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ORIGINAL ARTICLE

Favorable prognostic influence of T-box transcription factor Eomesodermin in metastatic renal cell cancer patients

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Abstract T-box transcription factors, T-box expressed in T cells (T-bet) encoded by *Tbx21* and Eomesodermin (Eomes), drive the differentiation of effector/memory T cell lineages and NK cells. The aim of the study was to determine the prognostic influence of the expression of these transcription factors in peripheral blood (pB) in a cohort of 41 metastatic (m) RCC patients before receiving sorafenib treatment and to analyze their association with the immunophenotype in pB. In contrast to *Tbx21*, in the multivariate analysis including clinical features, *Eomes* mRNA expression was identified as an independent good prognostic factor for progression-free survival (PFS, $p = 0.042$) and overall survival (OS, $p = 0.001$) in addition to a favorable ECOG performance status ($p = 0.01$ and $p = 0.008$, respectively). *Eomes* expression correlated positively not only with expression of T*bx21* and *TGFβ1* mRNA, but also with mRNA expression of the activation marker *ICOS*, and with in vivo activated HLA-DR$^+$ T cells. *Eomes* expression was negatively associated with TNFα-producing T cells. On protein level, Eomes was mainly expressed by CD56$^+$CD3$^-$ NK cells in pB. In conclusion, we identified a higher *Eomes* mRNA expression as an independent good prognostic factor for OS and PFS in mRCC patients treated with sorafenib.

Keywords Renal cell carcinoma · Memory T cells · Effector T cells · T-box expressed in T cells · Eomesodermin · Natural killer cells

Abbreviations

AP	Alkaline phosphatase
Ca	Corrected serum calcium concentrations
CAF	Cytokine and angiogenic factor
CCR	C–C chemokine receptor
CD	Cluster of differentiation
CT	X-ray computed tomography
CTL	Cytotoxic T cells
ECOG	Eastern Cooperative Oncology Group
Eomes	Eomesodermin
Hb	Hemoglobin
HLA-DR	Human leukocyte antigen class II
HMBS	Hydroxymethylbilane synthase
ICOS	Inducible T cell co-stimulator
IFN	Interferon
IL	Interleukin
mAb	Monoclonal antibody
MDSC	Myeloid-derived suppressor cells
MFI	Mean fluorescence intensity
mRCC	Metastatic renal cell cancer
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MSKCC	Memorial Sloan-Kettering Cancer Center score
mTOR	Mechanistic target of rapamycin, previously known as mammalian target of rapamycin
NK cells	Natural killer cells
NKT cells	Natural killer T cells

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OS	Overall survival
pB	Peripheral blood
PBGD	Porphobilinogen deaminase
PBMCs	Peripheral blood mononuclear cells
PFS	Progression-free survival
PMA	Phorbol 12-myristate 13-acetate
RECIST	Response Evaluation Criteria in Solid Tumors
RT-PCR	Reverse transcriptase polymerase chain reaction
T-bet	T-box expressed in T cells
T_{CM}	Central memory T cells
T_E	Effector T cells
T_{EM}	Effector memory T cells
TGF	Transforming growth factor
Th1	Type 1 T helper cells
TKI	Tyrosine kinase inhibitor
TNF	Tumor necrosis factor
Treg	Regulatory T cells
VEGF	Vascular endothelial growth factor

Introduction

Metastatic renal cell cancer (mRCC) is associated with an immunosuppressive phenotype characterized by increased frequencies of regulatory T cells (Treg), type 2 cells, and inflammatory cells like myeloid-derived suppressor cells (MDSC) and neutrophils. However, not only increased frequencies of immunosuppressive cells but also alterations in the frequency and the function of effector cells of both the adaptive and the innate immunity may play a role in tumor-induced immune dysbalance [1].

The T-box transcription factors T-box expressed in T cells (T-bet) and Eomesodermin (Eomes) have been well defined as key drivers not only of immune cell development but also of cytolytic function. They are highly expressed in type 1 immune cells such as Th1, Tc1, NK, NKT, and γδ T cells. Their expression increases as peripheral cells become more differentiated, with the exception for NK cells. The more differentiated $CD56^{dim}$ cells contain less Eomes than their predecessor $CD56^{bright}$ cells [2]. The balance of expression of the transcription factors Eomes and T-bet has been reported to influence whether $CD8^+$ T lymphocytes commit to memory or effector cells [3]. High T-bet expression seemed to favor effector cells [2]. T-bet levels declined as cells became more memory-like, whereas Eomes expression increased [2, 4]. The generation of a potent T cell memory response is important for mediating protective long-lasting immunity against tumors [5–7].

T-bet and Eomes drive Tc1 differentiation by preventing alternative $CD8^+$ T cell differentiation to Tc17 or Tc2 cell [8]. T-bet has been reported to be critical for the effector function of Th1 and NK cells, but to be only partially required for $CD8^+$ Tc1 cells. In contrast, Eomes, which is only weakly expressed in $CD4^+$ T cells, has been proposed to play a key role in the effector function of $CD8^+$ T cells [2, 9]. However, T-bet and Eomes act synergistically for eliminating cancer cells, but both the factors were not critical for the generation of systemic CTL activities against cancer cells. Instead, they were crucial for tumor infiltration by $CD8^+$ T cells by controlling migration of antitumor T cells to the tumor site through regulating chemokine receptors [2].

In NK cells, T-bet and Eomes fulfill complementary roles in lineage decision. Murine studies and co-expression analysis in humans support a sequential maturation model as Eomes is necessary for the generation and maintenance of mature NK cells, whereas T-bet is necessary to attain the most terminal stages of maturation [2, 5, 6].

