# Prognostic Value of Serum CA9 in Patients with Metastatic Clear Cell Renal Cell Carcinoma under Targeted Therapy

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**Abstract.** *Aim: Carbonic anhydrase 9 (CA9) has been found to be one of most powerful biomarkers for clear-cell renal cell carcinoma (RCC). The serum CA9 is detectable. The aim of this study was to evaluate the potential prognostic role of serum CA9 in patients with metastatic clear-cell RCC patients under targeted therapy. Patients and Methods: Serum samples came from the randomized phase 2 TORAVA trial. All patients received a targeted therapy (arm A designed as experimental group: temsirolimus and bevacizumab combination; arm B: sunitinib; arm C: interferon-alfa and bevacizumab). Seventy cases of metastatic clear-cell RCC were analyzed. There were 49 males and 21 females. The age ranged from 33.5 to 79.1 years with a median of 61.2 years. Serum samples were collected before treatment. Serum CA9 was quantified by enzyme-linked immunosorbent assay (ELISA). The correlation of the serum CA9 levels with the clinical parameters, treatment response and overall survival was analyzed. Overall survival estimates were calculated using the Kaplan-Meier method and compared by the log-rank test. Results: Serum concentrations of CA9 ranged between 0 and 897.3 pg/ml, with an average of 94.4±176.6 pg/ml. There was no association between serum CA9 and clinical parameters such as Eastern Cooperative Oncology Group (ECOG) Performance Status (p=0.367) or Motzer classification (p=0.431). The serum CA9 levels were lower in the response group (64.7±104.7 pg/ml) than the no-response group (108.2±203.8 pg/ml), but the difference was not statisticlly significant (p=0.366). For the patient group overall, the Kaplan-Meier survival curve showed that high serum CA9 levels were significantly associated with shorter overall survival (hazard ratio=2.65, 95% confidence interval=1.19-5.92, log-rank test p=0.0136). For the major group of patients treated with temsirolimus and bevacizumab, the Kaplan-Meier survival curve showed that high serum CA9 levels were significantly associated with shorter overall survival (p=0.0006). Conclusion: Serum CA9 levels may be of clinical interest to predict the outcome for patients under targeted therapy for metastatic clear-cell RCC. CA9 may be used to select patients with metastatic clear cell RCC for clinical trials.*

One third of patients with renal cancer present metastatic disease at the time of diagnosis and 30-40% of patients with localized renal cancer will develop a metastasis after surgery. Metastatic RCC is generally resistant to chemotherapy and to hormonal therapy. The medical treatment is immunotherapy, with a 10% response rate. Insights into the genetics and biology underlying RCC especially the role of the von Hippel-Lindau tumor suppressor gene (*VHL*) have provided the rationale for targeted therapy. At present, there are six targeted agents approved for the treatment of metastatic RCC: sorafenib, sunitinib, pazopanib, bevacizumab (in combination with interferon alpha), temsirolimus, and everolimus. The targeted therapy provides a 50% response rate, but with some severe side-effects and high cost (1). At present, the response to treatment is based on imaging examinations; only a marker that could predict the outcome of RCC is urgently needed.

CA9 is a transmembrane zinc metalloenzyme (2, 3). Besides its role in the regulation of pH, it has a function in cell adhesion, growth, and survival of tumor cells under normoxia and hypoxia (4). CA9 expression is induced by hypoxia. In non-neoplastic tissues, it has a very limited

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expression pattern. Despite its limited expression in normal tissues, CA9 is overexpressed in a variety of solid tumors. High level of its expression has been found in renal cancer, rectal cancer, bladder cancer, *etc*. (5-7). CA9 is highly expressed in clear-cell RCC due to the mutation of the *VHL* gene and is considered to be a powerful marker for clear-cell RCC (3). The information on serum CA9 of patients with cancer is very limited, but it has shown promising potential as a novel marker (8).

Besides the cell-associated form of this transmembrane protein, there is a soluble isoform of CA9 which is released by proteolytic cleavage and can be detected in the serum of patients with renal cancer (8-10). This form of CA9 may serve as an easily accessible marker to stratify patients for therapy. CA9 was found to be cleared from serum a few days after nephrectomy, suggesting a very promising marker to monitor therapy response and prognosis (9).

Traditional serum markers have been found in serum of patients with cancer. The well-known examples are carbohydrate antigen 125 (CA-125) for ovarian cancer and prostate specific antigen (PSA) for prostate cancer. These markers are used to assess therapy response and prognosis (11, 12). A rapid fall in CA-125 or PSA during treatment can predict a favorable prognosis. Unfortunately, there is no such marker available for metastatic RCC.

The aim of the current study was to evaluate the potential role of serum CA9 in predicting response and clinical outcome of patients with metastatic clear-cell RCC.

