ORIGINAL RESEARCH

Decreased mRNA expression of GATA1 and GATA2 is associated with tumor aggressiveness and poor outcome in clear cell renal cell carcinoma

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Abstract GATA-binding proteins 1 (GATA1) and 2 (GATA2) are zinc-finger transcription factors and belong to the GATA family proteins 1–6. *GATA1* interacts with the *TP53* tumor suppressor gene, and both GATAs have been shown to be involved in cell growth, apoptosis, and tumorigenesis of several solid tumors. GATA1 and GATA2 expression alterations are associated with poor survival and adverse clinicopathology in prostate and colorectal cancer, while the significance and prognostic value in clear cell renal cell carcinoma (ccRCC) has not been investigated as yet. We investigated relative messenger RNA (mRNA) expression levels of GATA1 and GATA2 in 77 ccRCC and 58 paired adjacent noncancerous renal tissues by quantitative real-time reverse-transcribed PCR. Relative mRNA expression levels were determined using the $\Delta\Delta$Ct method. GATA1 and GATA2 expression levels were significantly decreased in tumor tissues compared with normal tissues ($p<0.001$, paired t test). In univariate logistic regression analysis, decreased GATA1 and GATA2 expression levels were associated with advanced tumor disease ($p=0.005$ and 0.008), positive distant metastasis ($p=0.03$ and 0.001), and lymph node metastasis status ($p=0.011$ and 0.038). Reduced expression levels of GATA1 and GATA2 were associated with an increased risk of disease recurrence ($p=0.005$ and 0.006; hazard ratio=0.05 and 0.21). Pairwise bivariate analysis after adjusting for clinicopathological parameters revealed relative mRNA expression of GATA1, but not GATA2, as an independent candidate prognosticator for ccRCC. Our results support that *GATA1* and *GATA2* are involved in ccRCC tumor biology possibly affecting tumor development and aggressiveness.

Keywords *GATA1* · *GATA2* · Renal cell carcinoma · mRNA expression · Prognosis · Survival

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Background

Renal cell carcinoma (RCC) is one of the top ten causes of cancer deaths and the most lethal carcinoma of urological malignancies. The incidence of RCC has constantly increased during the past decades [1]. Clear cell RCC (ccRCC) is the most frequent histological subtype counting for approximately 75 % of all RCC histologies.

Molecular alterations occurring in ccRCC have been comprehensively analyzed [2]. Overall, a low number of genes with a mutation frequency of >30 %, such as the von Hippel-Lindau (*VHL*) and polybromo 1 (*PBRM1*) genes, have been identified. Instead, most tumors have been found to carry an individual spectrum of mutations making it impossible to identify usable mutation-based prognostic signatures as well as to draw simple functional conclusions. Alterations of the epigenetic network are frequently observed in ccRCC and particularly changes of DNA methylation patterns, mostly associated with altered expression levels of genes or

microRNAs, have been associated with functional alterations, clinicopathological parameters, survival, as well as therapeutic response of patients [3–5].

GATA1 and GATA2, zinc-finger transcription factors and members of the GATA family proteins 1–6, are known to be involved in cellular growth, differentiation, and apoptosis, especially in the hematopoietic lineage [6, 7]. A previous study showed that GATA1 interacts with TP53, and functional studies in erythroid cells suggested the existence of a reciprocal inhibition mechanism for TP53 and GATA1 expression [8].

Alterations of the TP53 gene are rarely found in RCC [2] and immunohistochemical analyses do not allow a direct clue to expression levels considering that mutant proteins have been found to show greater stability against degradation. On the other hand, different expression levels of TP53 in primary and metastatic RCC have been reported in several studies [9–11], proposing that an altered TP53 expression may promote disease progression of RCC albeit an impact on disease-free survival could not be demonstrated [12].

Variation of GATA2 expression has been suggested to be indicative for prognosis of several human solid malignancies [7]. Immunohistochemical detection of GATA2 overexpression in colorectal and prostate cancer was found to be associated with worse survival parameters albeit solely in univariate analysis [13, 14]. Expression array-based in silico analyses of gene omnibus breast cancer data [15] showed overlapping values for normal, primary, and metastatic breast cancer, a finding that resembles the TCGA breast cancer data set [16] (data not shown). Decreased expression levels as detected by northern blot analysis were found in high-stage neuroblastoma compared with low-stage tumors [17].

