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Single-nucleotide polymorphisms in DNA-repair genes and cutaneous melanoma

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ABSTRACT

Single-nucleotide polymorphisms in different DNA-repair genes are reported to modulate risk of various cancers including melanoma. We genotyped DNA from 1186 melanoma patients and 1280 healthy controls for 13 different polymorphisms in eight DNA-repair genes. Data analyses showed that none of the polymorphisms except T241M *XRCC3* was associated with an increased risk for cutaneous melanoma. Carriers of the variant alleles were associated with a decreased risk (OR 0.83; 95% CI, 0.79–0.98). Three additional polymorphisms together with T241M *XRCC3* that tagged the entire gene region and the neighbouring genes *KLC1*, *ZFYVE21* and *PPP1R13B* were not associated with the disease risk; neither were the inferred haplotypes. Imputation showed association of comparable magnitude with 11 non-genotyped neighbouring polymorphisms. Finally, the combination of results for all polymorphisms genotyped in the present study with published data suggests that none of the investigated polymorphisms was associated with melanoma susceptibility. We conclude that 13 non-synonymous polymorphisms in eight DNA-repair genes that are frequently investigated with respect to modulation of cancer risk in populations are not associated with susceptibility to cutaneous melanoma.

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1. Introduction

The incidence rate of cutaneous melanoma, which is etiologically linked to sun-exposure, has rapidly grown over the years [1]. The overall cumulative 5-year survival probabilities for all stages have increased to approximately 90%. However, metastasized melanoma due to intrinsic chemo-resistance is still categorized as an incurable disease with a high mortality rate [2,3]. Approximately 5–10% of melanoma cases are hereditary, a proportion of which are linked to highly penetrant germline mutations in the *CDKN2A* and *CDK4* genes [4]. Sporadic melanoma accounts for a majority of cases and is assumed to be caused by the interaction between low-penetrant genetic variants and environmental factors [5]. Environmental factors also influence the penetrance of germline mutations in familial cases [6,7]. While the association between risk for melanoma and variants in skin pigmentation genes has notably been confirmed in genome-wide association studies, the relevance of polymorphisms in other genes remains to be resolved [8,9].

Human DNA repair constitutes a critical mechanism that involves over 150 genes for the maintenance of genomic integrity [10]. The disruption of the repair system constitutes a causal factor for several disorders and for manifold increased cancer incidences. Different DNA-repair pathways operate on specific damages [11]. While small lesions are corrected by base-excision repair, nucleotide-excision repair is invoked for the removal of bulky lesions such as UV photoproducts and adducts formed by polycyclic aromatic hydrocarbons [12,13]. Double-strand breaks are repaired *via* the homologous recombination and non-homologous recombination pathways; the correction of DNA-replication errors involves the mismatch-repair pathway [14,15]. Various studies have shown large inter-individual variation in DNA-repair capacity, and individuals with low repair capacity are probably at an increased risk for developing different cancers including melanoma [16–18]. Non-synonymous single-nucleotide polymor-

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phisms within various DNA-repair genes, a potential source of variation in functionality of the human DNA-repair system, have been also associated with susceptibility to various cancers [19–22].

A number of studies have explored the association between risk for cutaneous melanoma and single-nucleotide polymorphisms in DNA-repair genes, including *XRCC1*, *APEX1*, *XPC*, *XPD*, *XPG* and *XRCC3* [23–35]. However, inadequate study sizes have led to inconclusive and contradictory outcomes. We carried out a large study with 1186 melanoma cases and 1280 control subjects, and combined our own results with published data.

2. Materials and methods

2.1. Cases and controls

Patients with histologically confirmed cutaneous melanoma were recruited in hospitals in Mannheim, Germany, and Valencia, Spain. All melanoma patients of European ethnicity who were referred to the Skin Cancer Unit, German Cancer Research Center Heidelberg, at the University Hospital Mannheim and the Department of Dermatology, Instituto Valenciano de Oncologia, Valencia, Spain, for treatment and follow-up of primary tumours or metastases between 2000 and 2007, were approached to participate in the study. The Skin Cancer Unit, Mannheim, Germany, is a tertiary referral center with special focus on advanced melanoma. The Department of Dermatology, Valencia, Spain, is a referral skin-cancer centre for the provinces of Valencia, Alicante, and Castellón, with a population of approximately 5 million people. Pathology reports of all primary tumours were reviewed. Data were available from 1368 patients. Among them, 1186 (513 German and 673 Spanish) patients had a histologically confirmed primary cutaneous melanoma. Written informed consent was obtained from all subjects and the study protocol was approved by the institutional ethics boards.

