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Genetic Deficiency of Complement Isoforms *C4A* or *C4B* Predicts Improved Survival in Metastatic Renal Cell Carcinoma

Ghazal I. Zafar[1], **Elizabeth A. Grimm**[1], **Wei Wei**[2], **Marcella M. Johnson**[2], and **Julie A. Ellerhorst**[1]

[1] Department of Experimental Therapeutics, University of Texas M. D. Anderson Cancer Center, Houston, TX, USA

[2] Division of Quantitative Sciences, University of Texas M. D. Anderson Cancer Center, Houston, TX, USA

Abstract

Purpose—Autoimmune phenomena during immunotherapy are associated with favorable outcomes for patients with metastatic renal cell carcinoma (RCC). We have reported improved survival for Stage IV RCC patients carrying autoimmunity-associated HLA class II haplotypes. We propose that the clinical benefit is mediated by products of other autoimmunity-associated genes linked to these haplotypes. One candidate gene is complement *C4*, which replicates as part of the RCCX module, can be present in multiple copies, and exists as *C4A* and *C4B* isoforms. Deficiencies of either isoform are associated with autoimmunity. The objective of this study was to test the hypothesis that *C4A* or *C4B* deficiency predicts improved survival for patients with RCC.

Materials and Methods—Total *C4* copy number was determined by simultaneous amplification of *RP1* and *TNXA/RP2* to quantitate RCCX modules. *C4A* and *C4B* alleles were distinguished by *Psh*AI RFLP.

Results—Genetic complotypes were determined for 61 patients. Individuals with a solitary copy of either *C4* isoform experienced longer survival. The median survival from the diagnosis of metastatic disease for patients with a solitary copy of *C4A* or *C4B* was 7.75 years vs. 1.25 years for the comparison group ($p = 0.001$), and was independent of the benefit derived from autoimmune class II genotypes.

Conclusions—We conclude that improved survival is seen for RCC patients with *C4A* or *C4B* deficiency, treated with cytokine therapy with/without surgery. These data support our hypothesis that RCC patients with autoimmune genotypes have favorable outcomes resulting from autoimmune mechanisms directed to the tumor.

Keywords

Renal cell carcinoma; complement; C4; autoimmunity

INTRODUCTION

Metastatic renal cell carcinoma (RCC) is generally resistant to most forms of systemic therapy. There is, however, a small subset of RCC patients who respond to immunotherapy, and some

Address correspondence to: Julie Ellerhorst M.D., Ph.D., Department of Experimental Therapeutics, Unit 362, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, Ph: 713-792-8990, Fax: 713-563-3531, Email: jaellerh@mdanderson.org.

of these patients are cured.[1] A fascinating correlate of clinical response to immunotherapy in this disease is the development of autoimmune phenomena, described in RCC patients treated with virtually all immunotherapeutics, but most commonly reported with interferon-α, interleukin-2, and CTLA-4 antibody.[2, 3] The spectrum of autoimmune events ranges from mild arthralgias to Hashimoto-like thyroid failure to potentially life-threatening colitis and hypophysitis. The well-documented correlation of these autoimmune phenomena with response and survival in RCC has led to speculation that anti-self mechanisms activated by the immunotherapeutic drugs are similarly directed to the metastatic tumor, resulting in tumoricidal effects. This concept also predicts that RCC patients who are genetically predisposed to autoimmunity are more likely to respond to immunotherapy and enjoy prolonged survival. Supporting this paradigm, we have reported significantly improved survival for stage IV RCC patients, treated with immunotherapy, who carry autoimmunity-associated HLA class II haplotypes.[4] Mechanistically, there are two explanations for our findings: the first is optimal tumor antigen presentation by these class II molecules; the second is genetic linkage of the true effector mechanism to these class II haplotypes. In exploring the second explanation, we have proposed the complement component C4 as a potential mediator of the observed favorable clinical outcomes.