The clinical significance of GATA1 and GATA2 expression changes in the context of ccRCC development and tumor progression has not been investigated so far.

In this study, we investigated whether alterations of GATA1 and GATA2 messenger RNA (mRNA) expression levels can be detected in ccRCC and associate with clinicopathological parameters or outcome of patients. We found that low GATA1 and GATA2 expression levels were correlated with adverse clinicopathology and shortened recurrence-free survival (RFS).

Materials and methods

Patients' characteristic and tissue specimens

In a cross-sectional and retrospective study, 77 ccRCC and 58 matched paired noncancerous normal renal tissue were sampled. The local ethic committees of Hannover Medical School and Eberhard Karls University of Tuebingen approved sample collection and study design, a general written consent was

obtained. TNM classification and histological grading (G) was done according to the Union for Cancer Control 2002 and Thoenes et al. [18] as described previously [19]. RCC tissues were obtained from open or laparoscopic nephrectomies or partial resections. Histopathological assessment of control sections was performed as described before [20]. Noncancerous, adjacent normal tissues (adN), i.e., morphologically normal kidneys were isolated with a minimum of 0.5 to 2 cm distant from the primary tumor lesion. Samples were directly snap-frozen and stored at −80 °C.

Localized and locally advanced RCC disease was defined as tumors with pT≤3b, lymph node (N), and metastasis (M) negative (N0, M0). Advanced tumors are tumors with pT=4 and/or lymph node positive (N+) and/or positive for distant metastasis (M+). Patients with G=2 were assigned to the intermediate group and were not considered as a parameter of low- (G1 and G1-2) vs. high-grade (G2–3 and G3) group comparisons. Time from surgery to the first progressive event counting for either a local recurrence or a new metastatic site detected by computed tomography scan was designated as RFS. Follow-up was available for 35 patients. Clinical and histopathological parameters are summarized in Table 1.

RNA isolation, cDNA synthesis, and quantitative real-time PCR analysis

RNA isolation from patient's renal tissues and from cultured primary tissue cells, i.e., renal proximal tubular epithelial cells (RPTEC) as controls, cDNA synthesis, and quantitative real-time PCR analysis (qRT-PCR) were performed as described before [21]. RNA isolation was performed using 20 cryo sections from each patient tissue and each with a thickness of 20 μm. Duplicate measurements for qRT-PCR analysis were performed using 384 sample plates, the ABI 7900 Fast Sequence Detection System, and an automated liquid handling system (FasTrans, AnalyticJena, Jena, Germany). Experimenters were blinded to survival and clinicopathological data.

For analysis of the GATA proteins, the following TaqMan® expression assays (Life Technologies, Foster City, CA, USA) were used: GATA1 (Hs01085823_m1), GATA2 (Hs00231119_m1). HPRT1 (Hs99999909_m1), GUSB (Hs00939627_m1), and RPL13A (Hs03043885_g1) were included as endogenous references in each run. The endogenous controls, HPRT1, RPL13A, and GUSB, were combined by dataAssist V2.0 software and "arithmetic mean" was used as a method of normalization. The cDNA obtained from RPTEC primary cell transcripts were utilized as biological controls. Each qRT-PCR run included blank and a no-template control. Relative expression levels were calculated using the delta-deltaCT (ΔΔCt) method [22, 23] and the SDS 2.3 Manager and dataAssist V2.0 software (Life technologies) as described previously [21].

Table 1 Clinicopathological parameters

Parameter	Cases	Percent
Cases in total	77	100
Sex		
Female	29	38
Male	48	62
Median age (years)	64	
Median tumor size (cm)	5.5	
T stage		
pT1	6	8
pT1a	19	25
pT1b	14	18
pT2	3	4
pT3	2	3
pT3a	9	12
pT3b/c	22	29
pT4	0	0
Not known	2	3
Lymph node status		
N0	68	88
N+	9	12
Status of metastasis		
M0	56	73
M+	21	27
Grade		
Low		
G1	14	18
G1–2	8	10
Intermediate		
G2	40	52
High		
G2–3	5	6
G3	10	13
Stage of disease		
Localized and locally advanced disease		
pT ≤3, N0, M0	34	44
Advanced disease		
pT=4 and/or N+, M+	43	56
Follow-up	35	43
Sex		
Female	13	17
Male	22	29
T stage		
T1	19	25
T2	2	3
T3	13	17
T4	0	0
Lymph node status		
N0	32	42
N+	3	4
Status of metastasis		