Also in the era of targeted therapies, there are several hints that immune dysbalance has a crucial influence on RCC course and that it might play a prognostic role. Not only markers of a systemic inflammatory response, such as neutrophils and C-reactive protein [7, 10, 11], but also immunosuppressive cells, as Treg, and soluble molecules, such as IL6, IL8, and VEGF [12–14], have been reported to have a negative prognostic impact on survival in RCC patients treated with targeted therapies. In contrast, we have previously shown that high *TGFβ1* mRNA expression levels in pB were associated with improved survival in mRCC patients treated with sorafenib [15].

However, little is known about the prognostic influence of factors regulating the effector function of the antitumor immune response in mRCC patients treated with targeted therapies. The aim of the study was to determine the prognostic influence of the expression of the T-box transcription factors T-bet, encoded by *Tbx21,* and Eomes in peripheral blood (pB) in the previously analyzed cohort of 41 mRCC patients before receiving sorafenib treatment on mRNA and protein level. Several reports about their expression in tumor tissue underline that they are important regulators for antitumor immune response and impact on prognosis [16–19].

Here, we identified *Eomes* mRNA expression as an independent good prognostic factor for progression-free survival (PFS) and overall survival (OS) likely due to its association with a favorable immune signature.

Materials and methods

Patients and blood samples

This study was carried out on 41 patients with histologically proven metastatic or unresectable RCC before receiving oral treatment with the multikinase inhibitor sorafenib as described in Busse et al. [15]. All patients had measurable

Table 1 Primer and probe sequences

PCR product	Primer and probe sequences
HBMS[a]	Fw 5′-TGCAGGCTACCATCCATGTCCCTGC-3′
187 bp	Rev 5′-AGCTGCCGTGCAACATCCAGGATGT-3′
Probes	5′-CGTGGAATGTTACGAGCAGTGATGCCTACC-Fluorescein-3′
	5′-LCRed640-TGTGGGTCATCCTCAGGGCCATCTTC-phosphate-3′
ICOS[a]	Fw 5′-ACAGGAGGATATTTGCATATTTATGA-3′
120 bp	Rev 5′-CCAACAAATAAGTATGCATCCC-3′
Probe	5′-FAM-TGCAGCCTTTGTTGTAGTCTGC-Tamra-3′
Eomes[b]	Fw 5′-ACTGGTTCCCACTGGATGAG-3′
160 bp	Rev 5′-CCACGCCATCCTCTGTAACT-3′
Probe	5′-FAM-AGGCGCAAATAACAACAACACCCAG-Tamra-3′
Tbx21[b]	Fw 5′-GGGAAACTAAAGCTCACAAAC-3′
337 bp	Rev 5′-CCCCAAGGAATTGACAGTTG-3′
Probe	5′-FAM-TGTGACCCAGATGATTGTGCTCCA-Tamra-3′
TGFβ1[a]	Fw 5′-CCCACAACGAAATCTATGAC-3′
314 bp	Rev 5′-GCTAAGGCGAAAGCCC-3′
Probes	5′-GGCACCCAGCGACTCG-Fluorescein-3′
	5′-LCRed640-AGAGTGGTTATCTTTTGATGTCACCG-phosphate-3′
IL10[a]	Fw 5′-CCTTCCAGTGTCTCGG-3′
357 bp	Rev 5′-TGGAGTACAGGGGCAT-3′
Probes	5′-AGGCGGGTGGATCACT-Fluorescein-3′
	5′-LCRed640-AGGTCAGGAGTTCCTAACCAG-phosphate-3′

[a] Primer and Probes were created using LightCycler Probe Design 2.0 Software (Roche)

[b] Primers according to [50], probes were created by using the online tool: eu.idtdna.com/PrimerQuest/Home/Index

disease and were included in European expanded access program. Treatment response was evaluated at baseline and every eight weeks thereafter or, as clinically indicated, by CT scans or MRI of the chest, abdomen, and brain following RECIST criteria [20]. Disease impact on patients daily living abilities was measured according to the ECOG Scale of Performance Status: 0—fully active, able to carry on all pre-disease performance without restriction; 1—restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work; 2—ambulatory and capable of all self-care but unable to carry out any work activities, up and about more than 50 % of waking hours [21]. To categorize the RCC patients into risk groups for predicting survival, the MSKCC for pretreated patients was used [22].

Approval by our institutional review board for investigation of prognostic and immunologic factors has been obtained, and all patients provided written informed consent before enrollment.

mRNA extraction, reverse transcription, and quantitative real-time PCR

After separation of human blood mononuclear cells by Ficoll-Isopaque density gradient centrifugation (Pharmacia, Erlangen, Germany), total RNA was extracted from mononuclear cells using the RNeasy Mini Kit including RNase-Free DNase Set (Qiagen, Hilden, Germany) according to the manufacturers' recommendations. RNA was converted to cDNA using the Omniscript Reverse Transcriptase Kit (Qiagen, Hilden, Germany). Quantitative real-time reverse transcriptase (RT)-PCR was performed by LightCycler Technology (Roche), and data were analyzed with the LightCycler software (version 3, Roche). All samples were run in duplicate, and the average value of both duplicates was used for quantification of gene expression.

Primer and probe sequences of *Tbx21, Eomes*, *transforming growth factor beta 1 (TGFβ1)*, *interleukin 10 (IL10)*, *inducible T cell co-stimulator (ICOS)*, and the housekeeping gene of *hydroxymethylbilane synthase (HMBS)*, also known as *porphobilinogen deaminase (PBGD)*, are listed in Table 1.

For the generation of standard curves, PCR products generated from cDNAs of all markers analyzed were cloned into the vector pCR2.1-TOPO (Invitrogen, Karlsruhe, Germany). A standard curve with three plasmid dilutions of duplicates was included in each respective PCR run. The quantification of serial plasmid dilutions yielded linear crossing point increases over a range of 6 logs (up to 10^{-5} pg/µl) for all markers analyzed.

