Optimization of sunitinib treatment in metastatic renal cell carcinoma : pharmacogenetic evidence and challenges

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CHAPTER 7

A genetic polymorphism in *CTLA-4* is associated with overall survival of sunitinib-treated patients with clear cell metastatic renal cell carcinoma

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Accepted by Clin Cancer Res

ABSTRACT

Purpose The survival of patients with clear cell metastatic renal cell carcinoma (cc-mRCC) has improved substantially since the introduction of tyrosine kinase inhibitors (TKIs). With the fact that TKIs interact with immune responses, we investigated whether polymorphisms of genes involved in immune checkpoints are related to the clinical outcome of cc-mRCC patients treated with sunitinib as first TKI.

Experimental Design 27 single nucleotide polymorphisms (SNPs) in *CD274* (PD-L1), *PDCD1* (PD-1) and *CTLA-4* were tested for a possible association with progression-free survival (PFS) and overall survival (OS) in a discovery cohort of 550 sunitinib-treated cc-mRCC patients. SNPs with a significant association ($p<0.05$) were tested in an independent validation cohort of 138 sunitinib-treated cc-mRCC patients. Finally, data of the discovery and validation cohort were pooled for meta-analysis.

Results *CTLA-4* rs231775 and *CD274* rs7866740 showed significant associations with OS in the discovery cohort after correction for age, gender and Heng prognostic risk group (HR=0.84, 95%CI: 0.72-0.98, p=0.028 and HR=0.73, 95%CI: 0.54-0.99, p=0.047, respectively). In the validation cohort, the associations of both SNPs with OS did not meet the significance threshold of $p<0.05$. After meta-analysis, *CTLA-4* rs231775 showed a significant association with OS (HR=0.83, 95%CI: 0.72-0.95, p=0.008). Patients with the GG-genotype had longer OS (35.1 months) compared to patients with an AG (30.3 months) or AA genotype (24.3 months). No significant associations with PFS were found.

Conclusions The G-allele of rs231775 in the *CTLA-4* gene is associated with an improved OS in sunitinib-treated cc-mRCC patients and could potentially be used as a prognostic biomarker.

INTRODUCTION

Renal cell cancer (RCC) is the most common type of kidney cancer in adults. Almost 70% of RCC is of clear cell histology and 20-25% of patients have metastatic spread by the time they are diagnosed with RCC [1, 2]. Even though new cases of RCC remain relatively few compared to other more frequent malignancies, the global incidence and mortality are steadily increasing at a rate of 2-3% per decade [2].

For decades, the outcome for metastatic RCC (mRCC) treatment with interleukin-2 and interferon-alpha was disappointing. With the discovery of *von Hippel-Lindau* (*VHL*) gene mutations in the majority of clear cell RCC, therapies targeting angiogenesis were introduced in early 2003 including tyrosine kinase inhibitors (TKIs), mammalian target of rapamycin (mTOR) inhibitors, and the anti-vascular endothelial growth factor (VEGF) monoclonal antibody bevacizumab. With an improved understanding of how tumor cells evade anti-tumor response and how T-cells can be redirected through activating or inhibiting receptors, the immune checkpoint pathway broadens the knowledge of the biological behavior of RCC. Checkpoint inhibitors, such as programmed death-1 (PD-1) inhibitors (pembrolizumab and nivolumab), PD-1 ligand (PD-L1) inhibitor (atezolizumab), and cytotoxic T-lymphocyte antigen 4 (CTLA-4) inhibitor (ipilimumab), have emerged as promising RCC treatments [3]. Given the fact that angiogenesis pathway inhibitors interact with immune responses [4, 5], the potential synergy between checkpoint inhibitors and angiogenesis inhibitors has led to several combination trials in mRCC, such as sunitinib or pazopanib combined with nivolumab [6].

Upon activation, some T-cell receptors (such as CD28) positively regulate T-cells, leading to tumor destruction, while others (such as PD-1 and CTLA-4) negatively regulate T-cells, resulting in prolonged tumor survival. When PD-1 binds to PD-L1, the activation of T cells is inhibited, resulting in the suppression of T-cell attack and tumor immune escape [7]. After binding to B7.1 (CD80) and B7.2 (CD86), CTLA-4 reduces the activated T-cell response to tumor cells, thereby failing to halt tumor progression [8]. Generally, there is a balance among the receptors which makes the immune response of T-cells maintain a proper intensity in order to protect normal cells from collateral damage [7]. However, overexpression of PD-L1 and CTLA-4 was observed in patients with many tumor types, including clear cell mRCC (cc-mRCC) [8-11].

Recently, the prognostic and or predictive value of aforementioned immune checkpoints has been evaluated in several tumor types. It was reported in RCC patients that higher PD-L1 expression was associated with a larger tumor size and a higher risk of death [10, 12, 13]. Subsequently, the association of higher PD-L1 tumor expression with shorter progression-free survival (PFS) and/or overall survival (OS) in mRCC patients treated by TKIs was reported by Choueiri *et al.* [14] and Fukuda *et al* [15]. The possible association between CTLA-4 expression and OS has been investigated in different tumor types with controversial results. A meta-analysis was conducted to pool all data, but consistent results were lacking possibly due to heterogeneity of the tumor types, differences in experimental methods (immunohistochemistry, ELISA or PCR) and varying sample sources (blood or tumor) used in the studies [16].

Thus far, there are no studies investigating the role of single nucleotide polymorphisms (SNPs) in genes related to the immune checkpoints in cc-mRCC patients. In this study, we investigated whether genetic variants within *CD274* (PD-L1), *PDCD1* (PD-1) and *CTLA-4* could be useful as prognostic biomarkers for sunitinib treatment outcome in cc-mRCC patients.