Table 1 (continued)

Parameter	Cases	Percent
M0	27	35
M+	8	10
Grade		
G1	3	4
G1–2	4	5
G2	19	25
G2–3	4	5
G3	5	6
Paired samples	58	75

Statistical analysis

For statistical calculations, the natural logarithms of relative expression levels (lnRQ) were used. All statistical analyses were conducted with the statistical software, R 3.02 [24]. The paired t test was performed to evaluate the mean expression differences in matched tumor and corresponding noncancerous tissue samples. Group comparisons within tumor tissues were carried out using univariate logistic regression analysis. Univariate and bivariate survival analyses were performed using the Cox proportional hazard regression model, and the Kaplan-Meier method was used for estimating survival curves. The optimum threshold for dichotomization of expression values was calculated using the Maxstat R package.

Pairwise bivariate survival analyses have been performed instead of multivariate survival analysis taking into account that a combination of a limited number of events with various covariates may lead to biased coefficients in statistical analyses [25, 26].

Results

GATA1 and GATA2 mRNA expression levels are decreased in ccRCC

Significant decreased mean mRNA expression levels of GATA1 and GATA2 were found when tumor (TU; mean lnRQs ± SD=−0.9±1.06 and −1.2±0.98) specimens were compared with matched adN tissues (mean lnRQs±SD of 0.14±1.44 and 0.68±0.66; each $p<0.001$; Fig. 1a, b).

GATA1 and GATA2 relative expression levels show association with clinicopathological parameters

Comparison of tumor subgroups using univariate logistic regression analyses revealed that reduced mRNA expression levels of GATA1 and GATA2 are statistically related with advanced stage of disease ($p=0.005$ and 0.008), positive
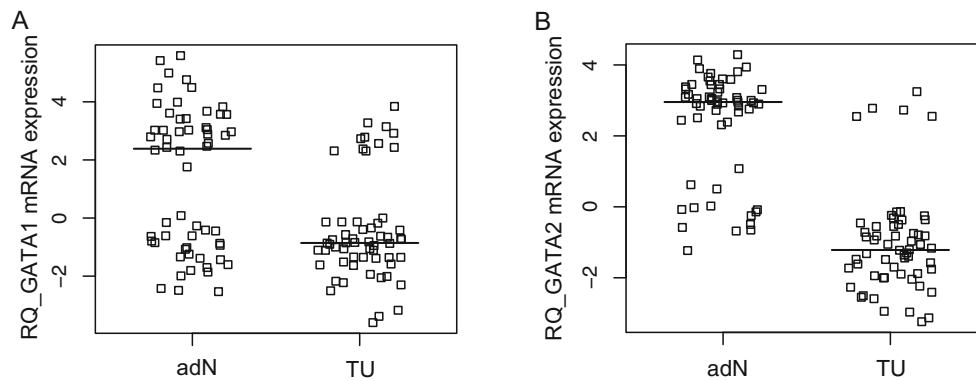
Fig. 1 GATA1 and GATA2 mRNA expression in paired clear cell renal cell carcinoma and noncancerous adjacent normal tissues. Relative GATA1 (a) and GATA2 (b) expression (RQ) values in adjacent normal (adN) compared with tumor (TU) tissues from ccRCC patients ($p<0.001$). A bimodal distribution of relative expression levels was observed both for GATA1 and GATA2 expression. Tumor tissues were characterized by a reduction of the number of tissues exhibiting high mRNA expression thus leading to significantly decreased mean expression levels in tumor tissues for both genes

status of distant metastasis ($p=0.03$ and 0.001), as well as positive lymph node metastasis ($p=0.011$ and 0.038). However, no statistical association was detected for the parameters age, tumor grade, and gender. The results are summarized in Table 2, and box plot graphics were used to illustrate relative mRNA expression differences in the analyzed subgroups (Fig. 2a, b).

GATA1 and GATA2 relative mRNA expression levels are associated with shortened RFS

The prognostic significance of GATA1 and GATA2 mRNA expression levels was analyzed using a univariate Cox proportional hazard regression model following calculation of the optimum cutoff value for relative GATA1 and GATA2 mRNA expression levels (lnRQs$=-0.85$ and -1.89). Univariate Cox regression revealed a statistical association of low mRNA expression levels both of GATA1 and GATA2 with a significantly shortened time to disease recurrence ($p=0.005$ and 0.006; hazard ratio (HR)$=0.05$ and 0.21; 95 %

confidence interval (95 % CI)$=0.01$–0.41 and 0.07–0.64; Table 3). A reduced RFS within the first 30 months was found in 11 out of 18 patients demonstrating GATA1 expression values below the cutoff and in six out of eight patients with GATA2 expression levels less than the calculated cutoff (Fig. 3a, b).

