Association of Inherited Variation in Toll-Like Receptor Genes with Malignant Melanoma Susceptibility and Survival

Andreas Gast1,2, Justo Lorenzo Bermejo2, Rainer Claus3, Andreas Brandt1, Marianne Weires1, Alexander Weber4, Christoph Plass3, Antje Sucker5, Kari Hemminki1, Dirk Schadendorf5*, Rajiv Kumar1**

1 Division of Molecular Genetic Epidemiology, German Cancer Research Center, Heidelberg, Germany, 2 Institute of Medical Biometry and Informatics, University of Heidelberg, Heidelberg, Germany, 3 Department of Epigenomics and Cancer Risk Factors, German Cancer Research Center, Heidelberg, Germany, 4 Junior Research Group Toll-like Receptors and Cancer, German Cancer Research Center, Heidelberg, Germany, 5 Department of Dermatology, University Hospital Essen, Essen, Germany

Abstract

The family of Toll-like receptors (TLRs) is critical in linking innate and acquired immunity. Polymorphisms in the genes encoding TLRs have been associated with autoimmune diseases and cancer. We investigated the genetic variation of TLR genes and its potential impact on melanoma susceptibility and patient survival. The study included 763 cutaneous melanoma cases recruited in Germany and 736 matched controls that were genotyped for 47 single nucleotide polymorphisms (SNPs) in 8 TLR genes. The relationship between genotype, disease status and survival was investigated taking into account patient and tumor characteristics, and melanoma treatment. Analysis of 7 SNPs in TLR2, 7 SNPs in TLR3 and 8 SNPs in TLR4 showed statistically significant differences in distribution of inferred haplotypes between cases and controls. No individual polymorphism was associated with disease susceptibility except for the observed tendency for TLR2-rs3804099 (odds ratio OR = 1.15, 95% CI 0.99–1.34, p = 0.07) and TLR4-rs2149356 (OR = 0.85, 95% CI 0.73–1.00, p = 0.06). Both polymorphisms were part of the haplotypes associated with risk modulation. An improved overall survival (Hazard ratio HR 0.53, 95% CI 0.32–0.88) and survival following metastasis (HR 0.55, 95% CI 0.34–0.91) were observed in carriers of the variant allele (D299G) of TLR4-rs4986790. In addition various TLR2, TLR4 and TLR5 haplotypes were associated with increased overall survival. Our results point to a novel association between TLR gene variants and haplotypes with melanoma survival. Our data suggest a role for the D299G polymorphism in the TLR4 gene in overall survival and a potential link with systemic treatment at stage IV of the disease. The polymorphic amino acid residue, located in the ectodomain of TLR4, can have functional consequences.

Introduction

Malignant melanoma, with its propensity to metastasize and chemoresistance, remains a fatal neoplasm. Spontaneous regression, a phenomenon likely mediated by the immune system, is more common in melanoma than in most other cancers. The role of host immune system in melanoma is evidenced by the association of autoimmune conditions such as thyroiditis, vitiligo or the appearance of autoantibodies with an improved prognosis in patients treated with interleukin 2 and/or interferon α therapy [1,2,3,4]. Variations in genes involved in critical pathways of the immune system might therefore influence the susceptibility to autoimmune diseases and subsequently melanoma susceptibility, progression and prognosis.

Toll-like receptors (TLRs), the mammalian homologues of the Drosophila Toll protein, are perceived to be sensors for recognition of a number of invading pathogens. TLRs are critical in linking innate and acquired immunity and in serving as detectors of infectious pathogens and cancer debris [5,6,7]. TLRs belong to the family of pattern-recognition receptors (PRRs) which are expressed, among others, on antigen-presenting cells like dendritic cells or T-cells. After induction through pathogen-associated molecules, TLRs transduce signals via distinct intracellular pathways leading to the activation of transcription factors such as NFkB, interferon regulatory factors (IRFs) or AP-1. Finally these transcription receptors trigger inflammatory responses such as the release of inflammatory cytokines and type I interferons [7,8,9].

Polymorphisms in genes encoding TLR associated with infectious and non-infectious diseases, including autoimmunity and cancer, have been studied extensively [10,11,12,13]. However, the variants in the genes have not been investigated for association with either melanoma susceptibility or disease outcome. In order to investigate the effect of polymorphisms in the TLR genes on melanoma susceptibility and survival, we genotyped 47 single nucleotide polymorphisms (SNP) in eight TLR genes in 763 cases from Germany and 736 ethnically matched controls.
Results

The two multiplex reactions contained 373 replicates from different SNPs that showed a concordance rate over >99%. The rate of undetermined samples by the Sequenom procedure was between 6 and 33% of the individual SNPs. Genotype distributions of all polymorphisms in controls did not show statistically significant deviation from Hardy-Weinberg equilibrium.