METHODS

Discovery cohort

The discovery cohort was composed of patients who participated in the EuroTARGET project [17]. In brief, the EuroTARGET is a multicentre observational study aiming to identify biomarkers for prediction of response to TKI treatment in mRCC, within which a hypothesis-free genome-wide association study (GWAS) was conducted on sunitinib efficacy in cc-mRCC patients. Thus, clinical information and germline DNA variant chip data (Illumina Human OmniExpress BeadChip) were available [17]. After quality control checks, 679,324 SNPs met the quality criteria applied (**Supplementary document 1**). A total of 6,540,327 SNPs were available after imputation by impute2 using the 1000 Genomes (phase 3 integrated data set of 2504 individuals) as reference panel. In the present study, patients with cc-mRCC who were treated by sunitinib as first TKI were included in the discovery cohort.

SNP selection

In order to capture the genetic variation in the target genes, genomic sequences of *CD274*, *PDCD1* and *CTLA-4* genes were retrieved from the 1000 Genomes Project (GRCh37.p13). For our study, we selected tagging SNPs using the SNP Tagger approach of the Haploview software package (version 4.2). Pairwise tagging with r^2 threshold 0.8 was used. Based on the 1000 Genomes Project, only SNPs with minor allele frequency (MAF) of 0.05 or higher were included. This approach allows to cover the common genetic variations in candidate genes [18], while reducing the number of tested SNPs.

Genotyping results of the selected tagging SNPs were extracted from the EuroTARGET project. To assess the quality of imputation, an estimated imputation "info" score was used, which was computed by impute 2 and took values between 0 and 1. An "info" score near 1 indicates that a SNP has been imputed with high certainty. SNPs with an "info" score lower than 0.9 were excluded from statistical analysis.

Validation cohort and genotyping

Positive hits were tested in an independent validation cohort consisting of cc-mRCC patients treated with sunitinib as first TKI. Details have been described previously [19]. In brief, patients were enrolled between 2004 and 2010 in five medical centers in The Netherlands and in the Cleveland Clinic Foundation Taussig Cancer Institute in the United States. Germline DNA was isolated from whole blood, serum, plasma or peripheral blood mononuclear cell samples and genotyped. A flowchart of the study design is shown in **Figure 1**. Genotyping methods are described in **Supplementary document 2**.

Statistical analysis

Study endpoints consisted of PFS and OS. PFS was defined as the time in months between the first day of sunitinib treatment and the date of progressive disease (according to RECIST version 1.1) or death due to cancer. If no progression was observed during sunitinib treatment, PFS was censored at the time of the last follow-up or death due to other reasons (whichever occurred first). OS was defined as the time in months between the first day of sunitinib treatment and the date of death or the date at which patients were last known to be alive. In the discovery cohort, associations between tagging SNPs and endpoints were univariately tested using an additive genetic model. Individual SNPs with

p-values lower than 0.1 were tested in a multivariable Cox-regression model together with well-established covariates age, gender and Heng prognostic risk group [20], which have been repeatedly associated with PFS and OS. Subsequently, individual SNPs with a p-value < 0.05 in the multivariable analysis were tested in the validation cohort using the same Cox-regression model and endpoint definitions. Finally, data from the discovery and validation cohort were pooled using a fixed effect meta-analysis. Patient characteristics between discovery and validation cohorts were compared by t-test, chi-square test, Mann-Whitney U test or Kaplan-Meier survival analysis with the log-rank test depending on the type of data. All tests were two-sided and carried out using SPSS Statistical Package for Windows (version 23.0 Armonk, NY: IBM Corp). R (version 2.3.2) and package MICE (Multivariate Imputation by Chained Equations) [21] were used to impute missing values for variables included in Heng prognostic risk group (WHO performance status, hemoglobin, neutrophil count, thrombocytes, calcium, and time from diagnosis until start of sunitinib).

Bioinformatic analysis

We used several bioinformatic tools to assess the possible functional relevance for genetic variants showing significant associations with endpoints in the final pooled analysis. The Genotype-Tissue Expression (GTEx, http://www.gtexportal.org/home/) portal was employed to identify potential associations between genetic variants and gene expression levels (eQTL) in all available tissues [22]. The OncoLnc (http://www.oncolnc.org/) [23] was used to link mRNA expression levels to the survival data from The Cancer Genome Atlas Kidney Renal Clear Cell Carcinoma (TCGA-KIRC) collection [24], which included 537 patients with different stages of cc-RCC.

RESULTS

Patient characteristics

A total of 550 and 138 sunitinib-treated cc-mRCC patients were included in the discovery and validation cohort, respectively. Patient characteristics for the discovery and validation cohorts are presented in **Table 1**. In brief, patients included in the discovery cohort were older (median age 63) than those in the validation cohort (median age 59). Nearly 93% of patients in the discovery cohort were classified in the intermediate or poor Heng prognostic risk group, whereas in the validation cohort only 72% belonged to these prognostic groups.

Consequently, patients in the validation cohort had longer PFS and OS compared to those in the discovery cohort. Only 86 (16%) patients in the discovery cohort and 14 (10%) patients in the validation cohort had one or more values missing as variables required for inclusion in a Heng prognostic risk group.

Discovery study on the associations of the selected SNPs with PFS and OS

In the discovery analysis, we selected a total of 34 tagging SNPs in *CD274*, *PDCD1* and *CTLA-4* according to our predefined tagging SNP approach. Genotype results for twenty-seven out of 34 tagging SNPs were imputed based on 1000 genomes project. Seven SNPs with "info" score lower than 0.9 were excluded from statistical analysis (**Supplementary Table S1**).

In the univariate analysis, *CTLA-4* rs231775 and *CD274* rs7866740 were associated with PFS and OS with p-values lower than 0.1 (**Supplementary Table S1**). After correction for age, gender and Heng prognostic risk group, *CTLA-4* rs231775 and *CD274* rs7866740 remained significantly associated with OS (hazard ratio (HR) = 0.84, 95%CI: 0.72-0.98, p=0.028 and HR=0.73, 95%CI: 0.54-0.99, p=0.047, respectively). The minor allele carriers of both SNPs had a better OS compared to that of major allele carriers. The association of *CTLA-4* rs231775 and *CD274* rs7866740 with PFS did not reach the significance threshold in the multivariate analysis (shown in **Table 2**).