To investigate whether the association between expression status and RFS is independent from clinicopathological parameters, GATA1 and GATA2 were analyzed in additional pairwise bivariate Cox regression analyses (Table 4; see Fig. 4a, b).

GATA1 mRNA expression levels remained a significant independent prognostic factor for disease recurrence in bivariate regression models (Table 4). For GATA1 expression, fairly constant HR as well as comparable significances were observed when comparing univariate and bivariate analyses, despite consideration of the strong univariate prognosticators metastasis and tumor grade. Beside GATA1 expression levels, the status of positive metastasis ($p=0.05$, HR$=3.01$; 95 % CI$=0.99$–9.08) and high tumor grade ($p=0.008$, HR$=5.88$;

Table 2 Statistical association of GATA1 and GATA2 mRNA expression levels with clinicopathological parameters

Variable	GATA1			GATA2		
	p value[a]	OR	95 % CI	p value[a]	OR	95 % CI
Stage of disease (Adv. vs. Loc.)[b]	**0.005**	0.54	0.35–0.83	**0.008**	0.51	0.31–0.84
Status of metastasis (M+vs. M0)	**0.030**	0.63	0.42–0.96	**0.001**	0.36	0.20–0.66
Tumor grade (high vs. low)[c]	0.080	0.67	0.43–1.05	0.252	0.73	0.42–1.25
Lymph node status (N+vs. N0)	**0.011**	0.47	0.26–0.84	**0.038**	0.51	0.31–0.84

Bold p values indicating the statistictical significance

OR odds ratio, *CI* confidence interval

[a] Univariate logistic regression analysis

[b] Localized (Loc.)=pT≤3, lymph node (N) and metastasis (M) negative (N0, M0), advanced (Adv.)=pT=4 and/or lymph node positive (N+) and/or positive for distant metastasis (M+)

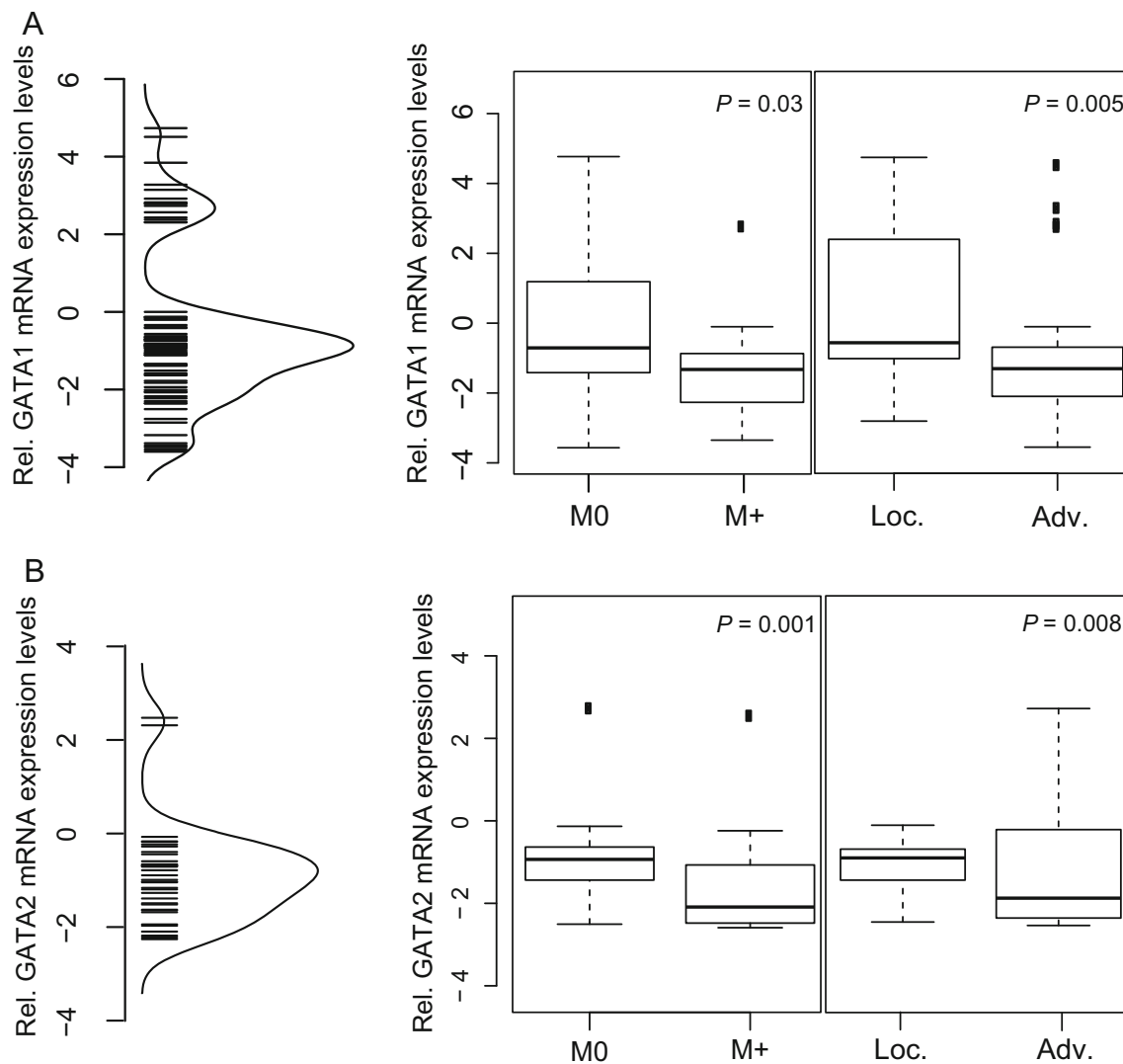[c] High vs. low tumor grade: G1 and G1–2 (low); G2–3 and G3 (high)