From each patient 2.5 mL blood was collected in a tube with EDTA. The detailed history of the disease of each patient in the German cohort was documented and stored in an electronic database (Achiever Medical Anyware, Achiever Software Ltd., Birmingham, UK), and the history of Spanish patients was documented and stored in a Microsoft Office Access database. German control subjects included 736 (368 males and 368 females) ethnically matched healthy individuals recruited at the Institute of Transfusion Medicine and Immunology Mannheim (Germany). Informed consent was obtained and the appropriate ethical board approved the study. Spanish control samples were obtained from 544 (335 males and 209 females) anonymous blood donors who were cancer-free individuals recruited at the Centro de Transfusión de la Comunidad Valenciana between April 2005 and November 2005. Due to the anonymity of blood donors, only age and gender, but no information on sun-exposure and skin phenotype was available for the study.

2.2. Genotyping

DNA was extracted from cryo-preserved blood by use of the DNA Blood MiniKit (Qiagen, Hilden, Germany) and genotyped for 13 different polymorphisms in eight different DNA-repair genes. The polymorphisms and genes investigated included *XPC* (A > C; K939Q; rs2228001 and C > T; A499 V; rs2228000), *ERCC2* (G > A; D312N; rs1799793 and A > C; K751Q; rs13181), *ERCC5* (C > G; D1104H; rs17655) in the nucleotide-excision repair pathway; *APEX1* (T > G; D148E; rs1130409), *XRCC1* (−77 T > C; rs3213245; G > A; R280H; rs25489 and G > A; R399Q; rs25487) in the base-excision repair pathway; *XRCC3* (−1843 A > G; rs1799794; Intron 5; G > A; rs861530; and C > T; T241 M; rs861539) in the double-strand break-repair pathway.

A region of 218 kb on chromosome 14q32.3 that included the *KLC1*, *XRCC3*, *ZFYVE21*, and *PPP1R13B* genes was identified for haplotype analysis by use of HapMap CEU data and Haploview 4.0 software. Within this region, three SNPs in the *XRCC3* gene and one SNP in *PPP1R13B* Intron 6 (C > T; rs7141928) tagged 94 SNPs (Supplementary Table 1 and supplementary Fig. 1).

Genotyping was performed with the 5′-nuclease allelic discrimination assay (TaqMan®) in 96-well format. TaqMan® primers and probes were purchased from Applied Biosystems, Foster City, CA. PCR was performed in 5–10-μL volume reactions with 5 ng DNA as a template, pre-made master mix and 0.5 × probe-primer mix. The initial temperature conditions for PCR were set at 50 °C for 2 min and 95 °C for 10 min followed by 35–45 cycles at 92 °C for 15 s and 60 °C for 1 min. Genotyping on amplified PCR products was scored by differences in VIC and FAM fluorescence level in the plate read operation on the ABI PRISM 7900HT sequence-detection system (Applied Biosystems, Foster City, CA) with SDS 1.2 software. Post-operation data were transferred as Microsoft Excel data and converted into genotype information.

Genotyping data were verified by direct DNA sequencing in a random selection of 5% of the samples; no discordance was observed. Sequencing reactions were performed with the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) in a 10-μL volume containing PCR product pre-treated with ExoSapIT® (Amersham Biosciences, Uppsala, Sweden) and two sequencing primers. The temperature conditions set for sequencing reactions were 96 °C for 2 min followed by 27 cycles at 96 °C for 30 s, 54 °C for 10 s and 60 °C for 4 min. Sequencing reaction products were precipitated with 2-propanol, washed with 75% ethanol, re-suspended in 25 μL water and loaded onto an ABI prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.3. Statistical analysis

Genotype frequencies of the SNPs included in the study were assessed for deviation from Hardy–Weinberg equilibrium with the χ^2 test. Heterogeneity of genotype frequencies between German and Spanish control populations was tested by the Mantel–Haenszel χ^2 test before combining both populations for analysis.