Validation study on the associations of polymorphisms with PFS and OS

In the validation cohort, the genotype call rate for *CTLA-4* rs231775 and *CD274* rs7866740 was 99.3% and 98.6%, respectively. Both SNPs were in Hardy-Weinberg equilibrium (HWE) (p>0.05). The MAF of both SNPs was similar in both the discovery cohort and the National Center for Biotechnology Information (NCBI) dbSNP database (CEU population, which represents Utah residents with Northern and Western European ancestry). Only four patients with the *CD274* rs7866740 GG-genotype were detected. As a consequence, a dominant model was used for the genetic association analysis.

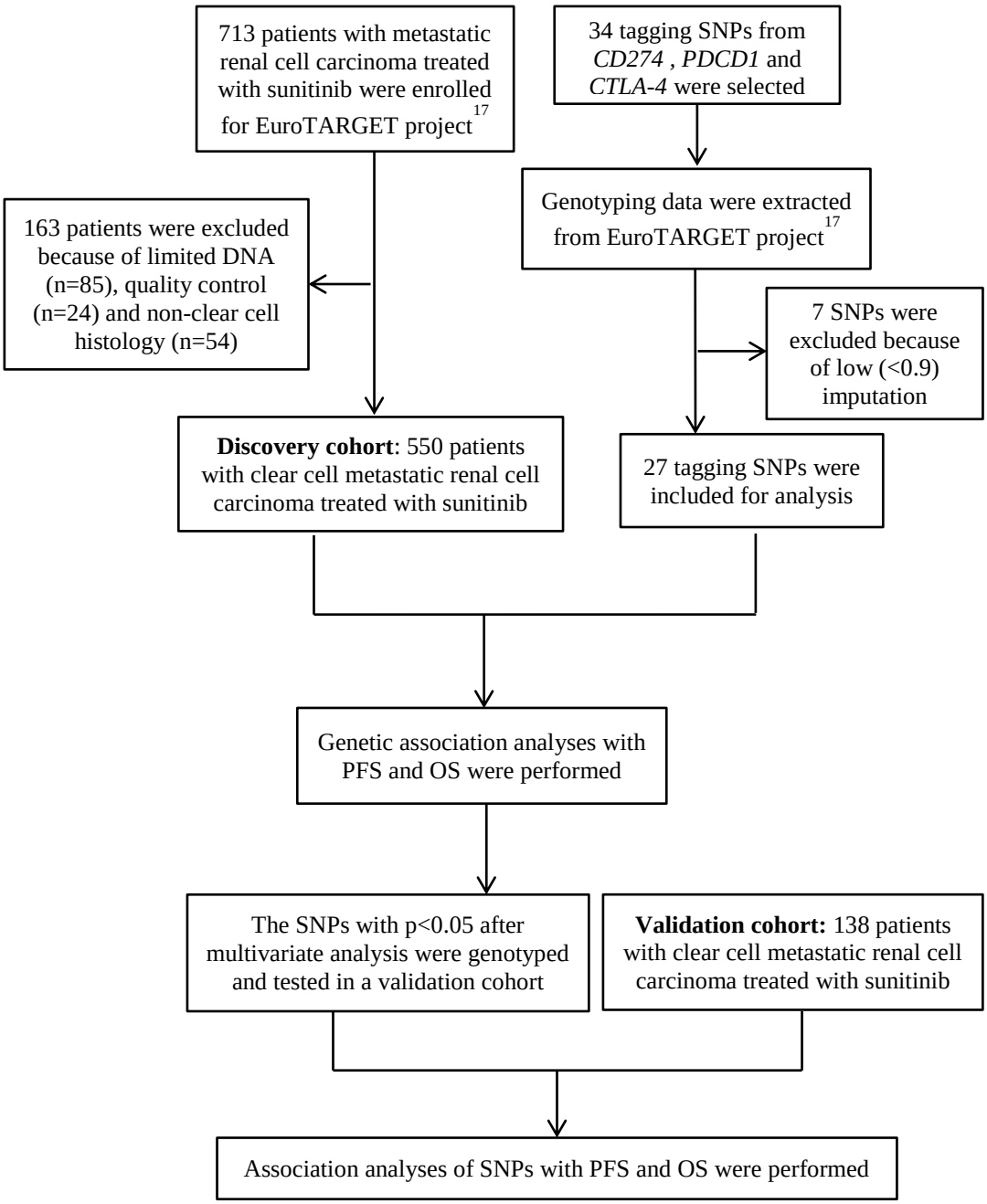

Figure 1 Flowchart of the study design

Table 1 Patient characteristics

Characteristics	Discovery cohort (n=550)	Validation cohort (n=138)	Pooled cohort (n=688)	p-value[#]
Median age at sunitinib start in years (range)	63 (33-87)	59 (28-80)	62 (28-87)	<0.05
Male	405 (74%)	97 (70%)	502 (73%)	0.453
Heng prognostic risk group[*]				
Good (0 risk factor)	38 (6.9%)	38 (27.5%)	76 (11.0%)	<0.05
Intermediate (1-2 risk factors)	291 (52.9%)	68 (49.3%)	359 (52.2%)	
Poor (3-6 risk factors)	221 (40.2%)	32 (23.2%)	235 (36.8%)	
Sunitinib starting dose				
50 mg	482 (87.6%)	134 (97.1%)	616 (89.5%)	
37.5 mg	46 (8.4%)	4 (2.9%)	50 (7.3%)	0.011
25 mg	20 (3.6%)	0	20 (2.9%)	
12.5 mg	2 (0.4%)	0	2 (0.3%)	
Mean number of metastatic sites	2.1	2.3	2.1	0.024
Progression-free survival				
Median in months	12.6	20.1	13.4	0.099
Number of events	341 (62.0%)	95 (68.8%)	436 (63.4%)	0.140
Overall survival				
Median in months	28.8	33.4	29.1	0.025
Number of deaths	341 (62.0%)	88 (63.8%)	429 (62.4%)	0.768

[*]The Heng prognostic risk group is based on six risk factors: WHO performance status (≥1), low hemoglobin (< lower limit of normal (LLN); for males LLN = 8.1 mmol/L or 13 g/dL, for females LLN = 7.1 mmol/L or 11.5 g/dL), high calcium (>2.5 mmol/L) and time from initial diagnosis to treatment with sunitinib (< 1 year), neutrophil count (> upper limit of normal (ULN)) and thrombocytes (> ULN) [20]. R (version 2.3.2) and package MICE (Multivariate Imputation by Chained Equations) [21] were used to impute missing values for variables included in Heng prognostic risk group.

