camp@yale.edu; Telephone +1 203 737 2517; Fax +1 203 737 5089.

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high dose cytokines are usually associated with toxicities which have limited their use to about 15% of patients (9–10). New agents, targets and approaches are therefore needed to improve outcome for RCC patients who are not responsive to these therapies or are not likely to tolerate them.

CD70 is a member of the tumor necrosis factor super family 7 (TNFSF7) and is a type II transmembrane surface antigen highly expressed on a small subset of activated memory T and B cells (11) or non lymphoid cells, such as stromal cells of the thymic medulla, and mature dendritic cells (12). CD70 expression is necessary for an effective immune response; it is required for T-cell activation, proliferation, or induction and has been implicated in the function of B lymphocytes, NK cytotoxic activity, and immunoglobulin synthesis (13–14). CD70 is the ligand for TNFRSF27 / CD27, a glycosylated transmembrane protein receptor, whose activation can lead to proliferation as well as apoptosis (15–16). Upon ligation to its receptor on B lymphocytes, CD70 promotes activation of B memory cells to differentiate into plasma cells (17). In T cells, CD70 allows differentiation of CTLs without the co-stimulation of the MHC-2/CD4 complex (18–19). CD70 has also been shown to enhance CD4+ memory T cell function and activate CD8+ T cells (11, 18).

CD70 is aberrantly expressed in hematologic malignancies and a number of solid tumors, including brain tumors, RCC, thymic carcinoma, nasopharyngeal carcinoma, ovarian, lung, colon and pancreatic cancer and melanoma (20–24). Despite the fact that renal tumors are highly immunogenic, effective immune responses often fail to develop and RCC successfully evades immune recognition by mechanisms that are not well understood. It has been shown that the tumor microenvironment can induce an apoptotic effect on tumor infiltrating lymphocytes (TILs) (25). In tumor cells, CD70 upregulation has a cytotoxic effect on B and T lymphocytes by inducing apoptosis through interaction with its receptor, CD27, leading to immune suppression (23, 26). The mechanism by which renal tumor cells expressing CD70 induce lymphocyte apoptosis remains unclear; Diegmann et. al suggest two possible mechanisms: either through direct contact between CD27 expressing lymphocytes and CD70 expressing RCC cells, or by CD70 cleavage and release into the tumor environment, where it can subsequently target tumor lymphocytes, interact with its CD27 receptor and initiate T cell apoptosis (26).

In two small cohort studies of 68 and 33 RCC patients respectively, CD70 was found to be specifically expressed in tumors but not in normal kidney tissue. Of the histological types of RCC, CD70 expression is reported to be highest in clear cell carcinoma, with expression significantly lower in the others (27–29).

Due to the aberrant upregulation of CD70 in RCC cells, its membrane surface expression, its limited normal tissue distribution, and the role it plays in lymphocyte suppression, pharmacologic targeting of CD70 is an approach that is under investigation for this disease (30). Clinical trials with CD70 targeting drugs are currently in early phases. The purpose of our study was to further characterize the expression patterns of CD70 in a larger cohort of RCC specimens including different histological subtypes, compare expression to normal kidney tissue, and study the association with prognosis and other pathological variables. To obtain a quantitative measure of CD70 expression in our cohort, we employed a method of Automated Quantitative Analysis (AQUA). This method is void of the bias associated with standard immunohistochemistry and has been validated in a number of prior studies (31).

MATERIALS AND METHODS

Cell lines and Western blots

A498, ACHN, Caki-1, Caki-2, 786-O were purchased from the American Type Culture Collection. HEK293T cell lines transiently transfected to overexpress CD70 (antigen standard for CD70), or a control vector (OriGene Technologies, Inc.) were utilized as a positive and negative controls respectively for CD70 immunoblotting. CD70 was detected with a monoclonal antibody (R&D Systems, Inc.,) at a concentration of 1:1000.