The *C4* gene, located on chromosome 6 in the mid-MHC region, telomeric to the HLA class II loci, exists as a component of the four-gene RCCX module (*RP1/2*, *C4*, *CYP21A/B*, *TNXA/B*). The majority of individuals carries one, two, or three RCCX modules per chromosome 6, and thus may have from two to six copies of *C4* per diploid genome.[5] Adding to the diversity of *C4* genotypes in the population are the two functionally distinct *C4A* and *C4B* isoforms. Proteolytic activation of the C4 protein, whether initiated by the classical or lectin activation pathway, leads to the generation of various anaphylatoxins, and, ultimately, to formation of the membrane attack complex which participates in damage to target cells and pathogens.[6]

The paradoxical association of a relative or absolute deficiency of either *C4* isoform with human autoimmune disease is well documented.[7, 8] Our theory of an association of autoimmunity-associated genotypes with improved outcomes for RCC patients predicts a beneficial effect from *C4* deficiency. In the present study, we test the hypothesis that a low copy number of *C4A* or *C4B* predicts improved survival for RCC patients with Stage IV disease.

PATIENTS AND METHODS

Patients

The study was approved by the M. D. Anderson Cancer Center (MDACC) IRB and conducted according to HIPAA guidelines. Informed consent was obtained from all individuals. The study population was derived from the same 80 patient cohort included in our previous report[4], of which 61 individuals had sufficient DNA remaining to complete the present study.

Determination of the *C4* copy number

The total *C4* copy number was determined for each patient by quantification of RCCX modules as described by Chung *et al.*[9], with minor modification. This methodology utilizes simultaneous amplification of *RP1* and a DNA fragment at the *TNXA-RP2* junction. Each reaction included 200 ng of genomic DNA, 2.5 mM $MgCl_2$, 600 nM RPa (forward primer for RP1), 600 nM RPb (reverse primer for *RP1* and *TNXA-RP2*), and 100 nM TNXc (forward primer for *TNXA-RP2*). Primer sequences were: RPa, 5′ CAAGAGAGGAGGCCTATCTTACCTGG3′; RPb, 5′ GCTCAAGCTGTGAGGAGAACT3′; TNXc, 5′TATCACAGGCTCTGGCCCCA3′ Conditions were 94°C 5 minutes, 1 cycle; 94°C 45 sec, 59°C 45 sec, 72°C 60 sec, 32 cycles; 72°C 10 min, 1 cycle. PCR products of 1.2 kb (*RP1*) and 1.0 kb (*TNXA-RP2*) were digitally

imaged and quantitated using Kodak Image Station 4000R (Eastman Kodak, Rochester, NY). The *RP1* band was given a fixed value of two copies, and the *TNXA-RP2* copy number was determined according to its ratio to *RP1*. The total *C4* copy number was calculated as the sum of these two quantities.

Determination of the *C4A:C4B* allelic ratio

The methodology for determining the allelic ratio of the *C4* isoforms was again based on the methods of Chung et al.[9] A 1110 base pair (bp) fragment of the *C4* gene was amplified with the following primers: Forward, 5′GCTCACAGCCTTTGTGTTGAA3′; Reverse, 5′ TTGGGTACTGCGGAATCCCC3′. Conditions were 94°C 5 minutes, 1 cycle; 94°C 30 sec, 58°C 45 sec, 72°C 60 sec, 34 cycles; 72°C 7 min, 1 cycle. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA), and eluted in a volume of 50 ul. This purified product was subjected to one additional round of amplification using ^{32}P-end-labeled reverse primer as follows: 94°C 5 minutes, 58°C 5 minutes, 72°C 10 minutes, 1 cycle. The labeled product was again purified as above and eluted in a volume of 30 ul. One half of the product (15 ul) was digested overnight with *Psh*AI. Restriction products were separated by agarose gel electrophoresis, transferred to a nylon membrane, and subjected to autoradiography from which digital images were prepared. Bands representing *C4A* (970 bp) and *C4B* (1100 bp) were quantitated by ImageQuant Software (Amersham Biosciences, Piscataway, NJ).

Statistical analysis

Survival was calculated from the diagnosis of metastatic disease. Survival curves were estimated using the Kaplan-Meier method. Log-rank test was used to compare survival curves of patients with different copy numbers of genes. Cox proportional hazards regression modeling was used to fit univariate and multivariate survival models and to identify the final model. All tests were two-sided and *p*-values of 0.05 or less were considered statistically significant. Statistical analysis was carried out using SAS version 9 (SAS Institute, Cary, NC) and S-Plus 7 (Insightful Inc., Seattle, WA).