[#]p-value shows the comparison between discovery and validation cohorts using t test, chi-square test, Mann-Whitney U test or Kaplan-Meier survival analysis with the log-rank test depending on the type of data.

In the validation study, none of the SNPs was significantly associated with PFS or OS either in the univariate analysis or after correction for age, gender and Heng prognostic risk group. Nevertheless, *CTLA-4* rs231775 showed the same direction of effect and comparable effect size for OS (HR=0.74, 95%CI: 0.55-1.01, P=0.057; **Table 2**) than that calculated in the discovery cohort (HR= 0.84, 95%CI: 0.72-0.98, p=0.028).

Pooled analysis of the associations of polymorphisms with PFS and OS

In the pooled cohort, *CTLA-4* rs231775 showed a significant association with OS (HR=0.83, 95%CI: 0.72-0.95, p=0.008). Patients with the GG-genotype had longer OS (35.1 months) compared to the OS of patients with the AG or AA genotype (30.3 months and 24.3 months, respectively). Kaplan-Meier plot for OS is presented in **Supplementary Figure S1**. No significant associations were found for PFS (shown in **Table 2**).

Functional effect

We utilized data from GTEx and OncoLnc to provide possible explanations for our interesting finding that *CTLA-4* rs231775 was associated with OS. In the fast majority of tissues in GTEx (Release V6p), GG-genotype of *CTLA-4* rs231775 was associated with a decreased mRNA expression of the *CTLA-4* gene compared to the AA and AG genotypes (**Supplementary Figure S2-A)**. Due to the strict significance threshold, the above association between *CTLA-4* rs231775 and mRNA expression was only observed in testis tissue ($p=1.0\times10^{-7}$, **Supplementary Figure S2-B**). By OncoLnc, OS was compared between cc-RCC patients with lower and higher *CTLA-4* mRNA expression. It was revealed that patients with lower *CTLA-4* mRNA expression (lower 50 percentile) had a longer OS (p=0.00255, **Supplementary Figure S3**).

Table 2 Results of the multivariable Cox-regression model for the association of genetic variants with PFS and OS in cc-mRCC patients treated with sunitinib as first TKI[a]

Protein	Gene	rs number	PFS		OS	
			HR (95%CI)[b]	p-value	HR (95%CI)[b]	p-value
Discovery cohort (n=550)						
PD-L1	*CD274*	rs7866740	0.77 (0.56-1.05)	0.093	0.73 (0.54-0.99)	0.047
CTLA-4	*CTLA-4*	rs231775	0.86 (0.74-1.01)	0.059	0.84 (0.72-0.98)	0.028
Validation cohort (n=138)						
PD-L1	*CD274*	rs7866740	1.03 (0.64-1.65)	0.911	1.35 (0.85-2.14)	0.197
CTLA-4	*CTLA-4*	rs231775	1.02 (0.78-1.35)	0.867	0.74 (0.55-1.01)	0.057
Pooled cohort (n=688)						
PD-L1	*CD274*	rs7866740	0.83 (0.65-1.07)	0.160	0.89 (0.69-1.14)	0.358
CTLA-4	*CTLA-4*	rs231775	0.88 (0.77-1.01)	0.073	0.83 (0.72-0.95)	0.008

[a]Multivariable analysis was adjusted by age, gender and Heng prognostic risk group.

[b]Major allele was the reference.

PFS, progression-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; cc-mRCC, clear cell metastatic renal cell carcinoma; TKI, tyrosine kinase inhibitor.

DISCUSSION

In the present study, we explored genetic variants in the checkpoint-related genes *CD274* (PD-L1), *PDCD1* (PD-1) and *CTLA-4* for a possible association with PFS and OS in cc-mRCC patients that received sunitinib as first TKI. The most important finding of our study is the identification of *CTLA-4* rs231775 as a potential prognostic biomarker for OS. Patients with the GG genotype showed an increased OS compared to those with the GA or AA genotype. To our knowledge, this is the first time that an association of the *CTLA-4* rs231775 genetic polymorphism with OS in this specific patient population is reported.

CTLA-4 rs231775 is located at position +49 in exon 1 of the *CTLA-4* gene and is a common nonsynonymous *CTLA-4* polymorphism. The +49 G allele encodes a Thr to Ala substitution at codon 17 in the signal peptide of the CTLA-4 protein [25]. Anjos *et al.* have found in a cell-free model of *in vitro* reconstitution of translation and endoplasmic reticulum processing that the G-allele of *CTLA-4* rs231775 was associated with inefficient glycosylation, which leads to a decrease in CTLA-4 expression in cell surface [26]. In addition, Sun *et al.* have demonstrated that CTLA-4-Ala (coded by the G-allele) had a lower capability to bind the CTLA-4 ligand and a weaker inhibitory effect on T-cell activation compared to the functional effects of CTLA-4-Thr (coded by the A-allele) [27]. In a large Chinese population they also found that subjects carrying the AA genotype were more susceptible for developing cancer than those with the GG genotype [27]. Either by the decrease of protein expression or through lower binding capability, both studies reach to the conclusion that the G-allele is associated with reduced inhibition of activated T-cells [27]. In addition, Ligers *et al.* have reported in patients with myasthenia gravis and multiple sclerosis that G-allele carriers showed a significant decrease of *CTLA-4* mRNA and protein expression [28]. Therefore, it seems likely that the G-allele of *CTLA-4* rs231775 is associated with a decrease of CTLA-4 expression.

