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## Differential Expression of Axl and Gas6 in Renal Cell Carcinoma Reflecting Tumor Advancement and Survival

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**Abstract Purpose:** Overexpression of the receptor tyrosine kinase Axl is implicated in several cancers. Therefore, we conducted this study to determine the expression of Axl and its ligand Gas6 in various renal cell carcinoma (RCC) types and in oncocytoma.

**Experimental Design:** Real-time quantitative reverse transcription-PCR was used to quantify tumor mRNA levels for Axl and Gas6 in a cohort ( $n = 221$ ) of RCC patients. Serum levels of soluble sAxl and Gas6 proteins were measured using specific ELISA assays ( $n = 282$ ). The presence of Axl protein in tumor tissue was evaluated by immunohistochemistry ( $n = 294$ ). Results were correlated to tumor-associated variables, clinical biochemical tests, and patient survival.

**Results:** Tumor Axl mRNA levels correlated independently to survival when assessed against tumor stage and grade. In the study group, the median cancer-specific survival of all RCC patients during 307 months of follow-up was 55 months (confidence interval,  $\pm 40.4$ ). The 25% of patients with lowest tumor Axl mRNA levels had significantly better survival than the rest ( $P = 0.0005$ ), with 70% of the patients still alive at the end of follow-up. In contrast, in patients with medium-high Axl mRNA, only 25% were alive at the end of follow-up. Tumor Gas6 mRNA levels correlated to survival, tumor-associated variables, and disease severity as did serum levels of soluble sAxl and Gas6 protein. However, no correlation between Axl protein in tumor tissue and survival was found.

**Conclusions:** Axl and Gas6 expression in RCC are associated with tumor advancement and patient survival. In particular, low tumor Axl mRNA levels independently correlated with improved survival.

Renal cell carcinoma (RCC) represents ~2% to 3% of all human cancers (1), being the most common cancer of the adult kidney (2). The prevalence is skewed between men and women (1.5:1), and RCC incidence peaks at 60 to 70 years of age (3). The only curative treatment of RCC is surgery (4). Today, smaller and less advanced RCC tumors are detected by imaging techniques, such as ultrasound and computed tomography, and nephron-sparing surgery of tumors <4 cm in diameter is an alternative that provides recurrence-free and long-term survival

rates (5). However, the outcome of metastatic RCC is poor and mortality remains very high with a median cancer-specific survival of 21 months (6, 7). Treatment of metastatic RCC, in addition to removal of primary tumor and resection of metastases, is palliative and includes systemic therapies, such as immunotherapy (e.g., IFN $\alpha$ ). Recently, a number of promising tyrosine kinase inhibitors are approved both in the United States and Europe for treatment of metastatic RCC (5).

RCC is composed of different tumor types, including conventional or clear cell RCC (cRCC; representing the majority of RCCs, 75-85%), papillary RCC (pRCC; 10-15%), chromophobe RCC (chRCC; 4-5%), collecting duct carcinoma (<1%), and unclassified RCC (5%; ref. 8). In ~4% to 5% of the cases, benign oncocytoma is diagnosed when RCC is suspected (9). The more aggressive types, cRCC and pRCC, originate from the kidney tubular epithelium, whereas the less aggressive chRCC and oncocytoma are derived from intercalated cells of the collecting duct (9). The RCC types are classified based on different histopathologic and specific genetic variations, where-in alteration in the von Hippel-Lindau tumor suppressor gene is one of the most common alterations responsible for cRCC, occurring in ~80% of these cases (9). Genetic alterations taking place in hereditary pRCC involve mutations in the proto-oncogenic receptor tyrosine kinase *c-Met* (10-15% *c-Met* mutations in the sporadic form of pRCC; ref. 9).

Axl receptor tyrosine kinase is another proto-oncogene that is abundantly expressed in human tissues (10) and, together with

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## Translational Relevance

Renal cell carcinoma (RCC) has long been recognized as a common tumor with dismal prognosis due to inadequate therapy of metastatic RCC. Despite the increased understanding of the underlying oncogenic events in RCC, no molecular markers are currently recommended for estimation of prognosis. Herein, we report a large study of potential molecular primary tumor and serum markers in RCC, namely the receptor tyrosine kinase Axl and its ligand Gas6. Both Axl and Gas6 expression are shown to be implicated in RCC tumor advancement and severity and correlated to patient survival. Furthermore, tumor Axl mRNA was an independent factor in multivariate analysis when assessed against RCC tumor stage and grade, wherein low tumor Axl mRNA was associated with longer patient survival.

Tyros3 and Mer, constitutes the TAM family of receptor tyrosine kinases that share the ligand Gas6 (product of the *growth arrest specific gene 6*; refs. 11–13). Together these receptors regulate many processes, such as cell proliferation, survival, cell adhesion, migration, blood clot stabilization, inflammation, cytokine release, and phagocytosis of apoptotic cells (10). Gas6 was originally found as a gene induced in cells during growth arrest (14). Axl was originally identified as a transforming gene in chronic myeloid leukemia patients (15, 16) and in a chronic myeloproliferative disorder (17) wherein Axl was suggested to be capable of transforming cells without intrinsic activating mutations. Axl has been shown to be overexpressed and to have mitogenic and prosurvival roles in a broad spectrum of human malignancies, e.g., breast, lung, colon, thyroid, and ovarian cancers (18–22). Recently, Axl was shown to mediate glioma cell proliferation, migration, and invasion (23), and in human gliomas, both Axl and its ligand Gas6 are frequently overexpressed and predict poor prognosis (24). Moreover, Axl affects multiple cellular behaviors required for neovascularization, such as endothelial proliferation, migration, survival, and tube formation *in vitro*, and regulates angiogenesis *in vivo* (25). Axl is a transmembrane protein, but soluble Axl (sAxl), consisting of the proteolytically released extracellular domain (26), is present in human serum as well (27). Gas6 is also present at low concentrations in circulation (27), and in both mouse and human serum Gas6 circulates in complex with sAxl (26).<sup>4</sup> The concentration profiles and biological implications of sAxl and Gas6 protein in serum in RCC patients are, thus far, not investigated.