## Patients and Methods

*Patients and serum samples*. Seventy serum samples from patients with metastatic clear-cell RCC were obtained. These serum samples were from the TORAVA trial. The TORAVA trial was a randomized phase II study aimed at determining the efficacy and safety of temsirolimus and bevacizumab combination in metastatic RCC. The TORAVA design has been recently described (13). Briefly, 171 patients who had untreated metastatic RCC were randomly assigned to three arms: arm A for the combination of bevacizumab (10 mg/kg every 2 weeks) and temsirolimus (25 mg weekly); arm B for sunitinib (50 mg/day for 4 weeks followed by 2 weeks off); or arm C for interferon-alfa (9 MIU three times per week) and bevacizumab (10 mg/kg every 2 weeks). Therefore, all patients received a targeted therapy. In the TORAVA trial, the patients were randomized using a 2:1:1 ratio of arm A, arm B and arm C. Another important objective of the TORAVA trial was to study the markers of prognosis for patients with metastatic RCC under targeted therapy. We limited our analysis of serum CA9 to clear-cell RCC. Seventy patients with a serum sample were available for analysis of CA9. These patients represented 38 in arm A (54.3%), 14 in arm B (20.0%) and 18 in arm C (25.7%). The characteristics of these patients were similar to those of the overall study population.

Before treatment, all patients had a detailed history taken, physical examination and baseline laboratory parameters. Pre-treatment baseline tumor status was evaluated with computed tomodensitometry (CT) of the brain, chest, abdomen and pelvis.

Table I. *Patients' characteristics*.

| | | |
|---|---|---|
| Age (years) | | |
| Mean | 59.9 | |
| Median | 61.2 | |
| Range | 33.5-79.1 | |
| | n | % |
| Gender | | |
| Male | 49 | 70.0 |
| Female | 21 | 30.0 |
| ECOG performance status | | |
| 0 | 43 | 61.4 |
| 1 | 25 | 35.7 |
| 2 | 2 | 2.9 |
| Motzer classification | | |
| Favorable prognosis | 17 | 26.2 |
| Intermediate prognosis | 41 | 63.1 |
| Poor prognosis | 7 | 10.8 |
| Unknown | 5 | |

Data collected included standard demographics and disease characteristics, first date of treatment, best response to treatment and date of progression, date of death or last follow-up. Patients were regularly followed-up after they entered onto treatment. Physical examinations, blood cell counts and imaging examinations were performed. Assessment of response was based on the Response Evaluation Criteria in Solid Tumors (RECIST) criteria (14). The clinical parameters are summarized in Table I.

*Quantitative analysis of serum CA9 levels*. Serum samples were collected before medical treatment. Serum CA9 was quantified according to our recent publication using the Quantikin Human CA9 Immunoassay (R and D Systems, Minneapolis, MN, USA) (8). Briefly, serum samples or standard control samples were incubated on microtiter plates coated with a specific antibody to CA9 for 2 h at room temperature. The plates were washed to remove the unbound antibody. After incubation of a conjugate solution, a substrate solution was added. Color development was stopped after 30 min. A microplate reader was used to determine colorimetric densities at 450 nm. Final results were calculated according to the standard curve. Results were expressed in pg/ml. The mean limit of quantification of this method was 2.28 pg/ml.

*Statistical analysis*. All statistical analyses were conducted using the SAS software (version 9.2; SAS, Cary, NC, USA). Non-parametric methods (Kruskal-Wallis one-way analysis of variance by ranks and Wilcoxon test) were used for testing correlations between serum CA9 and clinical parameters and comparing the difference in serum CA9 levels between the groups of responders and non responders. Overall survival (OS) was the time from randomization to death from any cause. OS was analyzed using Cox proportional hazards regression models and presented as Kaplan-Meier estimates with hazard ration (HR) and 95% confidence interval (CI). Difference in survival estimates between the patients dichotomized by serum CA9 level according to the median value (20.6 pg/ml), was assessed by a two-sided log-rank test.

