

**Title:** Polymorphisms in Nucleotide Excision Repair Genes and **Risk of Multiple Primary Melanoma:** the Genes Environment and Melanoma Study

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## Abstract

Polymorphisms in six genes involved in nucleotide excision repair of DNA were examined in a large, population-based case-control study of melanoma. Genotyping was conducted for 2485 patients with a single primary melanoma (controls) and 1238 patients with second or higher order primary melanomas (cases). Patients were ascertained from nine geographic regions in Australia, Canada, Italy and the United States. Positive associations were observed for *XPB* 312 Asn/Asn **versus** Asp/Asp [Odds ratio (OR) = 1.5, 95% Confidence Interval (CI) 1.2-1.9] and *XPB* 751 Gln/Gln **versus** Lys/Lys (OR = 1.4, 95% CI 1.1-1.7) genotypes and melanoma. The combined *XPB* Asn (A) 312 + Gln (C) 751 haplotype was significantly more frequent in cases (32%) compared with controls (29%) (P = 0.003) and risk of melanoma increased significantly with one and two copies of the haplotype (ORs 1.2, 95% CI 1.0-1.4, and 1.6, 95% CI 1.2-2.0, trend P = 0.002). No significant associations were observed for *HR23B* codon 249, *XPG* codon 1104, *XPC* codon 939, *XPF* codon 415, *XPF* nt 2063, *ERCC6* codon 1213 or *ERCC6* codon 1230. Odds ratios for *XPB* and *XPC* genotypes were stronger for melanoma diagnosed at an early age, but tests for interaction were not statistically significant. The results provide further evidence for a role of *XPB* in the etiology of melanoma.

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## Introduction

DNA repair plays a fundamental role in the maintenance of genomic integrity. The ability to repair DNA is quite variable within human populations [1], and decreased DNA repair has been associated with increased risk of a variety of human cancers, including melanoma [2]. A variety of somatic genetic alterations occur in melanoma, including mutations associated with exposure to ultraviolet (UV) radiation [3]. Several previous epidemiologic studies examined DNA repair capacity in risk of melanoma [for review, cf. 2, 4]. Hsu et al. [5] observed a higher level of bleomycin-induced DNA breaks in melanoma patients compared to controls. Roth et al. [6] reported higher loss of thymine dimer antigenicity in melanoma patients compared to controls. Wei et al. [7] and Landi et al. [8] used the host cell reactivation assay to evaluate repair of UV-induced DNA damage using lymphocytes from melanoma patients and cancer-free controls. Wei et al. [7] found that patients with melanoma had significantly lower mean DNA repair capacity (DRC) than controls; Landi et al. [8] did not find an overall case-control difference in DRC, but DRC was reduced among melanoma patients with low tanning ability and dysplastic nevi relative to the corresponding controls.

The principle pathway for repair of ultraviolet [UV]-induced DNA damage is nucleotide excision repair [NER]. NER removes pyrimidine dimers caused by UV radiation, bulky adducts and other helix-distorting DNA lesions [1, 9]. The earliest step in NER is recognition of DNA damage by a multi-protein complex containing XPC, HR23B **and centrin 2**, followed by recruitment of the transcription factor IIIH [TFIIH] complex of proteins [9]. The TFIIH complex, which contains XPD as a subunit, unwinds the DNA duplex around the damaged site. XPG binds to the multi-protein TFIIH complex and DNA, followed by recruitment of the XPF-ERCC1 complex. XPG and XPF-ERCC1 produce dual incisions 3' and 5' to the damaged site. After

release of the damaged DNA strand, the gap is filled by repair synthesis and ligation [1, 9]. ERCC6 participates in NER of oxidative DNA damage by forming complexes with RNA polymerase 1, TFIIH and XPG [10]. XPC is involved in up-regulation of *KINI7* and other UV-responsive genes involved in DNA replication [11]. The XPD protein also plays a role in initiation of RNA transcription by RNA polymerase II [12].