Axl and Gas6 expression in the human kidney is increased in inflammatory renal disease (28), and based on a study of Axl mRNA in 20 cases of cRCC, it has been proposed that Axl mRNA levels are increased in cRCC tissue (29). We have now conducted a large study to determine the expression of Axl and its ligand Gas6 at the mRNA level in RCC. Moreover, we have investigated the levels of sAxl and Gas6 protein in serum of RCC patients, as well as the presence of Axl in tumor tissue by immunohistochemistry.

## Materials and Methods

**Patient and primary RCC tumor information.** The study included 308 RCC patients in total and was approved by the ethical committee of Umeå University and the institutional review board. Each patient participated after providing informed consent. Physical examination, chest radiography, and computed tomography of the abdomen and chest during the last years were used to evaluate clinical stage for the patients. Tumor stage, after nephrectomy at the Department of Urology, Umeå University Hospital, between the years 1982 and 2003, was done according to the tumor-node-metastasis (TNM) classification system 2002 (30). The histopathologic nuclear grading was, according to Skinner et al. (31), based on the worst nuclear grade. RCC type was classified according to the Heidelberg consensus conference (2). Clinicopathologic characteristics of patients and tumors are shown in Table 1. Tumor size was defined as maximum diameter on computed tomography. Venous involvement was defined as invasion of tumor thrombus material in major renal veins and verified microscopically in tissue slices from the renal hilum. Capsule involvement was defined as invasion of the perinephric or renal sinus fat by tumor cells. None of the patients were treated with any RCC-specific therapy before nephrectomy. Among patients having metastatic disease at the time of surgery, 19 had radiation therapy, 11 had metastases resected, 14 received IFN treatment, and 24 received hormonal therapy (medroxyprogesterone acetate or tamoxifen), whereas 25 patients only were palliatively treated. In patients who recurred with metastatic disease after surgery, 20 had surgical resection of metastases, 28 received first line immunotherapy, 35 got hormonal therapy, 35 had radiation therapy, and 1 patient had first line treatment with a tyrosine kinase inhibitor (sunitinib), whereas the remaining patients only received palliative treatments. Patients were followed up according to a scheduled program, at least yearly by routine clinical follow-up, at Umeå University Hospital or by contacting patients directly. The last follow-up for all was conducted in June 2008. The follow-up time of alive patients was  $143.4 \pm 67.7$  mo ( $n = 67$ ). During the follow-up period, 169 patients died of their disease, 72 died of other causes, 7 were alive with disease, and 60 were alive with no evidence of disease.

**Preparation of tissue RNA.** For the study of mRNA levels, tumor samples from consecutive patients who underwent radical nephrectomy for RCC, collected between 1985 and 2003, were obtained immediately after surgical excision. The viable area of each tumor tissue was used for extraction of high-quality RNA, using the TRIzol reagent (Invitrogen), with the selection that only those having useful RNA could be included in the study ( $n = 221$ ). Histopathologically nonmalignant kidney cortex tissue ( $n = 44$ ), obtained furthest away from the tumor in the tumor-bearing kidneys, was used for comparative evaluation. RNA integrity was verified by ethidium bromide staining of 28S and 18S rRNA after agarose gel electrophoresis and Bioanalyzer (Agilent Technologies). RNA concentrations were quantified spectrophotometrically at a wavelength of 260 nm (DU640 spectrophotometer, Beckman Coulter). Each sample was rapidly frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis.

**Quantitative real-time reverse transcription-PCR.** One-step multiplex quantitative real-time reverse transcription-PCR (qRT-PCR) was done using primary tumor total RNA from 221 RCC patients and using total RNA from 44 matched histopathologically nonmalignant kidney cortex tissue serving as a reference. Amplifications of gene of interest and endogenous control gene were done in the same reaction tube. Gene-specific primers and Taqman probes were purchased from Applied Biosystems. To generate standard curves for quantitative analysis of each gene, cell lines that were reliable and had verified sources of mRNA for the particular gene were chosen. Tissue RNA was extracted as described above, and total RNA for Axl and Gas6 standards was extracted from cultured RCC 786-O cells (32) and stable Gas6-transfected HEK293 cells (33), respectively, using CellDirect One-Step qRT-PCR kit (Invitrogen). A master mix of Taqman MGB primers and

<sup>4</sup> C. Ekman, M. Tjwa, J. Stenhoff, et al. Gas6 is inhibited by binding to the soluble tyrosine kinase receptor Axl in human and mouse plasma, manuscript in preparation.