Fig. 2 Box plot illustration of mRNA expression levels of GATA1 and GATA2. **a** GATA1 expression levels were significantly reduced in metastasized (M+; *p*=0.03) and advanced disease (*Adv.*; *p*=0.005) of ccRCC. **b** A significantly reduced GATA2 expression level was also found in metastasized and advanced disease of ccRCC (*p*=0.001 and *p*=0.008). In both figure panels, the distribution of relative expression levels is illustrated by a Kernel distribution graph

95 % CI=1.56–22.1) were found to be independent predictors for a shortened RFS in bivariate regression models (Table 4).

Discussion

This study demonstrated that relative mRNA expression levels of GATA1 and GATA2 are significantly reduced in ccRCC. Moreover, both expression levels were found to be associated with adverse clinicopathological parameters, including advanced disease, positive status for distant, and lymph node metastasis in ccRCC patients.

GATA1 and GATA2 are members of the GATA1–6 transcription factor family and known to play a crucial role in differentiation of undifferentiated progenitor cells and proliferation and apoptosis in the hematopoietic system [6, 7].

However, the role of GATA1 and GATA2 in the tumorigenesis of other human solid malignancies has not been analyzed and associations of altered GATA1 or GATA2 expression levels with ccRCC tumorigenesis or aggressive subsets have not been reported as yet.

Therefore, taking into account the significant associations observed with advanced disease as well as lymph node and distant metastasis, our results suggest that GATA1 and GATA2 proteins may be involved in the development of aggressive ccRCCs. This hypothesis is further supported by the notion that decreased RFS associates with a reduced expression level of both markers. Interestingly, GATA1 remained an independent factor in bivariate Cox regression models even when survival models were adjusted for tumor grade, stage of disease, and status of distant metastasis, detected before as strong univariate predictors of RFS. Hence,

Table 3 Univariate statistical association of *GATA1* and *GATA2* mRNA expression levels and clinicopathological parameters with recurrence-free survival

	p value[a]	HR	95 % CI
GATA1 mRNA expression	**0.005**	0.05	0.01–0.41
GATA2 mRNA expression	**0.006**	0.21	0.07–0.64
Stage of disease (Adv. vs. Loc.)[b]	**0.030**	4.18	1.15–15.2
Status of metastasis (M+vs. M0)	**0.009**	4.27	1.43–12.8
Tumor grade (high vs. low)[c]	**<0.001**	9.48	2.92–30.8
Tumor stage (T1, T2 vs. >T2)	0.077	2.73	0.89–8.37
Lmyph node status (N+vs. N0)	0.398	1.92	0.42–8.72
Age[d]	0.155	0.42	0.13–1.38
Gender (male vs. female)	0.489	1.51	0.47–0.82