Table 2 Patients' characteristics

	N
Patient samples available	41
Male/female	26/15
Median age	65 (37–78)
Prior nephrectomy yes/no	39/2
Cytokine pretreatment yes/no	35/6
ECOG 0/1/2	14/19/8
MSKCC favorable/intermediate/poor	6/20/15
Hb normal/decreased	9/32
Corrected serum Ca normal/elevated	29/12
AP normal/elevated	34/7
Median PFS (95 % CI)	9.3 (5.2–13.3) months
Median OS (95 % CI)	17 (13.3–20.7) months
Median follow-up for progression-free patients	13.3 (2.3–27.8) months

Flow cytometric analysis

Cells were stained for surface antigens with fluorescence-conjugated monoclonal antibodies (mAbs) against CD3, CD4, CD45RA, CCR7, CD56, CD11b, CD14, HLA-DR (Biolegend, Fell, Germany), and CD8 (Becton–Dickinson, Heidelberg, Germany). Expression of transcription factors was measured by intracellular staining with mAbs against T-bet and Eomes (Biolegend, Fell, Germany, and eBioscience, Frankfurt a.M., Germany, respectively) following cell permeabilization by 0.1 % saponin. Samples were acquired on a FACSCantoII flow cytometer (Becton–Dickinson, Heidelberg, Germany), and data were analyzed by FlowJo software version 7.6.5 (TreeStar, Ashland, USA).

Statistical considerations

Nonparametric tests were used to evaluate the mRNA expression levels of *Eomes* and *Tbx21*, as well as protein expression of Eomes. The Mann–Whitney *U* test was used for comparing two groups, and the Kruskal–Wallis test was used for comparing more than two groups. The Wilcoxon rank-sum test was used to compare samples before treatment and after 8 weeks of treatment. Correlations were evaluated using the Spearman correlation test.

All tests were two-sided, and statistical significance was assumed if the null hypothesis could be rejected at the $p < 0.05$ level. Progression-free survival (PFS) was defined as from treatment start to the date of progression, death, or cutoff date for analysis. Overall survival (OS) was defined as from treatment start to death or cutoff date for analysis. Univariate Cox regression analysis was performed to examine the potential relationships between pre-treatment factors and survival. Multivariate analysis using a stepwise forward Cox regression technique was performed to investigate potential interactions between the entered covariates. Survival curves were estimated using the Kaplan–Meier method, and they were compared with the log-rank test. All statistical analyses were carried out using SPSS software (release 20.0).

Results

Patients

Patients' characteristics are listed in Table 2. Forty-one patients with mRCC received sorafenib for a median of 9 months (0.4–19.8 months). The overall response rate was 80.5 % with 9 partial responses and 24 stable diseases. The median follow-up for progression-free patients was 13.3 months (2.3–27.8 months).

mRNA expression of *Tbx21* and *Eomes* in peripheral blood

Transcripts of the housekeeping gene *HMBS* could be detected in all samples with a median transcript level of 7.87×10^{-2} pg/µl (range 2.03×10^{-2}–1.86×10^{-1} pg/µl). Therefore, all samples were regarded as informative.

Tbx21 and *Eomes* mRNA expression was detectable in pB of all 41 mRCC patients with a level up to 1.98×10^{-4} and 1.71×10^{-3}, respectively. Values were within the detection limit (up to 10^{-5} pg/µl) for both markers.

The median ratio of *Tbx21/HMBS* was 4.56×10^{-1} (range 1.71×10^{-3}–1.85) and of *Eomes/HMBS* was 2.2×10^{-1} (range 8.67×10^{-3}–1.12), respectively (Fig. 1).

Prior cytokine therapy as well as response to cytokine therapy had no influence on mRNA expression levels of *Tbx21* ($p = 0.928$ and $p = 0.757$, respectively) and *Eomes* ($p = 0.733$ and $p = 0.935$, respectively). There were no significant differences in mRNA expression levels of *Tbx21* ($p = 0.695$) and *Eomes* ($p = 0.867$) after 8-week treatment with sorafenib ($n = 28$, data not shown).

Correlation of *Tbx21* and *Eomes* mRNA levels with the immunophenotype in pB

We investigated whether *Tbx21* and *Eomes* mRNA expression levels were associated with a specific immunophenotype in pB. We analyzed mRNA expression levels of *ICOS* by RT-PCR and frequencies of HLA-DR$^+$ CD4$^+$ and CD8$^+$ T cells by flow cytometry to identify in vivo activated T cells. Moreover, we determined frequencies of effector/memory T cell subpopulations by flow cytometry by expression of CCR7 and CD45RA. *Eomes* and *Tbx21* mRNA expression showed a positive correlation