RESULTS

Study population

C4 genotyping was performed for 61 patients with metastatic RCC (Table I). The study subjects included 16 females and 45 males, reflecting the gender distribution of this malignancy. All patients had been treated with interferon-alpha, interferon-gamma, and/or interleukin-2 on phase II clinical trials conducted between the years 1990 and 1997. The study population was selected to represent four distinct clinical groups based on outcome to treatment. Group 1 consisted of seven individuals with metastatic RCC who achieved a major response and disease-free status, and who have remained disease-free for more than seven years without further intervention. Group 2 consisted of 11 patients who achieved major responses but, unlike Group 1, suffered relapses requiring further medical or surgical intervention. Group 3 included 10 patients who did not experience significant tumor reduction as a consequence of cytokine therapy, but, regardless, enjoyed prolonged survival of greater than 5 years. Group 4 was composed of 33 patients whose disease progressed through therapy and who ultimately succumbed to metastatic disease.

Establishment of *C4* complotypes

Determination of the complotype for each patient was a two-step process. First, the total copy number of *C4* genes was established by enumerating RCCX modules. The first module on each chromosome 6 is initiated by the *RP1* allele; subsequent modules begin with *RP2* (Figure 1). RCCX quantitation was based on simultaneous PCR amplification of a fragment of *RP1* and

the junction of *TNXA/RP2*. Because the diploid genome contains exactly two *RP1* genes, the *RP1* PCR product was assigned a value of two gene copies. The *TNXA/RP2* band, representing the additional RCCX modules, was quantitated relative to the two-copy *RP1* band. Step two of the complotyping process established the copy number of the individual *C4A* and *C4B* isoforms by restriction fragment length polymorphism analysis. The isoform ratio was adjusted to the total *C4* copy number to establish the total number of alleles of each isoform. An example of this calculation is demonstrated in Figure 2.

C4 complotype and survival

Our hypothesis predicted that *C4* deficiency, whether manifested as a low total copy number or a deficiency of a given isoform, would be associated with improved outcomes. As shown in Table II, this was indeed the case. Four copies of *C4* is the most common gene dose in the general population. RCC patients with *C4* deficiency, defined as fewer than four copies, survived longer than those with four or more copies (7.75 vs. 1.67 years, $p = 0.051$). With regard to *C4* isoform number, two copies of each is the norm. Preliminary analysis suggested that patients with exactly one copy of either *C4A* or *C4B* had prolonged survival. Because the survival times were similar, patients with a solitary copy of either isoform were combined into a single group for the final analysis. The median survival from the diagnosis of metastatic disease for patients with a solitary copy of *C4A* or *C4B* was 7.75 years vs. 1.25 years for the comparison group ($p = 0.001$, Fig 3). Similar results were found when survival was calculated from the initiation of treatment, with an identical *p*-value of 0.001 (data not shown).

A Cox proportional hazards model was used to carry out multivariate survival analysis with the total *C4* gene copy number and *C4* isoform data included in the model. The presence of a solitary isoform copy remained significant after adjusting for the total copy number; conversely, total copy number lost its borderline significant *p*-value (data not shown). Being the dominant factor associated with survival, isoform copy number was used for all additional analyses.

C4A/C4B Isoform Copy Number and Response to Immunotherapy

The association of isoform copy number with survival begged the question of whether this effect was mediated by therapeutic response, i.e., whether *C4* isoform deficiency in some way improved sensitivity to immunotherapy, which then influenced survival. On first glance at the data in Table III, one might come to this conclusion. Rates of isoform deficiency differed significantly among the four clinical groups ($p = 0.036$, Fisher's exact test), with the highest rates found in the patients with the best response (85.7%, Group 1) and the lowest rates in patients whose disease progressed through therapy (30.3%, Group 4). In contrast, however, the two intermediate groups displayed the opposite pattern. Group 3 patients, who lacked clinical response to immunotherapy but survived for years with longstanding stable disease, had a higher rate of isoform deficiency (60.0%) than Group 2 patients (45.5%) who responded to treatment, albeit transiently and/or incompletely. Thus, it appears that the influence of *C4* isoform deficiency on survival is not entirely a function of major clinical therapeutic response.