Bold p values indicating the statistical significance

Advanced (Adv.): pT=4 and/or lymph node positive (N+) and/or positive for distant metastasis (M+)

HR hazard ratio, *CI* confidence interval

[a] Univariate Cox regression analysis

[b] Localized (Loc.): pT≤3, lymph node (N) and metastasis (M) negative (N0, M0)

[c] High vs. low tumor grade: G1 and G1–2 (low); G2–3 and G3 (high)

[d] Dichotomized by the median age

our analysis identifies GATA1 mRNA expression as a candidate prognosticator for disease recurrence after curative surgery of ccRCC, therefore underlining the need of further confirmation in prospective studies with enlarged patient cohorts.

Moreover, our statistical analyses also raise the question how GATA1 may be functionally involved in the development of aggressive RCC tumors.

A GATA1 promoter polymorphism has been reported to contribute to tumor aggressiveness in breast cancer by increasing the survivin expression via promoter polymorphism [27]. The investigators found no difference between mRNA expression levels in normal and breast cancer tissues. By contrast, western blot and immunohistochemical analyses demonstrated increased signals in tumors suggesting GATA1 to contribute to the development of breast cancer. Interestingly, a reciprocal relationship between GATA1 expression and an inhibited *TP53* gene was found in erythroid cells, suggesting that an overexpression of GATA1 could lead to an inhibition of TP53 [8]. Of note, this mechanism could be bidirectional as GATA1 promoter activation can also be inhibited by TP53 [8]. This mechanistic link between TP53 and GATA1 such as the possible reciprocal relationship between TP53 and GATA1 promoter activation status in hematopoietic linage underlines the need to elucidate this interaction also in ccRCC. In RCC, a few studies demonstrated an increased protein level of mutant p53 and its association with adverse clinicopathology but not with cancer-specific survival [12]. These results were mainly obtained by IHC analyses, thus an implication to the molecular mechanism are hampered due to different stabilities of TP53 wild-type and mutant proteins. In breast cancer, a visual examination of TCGA expression data in the cancer browser did not reveal a clear difference of GATA1 expression levels. Conclusively, it is currently not clear whether common tumor-specific expression patterns, common mechanisms of GATA1/TP53 expression regulation, as well as a uniform relationship between expression alterations and clinical behavior exist in different tumor entities. Previous studies as well as our analyses highlight the requirement for detailed functional analyses of GATA1 in ccRCC.
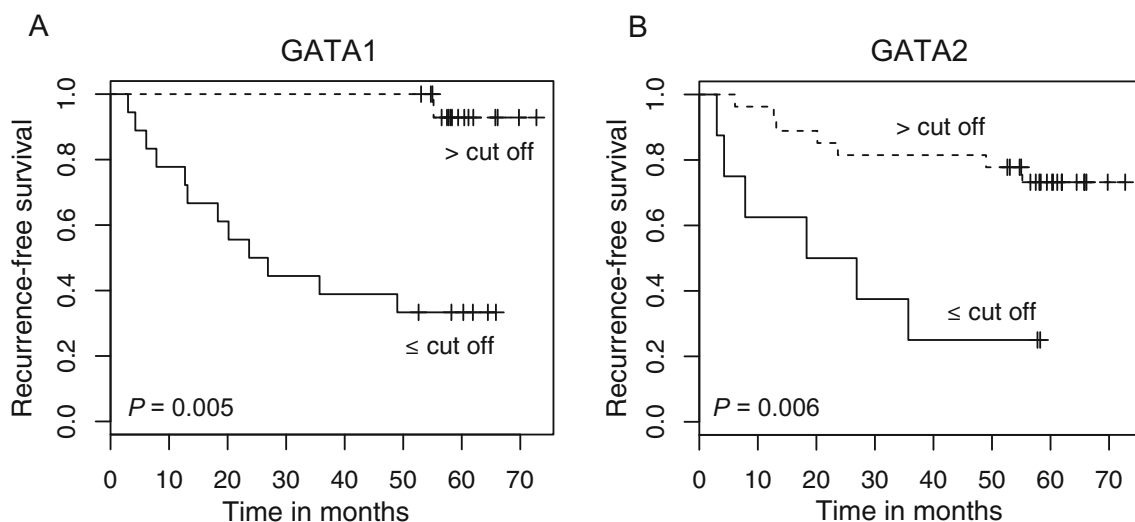