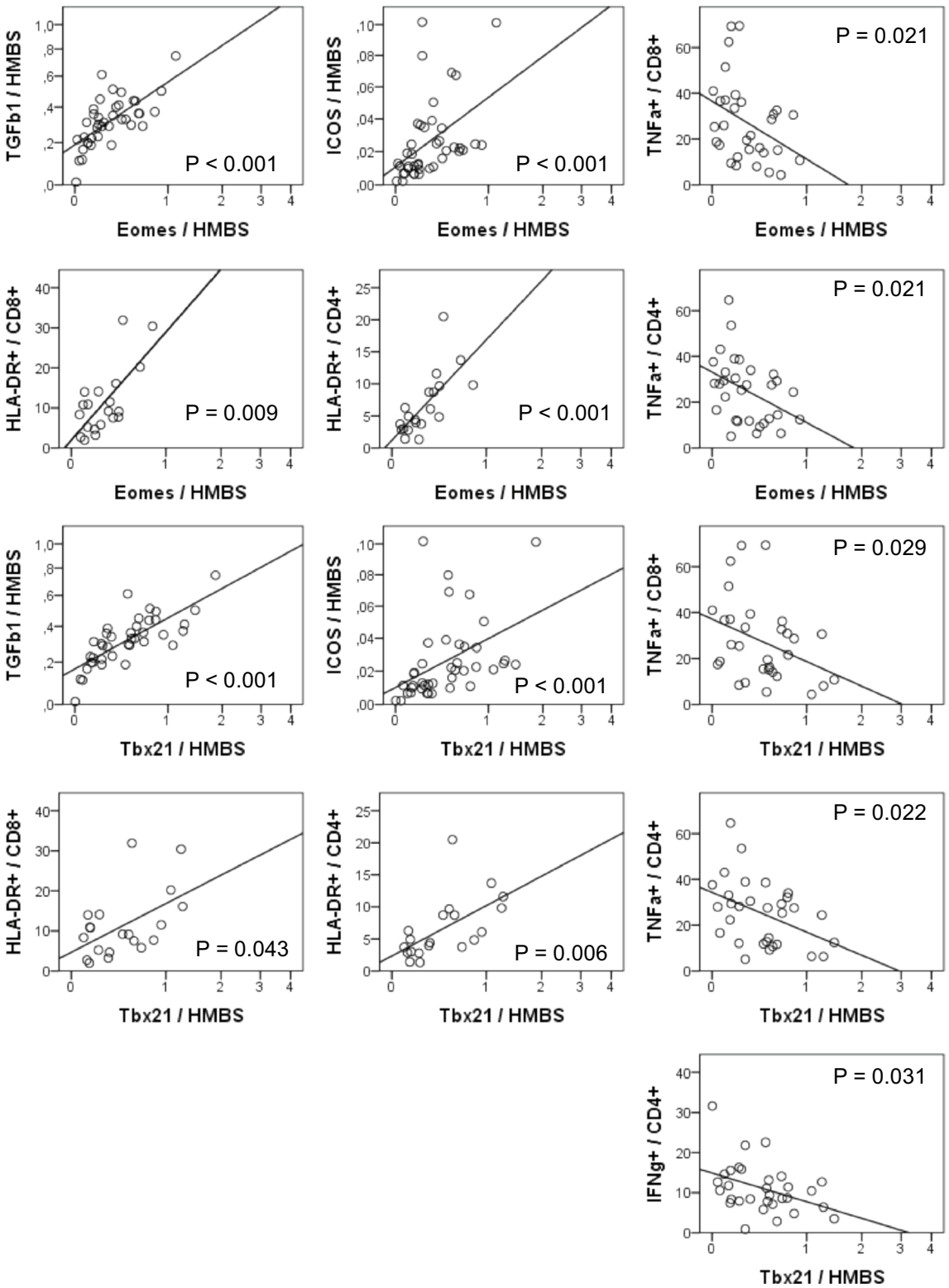

Fig. 1 Correlation of mRNA expression of *Eomes* and *Tbx21* with mRNA expression *of TGFβ1* and *ICOS* and with HLA-DR$^+$ T cells and cytokine-producing T cells. mRNA expression levels of *Tbx21*, *Eomes*, *TGFβ1*, and *ICOS* in PBMCs were determined by quantitative RT-PCR. The relative amount was expressed as ratio marker (pg/µl)/HMBS (pg/µl). The sample concentration was calculated using a plasmid standard curve. mRNA levels of all markers were within the respective detection range in all patient samples. HLA-DR surface expression on CD8$^+$ and CD4$^+$ T cells was determined by flow cytometry. IFNγ- and TNFα- producing T cells were measured by intracellular flow cytometry after in vitro stimulation of PBMCs by PMA/ionomycin

with mRNA expression of the activation marker *ICOS* ($p < 0.001$, $n = 41$), as well as with frequencies of HLA-DR$^+$ CD4$^+$ ($p < 0.001$ and $p = 0.006$, respectively, $n = 20$) and CD8$^+$ T cells ($p = 0.009$ and $p = 0.043$, respectively, $n = 20$), representing in vivo activated T cells (Fig. 1). mRNA expression levels of *Eomes* or *Tbx21* or the ratio of *Tbx21/Eomes* mRNA levels was not associated with frequencies of effector or memory CD4$^+$ or CD8$^+$ T cells.

In a previous study, we determined mRNA expression levels of immunosuppressive cytokines *IL10* and *TGFβ1* and frequencies of TNFα- and IFNɣ-producing CD4$^+$ and CD8$^+$ T cells after PMA/ionomycin treatment in vitro from the patients enrolled in this research study [23]. We included these data in our current analysis. We found that *Eomes* and *Tbx21* mRNA expression showed a positive correlation with mRNA expression of *TGFβ1* ($p < 0.001$, $n = 41$, Fig. 1). In contrast, *Eomes* and *Tbx21* mRNA expression levels were negatively correlated with TNFα-producing CD8$^+$ ($p = 0.021$ and $p = 0.029$) and CD4$^+$ T cells ($p = 0.021$ and $p = 0.022$, $n = 31$) after in vitro activation by PMA/ionomycin. Moreover, *Tbx21* mRNA expression was negatively correlated with INFɣ-producing CD4$^+$ cells ($p = 0.031$, Fig. 1).

To conclude, *Eomes* and *Tbx21* mRNA expression in pB correlated positively not only with markers of activated T cells producing less TNFα but also with mRNA expression of the immunosuppressive cytokine *TGFβ1*.