**Table 1.** Patient information and clinicopathologic variables in relation to RCC subtype and oncocytoma

| Variable                     | pRCC        | cRCC                   | chRCC        | Oncocytoma  |
|------------------------------|-------------|------------------------|--------------|-------------|
| Patients (%)                 | 35 (11.4)   | 250 (81.2)             | 14 (4.5)     | 9 (2.9)     |
| Patient sex %, male/female   | 65.7/34.3   | 59.2/40.8              | 50/50        | 55.6/44.4   |
| Patient age range (median)   | 25-82 (65)  | 34-85 (67)             | 36-80 (66.5) | 50-80 (66)  |
| TNM stage I + II/III/IV      | 18/10/7     | 102/61/86              | 8/4/2        | —           |
| Nuclear grade 1/2/3/4        | 5/11/16/3   | 9/148/124/67           | -/3/10/1     | —           |
| Tumor size mm range (median) | 25-250 (70) | 20-170 (80)            | 30-150 (88)  | 30-100 (40) |
| Vein invasion, no/yes        | 26/9        | 147/101                | 9/5          | 7/7-        |
| Capsule invasion, no/yes     | 24/9        | 161/85                 | 11/2         | 4/-         |
| Survival (1)/(2)             | 51(-)/47    | 41 ( $\pm 12.846$ )/42 | -/86         | -/100       |

NOTE: (1) indicates median survival months (CI), and (2) indicates the percentage of 5-y survival rate.

probes, reverse transcriptase, and polymerase was prepared and mixed with 40 ng sample RNA or with a series of known quantities standard RNA in a final volume of 25  $\mu$ L. The qRT-PCR reaction was done in an ABI PRISM 7900HT system (Applied Biosystems), with the following cycling conditions: reverse transcription for 15 min at 50°C, 2 min at 95°C, 40 cycles of 95°C for 15 s and 60°C for 60 s. Each measurement was done in duplicate, and the threshold cycle ( $C_t$ ) value was determined for each amplification curve. RNA template was omitted in negative controls, and standard curves for both genes of interest and housekeeping control gene were included in each run. From a screen of 11 potential candidates, the human  $\beta$ 2-microglobulin (*B2M*) was selected as the most consistent and reliable reference gene in human kidney samples (Supplementary Fig. S1A and B). For each sample, the mean of duplicate  $C_t$  values was converted to nanogram RNA from linear regression analysis using the relative standard curve method. Determined nanogram RNA values were then normalized to the endogenous reference nanogram *B2M* content of the same sample.

**Quantification of sAxl and Gas6 in human RCC serum.** Concentrations of sAxl and Gas6 serum protein in 282 RCC patients and 65 normal serum samples, retrieved from the NOBIDA biobank, established as a part of the Nordic Reference Interval Project (34, 35), was estimated using in-house developed ELISA assays specific for sAxl and Gas6, respectively. Serum samples were routinely obtained before any therapy from peripheral veins in fasting patients before 10 a.m. and stored at -80°C until analysis. Human sAxl and Gas6 serum levels were determined with sandwich ELISAs. The Gas6 ELISA, using a polyclonal goat antibody (AB885, R&D Systems) as catcher and biotinylated rabbit Gas6 antiserum as detector, was done as described with small modifications; PBS was replaced with TBS, 1% bovine serum albumin was replaced with 3% fish gelatin in TBS-Tween 20 [50 mmol/L Tris, 150 mmol/L NaCl (pH 7.4) containing 0.1% Tween 20 and 3% fish gelatin; ref. 27]. In the ELISA for sAxl, an immunoglobulin fraction of an in-house polyclonal rabbit antiserum (denoted Axl-#042), raised against purified recombinant sAxl expressed in a stable HEK293 cell line, was used as immobilized catcher (in Maxisorp microtiter plates; Nunc). The biotinylated immunoglobulin fraction of another in-house rabbit antiserum (Axl-#041) was used as detector. The ELISA was done using the same procedure as that described for the Gas6 ELISA (27). The details of the recombinant sAxl expression and the detailed characterization of the sAxl ELISA will be described elsewhere.<sup>5</sup> In brief, all incubation steps were carried out at room temperature, and every step was followed by washes with TBS-Tween 20. Samples and reagents were diluted in TBS-Tween 20 containing 3% fish gelatin. A dilution series of a known concentration of the recombinant sAxl was used as a standard, and wells with only TBS-Tween 20 with 3% fish gelatin were used as

negative controls. Samples were incubated overnight. For detection, the plates were incubated for 1 h with 3  $\mu$ g/mL of biotinylated anti-Axl-#041. After washes, the plates were incubated for 30 min with StreptABC/HRP (Dako) before development with OPD (Dako) and measurement of end point absorbance at 490 nm.

**Immunohistochemical analysis of Axl staining in a tissue microarray of RCC.** A tissue microarray was constructed by taking two valid tissue cores (0.6 mm) from each tumor block ( $n = 294$ ) and by inserting them into a recipient paraffin block (tissue microarray block). Each tissue microarray block included 98 tissue cores (49 cases in duplicates). Paraffin sections (4  $\mu$ m) were deparaffinized and subjected to antigen retrieval by boiling in 10 mmol/L citrate buffer (pH 6). The expression of Axl was detected using the Axl-#042 antiserum (4.4 mg/mL total IgG) at a dilution of 1:400. Immunohistochemical staining was done using the DAKO Techmate 500 staining equipment including Envision as secondary reagent, according to the instructions of the manufacturer (DAKO). For nuclear counterstaining, hematoxylin was used. An experienced pathologist evaluated the resulting staining pattern and the staining intensity. Defective cores, i.e., cores with absent tissue, large areas of necrosis, fat, and haemorrhages, and unstained cores, were omitted from the analysis, rendering 173 tumors that were used for analysis. Depending on staining intensity, Axl expression was divided into three groups; no, low, and high (0-2). The specificity of the Axl-#042 antiserum was evaluated by blocking specific Axl staining by preadsorption of antiserum with an excess of sAxl antigen (Supplementary Fig. S2A and B).

**Statistical analysis.** For statistical analysis of nonparametric data, the Mann-Whitney *U* test and Spearman rank correlation test were used. Survival curves were evaluated with the Kaplan-Meier method, and cumulative survival data were compared using the log-rank test. Multivariate prognostic analysis according to the Cox proportional hazard regression model was done to evaluate intervariate independence in relation to prognostic relevance. Survival time was measured from the date of nephrectomy to the date of death or the latest follow-up. All statistical tests were two sided, and the significance level was set at 0.05. Statistical analyses were done using the SPSS software package (version 16.0.2 for Macintosh; SPSS Institute). All assays were done blinded to the study end point.