Out of 47 TLR SNPs analyzed by Sequenom mass spectrometry TLR1-rs3923647 and TLR1-rs5743613, TLR2-rs5743704 and -rs5743708, TLR4-rs11536869 and -rs11536897 and TLR6-rs5743815 had minor allele frequencies <0.05; no carrier of variant allele for TLR9-rs5743846 was detected. In total, the genotyping results of 47 SNPs represent 99 polymorphisms in the selected TLR genes.

Case-control study

We did not observe any differences in the distribution of allele and genotype frequencies between melanoma cases and controls (Table S1, online only). The variant allele for TLR2-rs3804099 was associated with an increased risk (OR = 1.15, 95% CI 0.99–1.34, p = 0.07) and for TLR4-rs2149356 with a decreased risk (OR = 0.85, 95% CI 0.73–1.00, p = 0.06) of melanoma. However, for both, the association was not statistically significant.

Haplotype analysis

Analysis of 7 SNPs in TLR2, 7 SNPs in TLR3 and 8 SNPs in TLR4 showed statistically significant differences in distribution of inferred haplotypes between cases and controls (Table 1). The haplotype A-C-C-C-C-G-T of the TLR2 gene locus was associated with an increased risk compared to the consensus haplotype A-C-C-C-T-G-T (OR 1.31, 95%CI 1.03–1.65). This haplotype showed statistically significant differences in distribution of allele and genotype frequencies between cases and controls (Table 1). The variant allele for TLR4-rs2149356 with a decreased risk (OR = 0.85, 95% CI 0.73–1.00, p = 0.06) of melanoma. However, for both, the association was not statistically significant.

Polymorphisms in TLR genes and survival

Differences in survival according to genotype were investigated based on 622 patients with melanoma of the skin with known primary at first diagnosis without detectable metastasis (stage I/II) (339 males and 283 females out of 763 patients in total).

The OS from primary diagnosis was better in carriers of the TLR4-rs4986790 polymorphism than among non-carriers (n = 536). The median OS in carriers was 18.1 years (95% CI 12.1–24.4) compared to 11.0 years (95% CI 9.5–13.1) for non-carrier patients (log-rank p = 0.001, Table 2). The stratification for therapy showed that the HR was 0.41 (95% CI 0.24–0.69, p = 0.001) among patients receiving any therapy, compared to HR 0.35 (95% CI 0.05–2.59, p = 0.30) for patients that did not receive any therapy. The statistical non-significance in the latter could be attributed to small patient numbers.

Table 1. Distribution of the most common haplotypes within TLR2, TLR3 and TLR4 in German melanoma patients and German controls.

