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ORIGINAL ARTICLE

Differences in the frequencies of HLA-class I and II alleles between German patients with renal cell carcinoma and healthy controls

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Abstract The human leukocyte antigen (HLA) system is a major part of the human immune system and has an impact on tumor initiation, tumor progression, and immunosurveillance. Renal cell carcinoma tumors are considered to be immunogenic. Therefore, we studied the allele frequencies of four gene loci (HLA-A, -B, -C, and HLA-DR) in a cohort of German renal cell carcinoma (RCC) patients and in healthy controls. HLA-A-C were determined using serological methods, whereas HLA-C12, C14, C16, C18, and HLA-DR were characterized through the use of standard molecular biological methods. The occurrence of the HLA-C*12 allele was significantly increased in German RCC patients compared with healthy controls ($P < 0.005$; Fisher's exact test), whereas the occurrence of the HLA-DRB1*04 allele was significantly reduced in RCC patients compared with healthy controls ($P < 0.05$; Fisher's exact test). However, the presence of allele HLA-C*12 was not significantly associated with 10 year overall survival. We suggest that the frequency of HLA alleles can affect development of RCC and could add knowledge as predictive marker for future immunotherapies.

Keywords Renal cell carcinoma · HLA system · Prognosis · Allele frequency

Abbreviations

CI	Confidence interval
HLA	Human leukocyte antigen
KIRs	Killer cell immunoglobulin-like receptors
MHC	Major histocompatibility complex
OS	Overall survival
PBMCs	Peripheral blood mononuclear cells
RCC	Renal cell carcinoma

Introduction

Renal cell carcinoma (RCC) is the ninth most frequent cancer worldwide, and there were approximately 338,000 new cases diagnosed in 2012 [1]. Tumors of RCC are unusual among solid tumors as they are considered to be immunogenic tumors that are sensitive to immune attack [2, 3]. In all vertebrates, the major histocompatibility complex (MHC) has a major impact on the adaptive immune response against pathogens but also against tumor cells. It is well known that the human leukocyte antigen (HLA) system, the human version of the MHC, plays a role in the tumorigenesis and progression of RCC [4, 5]. The HLA-complex located on chromosome 6 is comprised of more than 200 genes, and more than 40 of these genes encode leucocyte antigens (reviewed in [6]). The HLA genes and

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their corresponding phenotypes that are involved in the immune response can be divided in two structurally and functionally distinct classes: class I and class II. The class I genes, with the so-called classical representatives being HLA-A, -B, and -C, are expressed by nearly all somatic cells, but their expression levels are tissue-specific. The class II genes, with the most prominent representative being HLA-DR and with different families (DM, DO, DP, DQ, or DR) and chains (A, B), are normally expressed by a subgroup of immune cells, such as B cells, activated T cells, macrophages, dendritic cells, and thymic epithelial cells (reviewed in [6]). To date, there are few studies that have compared the HLA allele distributions of healthy controls and RCC patients. Studies were performed either based on serological (conventional) typing or on molecular biological typing.

Kuntz et al. reported a higher frequency of HLA-A30/31 and HLA-B8 in German RCC patients but an absence of HLA-B17 compared with healthy controls (Kuntz et al. [7]). Kantor and coworkers described increased frequencies of the antigens HLA-B44 and HLA-DR8, but a deficit of HLA-DR1 in American RCC patients compared with healthy controls [7]. Again, using the serological HLA typing procedure, an increased frequency of the DR8 antigen and a decreased frequency of the DR1 antigen have been shown in RCC patients [8]. Onishi et al. revealed that the HLA antigens B35, B48, B60, DR6, DR8, and DR9 were expressed at a significantly lower rate in RCC patients than in control subjects [9]. Ozdemir et al. compared Japanese RCC patients with healthy controls using PCR-RFLP and identified a higher number of HLA-DRB*0101 and HLA-DRB1*0405 alleles in healthy controls than in RCC patients [10]. RCC patients with HLA-DRB*0101 and HLA-DRB1*0405 alleles tended to have lower tumor stages and to have less aggressive tumors than patients not exhibiting these alleles [10]. Yilmaz and coworkers compared Caucasian RCC patients with a healthy control group. They found that the HLA-A10, B44, DQ1, and DR10 antigens were significantly higher in the control group than in the patient group, whereas the HLA-A23 and DQ7 antigens were significantly higher in the RCC patients than in the control group [11]. Ozgur et al. found a higher frequency of the HLA-A1, HLA-A26, and HLA-DR11 alleles in Turkish RCC patients than in the control group, and they also found increased frequencies of A29 and DQ1 in healthy controls compared with RCC patients [12]. These results suggest a role of HLA alleles as susceptibility genes for RCC, associated with elevated or reduced risk of disease as well as their properties as disease modifiers.