C4A/C4B Isoform Copy Number and HLA Class II Haplotype

Previous data from our laboratory demonstrated that in this same patient population, improved survival was associated with the presence of components of either of two autoimmunity-associated HLA class II haplotypes: DRB1*0301/DQA1*0501/DQB1*0201 and DRB1*1501/DQA1*0102/DQB1*0602. The strongest survival benefit was associated with any combination of the two DQA1 alleles (*0102/*0501, *0501/*0501, or *0102/*0102).[4] Because the *C4* gene, in the mid-MHC region, is in the vicinity of the HLA class II locus, multivariate analysis was performed to determine if the survival benefit provided by a relative *C4* isoform deficiency was independent of the presence of these autoimmune class II haplotypes. For the

patient cohort included in the present study, the presence of two of the above DQA1 alleles was associated with improved survival from the diagnosis of metastatic disease ($p = 0.035$). However, when DQA1 and *C4* isoform deficiency were included in multivariate analysis, the Cox proportional hazard model showed only *C4* deficiency to be included in the final model, i.e., the copy number of *C4* isoforms is a more dominant predictor of survival than the DQA1 genotype (Table IV).

DISCUSSION

Autoimmunity is a poorly understood dysregulation of self-tolerance. There is a clear genetic component, particularly with loci in the mid-MHC region of chromosome 6. We have previously reported that autoimmunity-associated HLA class II haplotypes are associated with improved outcomes for RCC patients after treatment with cytokine therapy. We now demonstrate an even stronger and independent influence associated with relative genetic deficiencies of *C4A* and *C4B*, established correlates of autoimmune disease. It is notable that the survival benefit provided by complement deficiency extended to patients who did not respond to immunotherapy, in contrast to the HLA class II effect which appeared to be limited to responding patients. This would indicate that the survival benefit derived from complement deficiency may not depend on a major reduction of tumor burden, but instead, may result from tumor growth inhibition. Whether the influence of *C4* isoform deficiency on survival requires the presence of exogenous immune stimulation is an important question, but cannot be answered in the present study as all patients had received immunotherapy.

Intuitively, one would anticipate that the roles for *C4* isoform deficiency are similar in autoimmune tissue destruction and in control of metastatic renal cancer. Unfortunately, mechanisms by which *C4* deficiency contributes to autoimmunity are poorly understood and difficult to apply to the oncologic setting. One leading theory is based on the requirement for *C4* for the solubilization and clearance of immune complexes, which otherwise circulate for prolonged periods of time and deposit in healthy tissues.[10] A second theory, based primarily on animal models, derives from data suggesting that complement, bound to specific receptors on B-cells, is required for the induction and maintenance of self-tolerance, possibly by delivery of self-antigens to auto-reactive B-cells, which subsequently undergo deletion.[11, 12] These theories are not mutually exclusive and both mechanisms could result in tumor damage, whether through the deposition of immune complexes or targeting by auto-reactive B-cells.

It is interesting to note that overt autoimmunity is overwhelmingly a disease of women, and the incidence of RCC in women is substantially lower than in men. The prevalence of overt autoimmunity has not been reported for patients with RCC. However, we predict that a clinically relevant renal cancer will develop only with difficulty in the presence of active autoimmune disease, and that the prevalence of rheumatologic disorders in the RCC population is likely to be lower than in the general population. In keeping with this prediction, none of the patients in the study cohort carried the diagnosis of an autoimmune disease.

A shortcoming of our study is the use of gene copy number as a surrogate for C4 protein levels. Although the major determinant of circulating C4 levels is, in fact, the gene copy number, the correlation is not exact, and other factors influence protein levels.[5] Quantitation of circulating C4 protein and its isoforms is most commonly carried out by an immunofixation assay that performs best with fresh samples collected in EDTA.[13] These requirements preclude protein complotyping of our present study cohort, the majority of whom are deceased. However, studies are currently being designed for prospective C4 protein determination for Stage IV RCC patients. We will also examine levels in early stage RCC patients to determine if there is a beneficial effect of relative *C4* isoform deficiency in this patient subgroup as well. Additionally, it would be worthwhile to look for similar *C4* deficiencies in long-surviving

melanoma patients, another clinical group for which autoimmune phenomena are associated with therapeutic response.[14]

In conclusion, a body of data is accumulating from numerous studies supporting a role for autoimmune mechanisms in the control of advanced RCC. As the mechanisms of human autoimmunity are gradually deciphered, these findings should translate into improved clinical management of this malignancy.