| Gene | Haplotype | Cases (%) | Controls (%) | OR (95% CI) | P |
|------|-----------|-----------|--------------|-------------|---|
| TLR2 | A-C-C-C-T-G-T | 197 (12.9) | 220 (15.0) | Reference | 1.0 |
|      | T-C-A-C-T-G-T | 491 (32.3) | 496 (33.8) | 1.11 (0.88–1.39) | |
|      | A-C-C-C-C-G-T | 457 (30.0) | 391 (26.7) | 1.31 (1.03–1.65) | |
|      | T-T-C-C-C-G-T | 140 (9.2) | 141 (9.6) | 1.11 (0.82–1.50) | |
|      | A-C-C-C-C-C-G-T | 48 (3.2) | 51 (3.5) | 1.05 (0.68–1.63) | |
|      | T-C-A-C-T-A-T | 38 (2.5) | 33 (2.2) | 1.29 (0.78–2.13) | |
|      | T-C-C-C-T-G-T | 29 (1.9) | 24 (1.6) | 1.35 (0.76–2.40) | |
|      | T-T-C-C-T-G-T | 12 (0.8) | 11 (0.7) | 1.22 (0.53–2.82) | |
|      | other | 16 (1.0) | 15 (1.0) | Reference | – |
| TLR3 | C-G-A-C-C-C-A | 268 (17.6) | 256 (17.5) | Reference | 0.94 |
|      | C-A-G-C-C-G-G | 240 (15.8) | 211 (14.4) | 1.09 (0.84–1.40) | |
|      | C-G-G-T-A-C-G | 205 (13.5) | 187 (12.8) | 1.05 (0.81–1.36) | |
|      | C-G-G-C-A-G-C | 153 (10.1) | 176 (12.2) | 0.83 (0.63–1.09) | |
|      | T-G-G-C-C-C-G | 215 (14.2) | 175 (12) | 1.17 (0.90–1.53) | |
|      | C-G-G-C-C-A-A | 123 (8.1) | 128 (8.7) | 0.92 (0.68–1.24) | |
|      | C-G-G-C-C-G-G | 91 (6.0) | 77 (5.3) | 1.13 (0.80–1.60) | |
|      | C-G-G-C-C-G-C | 71 (4.7) | 75 (5.1) | 0.90 (0.63–1.31) | |
|      | C-G-G-T-A-G-G-G | 27 (1.8) | 45 (3.1) | 0.57 (0.35–0.95) | |
|      | C-G-G-C-C-G-G | 44 (2.9) | 39 (2.7) | 1.27 (0.79–2.06) | |
|      | C-G-G-C-C-G-A | 16 (1.1) | 20 (1.4) | 0.76 (0.39–1.51) | |
|      | T-G-G-C-C-C-A | 13 (0.9) | 14 (1.0) | 0.89 (0.41–1.92) | |
|      | C-G-G-C-C-G-A | 16 (1.1) | 13 (0.9) | 1.18 (0.55–2.49) | |
|      | other | 37 (2.4) | 53 (3.6) | Reference | – |
| TLR4 | A-T-C-A-A-G-G-G | 457 (30) | 405 (27.5) | Reference | 0.95 |
|      | A-C-C-G-A-G-G-G | 371 (24.4) | 357 (24.3) | 0.92 (0.76–1.12) | |
|      | A-T-A-G-A-G-G-C | 192 (12.6) | 237 (16.1) | 0.72 (0.57–0.91) | |
|      | A-C-C-G-A-G-G-T | 209 (13.7) | 215 (14.6) | 0.86 (0.68–1.09) | |
|      | A-T-A-G-G-G-G-C | 85 (5.6) | 77 (5.2) | 0.98 (0.73–1.37) | |
|      | G-T-A-A-G-G-G-C | 43 (2.8) | 52 (3.5) | 0.73 (0.48–1.12) | |
|      | A-T-A-G-A-G-G-T | 60 (3.9) | 40 (2.7) | 1.33 (0.87–2.03) | |
|      | A-T-A-G-A-G-A-C | 50 (3.3) | 39 (2.6) | 1.14 (0.73–1.76) | |
|      | A-C-A-G-A-G-G-G | 46 (3.0) | 40 (2.7) | 0.71 (0.28–1.81) | |

The TLR4-rs4986790 polymorphism was also associated with survival time following first metastasis (SFM). Carriers of the minor allele displayed a median SFM of 9.5 years (95% CI 3.3–11.1) compared to 2.8 years (95% CI 2.1–3.5) for non-carrier patients (log-rank p = 0.01, Figure 1B and Table 2). The effect was reflected in the Cox regression model with a HR of 0.55 (95% CI 0.33–0.91, p = 0.02) and adjustment for any therapy slightly decreased the estimate (HR 0.44, 95% CI 0.27–0.73, p = 0.001, Table 2). The stratification for therapy showed a statistically significant effect (HR 0.39, 95% CI 0.23–0.66, p = 0.001) only in patients that received therapy and not in patient that did not
receive any therapy (HR 0.62, 95% CI 0.08–4.86, p = 0.65). The TLR4-rs4986790 polymorphism did not associate with time from primary diagnosis to first metastasis (Table 2).

Figure 1. Overall survival (OS) and survival after metastasis (SFM) in German cutaneous melanoma patients according to the TLR4-rs4986790 (D299G) genotype. The Kaplan-Meier curves differentiate between carriers of the minor allele (AG+GG) and homozygote genotypes (AA) for (A) overall survival, log-rank P = 0.005 and (B) survival following the first metastasis, log-rank P = 0.01. Number of patients, both carriers and non-carriers, is given below the x-axis.

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Carriers of the variant allele of TLR2-rs3804100 (TC/CC) also showed a reduced OS (HR 0.55, 95% CI 0.29–1.05, p = 0.07). Adjustment for any therapy at any clinical stage did not show an
effect (HR 0.52, 95% CI 0.27–1.01, p = 0.05). We also evaluated the possible interaction between polymorphisms TLR4-rs4986790 and TLR2-rs3804100 regarding OS. Patients with the commonest genotype (rs4986790 = AA, rs3804100 = TT) showed the poorest prognosis. Among 13 patients with rs4986790 = AG and rs3804100 = TC/CC, no death was observed ten years after diagnosis. The survival heterogeneity among the four categories of individuals resulted in a probability value from the log-rank test of 0.01, which increased to 0.03 after inclusion of age, gender and Breslow thickness in a subsequent Cox regression. Other investigated polymorphisms did not associate with OS, SFM or MFS.