## Results

**Axl and Gas6 tumor mRNA levels in RCC.** Axl and Gas6 tumor mRNA levels were determined with qRT-PCR in a large cohort of RCC patients ( $n = 221$ ), displaying a representative distribution of RCC types (ref. 8; Table 1). Axl and Gas6 tumor mRNA levels differed between RCC types and the levels found in unaffected kidney cortex tissue (Fig. 1A and B). Thus, median tumor Axl mRNA levels were increased in pRCC, whereas median Gas6 mRNA was decreased in both pRCC and cRCC

<sup>5</sup> C. Ekman and B. Dahlbäck. Identification and quantification of the soluble form of the receptor tyrosine kinase Axl in human plasma, manuscript submitted.

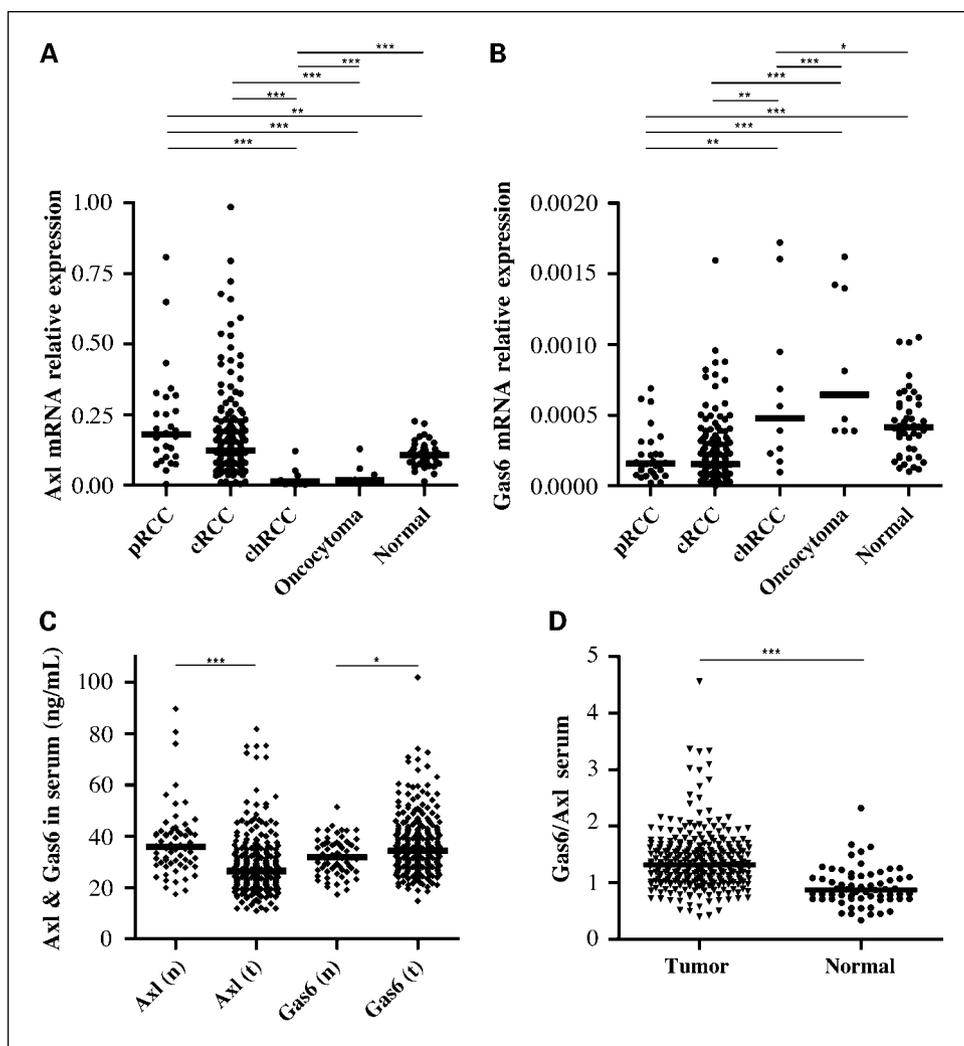
(Fig. 1A). In contrast, in chRCC and oncocytoma, Axl mRNA expression was very low, whereas Gas6 expression was slightly higher than that of unaffected kidney cortex tissue (Fig. 1A). No significant change of median expression of cRCC tumor Axl mRNA could be detected (Fig. 1A). In cRCC, there was a correlation between Axl and Gas6 mRNA levels ( $r_s = 0.297$ ,  $P < 0.001$ ; Supplementary Fig. S3A). Another noteworthy observation in cRCC was that the tumor Axl and Gas6 mRNA levels correlated positively with the Axl and Gas6 mRNA in their respective matched kidney cortex samples [ $r_s$  (Gas6) = 0.353,  $P = 0.044$  and  $r_s$  (Axl) = 0.347,  $P = 0.048$ ; data not shown].

**Axl and Gas6 protein levels in RCC.** The concentrations of sAxl and Gas6 protein in serum did not correlate to tumor Axl and Gas6 mRNA levels (Supplementary Fig. S4A and B), and there were no differences in serum levels of sAxl and Gas6 between the different RCC types (data not shown). However, compared with healthy controls, the serum concentrations of sAxl protein were lower (Fig. 1C) whereas those of Gas6 were higher in RCC (Fig. 1C), and the Gas6/sAxl ratios were higher in RCC patients than in controls (Fig. 1D). Although Gas6 and sAxl protein were changed in different directions compared with controls, there was a positive correlation between the two in RCC patients ( $r_s = 0.360$ ,  $P < 0.001$ ; Supplementary Fig. S3B).