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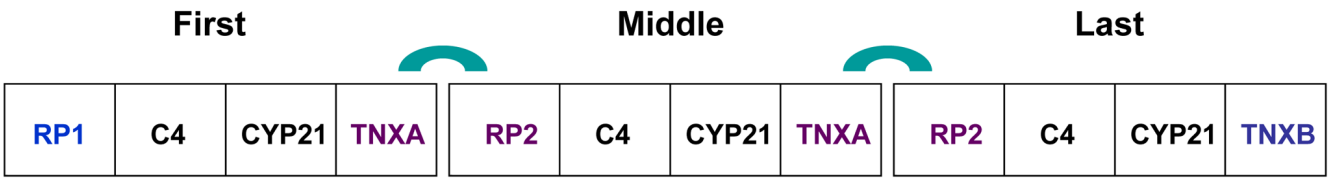

Figure 1.

Schematic diagram of the RCCX module. Each chromosome 6 can carry one, two, three, or (rarely) four consecutive modules. The *RP* gene of the first module is always the *RP1* allele; if additional RCCX modules are present, they are initiated by *RP2*. Similarly, the final RCCX module ends with *TNXB*, whereas the others carry *TNXA*. Components of the RCCX modules are not drawn to scale.

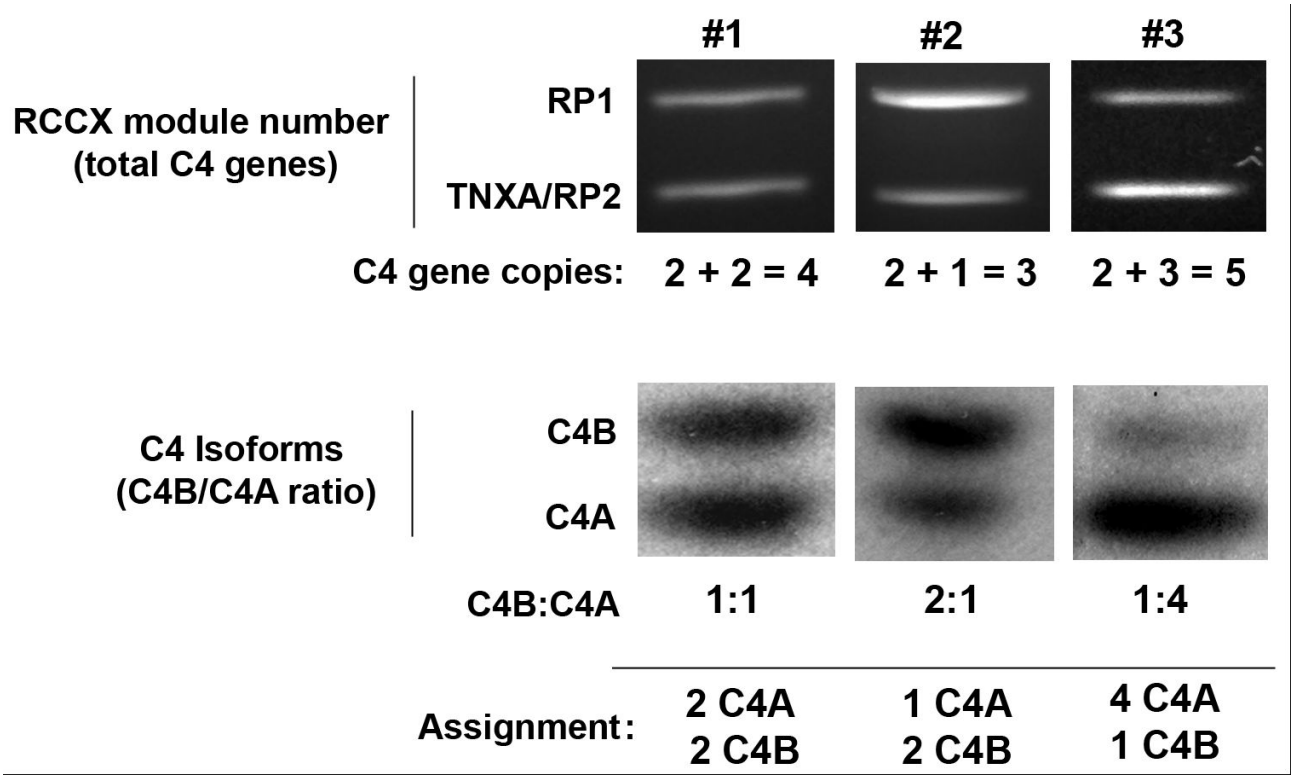

Figure 2.
Methodology for *C4* complotyping. Quantitation of RCCX modules yields the total *C4* copy number (top panel). After determining *C4B:C4A* ratios (bottom panel), the complete complotype can be ascertained. Data are shown for three individual patients.