### Discussion

TLRs, the mammalian homologues of Drosophila Toll protein, play a role as agents of innate and adaptive immunity in various diseases. We assumed that genetic polymorphisms in various TLRs were functional and influenced melanoma susceptibility and disease outcome. Our data showed that melanoma patients carrying the variant allele for the rs4986790 polymorphism in the TLR4 gene were associated with a prolonged OS. The increase in survival was confined to the period after the detection of a first metastasis, which implied that the effect of the polymorphism might possibly modulated dependent on therapy or alternatively by biological processes associated with melanoma cell migration and/or invasion. The patients in stage IV melanoma in most instances received a chemotherapy containing DTIC/temozolomide and interferons, which is known for long time not having any clinical effect on overall survival of the entire metastatic melanoma population [14,15].

One can speculate that the prolonged survival after first metastasis, which clinically is in most cases regionally spread, is mediated by more efficacious tumor control at the lymph node level and by interaction with the immune system. TLR4-rs4986790 polymorphism results in a change of amino acid D299G and it co-segregates with a T399I exchange, which are both located in the ectodomain of TLR4 [16]. The ectodomain of TLR4 is responsible for detecting of lipopolysaccharides from Gram negative bacteria in cooperation with an accessory molecule, myeloid differentiation factor 2 (MD-2). MD-2 forms a 1:1 complex with lipopolysaccharides, which is detected by the ectodomain of TLR4 [16]. Interestingly, the amino acid residues 299 and 399 of TLR4 gene were associated with a prolonged OS. The increase in survival was confined to the period after the detection of a first metastasis, which implied that the effect of the polymorphism might possibly modulated dependent on therapy or alternatively by biological processes associated with melanoma cell migration and/or invasion. The patients in stage IV melanoma in most instances received a chemotherapy containing DTIC/temozolomide and interferons, which is known for long time not having any clinical effect on overall survival of the entire metastatic melanoma population [14,15].

### Table 2. TLR4-rs4986790 genotype and overall survival (OS), survival following the first metastasis (SFM) and metastasis free survival (MFS).

| Genotype | Individuals | Median survival in years (95% CI) | Uncensored events (%) | Hazard Ratio (95% CI) | P |
|----------|-------------|-----------------------------------|-----------------------|-----------------------|---|
| OS       | AA          | 536                                | 11.0 (9.5–12.9)       | 161 (30.0)            | Reference |
|          | AG          | 81                                 | 18.1 (12.1–24.4)      | 19 (23.5)             | 0.53 (0.32–0.88) | 0.01 |
|          |             |                                    |                       | 0.53 (0.32–0.87)      | 0.01 |
| MFS      | AA          | 536                                | 7.2 (5.5–8.3)         | 239 (44.6)            | Reference |
|          | AG          | 81                                 | 10.6 (3.2–20.3)       | 39 (48.1)             | 0.81 (0.55–1.18) | 0.27 |
| SFM      | AA          | 222                                | 2.8 (2.1–3.5)         | 144 (64.9)            | Reference |
|          | AG          | 39                                 | 7.1 (3.3–11.1)        | 19 (46.2)             | 0.55 (0.33–0.91) | 0.02 |
|          |             |                                    |                       | 0.44 (0.27–0.73)      | 0.001 |

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**TLR Haplotype associations with survival time following first metastasis**

The TLR2 haplotype T-T-C-C-G-G-T (9.6%) was associated with an increased SFM compared to the common haplotype (HR 1.54 95% CI 1.04–2.28). Other significant associations with decreased SFM were seen for TLR4- A-T-A-G-G-A-G-T and TLR4-G-T-G-A-G-G-G-T (HR 1.39, 95% CI 1.03–1.88). Other associations of TLR haplotypes with MFS were not statistically significant.
and TLR signaling pathway genes found significant association between two polymorphisms with prostate cancer mortality that did not include \( TLR4 \)-rs4986790 [20]. Thus, even if modulated HMGB-1 signaling might be due to the effects of \( TLR4 \)-rs4986790, the outcome in terms of OS is difficult to predict as the TLR ligation can have pleiotropic effects on cellular differentiation and growth.