In immunohistochemical analysis, Axl staining was strong in human normal kidney, in particular in tubular epithelial cells (Supplementary Fig. S5D, right). In RCC tissue, Axl staining varied considerably between individual cases where no or both low and high intensities were found (Supplementary Fig. S5A-C and D, left). Of particular note, neither Axl tumor mRNA nor sAxl serum protein concentrations correlated to the intensity of the Axl immunohistochemical staining (Supplementary Fig. S6A and B).

**Axl and Gas6 mRNA and protein levels in RCC patients correlated with tumor associated clinical variables and biochemical analytes.** Correlation analyses were done to investigate whether Axl and Gas6 tumor mRNA and/or protein levels were related to clinicopathologic variables in patients with RCC (Table 2). Increased sAxl and Gas6 protein levels in serum correlated with more advanced tumor stage [ $P$  (Axl) = 0.037;  $P$  (Gas6) = 0.010], with nuclear grade [ $P$  (Axl) = 0.018;  $P$  (Gas6) < 0.001], and with tumor-thrombus extension factors related with worse outcome (vein invasion;  $P$  (Axl) = 0.001;  $P$  (Gas6) = 0.001), and furthermore, Gas6 also correlated with renal capsule invasion in all tumors ( $P = 0.013$ ). Decreased tumor Gas6 mRNA levels correlated with more advanced tumor stage ( $P = 0.03$ ) and more aggressive nuclear grade ( $P = 0.014$ ), whereas no correlation between tumor Axl mRNA levels and

**Fig. 1.** Axl and Gas6 tumor mRNA and sAxl and Gas6 serum protein in RCC. **A** and **B**, Axl and Gas6 tumor mRNA expression in various RCC subtypes and oncocytoma in relation to unaffected kidney cortex tissues ( $n = 44$ ). Expressions in kidney tumors ( $n = 221$ ) according to subtype along with respective median values. Levels were determined by qRT-PCR and expressed in arbitrary units as normalized against *B2M* reference gene. \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ . **C**, expression of sAxl and Gas6 protein in serum in various RCC subtypes and in oncocytoma (t, tumors), in relation to control human plasma ( $n$ , normal;  $n = 65$ ). Expressions in patient serum for all cancer subtypes together ( $n = 282$ ) along with their median values. Levels were determined by Axl-specific and Gas6-specific ELISA assays, respectively, and are expressed in absolute quantity of protein (ng/mL). \*\*\*,  $P < 0.001$ ; \*,  $P < 0.05$ . **D**, comparison of the ratio of Gas6/sAxl serum protein levels in RCC ( $n = 282$ ) compared with control plasma ( $n = 65$ ). Expression levels were determined as described in **C**.



**Table 2.** Tumor variables significantly associated with Axl and Gas6 tumor mRNA and serum levels of sAxl and Gas6 proteins

|                     | Axl               |                           | Gas6                       |                           |
|---------------------|-------------------|---------------------------|----------------------------|---------------------------|
|                     | Tumor mRNA (cRCC) | Serum protein             | Tumor mRNA (cRCC)          | Serum protein             |
| TNM stage           | —                 | <b>0.126*</b> 0.037 (273) | <b>-0.166*</b> 0.030 (172) | <b>0.155*</b> 0.010 (274) |
| Nuclear grade       | 0.111 0.146 (173) | <b>0.141*</b> 0.018 (280) | <b>-0.187*</b> 0.014 (172) | <b>0.229†</b> 0.001 (281) |
| Venous involvement  | —                 | <b>0.197†</b> 0.001 (278) | -0.115 0.135 (171)         | <b>0.199†</b> 0.001 (279) |
| Capsule involvement | 0.085 0.268 (170) | 0.096 0.113 (272)         | -0.105 0.176 (169)         | <b>0.151*</b> 0.013 (273) |

NOTE: Data given are as follows: Spearman correlation coefficient/*P* value (in italics)/number of cases (in brackets). Significant correlations are in bold font.

\*Correlation is significant at the 0.05 level (two tailed).

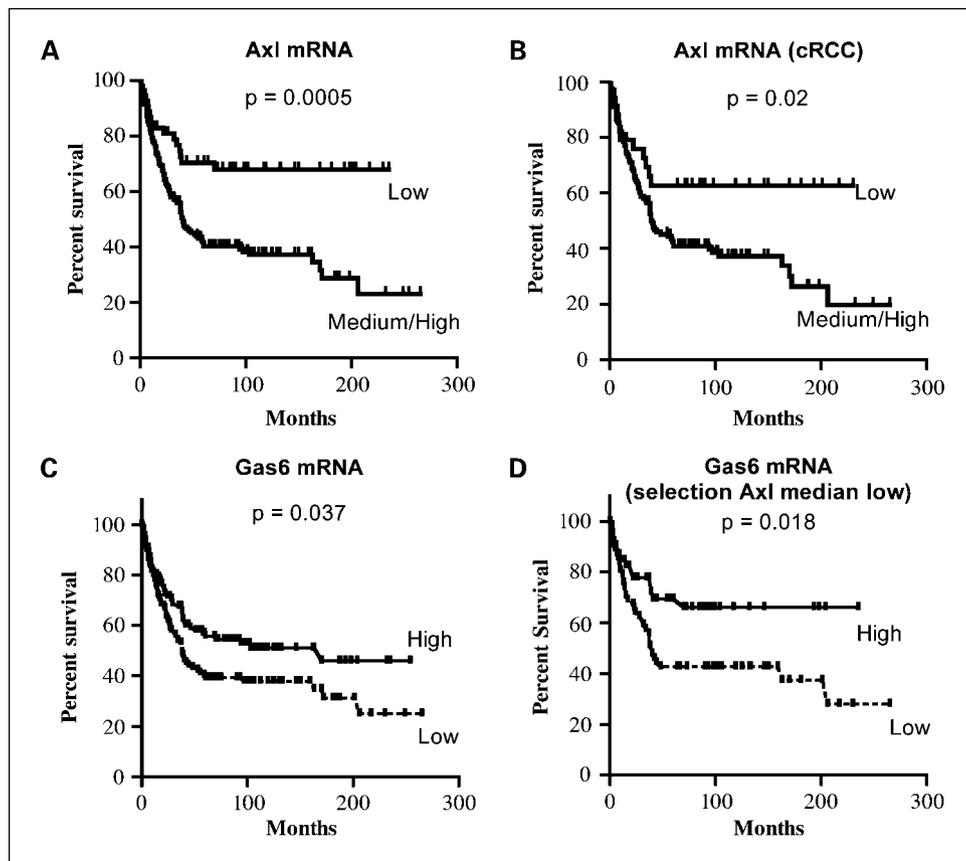
†Correlation is significant at the 0.01 level (two tailed).