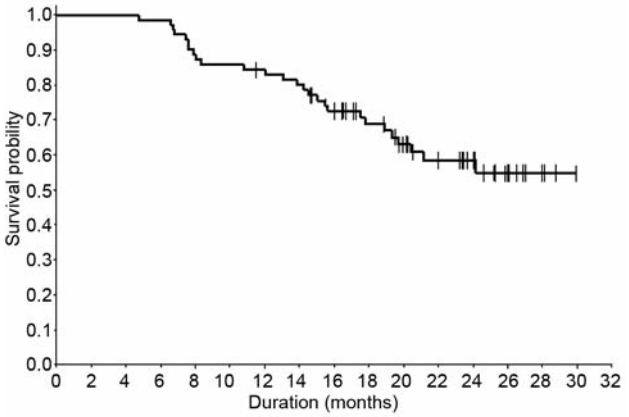


Figure 1. *The overall survival of patients (27 deaths during the follow-up).*

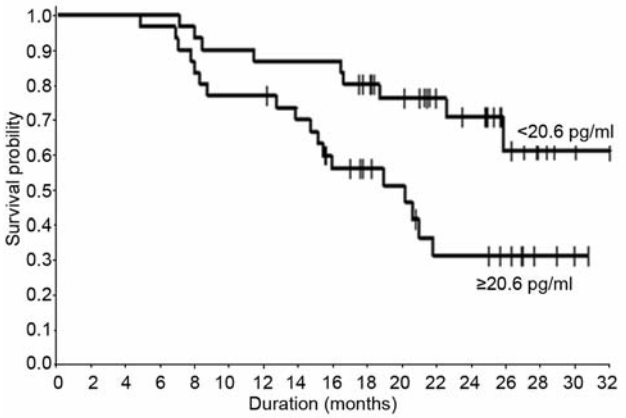


Figure 2. *The overall survival of all patients according to the level of serum CA9. The Kaplan-Meier survival curve showed a poorer prognosis of survival for patients in the high-CA9 group than those in the low-CA9 group (log-rank test, p=0.0136).*

## Results

*Correlation between CA9 level and clinical parameters*. Serum concentrations of CA9 ranged between 0 and 897.3 pg/ml, with an average of 94.4±176.6 pg/ml. The average serum CA9 was 91.8±162.4 pg/ml for the 43 patients with ECOG performance status of 0 and 98.7±200.2 pg/ml for the 27 patients with ECOG performance status of 1 or 2 (Wilcoxon non-parametric test, $p$=0.367). For Motzer classification, the serum CA9 level was 43.3±67.9 pg/ml for patients with favorable prognosis, 94.7±157.3 pg/ml for patients with intermediate prognosis and 271.8±363.0 pg/ml for those with weak prognosis. There was no association between serum CA9 and Motzer classification (Kruskall-Wallis test, $p$=0.431).

*CA9 level and treatment response*. According to the RECIST criteria, 22 patients were assessed as having a response to treatment and 46 patients no response to treatment. The serum CA9 levels were 64.7±104.7 pg/ml for the responding group and 108.2±203.8 pg/ml for non-responders. No statistically significant difference was observed between the groups (Wilcoxon non-parametric test, $p$=0.366).

*CA9 levels and survival analysis*. The median follow-up was 23.4 (range=4.7-29.9) months at the time of analysis. During the follow-up, 27 patients died from their disease. The curve of OS is shown in Figure 1. The median serum CA9 levels were used to divide the patients into high- and low- CA9 groups. The Cox univariate analysis is shownen in Table II. It indicates that the risk of death was higher (HR=2.65, 95% CI=1.19-5.92) in the group of patients with high CA9. There were 18 deaths in the high-CA9 group and 9 deaths in the low-CA9 group. OS according to serum CA9 level (Figure 2) revealed a poorer prognosis for the patients in the high-CA9 group (log-rank test, $p$=0.0136). No correlation was observed between CA9 level and the progression-free survival in these patients (data not shown).

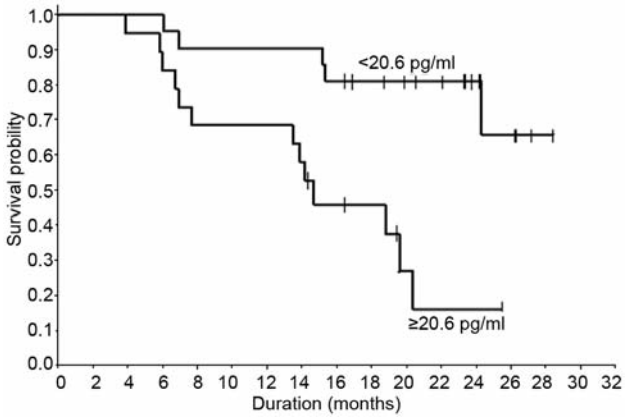


Figure 3. *The overall survival of experimental patients treated with temsirolimus and bevacizumab according to the level of serum CA9. The Kaplan-Meier survival curve showed a poorer prognosis of survival for patients in the high-CA9 group than those in the low-CA9 group (log-rank test, p=0.0006).*

For the major group of patients treated with temsirolimus and bevacizumab, for patients in the high-CA9 group, HR for OS was 5.45 (95% CI=1.19-5.92) (Table III). There were 13/18 deaths in the high-CA9 group and 5/20 deaths in the low-CA9 group. The Kaplan-Meier survival curve (Figure 3) demonstrated a poorer prognosis for patients in the high-CA9 group (log-rank test, $p$=0.0006).