We focused on HLA-DRB1, because there are several reports that show either an increased frequency of allele variants in RCC or in healthy controls depending on the ethnic origin of the patients. In addition, we decided to study HLA-C, because HLA-C alleles have not previously been studied in this context and, therefore, have not yet been reported as having an altered frequency in RCC patients compared with healthy controls.

Materials and methods

Patients

All the patients were treated at the Department of Urology of the Martin-Luther-University Halle-Wittenberg between 1995 and 2005, and they all gave written informed consent. Blood samples were collected at routine diagnostic examinations. The study was performed in compliance with the Helsinki Declaration. The use of the blood samples for research was approved by the Ethics Commission from the Medical Faculty of the Martin-Luther-University Halle-Wittenberg. An update of the follow-up data for the RCC patients was conducted in 2014.

HLA typing

The HLA typing of the patients and that of the volunteers included in the control group were performed using peripheral blood samples stabilized with Na-citrate buffer. The HLA-class I serotype comprising the loci HLA-A, -B, and -C was defined using commercial microlymphocytotoxicity typing trays (HLA-ABC Histo Tray, BAG Healthcare, Lich, Germany). Initially, the peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation. After two washing steps with isotonic phosphate-buffer saline, PBMCs were adjusted to approximately 2000 cells per microliter. Then, the PBMCs were incubated with a set of defined and pre-dropped anti-HLA sera in the cavities of the typing tray for 30 min at room temperature. After this antigen–antibody reaction, rabbit serum was added as a source of complement, and the suspension was incubated for another 60 min at room temperature. The results were visualized by adding a mixture of two fluorochromes. Ethidium bromide resulted in nucleic red staining of lyzed/dead cells. Cell death was as a consequence of the classical pathway's complement reaction initially induced by the binding of the specific anti-HLA antibodies (positive reaction). Acridine orange resulted in green staining of the vital cells not permeabilized by complement components (negative reaction) because of the lack of the respective HLA antigen. For the analysis, the percentage of red-stained cells was visually calculated in each cavity using an inverted fluorescence microscope. The percentage of dead cells was scored according to the scoring system of the National Institutes of Health (NIH) [score 1: <10% (negative); score 2: 10–20% (doubtfully positive); score 4: 20–50% (weakly

Table 1 Clinico-pathological data

Parameters	No. of patients (%)
Total	106
Gender	
Male	68 (64.2)
Female	38 (35.8)
Age (years)	
Range	34–91
Mean	68.9
Median	71.0
Tumor stage	
pT1	46 (43.4)
pT2	9 (8.5)
pT3	42 (39.6)
pT4	2 (1.9)
Unknown	7 (6.6)
Tumor grade	
G1	12 (11.3)
G2	51 (48.1)
G3	34 (32.1)
Unknown	9 (8.5)
Follow-up	
Range (months)	1–120
Mean	68.4
Median	81.5
Death of any cause (10 years OS)	
Alive	65
Died of any cause	41

pT pathological tumor stage

positive); score 6: 50–80% (positive); score 8: >80% (strongly positive)].

Genotypes of the HLA-class II loci (HLA-DRB1*, -DRB3* to -DRB5*), for HLA-class I loci (HLA-C12, C14, C16, and C18), and in some cases, single HLA-class I loci were additionally characterized using commercial PCR-SSP assays (Innotrain, Kronberg, Germany; Protrans, Ketsch, Germany). For the HLA-class I loci, the serological equivalent to the molecular biological characterization (PCR-SSP assays) is given. For this purpose, genomic DNA was extracted from the nuclei of peripheral blood cells using the procedure of Miller et al. [13]. All kits were used according to the manufacturers' instructions without any modifications.