tumor stage was found. No correlation between Axl immunohistochemical levels and any of the mentioned variables could be detected (data not shown).

To estimate the systemic effects of the kidney tumors, because paraneoplastic syndromes, including an abnormal liver function, anemia, low platelet count, and elevated erythrocyte sedimentation rate, are found in ~30% of patients with symptomatic RCC (5), the levels of hemoglobin, albumin, erythrocyte sedimentation rate, and C-reactive protein were correlated to the levels of sAxl and Gas6 protein in serum. Hemoglobin and albumin concentrations correlated negatively to both the sAxl and Gas6 serum protein levels ( $P < 0.001$ ; Supplementary Fig. S7A and B). In contrast, erythrocyte sedimentation rate and C-reactive protein correlated positively

to both sAxl and Gas6 serum protein concentrations ( $P < 0.001$ ; Supplementary Fig. S7C and D).

**Axl and Gas6 tumor mRNA and patient survival.** Median cancer-specific survival of all RCC patients during 307 months of follow-up was 55 months [confidence interval (CI),  $\pm 40.39$ ]. Although there was no simple linear relationship between RCC Axl mRNA levels and survival (Supplementary Fig. S8A and B), patients with the 25% lowest Axl mRNA stood out as a group with longer survival than the rest of the patients (Fig. 2A; Supplementary Fig. S8B). However, there was no difference in survival between patients with medium and high tumor Axl mRNA levels (Supplementary Fig. S8B). In the patient group with medium and high tumor Axl mRNA levels, the median survival during the follow-up was 40 months (CI,  $\pm 10.91$ ). In



**Fig. 2.** Low Axl and high Gas6 tumor mRNA levels correlate with longer survival. *A* and *C*, Kaplan-Meier curve analyses of patient survival in all RCC tumors and oncocytoma based on Axl (*A*) and Gas6 (*C*) tumor mRNA. Axl mRNA ( $n = 221$ ) levels were divided into low ( $\leq 25$ th percentile) and medium/high ( $> 25$ th percentile), respectively. Gas6 mRNA ( $n = 220$ ) levels were divided into low ( $\leq 50$ th percentile) and high ( $> 50$ th percentile), respectively. *B*, Kaplan-Meier curve analysis of patient survival exclusively in cRCC tumors based on Axl tumor mRNA. Axl mRNA ( $n = 173$ ) levels were divided as described in *A*. *D*, Kaplan-Meier curve analysis of patient survival in all RCC tumors and oncocytoma with selection for Axl mRNA low cases (median division;  $\leq 50$ th percentile) and based on Gas6 tumor mRNA. Gas6 mRNA ( $n = 111$ ) levels were divided as described in *C*. Patient survival is shown in months, and percentage survival for each group was compared using the log-rank test (pooled over strata). Ticks, censored cases. Significance of survival difference (*P*) is depicted.

contrast, in the "low Axl" group, 70% were alive at the end of follow-up (Fig. 2A). The improved survival time in the low Axl group was not a consequence of selection bias of less aggressive chRCC or of oncocytoma, because the same results were obtained in the specific cRCC group ( $P = 0.02$ ; Fig. 2B).

In contrast to what we found for tumor Axl mRNA, increased tumor Gas6 mRNA levels was associated with improved survival ( $P = 0.037$ ; Fig. 2C). In the "high Gas6" group the median survival was 170 months (CI, undefined), whereas the "low Gas6" group had median survival of 38 months (CI,  $\pm 9.756$ ). The combined Axl and Gas6 mRNA results yielded additional information. Thus, when analyzing the influence of Gas6 mRNA on survival in patients with either low or high tumor Axl mRNA expression (median division), we found that high tumor Gas6 mRNA further improved the survival of patients with median low levels of tumor Axl mRNA, with  $\approx 65\%$  surviving at the end of the follow up ( $P = 0.018$ ; Fig. 2D). However, tumor Gas6 mRNA levels did not influence the poor prognosis in patients with high tumor Axl mRNA levels (data not shown). In agreement with the survival analysis, patients that died of the disease had higher tumor Axl mRNA levels ( $P = 0.032$ ) and lower Gas6 mRNA levels ( $P = 0.021$ ) than those who stayed disease free (data not shown).