With the exception of two polymorphisms, \( TLR2 \)-rs3804099 and \( TLR4 \)-rs2149356, none other of the 47 variants in eight \( TLR \) genes included in this study showed association with risk of melanoma in comparison to the healthy controls. The variant alleles of the two polymorphisms showed a tendency towards association with susceptibility but not strong enough to rule out a chance finding. Nevertheless, previous reports have shown associations of the rs3804099 polymorphism in the \( TLR2 \) gene with sepsis in preterm infants [21]. It was also associated with reversal reaction in leprosy and tuberculosis in Ethiopian and Vietnamese populations [22,23]. On the other hand no association of this SNP could be found with normal tension glaucoma in a Japanese population or asthma in a German and a mixed German/Austrian population or type I diabetes mellitus in a Basque population [24,25,26,27]. Since the polymorphism is synonymous (N199N) and not linked with any other variant in the gene, the association with various diseases could be due to linkage with other SNPs outside the \( TLR2 \) gene region. The most commonly discussed polymorphisms in \( TLR2 \), the R677W and R753Q, have been shown to be associated, amongst other diseases, with leprosy, tuberculosis, staphylococcal infections, coronary restenosis and sepsis [11,12]. In our study the R677W could not be included due to immeasurable genotype, probably caused by a pseudogene located upstream of the true gene [28]. The R753Q variant was rather rare and did not show any association with melanoma susceptibility.

The variant allele of \( TLR4 \)-rs2149356, exhibited association with decreased risk of melanoma that was not statistically significant. However, the haplotype containing the same variant allele was associated with statistically significant decreased risk. \( TLR4 \)-rs2149356 is located in intron 2 of the gene and is linked with four other polymorphisms at the locus. One of which is located in intron 1 (rs1927911) and three (rs10116253, rs2737190, rs1927914) in the 5’ end of the gene. Therefore it is possible that SNPs in the 5’ end could possibly effect transcriptional regulation. In previous studies variants in the \( TLR4 \) gene have been shown to be associated with infectious and non-infectious diseases but rather inconsistently [10,29,30,31].

Table 3. Overall survival according to the commonest \( TLR2 \), \( TLR4 \) and \( TLR5 \) haplotypes.

| Gene | Haplotype | Frequency (%) | Uncensored events (%) | HR (95% CI) | \( P \) |
|------|-----------|--------------|-----------------------|-------------|--------|
| \( TLR2 \) | A-C-C-C-T-G-T | 121 (11.2) | 37 (3.4) | Reference | 0.11 |
| T-C-A-C-T-G-T | 367 (33.9) | 105 (9.7) | 0.86 (0.60–1.24) | | |
| A-C-C-C-G-T | 345 (31.9) | 100 (9.2) | 0.84 (0.58–1.24) | | |
| T-F-C-C-G-T | 104 (9.6) | 32 (3.0) | 1.07 (0.67–1.73) | | |
| A-C-C-C-C-G-C | 59 (5.5) | 9 (0.8) | 0.46 (0.22–0.95) | | |
| A-C-C-A-T-G-T | 33 (3.0) | 8 (0.7) | 0.62 (0.28–1.36) | | |
| T-C-A-C-T-A-T | 20 (1.8) | 1 (0.1) | 0.24 (0.03–1.78) | | |
| T-C-C-C-T-G-T | 20 (1.8) | 4 (0.4) | 0.58 (0.21–1.65) | | |
| other | 13 (1.2) | 6 (0.6) | — | — | — |
| \( TLR4 \) | A-T-C-A-A-G-G-T | 323 (29.9) | 82 (7.6) | Reference | 0.12 |
| A-C-C-G-A-G-G-T | 268 (24.8) | 85 (7.9) | 1.09 (0.77–1.53) | | |
| A-T-A-G-A-G-G-G | 150 (13.9) | 47 (4.3) | 1.40 (0.95–2.06) | | |
| A-C-C-G-A-C-G-T | 139 (12.9) | 35 (3.2) | 1.01 (0.67–1.50) | | |
| A-T-A-G-G-G-G-C | 68 (6.3) | 17 (1.6) | 0.60 (0.35–1.04) | | |
| A-T-A-G-A-G-G-T | 40 (3.7) | 16 (1.5) | 1.77 (1.02–3.08) | | |
| A-T-A-G-A-A-C | 32 (3.0) | 5 (0.5) | 0.44 (0.18–1.10) | | |
| G-T-A-G-A-G-G-T | 29 (2.7) | 9 (0.8) | 1.73 (0.83–3.60) | | |
| other | 33 (3.1) | 6 (0.6) | — | — | — |
| \( TLR5 \) | C-T-G-C | 241 (22.3) | 61 (5.7) | Reference | 0.32 |
| C-T-C-C | 395 (36.6) | 108 (10.0) | 1.18 (0.85–1.63) | | |
| C-G-G-C | 287 (26.6) | 86 (8.0) | 1.33 (0.91–1.93) | | |
| T-T-G-C | 96 (8.9) | 35 (3.2) | 1.60 (1.05–2.43) | | |
| C-T-C-T | 53 (4.9) | 12 (1.1) | 0.96 (0.51–1.83) | | |
| other | 8 (0.7) | 1 (0.1) | — | — | — |