Fig. 3 Kaplan-Meier plot for illustrating recurrence-free survival. The *solid line* shows the Kaplan-Meier curve for patients with **a** GATA1 and **b** GATA2 mRNA expression lower than or equal to the cutoff of −0.85 and −1.89 (natural logarithm), indicating patients with a shortened recurrence-free survival. The *dashed lines* illustrate the Kaplan-Meier curve for patients with mRNA expression levels above the cutoff. Low GATA1 and GATA2 mRNA expression phenotypes showed higher frequency of progression events within the first 35 months

Table 4 Association of GATA1 and GATA2 mRNA expression levels and clinicopathology with recurrence-free survival in bivariate Cox regression models

	GATA1			GATA2		
	p value[a]	HR	95 % CI	p value[a]	HR	95 % CI
mRNA expression	0.009	0.06	0.01–0.51	0.043	0.30	0.09–0.96
Stage of disease (Adv. vs. Loc.)	0.135	2.71	0.74–9.99	0.110	3.02	0.78–11.7
mRNA expression	0.008	0.06	0.01–0.49	0.135	0.35	0.08–1.39
Status of metastasis (M+ vs. M0)	0.050	3.01	0.99–9.08	0.211	2.39	0.61–9.40
mRNA expression	0.017	0.08	0.01–0.63	0.268	0.49	0.14–1.72
Tumor grade (high vs. low)[b]	0.008	5.88	1.56–22.1	0.003	7.18	1.92–26.7
mRNA expression	0.002	0.09	0.02–0.91	0.013	0.24	0.08–0.75
Tumor stage (T1, T2 vs. >T2)	0.152	2.28	0.74–7.03	0.163	2.25	0.72–7.00
mRNA expression	0.005	0.05	0.01–0.42	0.008	0.22	0.07–0.68
Lymph node status (N+ vs. N0)	0.657	1.39	0.29–6.45	0.700	1.35	0.28–6.36
mRNA expression	0.006	0.06	0.11–0.45	0.002	0.25	0.07–0.83
Age[c]	0.416	0.61	0.19–2.01	0.513	0.65	0.18–2.34
mRNA expression	0.003	0.05	0.01–0.38	0.005	0.20	0.06–0.61
Gender (male vs. female)	0.250	2.01	0.61–6.56	0.350	1.76	0.54–5.76

Adv. advanced tumors with pT=4 and/or lymph node positive (N+) and/or positive for distant metastasis (M+), *Loc.* localized and locally advanced tumors with pT ≤3. Lymph node (N) and metastasis (M) negative (N0, M0)

[a] Bivariate cox regression analysis hazard ratio (HR)

[c] High grade (G2–3 and G3). Low grade (G1 and G1–2)
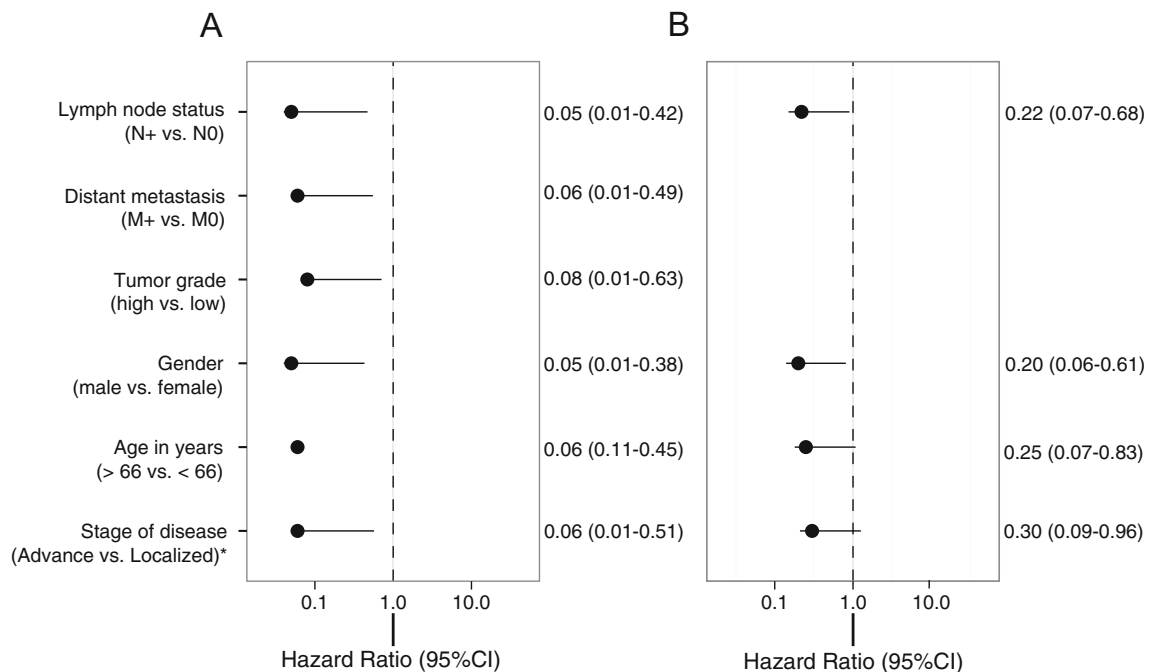
[d] Dichotomized by the median