In multivariate prognostic analysis according to the Cox proportional hazard regression model of tumor Axl and Gas6 mRNA, tumor Axl mRNA remained as an independent prognostic factor in relation to cancer-specific survival ( $P = 0.004$ ; CI, 1.278-3.712) whereas tumor Gas6 mRNA lost its prognostic information (Table 3).

**Axl and Gas6 protein levels and patient survival.** The levels of sAxl and Gas6 protein in serum from RCC patients correlated negatively to survival, i.e., having lower serum levels was beneficial for the patients [ $P$  (sAxl) = 0.048,  $P$  (Gas6) = 0.038; Fig. 3A and B]. Furthermore, serum levels of both sAxl and Gas6 protein were higher in RCC patients who died of the disease than in those who stayed alive and disease free [ $P$  (sAxl) = 0.014,  $P$  (Gas6) = 0.007; Fig. 3C and D]. Noteworthy, the intensity of Axl staining in tumor tissue did not correlate to survival (data not shown).

## Discussion

RCC is characterized by a variable clinical course and known for its unpredictable behavior (5, 9). Therefore, it is of great value to identify the fundamental molecular basis for the biological

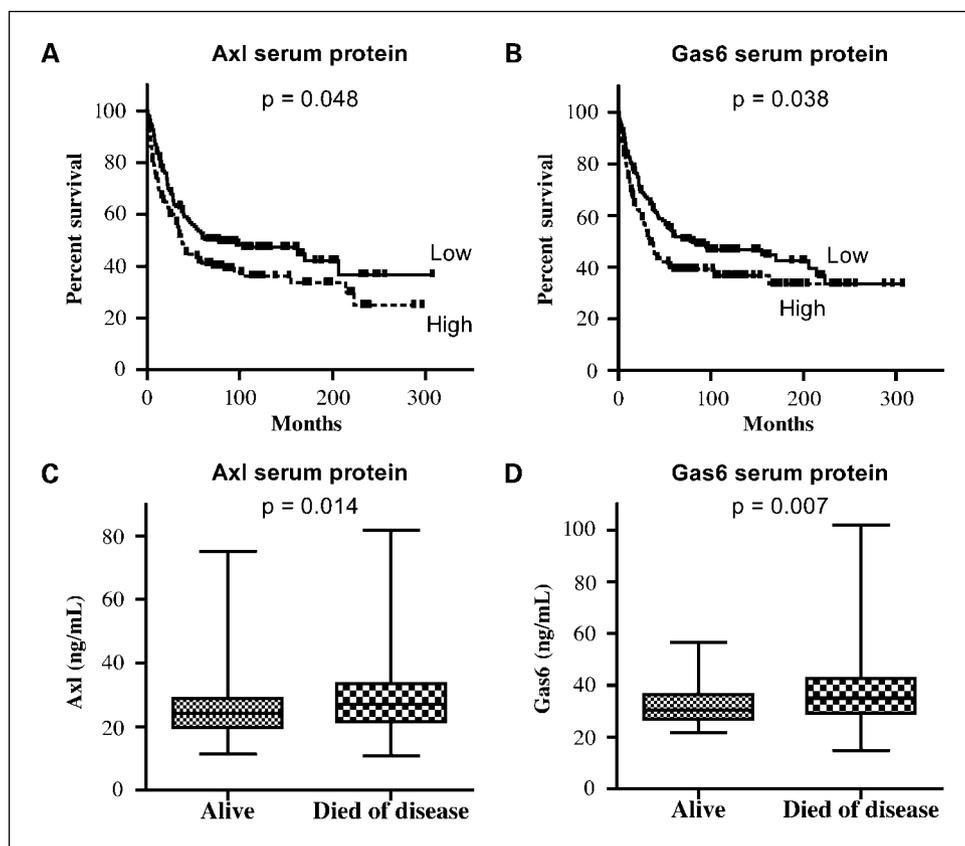
events underlying individual tumors. The 2002 TNM staging classification system (30), the Fuhrman grading system (36), and the histologic subtyping into different kidney tumors are generally accepted as the most important prognostic factors (5). However, it is currently unclear whether this system is optimal for prediction of survival in RCC. There have been considerable efforts to find other markers conclusively associated with risk of progressive disease. Markers currently under investigation are carbonic anhydrase IX, vascular endothelial growth factor (VEGF), hypoxia inducible factor, Ki67, p53, PTEN, E-cadherin, and CD44 (5). However, none of them is presently recommended for use in clinical routine, and thus, there is a great need for reliable prognostic and predictive markers.

In this report, low tumor Axl mRNA was shown to be an independent prognostic factor for RCC. Moreover, high tumor Gas6 mRNA was associated with improved survival. Combining Axl and Gas mRNA levels further defined a population of patients having low Axl and high Gas6 mRNA levels and good prognosis. The positive effects of high tumor Gas6 mRNA, together with the contrary expression patterns of tumor Axl and Gas6 mRNA in RCC, are intriguing because it has been shown in human glioma that high tumor Axl and Gas6 mRNA levels indicated worse prognosis (24) and because of observations that Gas6 binding to Axl stimulates proliferation and survival (37–39). Thus far, increased Gas6 mRNA correlating with favorable prognosis has only been reported in human breast cancer (40). Furthermore, in cRCC, tumor Axl mRNA levels were broadly distributed and the results are consequently more informative than those of a previous study of 20 patients that suggested Axl mRNA levels to be significantly up-regulated in cRCC (29). A methodologic difference that possibly can explain the divergent results is that tumor Axl mRNA was semiquantitatively estimated after RT-PCR by gel electrophoresis in the previous study, whereas we have used quantification by qRT-PCR.