Recent studies of DNA repair and human cancer have focused on common polymorphisms in genes involved in DNA repair and risk of melanoma and other cancers [13]. Nonconservative amino acid substitutions and other potential functional variants in DNA repair genes are common in human populations, including many of the genes involved in NER [1]. To date, **six** studies of NER gene polymorphisms and risk of melanoma have been published [14-19]. The results are not consistent across studies. We examined the association of single nucleotide polymorphisms [SNPs] in six NER genes (*XPD*, *HR23B*, *XPG*, *XPC*, *XPF*, and *ERCC6*) and melanoma as part of the Genes Environment and Melanoma [GEM] Study. We chose SNPs for evaluation based upon results of previous epidemiologic studies and information regarding observed or potential functional impact for each SNP. The GEM Study utilized a novel study design whereby patients with first primary diagnosis of melanoma served as controls, and patients with second or higher primary melanomas as cases. Odds ratios [ORs] calculated using GEM Study data estimate the relative risk of second or higher primaries among persons with single primary melanomas. The study included participants and investigators from Australia, Canada, Italy, and the United States.

## **Materials and Methods**

**Study Design and Participants.** The Genes Environment and Melanoma [GEM] Study is an international, multi-center case-control study of melanoma. The GEM Study population consists of incident cases of cutaneous melanoma identified in eight population-based cancer registries and one hospital center in Australia (New South Wales, Tasmania), Canada (British Columbia), Italy (Turin), and the United States (Michigan, North Carolina, Orange and San Diego Counties, and New Jersey) (Berwick M, et al., submitted). GEM Study controls were persons diagnosed with a first invasive primary melanoma during the year 2000. GEM Study cases were persons diagnosed with second or higher order invasive or *in situ* melanoma during the years 2000 to 2003. Inclusion of **patients with *in situ* melanoma in the case group (but not the control group)** was designed to avoid exclusion of participants who would have been diagnosed with an invasive subsequent primary if the *in situ* lesion had not been removed. Statistical analyses were repeated after excluding participants diagnosed with *in situ* disease (as described below). The study protocol was approved by the Institutional Review Board [IRB] at the coordinating center, Memorial Sloan Kettering Cancer Center [MSKCC], as well as IRBs at each participating institution.

Physician approval was sought prior to contacting eligible participants, and all study participants provided informed consent. A total of 3626 participants were enrolled, 1238 cases (multiple primaries) and 2485 controls (single primaries). **Enrollment according to study center was as follows: British Columbia, Canada (n = 160, 4.4%), Orange and San Diego Counties, California USA (n = 308, 8.5%), Michigan USA (n = 385, 10.6%), New Jersey USA (n = 343, 9.5%), New South Wales, Australia (n = 1282, 35.4%), North Carolina USA (n = 296, 8.2%), Ontario, Canada (n = 541, 14.9%), Tasmania, Australia (143, 3.9%),**

**Torino, Italy (n = 168, 4.6%).** A total of 97 controls developed additional primary tumors during the period of observation, and thus met the eligibility criteria as both cases and controls.

Participation rates among eligible subjects were 52% for cases and 54% for controls.

**Study participants were slightly younger (median age 59, range 7 to 97) compared to non-participants (median age 62, range 11 to 98), and participants were more likely to be female (44%) compared with non-participants (40%). Tumor thickness (Breslow depth) was greater among participants (median 0.56 mm) compared with non-participants (median 0.39 mm), but the difference was not statistically significant (Wilcoxon rank sum test P = 0.19).**

Participants were asked to provide 4-6 buccal swabs (94.3% of participants) or a blood sample (5.7%) for DNA extraction, and completed a self-administered questionnaire and one-hour telephone interview regarding medical history, phenotypic factors (hair color, eye color, tanning ability, freckling pattern, skin sensitivity to sun exposure) and other risk factors for melanoma. **Participants were asked about history of melanoma for each first-degree as well as more distant relatives. For the present analysis, family history was based upon first-degree relatives only.** Participants provided consent to obtain hematoxylin and eosin stained tumor sections from referring pathologists, and centralized review of histopathology for all tumors was conducted by a team of pathologists with expertise in melanoma.