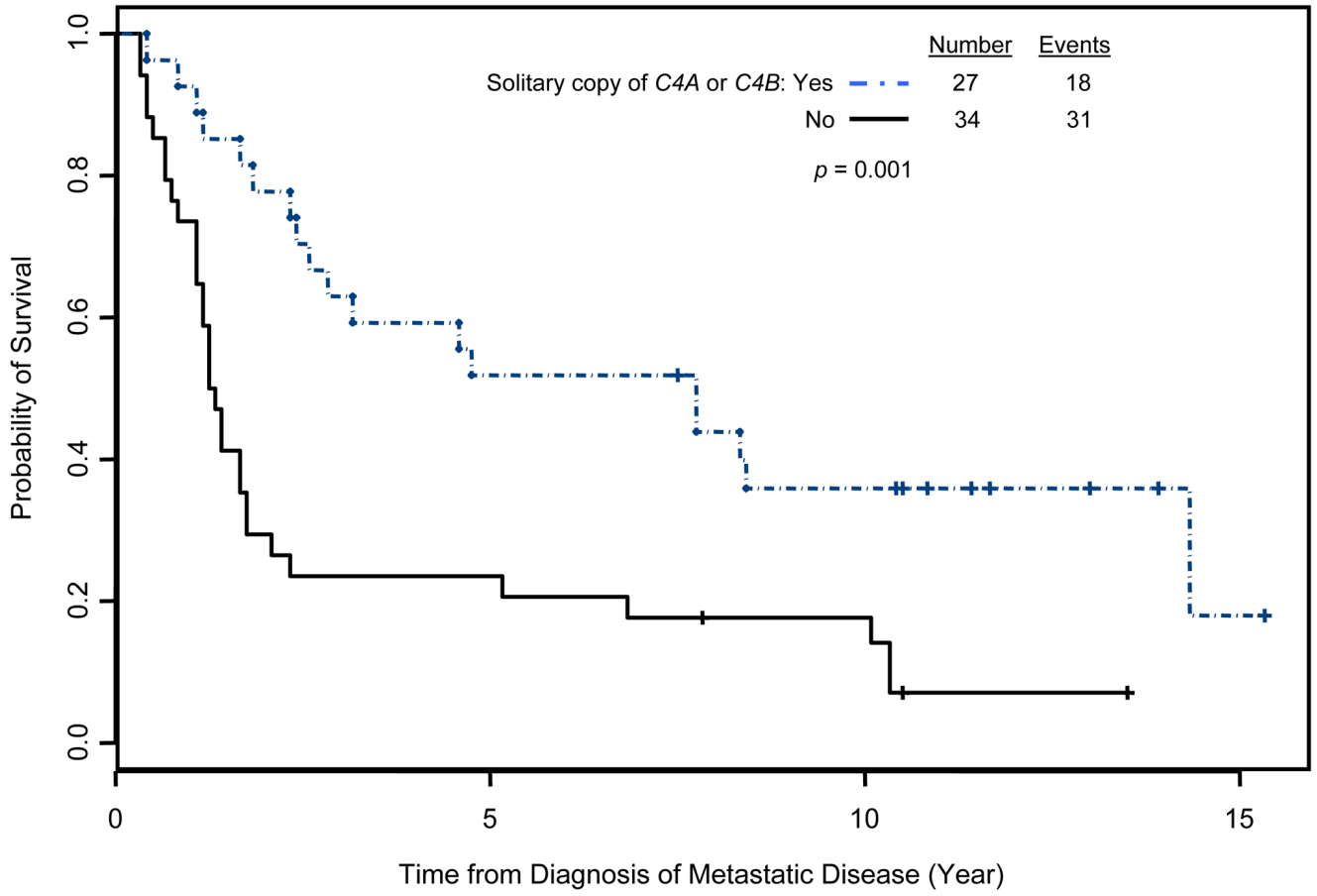

Figure 3.
Improved survival for RCC patients with a solitary copy of either *C4A* or *C4B*. Survival is measured from the diagnosis of Stage IV disease.