Fig. 4 Forest plot of hazard ratios (*HR*) and 95 % confidence interval (95 % CI) for reduced GATA1 (**a**) and GATA2 (**b**) mRNA expression levels and survival in association to the following subgroups: lymph node status, status of distant metastasis, tumor grade, gender, age, and stage of disease. The *vertical line* demonstrates no effect. The *black circles* demonstrate the HR with the corresponding 95 % CI indicated as *black lines to the right and to the left*. A worsen recurrence-free survival can be seen for both reduced GATA1 and GATA2 mRNA expression in association with adverse clinicopathological parameters

Intriguingly, the detection of GATA2 alterations has also been associated with prognosis in various solid tumors and increased expression levels likewise were found to promote the proliferation of breast cancer cells by stimulation of AKT phosphorylation due to phosphatase and tensin homolog inhibition [15]. Moreover, changed expression levels also occur in prostate and colorectal cancers as well as neuroblastoma, showing association with adverse clinicopathology, disease recurrence, and in case of neuroblastoma with a low-grade histopathological subtype [13, 14, 17]. Hence, GATA2 seems to be of potential clinical relevance for important solid tumors. As our data revealed that ccRCC patients with reduced GATA2 expression levels demonstrate significantly shortened periods of RFS as well as an association with adverse clinicopathology, GATA2 alterations consequently could also contribute to ccRCC tumorigenesis of a subset of tumors.

We also compared mRNA expression levels of GATA1, GATA2, and GATA5 and obtained a moderate correlation between GATA1 and GATA2 mRNA expression levels ($R=0.38$, $p<0.001$, data not shown) but found no correlation for GATA1 or GATA2 and GATA5 ($R=-0.05$ and -0.05, data not shown). Thus, from a statistical point of view, a coregulation of expression is not supported by our data, which could be in line with findings functionally differentiating the GATA1, GATA2, and GATA3 transcription factors from the GATA4, GATA5, and GATA6 proteins.

This study is limited due to the retrospective, nonrandomized design and the comparatively low number of follow-up cases after nephrectomy.

Thus, T4 stage was not represented in the advanced tumor group and univariate analysis of stage revealed no significance, thus limit the results of our corresponding bivariate survival analysis. Moreover, establishing GATA1 as a prognostic factor would require analysis of an independent validation patient cohort, optimally recruited in a multicenter study design.

On the other hand, our findings suggest a contribution of GATA1 and GATA2 proteins to the progression of ccRCC toward more aggressive molecular subtypes of tumors. Considering the low number of frequently mutated genes found in ccRCC and the corresponding lack of mutational information regarding progression of tumor, our results emphasize the relevance of future functional studies characterizing the role of GATA1 and GATA2 in the cellular signaling of ccRCC, possibly providing both new insights in tumor biology of aggressive tumors as well as new targets for molecular intervention.

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Author contributions

IP wrote the manuscript, prepared the figures, and participated in the study design. ND carried out the mRNA expression analyses and participated in the manuscript preparation. JH and AS assembled histopathological, clinicopathological, and survival data. JH performed isolation and characterization of tissue samples and assembly of patients. MWK, CvK, HT, and MAK assisted with general scientific discussion. JS identified the candidate genes, conceived of the study, developed the study design and analytical assays, constructed and ran the clinical database, performed statistical analyses together with RS, and participated in manuscript and figure preparation. All authors read and approved the final manuscript.