**DNA extraction.** Biologic samples were mailed to the Molecular Epidemiology Laboratory at MSKCC for DNA extraction. DNA was isolated from buccal swabs using the Puregene DNA isolation kit (Gentra System Inc., Minneapolis MN), replacing glycogen with rRNA (10 µg/µl) for the DNA precipitation step. DNA was extracted from peripheral blood lymphocytes using

the Qiagen QIAamp DNA Blood Kit (Qiagen Inc., Valencia CA) following manufacturer's recommendations. DNA samples were missing on 3 cases and 7 controls. The total number of DNA samples available for genotyping was 3713 (1235 cases, 2478 controls).

**Genotyping of SNPs.** Genotyping was conducted in the High-throughput Genotyping Core Laboratory at UNC Chapel Hill using the ABI 7700 Sequence Detection System, or "Taqman"<sup>™</sup> assay (Applied Biosystems). The following SNPs were genotyped: *XPD* (*ERCC2*) codon 312 (rs 1799793), *XPD* codon 751 (rs 13181), *HR23B* codon 249 (rs1805329), *XPG* (*ERCC5*) codon 1104 (rs 17655), *XPC* codon 939 (rs 2228001), *XPF* (*ERCC4*) codon 415 (rs 1800067), *XPF* 2063 (rs 1799797), *ERCC6* codon 1213 (rs 2228527), and *ERCC6* codon 1230 (rs 4253211). Primer and probe sequences and annealing temperatures for each genotyping assay are listed in Table 1. Alternative names for each locus are also provided in Table 1. Probes were labeled on the 5' end with either FAM or VIC (PE Biosystems). Probes were labeled on the 3' end with the quencher dye 6-carboxy-*N,N,N',N'*-tetramethylrhodamine [TAMRA].

Polymerase chain reaction [PCR] reactions were performed in 15 µl reaction volumes in 96-well plates. Reactions contained 0.7X ABI Universal Master Mix, 200 nM of each allele specific probe, 900 nM of each primer, and 15 ng of genomic DNA. After reactions tubes were set up, amplification was performed using a Perkin-Elmer GenAmp 9700 thermocycler. Reaction tubes were placed into the thermocycler after the temperature had reached 50°C. The amplification was performed using the following conditions: 50°C for 2 minutes (AmpErase UNG Activation), 95°C for 10 minutes (AmpliTaq Gold Activation), and 40 cycles of 92°C for 15 seconds (denature) and 56°C, 60°C or 62°C (Table1) for 1 minute (anneal/extend). Genotypes were read using the ABI 7700 in allele discrimination mode for end-point detection.

Samples that failed to amplify were repeated. Those samples that failed to amplify on the second run were scored as missing. Missing genotypes for each loci were as follows: *XPD* codon 312 (59 cases, 81 controls), *XPD* codon 751 (23 cases, 42 controls), *HR23B* codon 249 (35 cases, 73 controls), *XPG* codon 1104 (42 cases, 70 controls), *XPC* codon 939 (26 cases, 39 controls), *XPF* codon 415 (27 cases, 33 controls), *XPF* 2063 (20 cases, 33 controls), *ERCC6* codon 1213 (28 cases, 70 controls), *ERCC6* codon 1230 (20 cases, 42 controls). Assays were repeated for a random 10% sample and results were identical to the initial analysis for each locus. For each genotyping assay, DNA samples from the Coriell tissue repository (Coriell Institute for Medical Research, Camden NJ) that had previously been sequenced at the National Cancer Institute (<http://www.nci.snp500.gov>) were used as positive controls.

**Statistical Methods.** Data were collected and sent to the coordinating center at MSKCC, where all statistical analyses were conducted. Departures from Hardy-Weinberg equilibrium were evaluated by calculating expected genotype frequencies among controls based on observed allele frequencies and comparing the expected frequencies to observed genotype frequencies using  $\chi^2$  tests. Differences between allele or genotype frequencies in cases and controls were estimated using  $\chi^2$  tests or Fisher's Exact tests when expected counts were less than 5. Tests for statistical significance were two-sided with an alpha level of 0.05.