Statistical analysis

The differences between HLA allele frequencies in RCC patients and healthy controls were determined using the Fisher's exact test. For survival analyses, the overall survival (OS) was defined as the interval between diagnosis and death of the RCC patients or the last valid follow-up information. Statistical analyses of the associations between HLA-C*12 and prognosis were performed using Kaplan–Meier analysis (log-rank test). All calculations were performed using the SPSS 23.0 statistical package (SPSS-Science, Chicago, IL).

Results

Frequency of HLA-C and -DRB1 alleles

We studied the occurrence of 16 HLA-A, 30 HLA-B, 14 HLA-C, and 15 HLA-DRB1 alleles in 106 German RCC patients and 201 healthy controls (Table 1). There was no significant difference between the presence of HLA-A and HLA-B alleles in RCC patients compared to healthy controls (Tables 2, 3). One HLA-C allele, HLA-C*12, showed a significantly higher frequency in RCC patients than in healthy controls ($P < 0.005$; Fisher's exact test; Table 4). Furthermore, the HLA-DRB1*04 allele occurred more frequently in healthy controls than in RCC patients ($P < 0.05$; Fisher's exact test; Table 5), and allele C*07 exhibited an analogous distribution, although not significantly ($P < 0.06$). All other studied alleles of the gene loci HLA-C and HLA-DRB1 did not display any different distribution between the RCC patients and healthy controls (Tables 4, 5).

Table 2 Percentages of HLA-A* alleles in German RCC patients and healthy controls

Alleles	Control $n = 201$ in %	RCC $n = 106$ in %	Fisher's exact test	Pearson's χ^2 value
A*1	13.4	12.3	NS	0.167
A*2	30.1	34.4	NS	1.207
A*3	13.2	14.6	NS	0.243
A*11	7.2	5.7	NS	0.538
A*23	3.0	3.3	NS	0.046
A*24	8.7	8.5	NS	0.008
A*25	5.0	3.8	NS	0.460
A*26	3.2	2.8	NS	0.075
A*28	3.7	5.2	NS	0.727
A*29	2.5	1.9	NS	0.225
A*30	3.0	1.4	NS	1.435
A*31	2.7	1.4	NS	1.087
A*32	1.7	2.4	NS	0.276
A*33	1.5	1.9	NS	0.135
A*34	0.2	0.0	NS	0.528
A*66	0.7	0.5	NS	0.162

Table 3 Percentages of HLA-B* alleles in German RCC patients and healthy controls

Alleles	Control $n=201$ in %	RCC $n=106$ in %	Fisher's exact test	Pearson's χ^2 value
B*7	12.2	10.4	NS	0.460
B*8	9.0	7.5	NS	0.365
B*13	3.5	2.8	NS	0.192
B*14	1.2	1.4	NS	0.030
B*15	0.2	0.0	NS	0.530
B*18	5.2	4.2	NS	0.293
B*27	3.7	7.1	NS	3.314
B*35	12.0	12.7	NS	0.076
B*37	1.0	0.5	NS	0.474
B*38	1.7	3.3	NS	1.505
B*39	1.7	0.9	NS	0.617
B*40	0.2	0.5	NS	0.211
B*41	1.7	1.4	NS	0.094
B*44	13.7	14.6	NS	0.095
B*45	0.7	0.0	NS	1.594
B*47	0.2	0.0	NS	0.530
B*49	1.7	0.9	NS	0.617
B*50	1.5	1.4	NS	0.006
B*51	6.0	5.7	NS	0.026
B*52	1.2	0.5	NS	0.860
B*53	0.0	1.4	NS	5.702
B*55	1.0	0.9	NS	0.004
B*56	1.5	0.5	NS	1.290
B*57	5.0	3.3	NS	0.936
B*58	0.5	0.9	NS	0.423
B*60	6.0	7.1	NS	0.277
B*61	0.7	2.4	NS	2.792
B*62	6.5	6.6	NS	0.003
B*63	0.0	0.5	NS	1.895
B*75	0.0	0.5	NS	1.895

Survival analysis

Kaplan–Meier analysis was performed to study the association of the frequency of HLA-C*12 allele with 10 year OS. RCC patients without the HLA-C*12 allele had a mean 10 year OS of 87 months (95% CI 78–96 months), whereas those with the HLA-C*12 allele had a mean survival of 72 months (95% CI 36–107 months; log-rank test), but this difference of 15 months was not significant.