Tissue Microarray (TMA) Construction

TMA's were constructed using RCC cores from 330 patients, 294 with matching adjacent normal renal tissue, each measuring 0.6 mm in diameter, spaced 0.8 mm apart on slides. Tumors were represented by two cores from different areas of the specimen. Specimens and clinical information were collected with approval of a Yale University Institutional Review Board. Histological subtypes included clear cell (71%), papillary (14%), chromophobe (2%), mixed histology (4%), oncocytomas (6%), and sarcomatoid tumors (3%). Oncocytomas were excluded from survival analyses due to their uniformly good outcome. Fifty six percent (56%) had stage I disease, 8% had stage II, 8% had stage III disease, and 28% had stage IV disease. Twelve percent were Fuhrman nuclear grade I, 52% grade II, 27% grade III and 9% grade IV. Specimens were resected between 1987 and 1999, with follow-up time of 2–240 months (median–89.7). Age at diagnosis was 25–87 years (median–63). None of these patients were treated with sunitinib, sorafenib, pazopanib, bevacizumab, everolimus or temsirolimus, however, a small subset received interferon or interleukin-2.

Immunohistochemistry

Staining was performed for AQUA as described (31). Slides were incubated with mouse monoclonal anti-human CD70, (at a 1:40 dilution). Goat anti-mouse HRP-decorated polymer backbone (Envision, Dako North America, Carpinteria, CA) was used as a secondary reagent. To create a tumor mask, slides were simultaneously incubated with rabbit anti-cytokeratin (Dako) at 1:100, and visualized with mouse anti-rabbit secondary antibody conjugated to Alexa 488 (Molecular Probes, Inc., Eugene, OR). The target antibody was visualized with Cy5-tyramide (Perkin-Elmer, Boston, MA).

Automated Image Acquisition and Analysis (AQUA)

Images were acquired and analyzed using algorithms that have extensively been described²⁰. Briefly, monochromatic, high-resolution (1024 × 1024 pixel) images were obtained of each histospot. Tumor was distinguished from stroma by the cytokeratin/streptavidin signal. Coalescence of cytokeratin at the cell surface was used to localize cell membranes, and DAPI was used to identify nuclei. CD70 showed cytoplasmic/membranous staining only, and no nuclear staining was seen. The target signal (CD70) from the pixels within the membrane and cytoplasm was normalized to the area of tumor mask and scored on a scale of 0–255 (the AQUA score).

Array Validation and Statistical Analysis

To account for intra-tumor heterogeneity, two separate slides, each containing a core from a different area of the tumor for each patient, were utilized. CD70 AQUA scores from different TMAs were averaged after mean normalization. For patients with only one interpretable core, the single score was used for analysis. No associations between scores and tissue age or array position (row/column) were identified. TMA histospots were deemed uninterpretable if they had insufficient tumor, loss of tissue, or abundant necrosis. Of the

360 patient tumor histospots on the array, 326 were interpretable for both cores. Statview 5.0 and JMP 5.0 software was used (SAS Institute, Cary, NC). The prognostic significance of parameters was assessed using the Cox proportional hazards model with RCC-specific survival as an endpoint. The associations between continuous AQUA scores of the target expression and pathological parameters were assessed using ANOVA.

RESULTS

Antibody validation

The anti CD70 antibody was validated by immunoblotting of lysates from a panel of renal cell carcinoma cell lines and lysates from HEK293T cells transiently transfected to overexpress CD70 (antigen standard for CD70) or a negative control vector (Supplemental Figure 1). The antibody was highly specific for its target, revealed on a Western blot by three bands representing the monomeric (20.9kDa), dimeric (42kDa) and trimeric (63kDa) forms of the protein, when compared to an antigen standard for CD70. CD70 was expressed in two of the five RCC cell lines.

Tumor expression

Figure 1 (upper and lower panels respectively) shows examples of strong and weak immunoreactivity of CD70 in two histospots; staining within the tumor mask within a histospot was fairly homogenous. The expression of CD70 between core samples taken from different parts of the same tumor were highly correlated ($R = 0.76$; $R^2 = 0.58$, $P < 0.0001$) (Figure 2A). AQUA scores ranged from 0.31 to 122.43, with a mean of 23.39 for tumor tissue, and from 2.54 to 26.79 for normal tissue, with a mean of 12.70.