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In conclusion, on the role of variants in eight TLR genes in malignant melanoma show an association of one variant in the TLR4 gene with the disease survival. Though we did not find any clear association for any polymorphism with the disease susceptibility, for two SNPs we did find a tendency for such an association that also extended into a frequent haplotype in TLR4 gene.

Materials and Methods

Patients and controls

763 patients of German origin (418 male, 345 female) with cutaneous melanoma were recruited at the Skin Cancer Unit at the Mannheim University Hospital, Germany, according to eligibility criteria that included histologically confirmed melanoma of the skin; AJCC stage 0 (in situ melanoma, 10), I (253), II (112) and IV (11) disease and 19 tumors had an unknown stage (Table 4). Median and mean age of the melanoma cases at diagnosis was 55 and 54.1 years, respectively. Mean Breslow thickness was 2.14 mm (95% confidence interval 1.97 to 2.31 mm).

Table 4. Characteristics of the German patients included in the study.

|                      | All patients | Patients with AJCC stage 0, I or II at first diagnosis (FD) |
|----------------------|--------------|-------------------------------------------------------------|
| No.                  | 763          | 622                                                         |
| Gender               |              |                                                             |
| Male                 | 418 (54.8%)  | 341 (38.8%)                                                 |
| Female               | 345 (45.2%)  | 281 (45.2%)                                                 |
| Age at first diagnosis(FD) |          |                                                             |
| Median               | 55           | 54                                                          |
| Mean                 | 54.1         | 54.9                                                        |
| Breslow thickness (mm) |            |                                                             |
| Mean (95% CI)        | 2.14 (1.97–2.31) | 1.81 (1.66–1.96)                                           |
| Ulceration of primary tumor |         |                                                             |
| Yes                  | 130 (17.0%)  | 92 (14.8%)                                                  |
| No                   | 185 (24.3%)  | 159 (25.6%)                                                 |
| unknown              | 448 (58.7%)  | 371 (59.7%)                                                 |
| AJCC stage at FD     |              |                                                             |
| 0                    | 10 (1.3%)    | 10 (1.6%)                                                   |
| I                    | 396 (51.9%)  | 396 (63.7%)                                                 |
| II                   | 216 (28.3%)  | 216 (34.7%)                                                 |
| III                  | 111 (14.6%)  | –                                                           |
| IV                   | 11 (1.4%)    | –                                                           |
| unknown              | 19 (2.5%)    | –                                                           |
| Metastasized during follow-up |      |                                                             |
| Yes                  | 237 (31.1%)  | 237 (38.1%)                                                 |
| No                   | 170 (22.3%)  | 127 (20.4%)                                                 |
| Deceased during follow-up |          |                                                             |
| No therapy           | 408 (53.5%)  | 364 (58.5%)                                                 |
| Any therapy          | 353 (46.3%)  | 258 (41.5%)                                                 |
| Chemotherapy         | 28 (3.7%)    | 17 (2.7%)                                                   |
| Immunotherapy        | 129 (16.9%)  | 98 (15.8%)                                                   |
| All other therapy combinations | 199 (26.1%)  | 143 (23.0%)                                                 |

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Blood samples from case subjects were taken during the first appointment at the Skin Cancer Unit. Controls included 736 healthy individuals (368 male and 368 female) recruited at the Institute of Transfusion Medicine and Immunology in Mannheim, Germany. Median and mean age of the controls were 61 and 60.3 years, respectively. The ethical approval for the study was granted by Ethics Commission of the Faculty for Clinical Medicine of Ruprecht-Karls-Universität, Heidelberg and written informed consent was obtained from all study participants. DNA was extracted from blood samples using Qiagen mini-preparation kits.