Eomes mRNA and ECOG were independently associated with PFS and OS

Data of 41 patients were available to analyze the influence of clinical features and mRNA expression levels of *Eomes* and *Tbx21* on survival. In 23 patients, immune cell subpopulations were measured by flow cytometry, and their influence on survival was analyzed. Clinical features considered for univariate analysis were selected on the basis of previously identified factors that impact on survival of mRCC patients [22, 24] and included Eastern Cooperative Oncology Group (ECOG) performance status score, hemoglobin (Hb), alkaline phosphatase (AP), and corrected serum calcium (Ca) concentrations. Decreased Hb, elevated Ca, AP serum concentrations, and a high ECOG performance status score had a negative prognostic influence on PFS and OS. However, this was only significant for ECOG performance status ($p < 0.001$; Table 3; Fig. 2). Prior cytokine therapy as well as response to cytokine therapy had no influence on PFS and OS.

Univariate Cox regression analysis (Table 3) revealed a positive prognostic influence of *Tbx21* and *Eomes* mRNA expression on PFS ($p = 0.012$ and $p = 0.008$, respectively) and OS ($p = 0.057$ and $p = 0.007$, respectively). This is reflected in the survival curves (Fig. 2, Kaplan–Meier, $n = 41$).

Table 3 Univariate and multivariate Cox regression analysis

	Univariate, *p* value		Multivariate, *p* value	
	PFS	PFS	OS	PFS
Hb (decreased vs. not decreased)	0.308	0.241	n.i.	n.i.
AP (elevated vs. not elevated)	0.379	0.247	n.i.	n.i.
Ca (elevated vs. not elevated)	0.935	0.764	n.i.	n.i.
ECOG (0 vs. 1 vs. 2)	**0.004**	**<0.001**	**0.011**	**<0.001**
Tbx21 mRNA level	**0.012**	**0.057**	0.628	0.16
Eomes mRNA level	**0.008**	**0.007**	**0.016**	**0.009**

Statistically significant *p*-values are printed in bold

n.i. not included

In a multivariate Cox proportional hazards model, containing those clinical features with *p* value ≤0.2 upon univariate analysis and mRNA levels of *Eomes* and *Tbx21* (Table 3), a low ECOG and higher *Eomes* mRNA expression were independently associated with favorable PFS ($p = 0.011$ and $p = 0.016$, respectively) and favorable OS ($p < 0.001$ and $p = 0.009$, respectively). This was also observed when patients without prior cytokine therapy were excluded (PFS $p = 0.015$ and $p = 0.002$, respectively, and OS $p = 0.001$ and $p = 0.011$, respectively).

Eomes was particularly expressed by NK cells in peripheral blood

We asked whether there was a specific Eomes-expressing immune cell population with favorable impact on survival. Protein expression of Eomes was analyzed by flow cytometry in pB samples of 25 patients. As Eomes$^+$ cells/total lymphocyte population had no influence on OS and PFS, we looked in more detail at lymphocyte subpopulations, especially at CD8$^+$CD3$^+$ T cells and CD56$^+$CD3$^-$ NK cells (Fig. 3a, b), because their differentiation and effector function are regulated by Eomes [2, 8, 9].

Eomes was expressed particularly in the CD56$^+$CD3$^-$ NK cells with a median frequency of 51.2 % (range 0.12–81.1 %). In the CD8$^+$CD3$^+$ T cell population, the median frequency of Eomes$^+$ cells was 16.4 % (1.15–56.3 %) and was significantly lower compared to NK cells ($p < 0.001$, Fig. 3b). In contrast, compared to CD8$^+$CD3$^+$ T cells, the median frequency of Eomes$^+$ cells in the CD4$^+$CD3$^+$ lymphocyte population (Fig. 3b) was only 2.7 % (0–39.7 %, $p < 0.001$). This is in line with previous reports [2].

As Eomes is known to regulate the balance between effector and memory T cells [3], we explored its expression in effector/memory populations of CD8$^+$ T cells. Frequencies of Eomes$^+$ cells differed in memory and effector T cells ($p = 0.043$). They were higher in CD45RA$^+$CCR7$^-$ effector T cells (T_E) with a median frequency of 29 % (range