Discussion

We studied the frequencies of HLA-A, HLA-B, HLA-C, and HLA-DRB1 alleles in German RCC patients and healthy controls. Interestingly, the HLA-C*12 allele occurred more frequently in the RCC patients than in the healthy controls, whereas the HLA-DRB1*04 allele was detected at a higher frequency in the healthy controls than in the RCC patients. The frequency of single HLA-C alleles had not yet been studied in RCC patients. However, HLA-C*12 belongs to the C1 group of alleles with corresponding phenotypes recognized by killer cell immunoglobulin-like receptors (KIRs) KIR2DL2/3. HLA-C1 alleles have been reported to occur with significantly decreased frequency in kidney cancer patients representing ligands for KIR2DL2/3 [14]. Furthermore, upon treating metastasized RCC patients with natural killer cells from allogenic donors that were HLA-C matched, to enable HLA-C and KIR-mediated natural killer (NK) cell inhibition, one out of eleven patients has been reported to have an objective regression of lung metastases [15]. Moreover, studying NK activity in RCC patients with different HLA-C alleles, allogeneic NK cells were found to be more cytotoxic to tumor targets mismatched for KIR ligands than their KIR ligand-matched counterparts [16]. We did not find that an increased frequency of HLA-C*12 in RCC was associated with poorer OS. A reduced expression of HLA-A, HLA-B, HLA-C, and non-classical HLA-G was associated with poorer survival in esophageal cancer, whereas in gastric and colorectal cancer, the prognostic impact of these classical and non-classical HLA-class I antigens remains conflicting [17]. However, Goodyear et al. described CD8+ T-cell recognition of a cancer testis antigen peptide presented through HLA-Cw7 in patients with multiple myeloma that was associated with a better survival [18]. In general, an increased allele HLA-C*12 frequency in RCC patients compared to healthy controls cannot predict an association with poor prognosis in RCC patients and further studies are necessary to investigate the role of HLA-C*12 in RCC.

For a small group of metastasized RCC patients treated with cytokines, HLA-class II haplotypes (DRB1*0301/DQA1*0501/DQB1*0201 and DRB1*1501/DQA1*0102/DQB1*0602) predicted a favorable outcome [19]. However, these alleles are haplotypes known to be associated with autoimmune diseases, and the authors suggest autoimmune mechanisms of cytokine-induced anti-tumor activity in these patients [19]. An association between anti-tumor and autoimmune phenomena has also been suggested for treating patients with anti-CTLA antibodies [20]. A recent case report described a metastatic melanoma patient with HLA-C*06 (and the autoimmune disease psoriasis) who exhibited spontaneous regression of the metastasis [21].

Our finding of an increased frequency of HLA-DRB1*04 in healthy controls compared with German RCC patients is comparable with the report for this allele in a Japanese RCC cohort [10].

Why could it be important to study the frequency of HLA alleles? Several studies have shown that the

Table 4 Percentages of HLA-C* alleles in German RCC patients and healthy controls

Alleles	Control $n=170$ in %	RCC $n=97$ in %	Fisher's exact test	Pearson's χ^2 value	RR (95% CI)
C*1	5.0	2.6	NS	1.835	
C*2	5.9	9.8	NS	2.791	
C*3	16.2	16.5	NS	0.009	
C*4	15.3	19.6	NS	1.625	
C*5	9.1	7.7	NS	0.301	
C*6	11.8	8.8	NS	1.167	
C*7	33.8	25.8	NS	3.749	
C*8	1.2	1.5	NS	0.131	
C*12	0.6	4.6	*P < 0.005*[1]	10.047	2.3 (1.7–3.1)
C*14	0.0	1.5	NS	5.287	
C*15	0.3	0.0	NS	0.572	
C*16	0.3	0.5	NS	1.162	
C*17	0.6	0.5	NS	0.012	
C*18	0.0	0.5	NS	1.756	

The Bonferroni-adjusted threshold for significance is at $\alpha=0.0036$ for HLA-C*