To assess the difference in expression between nonmalignant tissue from the nephrectomy specimen and corresponding tumor, we used paired *t* tests. The mean expression of CD70 was almost twice as high in RCC when compared to benign tissues (23.4 vs. 12.7, $P < 0.0001$) (Supplemental Figure 2).

40% of all tumor specimens had scores above the 95th percentile of the normal tissue AQUA score distribution which corresponds to a value of 20.1. Among the histologic subtypes, 54% of all sarcomatoid tumors, 49% of the clear cell, 19% of the papillary and 5% of the oncocytomas had AQUA scores above this value. While clear cell and sarcomatoid samples globally show the highest level of expression of CD70, we note that there are a significant number of tumors within these two histological subtypes with low (below the 95th percentile of the normal tissue AQUA score) levels of expression of CD70.

To compare CD70 expression across histologic subtypes, we used ANOVA. Expression of CD70 was significantly higher in sarcomatoid and clear cell tumors, and less elevated in oncocytomas ($p < 0.0001$) (Figure 2B). The mean differences between subtypes are shown in Supplemental Table 1. Given the range of CD70 expression in papillary carcinomas (1.03 to 115.9), we further divided these tumors into type 1 and type 2 subsets (representing 48.7% and 52.3% of the tumors, respectively). There was a trend towards higher CD70 expression in type 2 papillary tumors (mean AQUA score of 9.13 vs. 21.15, respectively); however this difference did not achieve statistical significance ($P = 0.0724$). No association was found between the expression of CD70 and stage or nuclear grade.

To assess the association between CD70 expression and survival, we dichotomized the continuous CD70 AQUA scores into “high” and “low” expression, using the 95th percentile value of normal tissue AQUA score as a cutpoint. By Cox univariate survival analysis of dichotomized AQUA scores, we found that high CD70 expression (scores above the 95th percentile value) correlated with decreased survival in the clear cell subset of specimens,

with borderline significance by log-rank statistics ($P = 0.0432$). On multivariate analysis, CD70 expression did not retain its independent prognostic value. The only variables associated with survival by multivariate analysis were stage and nuclear grade ($P < 0.0001$ and $P = 0.005$, respectively). There was no correlation between high CD70 expression and survival in any other histological subsets of patients ($P > 0.05$).

DISCUSSION

In this work, we assessed the expression patterns of CD70 in a large cohort tissue microarray of RCC specimens and adjacent non-malignant tissue. Expression of CD70 in RCC tumors is variable, but averages twice of that seen in normal tissue. High CD70 expression is a predictor of decreased survival on univariate analysis in the clear cell subset of RCC tumors, but did not retain its prognostic significance on multivariable analysis and was not associated with grade or stage.

Elevated CD70 expression in RCC specimens compared to normal tissue and specifically in the clear cell, sarcomatoid and papillary type II subtypes, may have important therapeutic implications. RCCs are characterized by relative resistance to chemotherapy and radiation therapy, and despite progress made in recent years in treating metastatic disease with VEGF pathway targeting therapies, most of these patients eventually succumb to metastatic disease. Therefore, new prognostic biomarkers and therapeutic targets for RCC are needed. The association between high CD70 expression and decreased survival in some (but not all) RCC specimens further suggests that CD70 might be a valuable therapeutic target in this disease. We note, however, that not all RCCs express high levels of CD70 (>95% of normal levels).

RCC sometimes responds to immune-stimulating therapies; although it often effectively escapes immune recognition through expression of immunosuppressive mediators. The ability to avoid immune recognition through the apoptosis of tumor infiltrating lymphocytes is essential for the proliferation of RCC (26). Little is known about the mechanisms implicated in this phenomenon and therefore, the discovery of immunogenic molecules associated with RCC and their upstream and downstream mediators is crucial for development of effective treatments. Several studies in the literature support a role for CD70 in the malignant process in lymphomas and some solid tumors. Upregulation of CD70 in clear cell and sarcomatoid RCCs, as well as in a smaller proportion of papillary tumors, suggests a possible involvement of this protein in the immune escape.