We sought evidence that decreased CTLA-4 expression contributes to an improved OS. CTLA-4 is thought to play a negative regulatory role after binding with its ligands. Hence, it could be hypothesized that low CTLA-4 expression leads to low ligand binding, because of which T-cells can only be weakly inhibited facilitating an enhanced autoimmune response of possible benefit for the patient with cancer. We, therefore, investigated the relationship of *CTLA-4* mRNA expression levels with OS by using the TCGA-KIRC

dataset [23, 24], which revealed that ccRCC patients (regardless of tumor stage) with lower *CTLA-4* mRNA expression had longer OS. Combining all findings, the G-allele of *CTLA-4* rs231775 is indeed associated with lower *CTLA-4* mRNA expression, and lower mRNA expression links to longer OS, which is consistent with our results.

Our finding provides insight into RCC prognosis and corroborates the current strategies of new drug development for RCC. In this respect, ipilimumab, which is designed to inhibit the CTLA-4 protein, has demonstrated clinical efficacy and manageable toxicity as monotherapy or combined with nivolumab in mRCC [29]. To date, there are no studies investigating the role of *CTLA-4* polymorphisms or CTLA-4 protein expression on ipilimumab outcome in mRCC patients. However, in patients with melanoma, Breunis *et al.* have assessed seven *CTLA-4* polymorphisms with ipilimumab response. In responding patients there were proportionally more rs231775 A-allele carriers than G-allele carriers, while there was no difference in non-responding patients (OR=0.39, 95%CI: 0.18-0.82, p=0.009) [30]. If confirmed in mRCC patients on the basis of the results presented here, rs231775 might be important for the optimization of ipilimumab treatment of mRCC.

In the present study in cc-mRCC patients that received sunitinib as first TKI, we were able to demonstrate an association of *CTLA-4* rs231775 with OS, but not with PFS. Interestingly, Song *et al.* have also identified *CTLA-4* rs231775 is a prognostic biomarker in patients with advanced non-small cell lung cancer [31]. Patients with a GG or GA genotype experienced a significantly longer OS than those with an AA genotype after correction for many covariates among which was treatment [31]. These findings strongly suggest that the *CTLA-4* genetic variant is more of influence on the biological behaviour of the disease than with the effects of treatment, implying the prognostic role of this polymorphism. Introduction of checkpoint inhibitors in the treatment of cancer patients may, however, change the relevance of *CTLA-4* rs231775 for PFS.

Polymorphisms in *PDCD1* (PD-1) and *CD274* (PD-L1) are thought to be promising genetic candidates to explain differences in immunosuppressive function. It has been reported that *PDCD1* rs10204525 was associated with OS in 439 patients with locoregional gastric cancer [32] as well as in 668 patients with resectable colorectal cancer [33], suggesting that it may serve as a prognostic factor. In our patient population of metastatic disease, however, we did not observe any significant association of *PDCD1* rs10204525 (which was captured

by *PDCD1* rs41386349) with survival outcome. *CD274* rs4143815 has been reported to be significantly associated with worse OS in a total of 354 patients with non-small cell lung cancer who underwent curative resection [34]. We observed an association of *CD274* rs7866740 with OS in the discovery cohort, but failed confirmation in the final analysis. Owing to the fact that genotype of *CD274* rs7866740 was imputed and the MAF was relatively small, the significant association with *CD274* rs7866740 in the discovery cohort might be detected by chance. Future studies should focus on the relevance of these SNPs in patients with no/microscopic disease after surgery *vs* those with advanced disease.

Whereas previous studies have focused on SNPs in genes of the VEGF signalling pathway, we are the first to explore the potential association of the genetic variability in three genes encoding immune checkpoints with the outcome in sunitinib-treated patients. Although we did not formally confirm the association in the validation cohort, the meta-analysis showed similar results to those of the discovery cohort and with stronger statistical evidence. In addition, a biological and mechanistic rationale was provided using expression and survival data from GTEx [22] and TCGA [23]. However, we should interpret results from GTEx and TCGA carefully. It is because the significant association of genotype with CTLA-4 expression is found in testis not in kidney tissue (only 39 samples available in GTEx) or T-cells in microenvironment. Moreover, TCGA-KIRC dataset include patients with different stages and follow-up period is not long enough. As a result, the plot in **Supplementary Figure S3** might be changed when tumor stage is taken into account and after enough follow-up period. The possible reasons of the failed validation could be the relatively small sample size as well as differences in patient characteristics between the two cohorts. Due to the need for imputation, adjustment of PFS and OS might slightly be distorted. To reduce this chance, a multiple imputation procedure instead of a single imputation was used. Moreover, imputation was implemented in 16% and 10% patients in the discovery and validation cohort, respectively, which could be considered negligible. The significance threshold in the discovery study was not adjusted by the strict Bonferroni correction in this exploratory study, since Bonferroni correction will increase the type II error (the chances of false-negative results) [35]. In our opinion, an inflated type I error due to multiple comparisons is better solved by a validation study than by a Bonferroni correction.

In conclusion, data from the present study show that the G-allele of *CTLA-4* rs231775 is associated with improved OS in patients with cc-mRCC receiving sunitinib, suggesting this polymorphism may serve as a prognostic marker.