NS not significant, *RR* relative risk

Significant value is in italics

[1] $p = 0.0004$ (C*12) The Bonferroni-adjusted threshold for significance is at $\alpha=0.0008$ for all HLA-class I alleles (HLA-A, -B, -C)

Table 5 Percentages of HLA-DRB1* alleles in German RCC patients and healthy controls

Alleles	Control $n=201$ in %	RCC $n=105$ in %	Fisher's exact test	Pearson's χ^2 value	RR (95% CI)
DRB1*01	11.7	13.3	NS	0.333	
DRB1*03	10.2	8.1	NS	0.727	
DRB1*04	15.0	9.0	*P < 0.05*[1]	4.283	0.7 (0.4–0.99)
DRB1*05	0.2	0.0	NS	0.525	
DRB1*06	0.2	0.0	NS	0.525	
DRB1*07	12.0	11.9	NS	0.001	
DRB1*08	2.2	3.8	NS	1.248	
DRB1*09	1.2	2.4	NS	1.101	
DRB1*10	0.7	0.5	NS	0.157	
DRB1*11	13.0	13.8	NS	0.085	
DRB1*12	2.7	2.4	NS	0.071	
DRB1*13	12.5	17.6	NS	2.994	
DRB1*14	2.7	2.4	NS	0.071	
DRB1*15	12.2	13.3	NS	0.155	
DRB1*16	3.2	1.4	NS	1.777	

Significant value is in italics

The Bonferroni-adjusted threshold for significance is at $\alpha=0.0033$ for HLA-DRB1*

[1] $p = 0.0015$ (DRB1*04)

occurrence of different HLA alleles may have either a predisposing effect for RCC or a protective effect against RCC. In their recent review article, Casey et al. suggested that the effectors of the immune system are involved in the mechanism of tumor regression upon targeted oncogene inactivation [22]. Targeted therapies are able to activate immune effectors, e.g., CD4+ T cells and CD8+ T cells, and/or influence MHC class I antigen presentation [22]. A treatment with anti-CTLA-4 antibodies has been shown to increase the HLA-DR expression on CD4+ and CD8+ T cells [20]. In addition, immunotherapies based on the blockade of immunosuppressive

signals, such as CTLA4 and PD-1/PD-L1, allow the immune system to regain control over the progression of various tumors [23]. In an initial phase 1 trial (IgG4 anti-PD-1 antibody) that exclusively included very late stage patients with progressive disease, cases with tumor regression, including mixed responses, partial responses, and a complete response, were observed in colon cancer, melanoma, lung cancer, and renal cancer. The observed courses of the diseases were also associated with significant increases in lymphocyte infiltration into metastatic tumor deposits [23]. Most interestingly, there are several current clinical studies based on PD-1 blockade in RCC (reviewed in [24]). Notably, a recent study reported the differential expression of PD-L1 between primary and metastatic sites in clear cell RCC [25] that may also affect response to therapy. Recently, Kostine et al. showed that PD-L1 expression positively correlated with other immune parameters, such as a high number of tumor-infiltrating lymphocytes ($P = 0.014$) and expression of HLA-class I antigens ($P = 0.024$) in chondrosarcoma [26]. Altogether, it would be of high interest to study the relationship between the response to targeted and/or immunotherapy and the occurrence of different HLA alleles in RCC patients.

Many studies have investigated the expression of HLA molecules in human tumors and have emphasized the importance of these molecules in immune surveillance. Patients with HLA-class I-positive RCC detected by immunohistochemical staining with an antibody (EMR8-5) directed against the heavy chains of all HLA-A, -B, and -C phenotypes showed longer recurrence-free survival than those with down-regulated expression [27]. Furthermore, in clear cell RCC, the down-regulation of HLA-class I expression is associated with tumor progression and poor prognosis [28]. The detection of HLA-class II expression in nine RCC specimens by immunohistochemical staining using an anti-HLA-DR alpha-chain antibody showed that all investigated samples revealed class II-positive tumor cells [29]. In this study, we suggest that the investigation of the frequency of HLA alleles may have a predictive value for RCC patients.

In summary, we detected an increased occurrence of the HLA-C*12 allele and a decreased frequency of HLA-DRB1*04 in German RCC patients compared with healthy controls. However, the occurrence of the HLA-C*12 allele was not associated with poorer prognosis of the RCC patients carrying this genotype.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

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