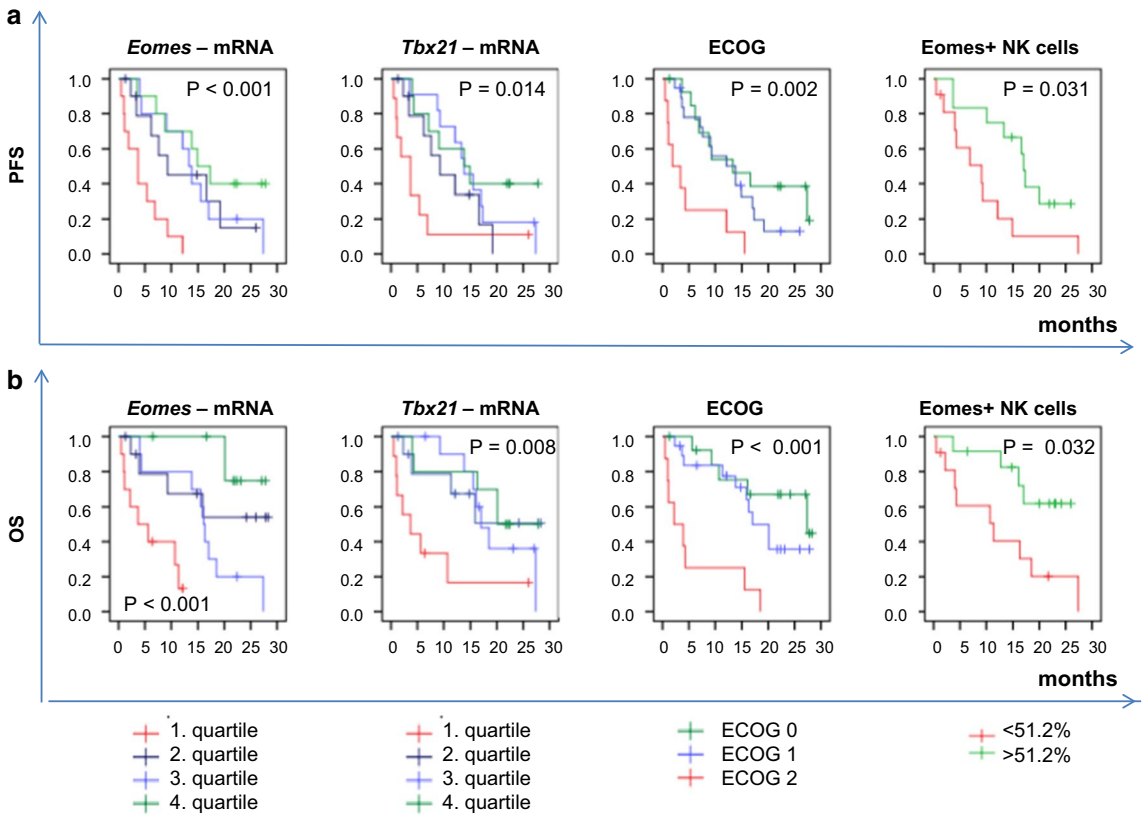

Fig. 2 Kaplan–Meier curves for *Tbx21* mRNA, *Eomes* mRNA, ECOG, and Eomes$^+$/CD56$^+$CD3$^-$ NK cells. mRNA was derived from PBMCs. mRNA expression levels were divided into quartiles with low (1. quartile), intermediate low (2. quartile), intermediate high (3. quartile), and high (4. quartile) mRNA expression. Eomes$^+$/CD56$^+$CD3$^-$ NK cells were determined by intracellular flow cytometry and divided into two groups based on the median frequency of Eomes$^+$ cells (51.2 %) in the CD56$^+$CD3$^-$ population. Survival curves were compared with the log-rank test. **a** *PFS* progression-free survival, b *OS* overall survival

6–72.8 %) and CD45RA$^-$CCR7$^-$ effector memory T cells (T_{EM}) with a median frequency of 17.5 % (range 3–47.5 %) compared to CD45RA$^-$CCR7$^+$ central memory T cells (T_{CM}) with a median frequency of 4.7 % (range 0–30.4 %) and CD45RA$^+$CCR7$^+$ naïve T (T_{naive}) cells with a median frequency of 4.1 % (range 0–30.6 %, Fig. 3c). To determine Eomes protein expression levels, we analyzed median fluorescence intensities (MFI) of Eomes (Fig. 3d). T_E and T_{EM} had higher MFI of Eomes [MFI 84 (range 0–158) and 122 (range 0–210)], respectively, compared to T_{CM} and T_{naive} cells [MFI 28 (range 0–76) and 27 (range 0–86), respectively, $p < 0.001$]. However, Eomes MFI was highest in CD56$^+$CD3$^-$ NK cells (MFI 225 [range 0–387]) compared to CD8$^+$ T cell effector/memory subpopulations ($p < 0.001$).

Univariate Cox regression analyses revealed that neither frequencies of CD56$^+$CD3$^-$ NK cells nor frequencies of effector/memory CD8$^+$ T cells had an influence on PFS or OS ($n = 23$, data not shown). However, a high frequency of Eomes-expressing cells in the CD56$^+$CD3$^-$ cell population was significantly associated with increased PFS ($p = 0.035$) and OS ($p = 0.028$). This is also reflected in the Kaplan–Meier curves (Fig. 2). Due to the low number of patient samples ($n = 23$), frequencies of Eomes$^+$CD56$^+$CD3$^-$ cells were not included in the multivariate Cox model.

Discussion

Here, we demonstrated that mRNA expression levels of T-box transcription factors *Eomes* and *Tbx21* in pB impact on survival in sorafenib-treated mRCC patients, with *Eomes* mRNA expression being an independent prognostic factor for OS and PFS.

Treatment options for patients with RCC have significantly improved in recent years. Nonetheless, mRCC treated with the new targeted therapies will progress in the majority of patient's overtime with variable course. Therefore, there is a need to determine patient's risk of cancer progression in order to stratify for treatment. Heterogeneity of clinical and laboratory features is acknowledged to be a dominant factor that impact on survival of RCC