Table II. *The univariate analysis of the Cox model for all patients.*

| Variable | Coding | N (deaths) | HR | 95% CI |
|---|---|---|---|---|
| CA9 median | <20.6 | 35 (9) | | |
| | ≥20.6 | 35 (18) | 2.648 | 1.185-5.916 |

HR: Hazard ratio; CI: confidence interval; CA9: carbonic anhydrase 9.

Table III. *The univariate analysis of the Cox model for experimental patients treated with temsirolimus and bevacizumab.*

| Variable | Coding | N (deaths) | HR | 95% CI |
|---|---|---|---|---|
| CA9 median | <20.6 | 20 (5) | | |
| | ≥20.6 | 18 (13) | 5.452 | 1.883-15.783 |

HR: Hazard ratio; CI: confidence interval; CA9: carbonic anhydrase 9.

## Discussion

Immunotherapy provides 15-20% partial and 1-5% complete response rates in the treatment for metastatic RCC. Since 2006, molecular-targeted therapy has changed treatment strategies. Targeted therapy has led to better results compared to immunotherapy (1), but has raised new questions. The response to treatment is dependent on repeated imaging examinations, which is costly and subjective. A marker which could reduce the imaging examinations, predict prognosis and choose the best candidates for treatment would be very welcome. Another question is how to select patients for clinical trials. Research in the area of markers of prediction or prognosis is very urgently needed alongside the increasing use of targeted therapy (15-18)

CA9 is mainly studied by immunohistochemistry in tumor tissues. The advantage of using a serum sample over immunohistochemistry on biopsy is that an ELISA test is quick and quite standardized (10). Serum can easily be collected at several time points during the course of the disease for longitudinal assessment of potential markers. Serum CA9 could be a potential novel marker for patients with cancer. In our previous study, we found that the serum levels of CA9 were elevated and had a prognostic value in patients with a localized clear-cell RCC, compared with healthy individuals (8). CA9 was cleared away from serum a few days after nephrectomy, suggesting its value as a promising marker for cancer (9). Recently, Pena *et al.* showed that the plasma level of CA9 was correlated with prognosis of metastatic RCC (17). They found that an elevated plasma CA9 level correlated with poor survival. However, whether serum CA9 provides information on the prognosis of patients with metastatic clear-cell RCC under targeted therapy is unknown.

In the present study, the serum concentration of CA9 in patients with metastatic clear-cell RCC varied widely. We observed that serum CA9 did not correlate with clinical parameters. Furthermore, we did not find any association between the level of serum CA9 and the treatment response defined by RECIST. We observed that a high level of CA9 was associated with a poor OS, thus suggesting that serum CA9 levels could allow for discrimination of metastatic RCC with poor outcome. Therefore, it is reasonable to speculate that serum CA9 could reflect some aspect of the tumor biology.

Circulating endothelial cells and soluble isoforms of vascular endothelial growth factor (VEGF) have been reported to be predictors of treatment response in patients with metastatic renal cancer (16, 19). In the present study, serum CA9 did not correlate with tumor response, as documented by RECIST. As serum CA9 may be mainly tumor-derived, its changes during treatment are expected to reflect the balance resulting from a complex interplay between the biological actors and the therapeutic agent. These markers may not be as informative for tumor shrinkage. Clinical results of targeted therapy have shown little evidence of tumor shrinkage and have rather suggested a cytostatic effect. Farace *et al.* found that the levels of circulating CD45(dim)CD34(+)VEGFR2(+) progenitor cells did not correlate with treatment response, but were associated with treatment prognosis (15). They believe that the tumor response criteria by RECIST may not be a good indicator of the clinical benefit of targeted therapy (15). Survival may be a more appropriate end-point for marker evaluation. In the present study, we did not find a relationship between the progression-free survival and serum CA9 level. Whether progression-free survival is a good surrogate marker of OS has not been confirmed in metastatic clear-cell RCC (20).

There are several limitations to our study. Serum samples were not available to study the CA9 levels at different time points during the treatment. Furthermore, there is no single-predictive marker for targeted therapy. Rather, a panel of biomarkers has to be considered in order to obtain a detailed picture of the effectiveness of the targeted therapy. Future studies in a cohort of patients with metastatic RCC undergoing a targeted therapy are needed to confirm our results and to determine the eventual prognostic value of CA9.

In conclusion, this study provides data on serum CA9 levels in patients with metastatic clear-cell RCC under targeted therapy. We found that the serum CA9 level was associated with the OS. The serum CA9 may be of clinical utility to predict the outcome of patients treated by targeted therapy.

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