Unconditional logistic regression was used to calculate ORs and 95% Confidence Intervals [CIs] for NER genotypes and melanoma. ORs in the GEM Study compare cases with higher order primary melanomas to controls with single primaries. When controls are incident cases from a defined population base and cases are incident higher order primaries from the same population base, the OR comparing cases and controls is an indirect estimate of the same

parameter that would be obtained from a case-control study of the same population base [20].

ORs were adjusted for age, sex, center, and an age-sex interaction term. The age-sex interaction term was required since the population incidence of melanoma is higher in younger women than men, but lower in women compared to men at higher ages. Beta coefficients for NER genotypes changed by less than 10% after adjustment for phenotypic factors, **family history** and other covariates (data not shown).

Tests for linear trend in ORs were conducted for each NER gene separately by coding the number of variant alleles as an ordinal variable (0, 1, 2) and calculating P-values for regression coefficients in logistic regression models. Similarly, tests for linear trend were conducted combining genotypes for all NER genes: the total number of variant alleles was summed across all loci and coded as an ordinal variable (0, 1, 2...10+). Stratified analysis was used to investigate modification of ORs by age at diagnosis. Age at diagnosis was defined as the age at first diagnosis for controls and the age at most recent diagnosis for cases. Likelihood ratio tests [LRTs] were performed to evaluate age-genotype interactions. ORs for NER genes were also calculated after stratification on phenotypic index. Phenotypic index was a five level ordinal variable that combined information regarding hair color, eye color and propensity to tan.

SAS Genetics (version 9.0; SAS, Cary NC) was used to estimate *XPD* codon 312 + codon 751, *XPF* codon 415 + nt2063, and *ERCC6* codon 1213 + codon 1230 haplotype frequencies, and to compare haplotype frequencies in cases and controls. Haplotype estimates from SAS Genetics are based upon the Expectation-Maximization (EM) algorithm. Lewontin's  $D'$  value, an estimate of the extent of linkage disequilibrium, was calculated using SAS Genetics. P-values comparing haplotype frequencies in cases and controls were calculated using the Haplotype-Score Statistic

of Schaid et al. [21]. Relative risks and trend tests for individual haplotypes were calculated using the method proposed by Venkatraman et al. [22].

## Results

Characteristics of GEM Study cases and controls are presented in Table 2. Cases were generally older than controls, reflecting the fact that one must experience a first melanoma before being at risk for a subsequent melanoma. Cases also showed a male predominance, reflecting the higher incidence of melanoma in men [23]. **Family history of melanoma was more common among cases compared to controls.** Breslow depth was smaller for cases, largely due to our decision to include as eligible cases patients with a subsequent *in situ* primary melanoma. **The proportion of participants under the age of 30 was similar for North America (4.8%) compared to Australia (3.9%); however, the proportion of participants age 70 and over was higher for Australia (37.0%) compared to North America (24.5%) (P < 0.001).**

Genotype frequencies, allele frequencies and ORs for melanoma are presented for each NER genetic polymorphism in Table 3. **Genotype frequencies were similar across study centers. For example, comparing participants from North America and Australia, there were no statistically significant differences in genotype frequencies for XPD 312 (P = 0.41) or 751 (P = 0.44).** Genotype and allele frequencies in GEM Study controls were within the range of previous reports for unaffected whites (controls) from Europe and the United States. For XPD, control genotype frequencies in previous reports ranged from 10 -19% for codon 312 Asn/Asn [4, 13, 24-26] and from 10-17% for codon 751 Gln/Gln [13, 24-28]. Reported values were 3% for HR23B Val/Val [<http://www.nci.snp500.gov>], 5 to 7% for XPG codon 1104 His/His [17, 28-30], **13-15%** for XPC codon 939 Gln/Gln [**19, 25, 28**], 0% for XPF 415 codon 415

Gln/Gln [31, <http://www.ncbi.nlm.nih.gov>], 6% for *ERCC6* codon 1213 Gly/Gly [<http://www.ncbi.nlm.nih.gov>], and 0% for *ERCC6* codon 1230 Pro/Pro [<http://www.ncbi.nlm.nih.gov>]. The only available genotype frequency for *XPF* nt 2063 A/A was 11% among cadaver controls in the study of Winsey et al. [14].