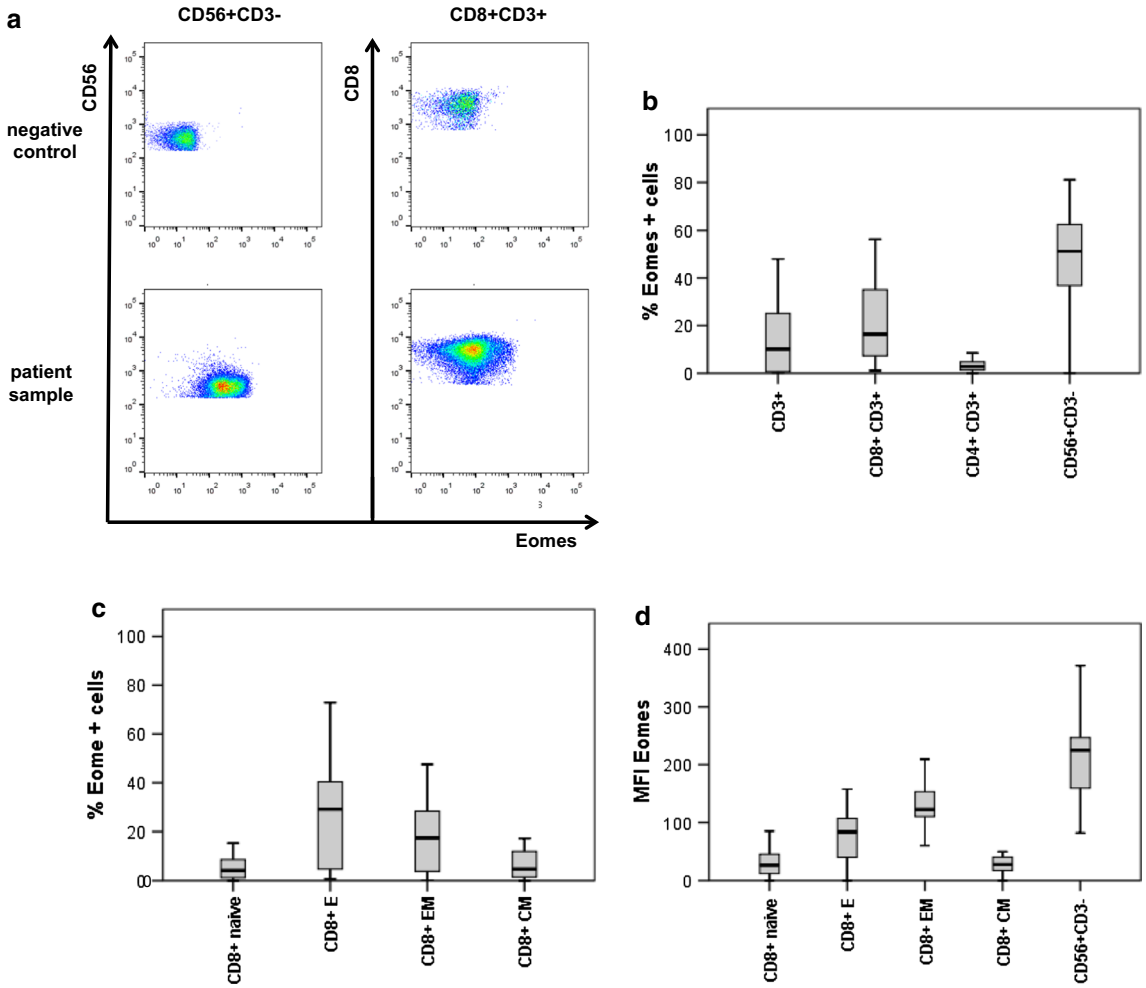

Fig. 3 Expression of Eomes in peripheral lymphocyte subpopulations of mRCC patients. Eomes expression was determined by intracellular flow cytometry staining in lymphocyte subpopulations as indicated. The *box/whisker graphs* display 25–75 % (*box*) and 10–90 % (*whisker*). The *line in the box* represents the median value. **a** Exemplary flow cytometry plots from one patient showing Eomes expression in $CD56^+CD3^-$ NK cells and $CD8^+CD3^+$ T cells. **b** Frequency of Eomes-expressing cells in $CD3^+$ T cells, $CD8^+CD3^+$ T cells, $CD4^+CD3^+$ T cells, and $CD56^+CD3^-$ NK cells. **c** Frequency of Eomes-expressing $CD8^+$ T cells within $CD45RA^+CCR7^+$ T_{naive} cells, $CD45RA^+CCR7^-$ T_E, $CD45RA^-CCR7^-$ T_{EM}, and $CD45RA^-CCR7^+$ T_{CM}. **d** Eomes median fluorescence intensity (MFI) values in $CD8^+$ memory/effector subpopulations and in $CD56^+CD3^-$ NK cells

patients [22, 25]. In addition to tumor tissue biomarkers, various soluble molecules and cellular populations in pB have been identified as potential biomarkers [13, 26, 27]. Here, we could show that higher *Eomes* mRNA expression levels were an independent good prognostic factor for PFS and OS, in addition to a favorable ECOG. Several reports about their expression in tumor tissue underline that they are important regulators for antitumor immune response [8, 16–19]. *Eomes* mRNA expression positively correlated with mRNA expression of *Tbx21*. However, *Tbx21* mRNA was not an independent prognostic factor in the multivariate analysis. The T-box transcription factors Eomes and T-bet, encoded by *Tbx21*, are master regulators of effector cells in adaptive and innate immunity. They were found not only to be important to regulate the balance between memory and effector T cells but also to maintain effector function in long-term memory $CD8^+$ T cells [2, 4]. However, we found neither influence of cytokine-producing Tc1 cells or Th1 nor influence of memory T cells that have been reported to be crucial for long-term antitumor immune response.

Therefore, we asked whether the favorable prognostic role of *Eomes* mRNA expression on survival might be related to a specific Eomes-expressing cell population. Thus, we analyzed Eomes expression on protein level in the whole lymphocyte population and in lymphocyte subpopulations known to express Eomes. However, neither frequencies of Eomes-expressing lymphocytes nor Eomes-expressing memory/effector T cell subpopulations influenced survival. Interestingly, frequency of $Eomes^+$ cells/$CD56^+CD3^-$ NK cell population, most likely

representing mature NK cells [2, 5, 28], had a positive prognostic influence on PFS and OS in univariate analysis. Gordon et al. could show that mature Eomes^{+}DX5^{+} NK cells expressed modestly more *perforin1* mRNA than immature Eomes^{-}TRAIL^{+} NK cells, consistent with a role for Eomes in inducing *perforin1* [3, 5, 9]. Moreover, CD56^{dim}CD3^{-} cells, which are normally found in pB, were described to exhibit rather cytolytic and less immunoregulatory functions by cytokine production [2, 29]. However, due to the low sample number, multivariate analysis was not conducted. Therefore, these results must be regarded as descriptive and might only give a hint that also innate immunity, especially mature Eomes^{+} cytotoxic NK cells, plays a critical role in tumor immune response in RCC [30].

It seemed to be more likely that the favorable prognostic influence of higher *Eomes* mRNA levels was not related to a specific Eomes-expressing cell population but rather to the fact that higher *Eomes* mRNA expression might be part of a favorable immune signature.