Multivariate logistic regression was used to estimate adjusted odds ratio (OR) for the association between melanoma and alleles/genotypes/haplotypes; the covariates age, gender and nationality (German or Spanish) were included in the model. Statistical analyses were implemented using the SAS® version 9.1 software (SAS Institute Inc., Cary, NC). The haplotype procedure of SAS/Genetics Software was used to estimate haplotype frequencies in cases and controls separately, and to infer the possible haplotype combinations for each individual. Relationship between haplotypes and melanoma risk were summarized as global *p*-values.

There is evidence that genetic association studies may benefit from combining information across SNP markers and from exploiting existing catalogues of variation [36]. We imputed genotypes based on HapMap data to detect possible associations with genetic variants that were not typed in our study. Imputation relied on inference of haplotypes by means of the expectation-maximisation (EM) algorithm in the presence of partially incomplete data. In brief, missing alleles were excluded from the calculation of allele frequencies. In the E-step, frequencies of partially missing genotypes were updated looping through all possible genotypes. In the M-step, all existing haplotypes that have alleles identical to the non-missing alleles of this haplotype were updated. The certainty of imputation of genotypes was evaluated by cross-validation and it was represented by minus the logarithm of the probability value (Pval) for Cohen's Kappa between true and imputed genotypes (Supplementary Fig. 2). The selection of the region for the subsequent association analyses relied on the visual inspection of recombination rates and imputation accuracies around the genotyped SNPs. Uncertainty in the imputed genotypes was taken into account in the logistic regression by bootstrapping from the multinomial distribution of the expected genotypes given the observed, directly genotyped variants (1000 replicates). The estimated *p*-values referred to a model-free, three-genotype model.

A random effects model was used to combine our own results and published data. Results were represented by standard meta-analysis plots: confidence intervals for each single study were indicated by horizontal lines, single OR by squares and summary estimates by diamonds with horizontal limits at confidence limits and width inversely proportional to the standard error. Meta-analyses were performed using the package *rmeta* in the free software environment for statistical computing R. We assumed that the identified studies were random samples from a general study population and used a random effects model to summarize OR estimates. In addition we evaluated the consistency of this assumption using Woolf's test under a fixed effects model, investigated study-specific contributions to heterogeneity with a leave-one-out approach and hypothesized on the role of publication bias in global heterogeneity.

3. Results

The distributions of genotyped polymorphisms in German and Spanish controls did not show deviation from Hardy–Weinberg equilibrium. The Mantel–Haenszel test identified heterogeneity between German and Spanish controls for the *XRCC3* Intron 5 polymorphism ($p = 0.02$), but not for any other investigated SNP. The mean ages at diagnosis of melanoma in the German and Spanish study populations were 57.9 and 50.9 years, respectively. The mean ages of controls were 60.3 years in Germany and 37.1 years in Spain. The proportion of females in the German cases and controls was 44% and 50%, respectively. The corresponding proportions in cases and controls from Spain were 56% and 38%, respectively. The frequency of variant allele carriers for the T241M *XRCC3* polymorphism was significantly lower among cases than among controls (OR 0.83, 95% CI 0.70–0.98). With this one exception, no differences in allele or genotype frequencies for any other polymorphism were observed between cases and controls (Table 1).

The genotyped variants included two polymorphisms in the *XPC* gene, two in the *ERCC2* gene, three in the *XRCC1* gene and four in the *XRCC3-PPP1R13B* genes. Haplotype analyses revealed no differences in haplotype frequencies between melanoma cases and controls (Supplementary Table 2).

Recombination rates in the 500-kb region centered on the four SNPs genotyped in the *XRCC3-PPP1R13B* was low, and the results on certainty of imputation suggested that many

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