Selection of SNPs

Selection of SNPs in eight human TLR genes was done by inclusion of known non-synonymous SNPs, those in regulatory regions or reported by other investigators. Additionally tagging SNPs for each gene region were selected from HapMap data using the criteria, a) exclusion of individuals with >50% missing genotypes, b) pair-wise r2>0.8 for each SNP pair and c) minor allele frequencies >5%. Of the eight TLR genes, three were located in a gene cluster on chromosome 4p14 (TLR6-TLR1- TLR10). For tagging analysis, the region from rs10024216 to rs6351668 (69.5 kb) was selected (Figure S1, online only). TLR2 and TLR3 are located on chromosome arm 4q; these two genes were about 32 Mb apart from each other and were not linked. TLR4 is on chromosome 9q23; TLR5 on 1q41 and TLR9 on 3p21. In total we selected 47 SNPs that were informative for 99 polymorphisms in 8 genes. The selected SNPs were grouped into 2 multiplex assays (W1 and W2) for the Sequenom mass array platform (Sequenom, San Diego, CA, USA, Table S2 and S3, online only). In addition, the TLR1-rs4986790 (D299G) SNP was selected for genotyping using an allelic discrimination method.

Genotyping

Genotyping was carried out with the Sequenom method. In two multiplex PCR (with the 25 or 23 primer pairs, respectively) DNA fragments with SNPs were amplified using 10 ng DNA as template in 5 µl volume. Genotype analysis was performed by the Sequenom TYPER 4.0 software. Validation of genotype data for SNPs that showed statistically significant association (TLR2-rs3804999 and TLR1-rs2149356) was done by allelic discrimination method and DNA sequencing.

Statistical analysis

All statistical calculations were carried out using SAS version 9.2 (SAS Institute, Cary, NC, USA). Odds ratios (OR) with the corresponding 95% confidence intervals (95% CI) for assessment of the association between risk and genotype were based on logistic regression adjusted for age, gender and Breslow thickness. The haplotype procedure of SAS genetics was used to estimate haplotype frequencies in cases and controls separately, and to infer possible haplotype combinations for each individual. The evidence of association between genotype/haplotype and melanoma risk were summarized by probability values.

Overall survival (OS) was defined as the time (in years) from date of first diagnosis until death or last patient contact. Metastasis free survival (MFS) was the time from diagnosis of primary melanoma until the first metastasis (either regional or distant). Survival following first metastasis (SFM) was defined as the time from first metastasis to death or last patient contact. Alive patients at the latest visit/contact were considered censored. Univariate survival curves were based on the Kaplan-Meier method and statistical significance was quantified by log-rank tests. Genotype-specific survival differences were adjusted for age, gender and Breslow thickness, based on a proportional hazard regression.
(Cox) model for each of the polymorphisms. Ulceration status was not included in the multivariate analysis due to unavailability of complete data. Subsequently, the analysis of the potential association between genotype and survival was adjusted for therapy administered in stage IV. We considered the start time and duration of therapy, treating therapy as a time-dependent covariate. The possible interaction between therapy and genotype was explored by including the interaction in the Cox regression and, additionally, by stratification of patients into therapy groups.

Association of haplotypes with survival time was carried out by Cox regression. Thereby, we assumed that individuals carry the most likely combination of possible haplotypes and that the effects of haplotypes interact multiplicatively. Hazard ratios (HR) were calculated taking the consensus haplotype as reference. If the frequency of the consensus haplotype was low, the most common haplotype was taken as reference.

**Supporting Information**

**Figure S1** Linkage disequilibrium (LD) plot of the 69-kb TLR6-TLR1-TLR10 gene cluster on chromosome 4p14. The plot was drawn using Haploview software 4.2 and based on HapMap homepage. It shows r² values, the higher r², the darker the box. LD blocks are depicted as triangles. Positions of the TLR genes and SNPs are shown in the upper part of the figure.

**Table S1** Genotype distributions of TLR SNPs in malignant melanoma of the skin in patients and controls.

**Table S2** Toll-like receptor genes and polymorphisms.

**Table S3** List of iplex primer and masses.

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**Author Contributions**

Conceived and designed the experiments: AG KH DS RK. Performed the experiments: AG RC AS. Analyzed the data: AG JL AB MW AW RK. Contributed reagents/materials/analysis tools: AG CP AS KH DS RK. Wrote the paper: AG RK AW KH DS RK.

**References**

1. Kalialis LV, Drzewiecki KT, Klyver H (2009) Spontaneous regression of metastases from melanoma: review of the literature. Melanoma Res 19: 275–282.