Table I

Characteristics of study population by group

		Group 1 Disease-Free *n* = 7		Group 2 Response/Relapse *n* = 11		Group 3 Stable Disease *n* = 10		Group 4 Disease Progression *n* = 33	
		n	**(%)**	***n***	**(%)**	***n***	**(%)**	***n***	**(%)**
Gender	Male	5	(71.4)	7	(63.6)	6	(60.0)	27	(81.8)
	Female	2	(28.6)	4	(36.4)	4	(40.0)	6	(18.2)
Race	Caucasian	7	(100.0)	8	(72.7)	8	(80.0)	29	(87.8)
	Hispanic	0	(0.0)	3	(27.3)	1	(10.0)	2	(6.1)
	Black	0	(0.0)	0	(0.0)	1	(10.0)	2	(6.1)
Stage IV Age (yrs)	Median	52		47		61.5		50	
	Range	40–70		40–70		35–74		30–65	
No. disease sites	1	4	(57.1)	10	(90.9)	6	(60.0)	14	(42.4)
	2	3	(42.9)	0	(0.0)	4	(40.0)	13	(39.4)
	≥3	0	(0.0)	1	(9.1)	0	(0.0)	6	(18.2)
Lung, lymph node only	yes	4	(57.1)	8	(72.7)	3	(30.0)	15	(45.5)
	no	3	(42.9)	3	(27.3)	7	(70.0)	18	(54.5)
Treatment	IFN alone	3	(42.9)	3	(27.3)	7	(70.0)	2	(6.1)
	IL-2 alone	0	(0.0)	0	(0.0)	0	(8.3)	2	(6.1)
	IL-2 + IFN	4	(57.1)	8	(72.7)	3	(30.0)	26	(78.8)
	IL2 + TNF	0	(0.0)	0	(0.0)	0	(0.0)	3	(9.0)
Stage IV Surv (mos)	Median	137		55		124		14.5	
	Range	94+–167+		4–162+		62–184+		4–57	

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Table II

Summary of complotyping data.

	N (%)	Median survival in years from Stage IV diagnosis (95%CI)	*p**
Total *C4* copy number			
≥ 4	46 (75.4)	1.67 (1.25, 2.83)	
< 4	15 (24.6)	7.75 (2.58, NA)	0.051
Solitary isoform copy			
No	34 (55.7)	1.25 (1.17, 1.75)	
Yes	27 (44.3)	7.75 (2.83, NA)	0.001

* Log-rank test. NA, not applicable; CI, confidence intervals

Table III

Association of isoform complotype with clinical group

		Number	Median survival in years from Stage IV diagnosis (range)	Number with solitary copy of either *C4* isoform (%)
Group	1	7	11.4+ (7.8+–13.9+)	6 (85.7)
	2	11	4.6 (0.3–13.5+)	5 (45.5)
	3	10	10.3 (5.2–15.3+)	6 (60.0)
	4	33	1.3 (0.3–4.8)	10 (30.3)

Table IV

Univariate and multivariate Cox proportional hazards regression models for survival from diagnosis of stage IV disease, incorporating the presence of autoimmune DQA1 alleles and solitary copies of the genes for *C4A* or *C4B*

		Univariate Model				Multivariate Model			
Variable	**n**	**HR**	**95% LL**	**95% UL**	***p*-value**	**HR**	**95% LL**	**95% UL**	***p*-value**
DQA1 alleles: both autoimmune									
No	46	1.00				1.00			
Yes	15	0.440	0.205	0.944	0.035	0.591	0.267	1.309	0.19
Solitary copy of C4A or C4B									
No	34	1.00				1.00			
Yes	27	0.373	0.205	0.681	0.0013	0.428	0.229	0.800	0.0078

Abbreviations: HR, hazard ratio; 95% LL, lower limit of the 95% confidence interval; 95% UL, upper limit of the 95% confidence interval