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Supplementary document 1 Quality control

Quality control (QC) checks were performed using software R version 3.2.3, and PLINK software, version 1.07. Individuals were excluded from analyses based on an individual genotype call rate < 97%, gender mismatch between reported and estimated sex based on genotypes of the X-chromosome (using PLINK), or excess of heterozygous genotypes as measured by the inbreeding coefficient. An inbreeding statistic of $F > 0.1$ was judged to be outlying and individuals were removed from the analysis. Genetic markers were excluded based on a single nucleotide polymorphisms (SNPs) call rate < 97%, minor allele frequency (MAF) < 1%, and a p-value $\leq 10^{-7}$ for the Hardy-Weinberg equilibrium (HWE) goodness-of-fit test. After exclusion of individuals and markers in these marginal QCs, the remaining set was used for integrative QC assessment. In order to evaluate the possibility of population stratification or outliers, multidimensional scaling (MDS) analysis was performed in PLINK. In addition, pairwise identity by state (IBS) statistics were calculated to assess duplicates. Both MDS and IBS were computed using PLINK. Individuals that were identified as outliers based on IBS clustering or the MDS analysis were excluded from the association analyses.

The observed individual genotype call rates varied between 99.2 to 100% and therefore meets the quality criteria. Based on further quality control steps, 24 patients were excluded from analysis. Genders were specified with a sample mix-up rate of 0.2% which means that 2 patients were removed prior to analyses. MDS showed that 20 patients were recognized as outliers and were therefore removed from analyses. In addition, 2 patients were excluded because of an inbreeding coefficient of $F > 0.1$.

Supplementary document 2 Genotyping methods

Genotyping of *CTLA-4* rs231775 was performed by high-resolution melting (HRM) analysis following PCR amplification. Primer sequences were as follows: forward 5'-CACAAG GCTCAGCTGAACC-3' and reverse 5'-GTGCAGGGCCAGGTCCTG-3'. The PCR amplification was performed on Labcycler PCR instrument (SensoQuest GmbH, Germany. The PCR program was 94°C for 15 min, 40 × (95°C, 20s; 60°C, 40s; 72°C, 30s), 72°C for 10 min, 95°C for 1 min, followed by a final hold at 25°C. Samples were then transferred to the LightScanner® system (BioFire Defense, USA). The LightScanner

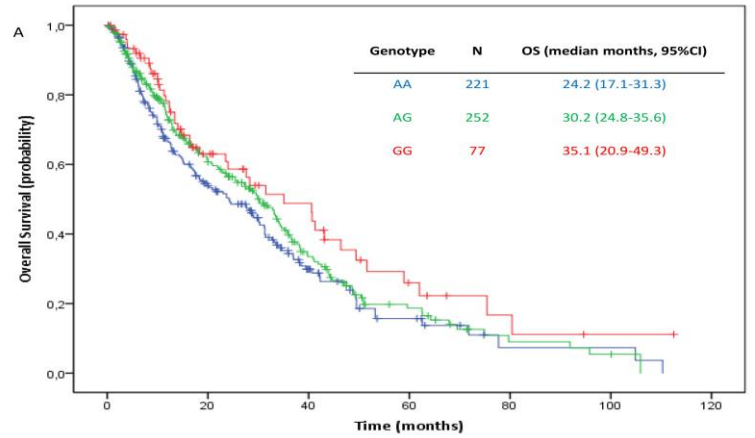
protocol was set up as start temperature 55°C, end temperature 98°C and hold temperature 50°C.

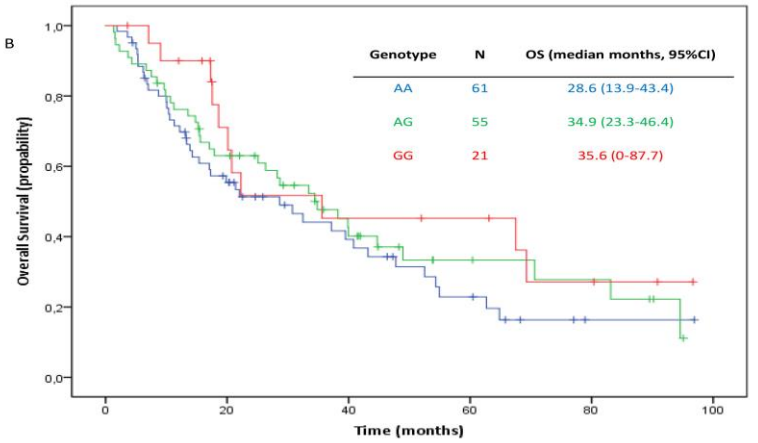
Genotyping of *CD274* rs7866740 was performed by pyrosequencing (PyroMark Instrument, Sweden). The forward primer (5'-CGATTTCACCGAAGGTCAGG-3') and reverse primer (5'-GAGGAACAACGCTCCCTACC-3'-biotin) were used to amplify DNA fragment by PCR. The forward sequencing primer (5'-GAGCAGCTGGCGCGT-3') was applied to detect the target region. The PCR amplification was performed on Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific, USA), and the PCR program was carried out as: 95°C for 15 min, 40 × (94°C, 30s; 55°C, 30s; 72°C, 30s), 72°C for 10 min, followed by a final hold at 25°C. Then, the PCR mixture was immobilized by streptavidin beads, denaturated by 0.2 M NaOH, and released into sequencing primer. The results of pyrosequencing reaction were analyzed on a PSQ 96MA Pyrosequencer (Pyrosequencing AB, Sweden).