Axl/Gas6 signaling is involved in directed cell migration of smooth muscle cells in the context of remodeling of the vessel wall after vascular injury and for endothelial cells during tumor angiogenesis (25, 41). Thus, Axl has recently been shown to affect multiple cellular behaviors required for neovascularization *in vitro*, and loss of Axl expression in tumor cells blocked the growth of solid human neoplasms in an *in vivo* breast carcinoma model (25). As such, this angiogenic potency of Axl/Gas6 signaling might contribute to tumor progression in RCC, wherein vascularization plays an important role (9) and could be one explanation for a worse outcome of patients with higher

**Table 3.** Multivariate prognostic analysis according to the Cox proportional hazard regression model

|                                      | Exp(B) | P      | 95% CI for Exp(B) |
|--------------------------------------|--------|--------|-------------------|
| <b>Prognostic factor</b>             |        |        |                   |
| Tumor Axl mRNA                       | 2.107  | 0.007  | 1.231-3.605       |
| Tumor Gas6 mRNA                      | 1.258  | 0.248  | 0.852-1.856       |
| Age (<65 y, >65 y)                   | 0.754  | 0.148  | 0.515-1.105       |
| Sex (male versus female)             | 1.280  | 0.216  | 0.866-1.893       |
| Tumor diameter (<80 mm, >80 mm)      | 1.082  | 0.683  | 0.742-1.577       |
| Grade (I + II versus III + IV)       | 1.414  | 0.196  | 0.836-2.394       |
| Stage (I + II versus III + IV)       | 12.143 | <0.001 | 6.666-22.121      |
| <b>After final stepwise analysis</b> |        |        |                   |
| Tumor Axl mRNA                       | 2.178  | 0.004  | 1.278-3.712       |
| Stage (I + II versus III + IV)       | 12.095 | <0.001 | 6.858-21.332      |



**Fig. 3.** Low sAxl and Gas6 serum protein levels in RCC correlate with longer survival. *A* and *B*, Kaplan-Meier curve analysis of patient survival in all RCC tumors and oncocytoma based on sAxl (*A*) and Gas6 (*B*) protein in serum, respectively. sAxl ( $n = 282$ ) and Gas6 ( $n = 283$ ) protein levels were divided into low ( $\leq 50$ th percentile) and high ( $> 50$ th percentile) categories. Patient survival is shown in months, and percentage survival for each group was compared using the log-rank test (pooled over strata). Ticks, censored cases. Significance of survival difference ( $P$ ) is depicted. *C* and *D*, comparison of sAxl (*C*) and Gas6 (*D*) protein expression levels in serum of RCC patients who stayed alive and disease-free after tumor nephrectomy, with those levels in RCC patients who had their tumor removed, but eventually died of the disease. Quantifications of sAxl [ $n$  (alive) = 55,  $n$  (died of disease) = 159] and Gas6 [ $n$  (alive) = 55,  $n$  (died of disease) = 160] protein concentrations in serum were determined by use of Axl-specific and Gas6-specific ELISA assays, respectively, and are expressed in absolute quantity of protein (ng/mL).

Axl mRNA levels. Still intriguing is the observation that high tumor Gas6 mRNA levels correlated with longer survival. Recently, Gas6 via Axl was shown to have inhibitory effects on the VEGF receptor-driven angiogenic program (42), the importance of which, especially in cRCC angiogenesis wherein the majority of cases are von Hippel-Lindau-defective with increased levels of hypoxia-inducible factor- $\alpha$  target genes such as VEGF (9), is well known (43, 44). Therefore, high RCC Gas6 mRNA levels could be beneficial by possibly inhibiting this VEGF-driven angiogenesis. Furthermore, prolonged Gas6 stimulation results in Axl internalization and lysosomal degradation (45), and this Gas6-dependent Axl down-regulation over time could possibly contribute to the better prognosis of RCC patients with high tumor Gas6 levels.

Unfortunately, we cannot report any information about the phosphorylation status of Axl in tumor tissues, which would be informative with respect to receptor activation. However, it should be emphasized that it was Axl at the mRNA level that correlated to survival time of RCC patients and that Axl protein in tumor tissue did not.

The Gas6 system has been implicated in primary haemostasis, and Gas6<sup>-/-</sup> mice are rescued from induced thrombosis (46). Possibly, the skewed balance between sAxl and Gas6 protein in serum and, thus, the assumed risk of increased thrombus formation, likewise the various correlations of sAxl and Gas6 serum protein to several tumor risk factors, could contribute to the poor outcome of patients with RCC. Indeed, lower levels of sAxl and Gas6 protein in serum correlated to longer survival in RCC patients. Furthermore, we have verified that Gas6 protein in serum is correctly  $\gamma$ -carboxylated (data not

shown), as Gas6 that is not would not be functional. The mechanisms regulating the shedding of sAxl protein from cells and the Gas6 protein levels in serum under healthy conditions and in malignancies are unknown. We find it unlikely that the sAxl and Gas6 protein in the serum of RCC patients were derived from the tumor tissue because there were no correlations between Axl and Gas6 mRNA levels in RCC and corresponding sAxl and Gas6 protein levels in serum. Possibly, sAxl and Gas6 are derived from endothelial cells, fibroblasts, and/or vascular smooth muscle cells (13) and aberrantly released into the circulation in response to the disease.

In conclusion, we report that tumor Axl and Gas6 mRNA levels and sAxl and Gas6 serum protein levels correlated to survival of patients with RCC. Particularly noteworthy was the observation that low tumor Axl mRNA in RCCs at time of nephrectomy remained as an independent survival factor after multivariate prognostic analysis. Altogether, our study contributes to a greater understanding of the complicated pathobiology of RCC, wherein the discovery of new molecular markers for use in prognosis and/or medical intervention is highly desired.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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