Higher *Eomes* mRNA expression correlated not only positively with *Tbx21* mRNA but also positively with *TGFβ1* mRNA expression. This seemed to be contradictory, because *TGFβ1* is known to have various immunosuppressive properties. However, we previously demonstrated that high *TGFβ1* mRNA expression in pB was associated with improved survival in mRCC patients treated with sorafenib [15]. Therefore, *TGFβ1* mRNA might be part of this favorable immune signature.

How can this be explained? Both Eomes and T-bet are highly expressed in type 1 immune cells, and not only important for their development, but for their cytolytic function. Therefore, high *Eomes* and *Tbx21* mRNA expression might reflect an effective antitumor immune response. Activated CD8^{+} T cells express *TGFβ1* mRNA and produce TGFβ [31]. TGFβ is not only produced by T cells. In our patient cohort, monocytes seemed to be the major source of TGFβ production [15]. *TGFβ1* mRNA might be induced in immune cells as a feedback mechanism to avoid inflammatory overreaction that might cause immunosuppression, angiogenesis, and tumor progression [32].

Previous studies have demonstrated that endogenous and exogenous TGFβ regulate T cell differentiation and homeostasis. By differentially regulating Eomes and T-bet expression, TGFβ1 blocked central memory T cell development in favor of effector memory T cells [31]. Moreover, depending on the cytokine environment, TGFβ induced Treg, Th9 and Th17 and inhibited IL12-induced Th1 development. Nevertheless, in the presence of IL4, TGFβ1 enhanced IFNγ-induced CD103^{+} Th1 through induction of both Eomes and T-bet [33, 34]. Therefore, it might enhance antitumor immune response. CD103^{+} Th1 preferentially expressed Eomes, and both IFNγ-induced T-bet and TGFβ were required for Eomes expression [33]. This might explain the positive correlation of *TGFβ1* mRNA expression with mRNA expression of *Eomes* and *Tbx21*.

Moreover, *Eomes* and *Tbx21* mRNA levels were associated with higher mRNA expression of the activation marker *ICOS* and with higher frequencies of HLA-DR^{+}-activated T cells, but also with T cells producing less TNFα. Whether those cells have higher cytolytic activity due to perforin or FasL expression and less immunoregulatory or inflammatory function needs to be determined [3, 5, 9, 35]. It is interesting in this context that not only immunosuppressive cells like Treg [12] but also clinical and laboratory factors associated with inflammation like neutrophils, C-reactive protein, and TNFα serum levels [7, 10, 11, 36–40] are known to have a negative impact on survival.

Surprisingly, *Tbx21* mRNA expression is associated with less IFNɣ-producing CD4^{+} T cells, although T-bet is known as a master regulator of IFNɣ expression in Th1 [41]. However, *Tbx21* mRNA expression in PBMCs does not necessarily correlate with *Tbx21* mRNA expression or with T-bet protein expression in CD4^{+} T cells. T-bet protein expression in CD4^{+} T cells did not show any negative correlation with IFNγ production or significant positive correlation (data not shown). Nevertheless, although T-bet is crucial for IFNɣ production in T cells, the overall cytokine environment might modify not only T cell development but also their function. Ylikoski et al. [42] demonstrated that human CD4^{+} T cells, stimulated by IFNɣ or IL12, expressed the same amounts of T-bet, but they differed in IFNγ production. Moreover, the effect of TGFβ1 on IFNɣ production and T-bet expression in human CD4^{+} T cells has been reported to be dependent on the cytokine environment [42]. In mouse CD4^{+} T cells, IFNɣ suppression is mediated by T-bet at recall stimulation but not at priming [43]. Therefore, *Tbx21* mRNA level and T-bet protein expression do not necessarily correlate with IFNɣ production.

Whether a higher *Eomes* mRNA level as an indicator of a favorable immune signature is a useful marker alone or in combination with other already established clinicopathological factors or with other circulating angiogenic biomarkers [27, 44], needs to be investigated in larger patient cohorts in a prospective study.

These angiogenic biomarkers may include VEGF and VEGF-related proteins or cytokines. Baseline VEGF has been identified as an independent negative prognostic marker for survival in patients treated with targeted therapies or IFNα [45–47]. However, regarding sorafenib therapy, higher baseline VEGF levels may be associated with better clinical outcome with sorafenib therapy [48]. Among cytokines, IL6 seems to be the most promising marker despite so far mainly analyzed in sunitinib-treated and pazopanib-treated patients [13]. High concentrations

of IL6 were predictive of improved relative PFS benefit from pazopanib [14]. Besides specific angiogenic factors (CAF), multi-CAF signatures might also be considered. A phase II study comparing first-line sorafenib with sorafenib plus IFNα in advanced RCC identified a six-marker baseline CAF proangiogenic signature [osteopontin, vascular endothelial growth factor (VEGF), carbonic anhydrase 9, collagen IV, VEGF receptor-2, and tumor necrosis factor-related apoptosis-inducing ligand] that correlated with PFS benefit [49].

Conclusions

We identified higher *Eomes* mRNA expression as an independent good prognostic factor for OS and PFS in our patient cohort treated with sorafenib. This might be due to its association with a favorable immune signature. Further investigations in a larger patient cohort are warranted to evaluate whether *Eomes* mRNA expression in pB is a clinically usable prognostic marker that provides prognostic information beyond that of standard clinicopathological factors. Moreover, it would be interesting to determine the prognostic role of *Eomes* expression in patients treated with mTOR inhibitors and TKIs like sunitinib that might differentially modulate tumor-induced inflammation and *Eomes* expression.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest including any financial, personal, or other relationships with other people or organizations within that could inappropriately influence their work.

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