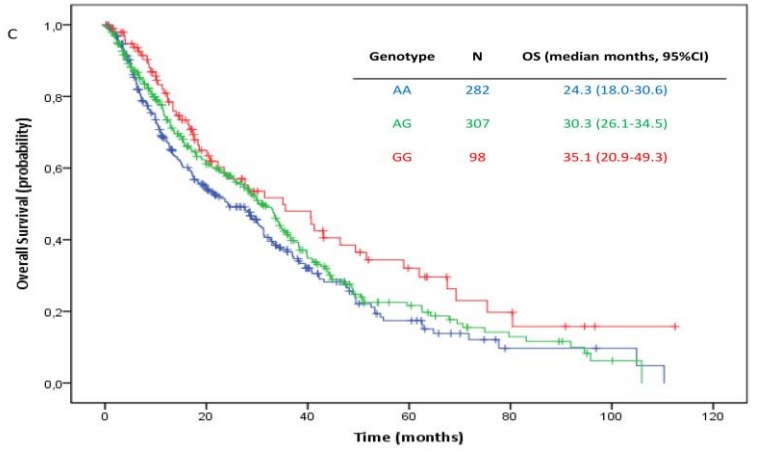
Supplementary Table S1 Selected tagging SNPs

Gene	rs number	Minor allele frequency in discovery cohort	"Info" score	Include (Y/N)
PDCD1(n=11)	rs11568821 C>T	0.133	0.989	Y
	rs28539662 G>A	0.182	0.897	N
	rs28680420 C>T	0.322	0.921	Y
	rs35399295 C>T	0.222	1	Y
	rs41386349 G>A	0.062	0.974	Y
	rs55829775 G>C	0.046	0.706	N
	rs7419333 C>G	0.356	0.995	Y
	rs7419870 A>G	0.576	0.957	Y
	rs7560086 G>A	0.174	0.86	N
	rs7565639 C>T	0.656	0.961	Y
	rs7603052 T>C	0.627	0.925	Y
CD274 (n=21)	rs10114060 G>A	0.267	0.99	Y
	rs10122089 C>T	0.519	0.986	Y
	rs16923173 G>A	0.043	1	Y
	rs17804441 T>C	0.250	1	Y
	rs1970000 C>A	0.507	0.95	Y
	rs2297136 G>A	0.53	1	Y
	rs2297137 G>A	0.234	0.935	Y
	rs2890657 G>C	0.202	0.986	Y
	rs2890658 C>A	0.096	1	Y
	rs3780394 A>C	0.073	0.98	Y
	rs41303227 C>T	0.064	0.88	N
	rs4143815 G>C	0.288	1	Y
	rs4742098 A>G	0.252	0.976	Y
	rs59906468 A>G	0.240	0.929	Y
	rs7023227 C>T	0.613	0.98	Y
	rs74725413 A>C	0.028	0.761	N
	rs74791855 T>C	0.044	0.823	N
	rs76778936 T>G	0.039	0.807	N
	rs7866740 C>G	0.092	0.948	Y
	rs78745832 C>T	0.096	0.941	Y
	rs79855302 G>A	0.046	0.936	Y
CTLA-4 (n=2)	rs231775 A>G	0.366	1	Y
	rs231777 C>T	0.867	0.996	Y

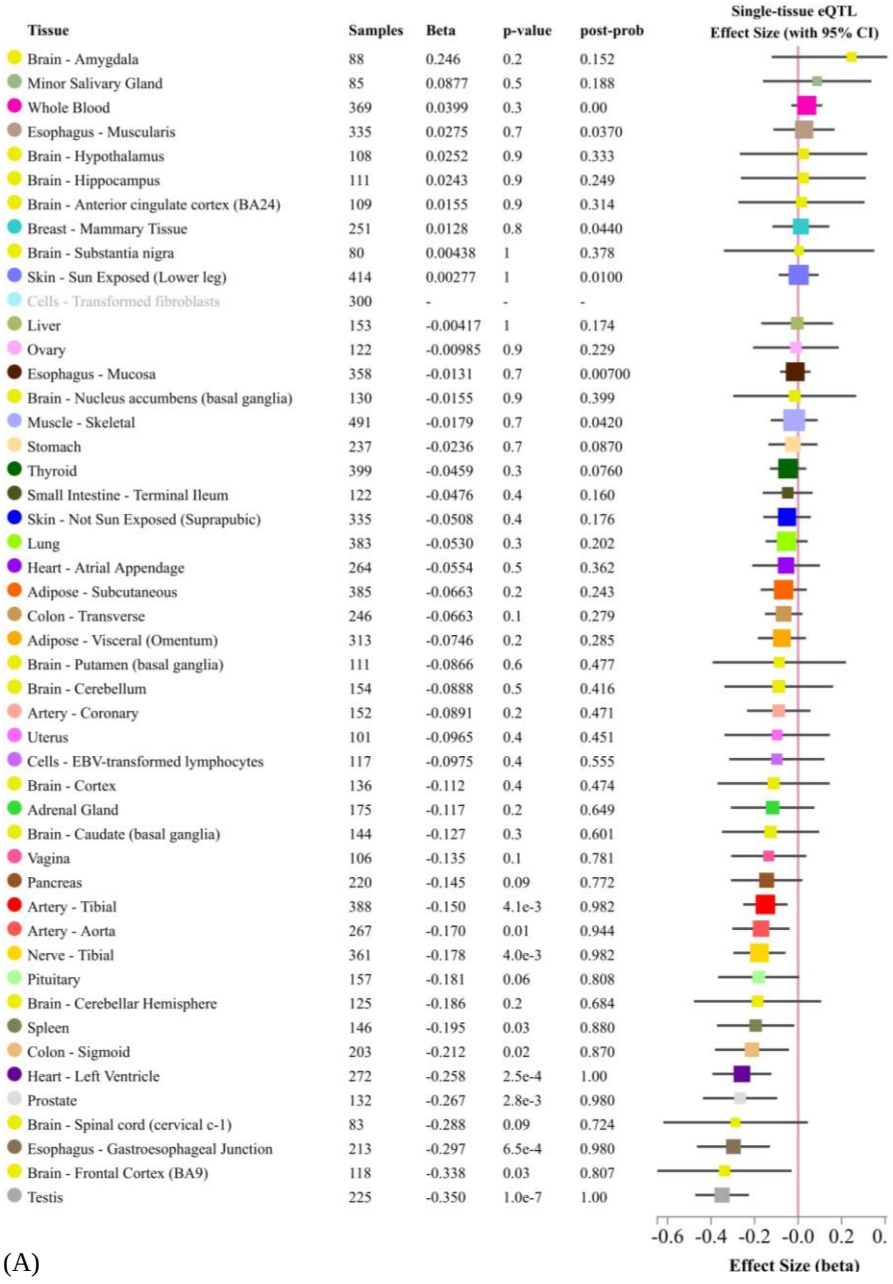
Supplementary Figure S1 Kaplan-Meier plot for overall survival by genotype of *CTLA-4* rs231775 in discovery cohort (A), validation cohort (B) and combined cohort (C).