Tumor antigens specifically expressed on neoplastic cells and not on adjacent normal tissue cells are attractive targets for drug development. Moreover, therapy using drug- or toxin-conjugated monoclonal antibodies may increase the therapeutic index. Binding of an anti-CD70 antibody to CD70 expressed on the surface of tumor cells results in the rapid internalization of the antibody-receptor complex, a phenomenon that can be exploited to mediate cell killing via a toxic immunoconjugate (28). Antibody-drug conjugates targeting CD70, consisting of either cleavable or un-cleavable linkers, are being studied as a vehicle to deliver potent cytotoxic drugs to CD70⁺ malignant cells, and have been shown to have potent antitumor activity *in vitro* and in solid tumor xenograft models, including RCC tumors (30, 32).

Novel therapeutic agents, including SGN-70, SGN-75 (Seattle genetics, Inc.) and MDX-1203 (Medarex, Inc.), targeting CD70 are currently being developed. SGN-70 is an unconjugated antibody that hinders the binding of CD70 to its receptor and acts as a mediator for Fc-dependent antibody effector functions (30). SGN-70 induces tumor cell lysis via antibody-mediated cellular cytotoxicity, complement fixation and enhanced uptake by phagocytic cells. SGN-75 is a humanized anti-CD70 monoclonal antibody attached to a

potent, synthetic drug, monomethyl auristatin F (MMAF). SGN-70 and SGN-75 were found to significantly decrease tumor burden and prolong survival of RCC tumor-bearing mice, and are currently in early phase clinical studies for autoimmune disease patients or RCC and non-Hodgkin lymphoma patients, respectively (32–34). MDX-1203 is an antibody-drug conjugate in phase I clinical trials for patients with metastatic clear cell RCC (ccRCC) and B-Cell Non-Hodgkin's Lymphoma. The antibody consists of the fully human anti-CD-70 conjugated to a DNA alkylating cytotoxic drug.

The association between CD70 expression levels in RCC histological subtypes and response to any of these compounds is unknown and needs to be addressed in future studies. Our results show that while expression of CD70 is globally higher in clear cell RCC and sarcomatoid tumors, not all clear cell and sarcomatoid tumors harbored high levels. Furthermore, although papillary tumors expressed relatively low levels of CD70 on average, there was a subset of papillary tumors, particularly type II tumors, that had elevated expression.

In conclusion, our results support prior smaller studies showing that CD70 can be aberrantly expressed in RCC, and support further evaluation of antibody-drug conjugates targeting CD70 in RCC tumors. Furthermore, because of the variation in CD70 expression among tumor types, patient selection and stratification for these therapies should be based on target expression rather than histologic subtype. The association between CD70 expression and the efficacy of CD70 targeting drugs, preferably using quantitative or semi-quantitative methods, warrants further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants R0-1 CA158167 (to H. Kluger), R0-1 CA129034 (to F. Waldman) and by American Cancer Society Award M130572 (to H. Kluger).

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Figure 1.

Cytoplasmic CD70 expression in RCC histospots using Cytokeratin to define the tumor mask, DAPI to define the nuclear compartment, and Cy5 for the target. Merged images displayed in the left half of panels A and B show CD70 within the nuclear compartment and within the cytoplasmic compartment within the tumor mask at 10× magnification. The right half of the panels shows expression of CD70 at 60× magnification. Panel A shows a positive/high expression of CD70, while panel B depicts low expression levels.

Figure 2.

A) Regression plot from the scores derived from the set of renal arrays for CD70. The Y-axis represents AQUA scores from one slide and the X axis from the second slide. Each array contains histospots from the same patients, taken from different areas of the tumor. The linear regression line has an r-value of 0.76 ($P < 0.0001$). **B**) Box plot representing the AQUA scores for CD70 expression levels in the five histological types of renal cell carcinoma, reflecting the results from the ANOVA analysis. The horizontal bars represent the 95th, 75th, 50th, 25th, and 5th percentiles (top to bottom), and the notch demonstrates the 95% confidence interval around the mean. Clear Cell and Sarcomatoids show the highest

level of expression of CD70. There is also a significant subgroup of clear cell and sarcomatoid tumors with low levels of expression of CD70. The overall ANOVA P-value was <0.0001. T-tests between individual subsets are shown in supplementary table 1. The number of tumors in each subset is listed in parentheses.