2. Atkins MB, Mier JW, Parkinson DR, Gedda JA, Berkman EM, et al. (1988) Hypothyroidism after treatment with interleukin-2 and lymphokine-activated killer cells. N Engl J Med 318: 1557–1563.

3. Gogas H, Ioannovich J, Dafni U, Stavropoulou-Giskas C, Frangka K, et al. (2006) Prognostic significance of autoimmunity during treatment of melanoma with interferon. N Engl J Med 354: 799–798.

4. Phan GQ, Attia P, Steinberg SM, White DE, Rosenberg SA (2001) Factors associated with response to high-dose interleukin-2 in patients with metastatic melanoma. J Clin Oncol 19: 3477–3483.

5. Akira S, Takeda K, Kaiso T (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2: 675–680.

6. Rallabhandi P, Bell J, Boukhvalova MS, Medvedev A, Lorenz E, et al. (2006) Polymorphisms in Toll-like receptor genes and risk of cancer. Oncogene 27: 244–252.

7. Atkins MB, Mier JW, Parkinson DR, Gedda JA, Berkman EM, et al. (1988) Hypothyroidism after treatment with interleukin-2 and lymphokine-activated killer cells. N Engl J Med 318: 1557–1563.

8. Kumar H, Kawai T, Akira S (2009) Toll-like receptors and innate immunity. Adv Exp Med Biol 560: 11–18.

9. Wang RF, Miyahara Y, Wang HY (2008) Toll-like receptors and immune regulation: implications for cancer therapy. Oncogene 27: 181–189.

10. Misch EA, Hawn TR (2008) Toll-like receptor polymorphisms and susceptibility to human disease. Clin Sci (Lond) 114: 347–360.

11. Schwartz DA, Cook DN (2005) Polymorphisms of the Toll-like receptors and human disease. Clin Infect Dis 41 Suppl 7: S403–407.

12. Malhotra D, Relhan V, Reddy BS, Bamezai R (2005) TLR2 Arg677Trp polymorphism in human TLR2 is associated with increased susceptibility to tuberculosis meningitis. Genes Immun 6: 422–428.

13. El-Omar EM, Ng MT, Holden GL (2008) Polymorphisms in Toll-like receptor genes and risk of cancer. Oncogene 27: 244–252.

14. Thuong NT, Hawn TR, Thwaites GE, Chau TT, Lam NT, et al. (2007) A polymorphism in human TLR2 is associated with increased susceptibility to tuberculosis meningitis. Genes Immun 6: 422–428.

15. Nakamura J, Meguro A, Ota M, Nomura E, Nishide T, et al. (2009) Association of toll-like receptor 2 gene polymorphisms with type I diabetes mellitus in the Basque population. Ann N Y Acad Sci 1079: 268–272.

16. Bochud PY, Hawn TR, Siddiqui MR, Sauderson P, Britton S, et al. (2008) Toll-like receptor 2 (TLR2) polymorphisms are associated with reversal reaction in leprosy. J Infect Dis 197: 253–261.

17. Wang HY, Wang RF (2008) Toll-like receptor polymorphisms and susceptibility to human disease. Clin Sci (Lond) 114: 347–360.

18. Eigentler TK, Caroli UM, Radny P, Garbe C (2003) Palliative therapy of disseminated malignant melanoma: a systematic review of 41 randomised clinical trials. Lancet Oncol 4: 748–759.

19. Agarwala SS (2009) Current systemic therapy for metastatic melanoma. Expert Rev Anticancer Ther 9: 587–595.

20. Park BS, Song DH, Kim HM, Choi MS, Lee H, et al. (2009) The structural basis of lipopolysaccharide recognition by the TLR4-MD2 complex. Nature 459: 1191–1193.

21. Kallashhanda P, Bell J, Boudkalova MS, Medvedev A, Lorenz E, et al. (2006) Analysis of TLR4 polymorphic variants: new insights into TLR4-MD2/CD14 stoichiometry, structure, and signaling. J Immunol 177: 322–332.

22. Kalialis LV, Drzewiecki KT, Klyver H (2009) Spontaneous regression of metastases from melanoma: review of the literature. Melanoma Res 19: 275–282.

23. Agarwala SS (2009) Current systemic therapy for metastatic melanoma. Expert Rev Anticancer Ther 9: 587–595.

24. Park BS, Song DH, Kim HM, Choi MS, Lee H, et al. (2009) The structural basis of lipopolysaccharide recognition by the TLR4-MD2 complex. Nature 459: 1191–1193.