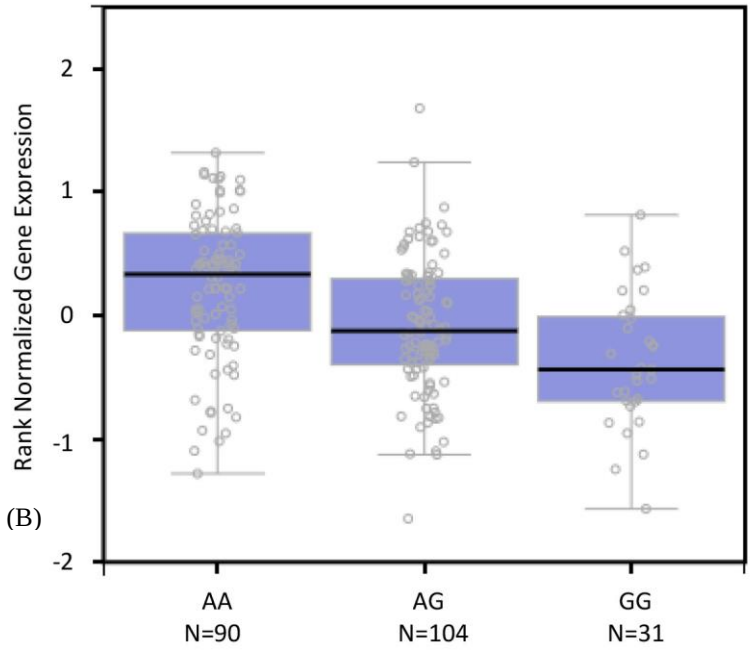


Supplementary Figure S2 The association of *CTLA-4* rs231775 with *CTLA-4* gene expression in multiple tissues (A) and in testis tissue (B, $p=1.0\times10^{-7}$) using data from the Genotype-Tissue Expression (GTEx) dataset[22].



Tissue	Samples	Beta	p-value	post-prob
Brain - Amygdala	88	0.246	0.2	0.152
Minor Salivary Gland	85	0.0877	0.5	0.188
Whole Blood	369	0.0399	0.3	0.00
Esophagus - Muscularis	335	0.0275	0.7	0.0370
Brain - Hypothalamus	108	0.0252	0.9	0.333
Brain - Hippocampus	111	0.0243	0.9	0.249
Brain - Anterior cingulate cortex (BA24)	109	0.0155	0.9	0.314
Breast - Mammary Tissue	251	0.0128	0.8	0.0440
Brain - Substantia nigra	80	0.00438	1	0.378
Skin - Sun Exposed (Lower leg)	414	0.00277	1	0.0100
Cells - Transformed fibroblasts	300	-	-	-
Liver	153	-0.00417	1	0.174
Ovary	122	-0.00985	0.9	0.229
Esophagus - Mucosa	358	-0.0131	0.7	0.00700
Brain - Nucleus accumbens (basal ganglia)	130	-0.0155	0.9	0.399
Muscle - Skeletal	491	-0.0179	0.7	0.0420
Stomach	237	-0.0236	0.7	0.0870
Thyroid	399	-0.0459	0.3	0.0760
Small Intestine - Terminal Ileum	122	-0.0476	0.4	0.160
Skin - Not Sun Exposed (Suprapubic)	335	-0.0508	0.4	0.176
Lung	383	-0.0530	0.3	0.202
Heart - Atrial Appendage	264	-0.0554	0.5	0.362
Adipose - Subcutaneous	385	-0.0663	0.2	0.243
Colon - Transverse	246	-0.0663	0.1	0.279
Adipose - Visceral (Omentum)	313	-0.0746	0.2	0.285
Brain - Putamen (basal ganglia)	111	-0.0866	0.6	0.477
Brain - Cerebellum	154	-0.0888	0.5	0.416
Artery - Coronary	152	-0.0891	0.2	0.471
Uterus	101	-0.0965	0.4	0.451
Cells - EBV-transformed lymphocytes	117	-0.0975	0.4	0.555
Brain - Cortex	136	-0.112	0.4	0.474
Adrenal Gland	175	-0.117	0.2	0.649
Brain - Caudate (basal ganglia)	144	-0.127	0.3	0.601
Vagina	106	-0.135	0.1	0.781
Pancreas	220	-0.145	0.09	0.772
Artery - Tibial	388	-0.150	4.1e-3	0.982
Artery - Aorta	267	-0.170	0.01	0.944
Nerve - Tibial	361	-0.178	4.0e-3	0.982
Pituitary	157	-0.181	0.06	0.808
Brain - Cerebellar Hemisphere	125	-0.186	0.2	0.684
Spleen	146	-0.195	0.03	0.880
Colon - Sigmoid	203	-0.212	0.02	0.870
Heart - Left Ventricle	272	-0.258	2.5e-4	1.00
Prostate	132	-0.267	2.8e-3	0.980
Brain - Spinal cord (cervical c-1)	83	-0.288	0.09	0.724
Esophagus - Gastroesophageal Junction	213	-0.297	6.5e-4	0.980
Brain - Frontal Cortex (BA9)	118	-0.338	0.03	0.807
Testis	225	-0.350	1.0e-7	1.00

(A)



Supplementary Figure S3 The survival curve shows the association of *CTLA-4* gene expression with overall survival in patients with clear cell renal cell carcinoma (p=0.00255) through OncoLnc, which links The Cancer Genome Atlas (TCGA) survival data to mRNA expression levels.[23] (low: lower 50 percentile, high: upper 50 percentile)