Statistically significant departures from Hardy-Weinberg equilibrium were observed for three loci: *HR23B* genotypes in GEM Study controls ( $P = 0.004$ ), where less Val/Val homozygotes were observed ( $n = 71, 3\%$ ) than expected ( $n = 96, 4\%$ ); *XPC* 939 in GEM Study controls ( $P = 0.009$ ), less Gln/Gln homozygotes ( $n = 402, 16\%$ ) than expected ( $n = 430, 18\%$ ); and *XPG* 1104 in GEM Study cases ( $P = 0.03$ ), more His/His homozygotes ( $n = 73, 6\%$ ) than expected ( $n = 58, 5\%$ ). The remaining loci did not exhibit significant departure from Hardy Weinberg equilibrium in GEM Study cases or controls.

Statistically significant differences in allele frequencies between cases and controls were observed for *XPD* codon 312 and codon 751 (Table 3). ORs were modestly elevated for *XPD* genotypes and melanoma, and trend tests were statistically significant. **Trend tests for *XPD* were statistically significant for North American but not for Australian participants, which may be attributable to lower statistical power in analyses stratified by study center.** The remaining case-control differences in allele frequencies were not statistically significant. ORs were modestly elevated for *HR23B*, *XPG* and *XPF* 415 genotypes and melanoma, but trend tests were not significant. **ORs were unchanged when we adjusted for family history of melanoma as a covariate (data not shown).** **ORs were similar after stratification on family history: for example, the OR for *XPD* 312 Asn/Asn versus Asp/Asp was 1.4 (95% CI 0.8-2.5) among participants with a family history, and 1.5 (95% CI 1.2-2.0) in those without.**

The corresponding ORs for *XPD* 751 Gln/Gln versus Lys/Lys were 1.3 (95% CI 0.7-2.4) and 1.3 (95% CI 0.7-2.4).

The GEM study design has the potential for false positive associations between genotype and risk of melanoma if genotype is associated with case survival [20]. GEM Study controls are incident cases of melanoma, while patients at risk of a subsequent primary represent prevalent cases. Thus, ORs comparing cases and controls could be biased upward if genotype is related to increased patient survival, and downward if genotype is related to decreased survival. We tested this possibility by comparing the distributions of time from first diagnosis to second diagnosis among cases according to NER genotypes. There were no statistically significant differences in the interval between diagnoses according to NER genotypes (data not shown). The fact that GEM Study cases included not only patients with a second primary melanoma, but also incident cases with higher order primaries, could increase the magnitude of the ORs if NER genotypes are associated with increasing multiplicity of melanoma. ORs could also be affected by including cases with *in situ* melanoma if NER genotypes influenced the transition from *in situ* to invasive disease. To address these issues, separate analyses were conducted excluding cases with higher order primaries and *in situ* melanoma in order to determine whether ORs were altered for NER genotypes. ORs were essentially unchanged when we restricted the analysis to cases with second primaries only, and when we excluded cases with *in situ* melanoma (data not shown).

*XPD* haplotype frequencies and ORs for combined *XPD* genotypes and melanoma are presented in Table 4. Significant linkage disequilibrium was observed between alleles at *XPD* codon 312 and 751 in both cases and controls. A higher frequency of the *XPD* Asn (A) 312 +

Gln (C) 751 haplotype was observed in cases compared to controls (Table 4a). ORs for melanoma were elevated among participants with two copies of the *XPD* Asn (A) 312 + Gln (C) 751 haplotype. **Phase is ambiguous for double heterozygotes, therefore haplotypes were imputed by assigning these individuals to haplotype groups in proportion to the probabilities estimated by the EM algorithm. The numbers of individuals with imputed haplotypes are included in parentheses.** The trend test was statistically significant for increasing number of the variant haplotype (Table 4b). There were no statistically significant case-control differences in haplotype frequencies for *XPF* codon 415 + nt 2062 or *ERCC6* codon 1213 + codon 1230 (data not shown).

ORs for NER genotypes and melanoma stratified by age at diagnosis are presented in Table 5. ORs were higher for melanoma diagnosed before age thirty compared with other age groups for *XPD* codon 312, *XPD* codon 751, and *XPC* codon 939. The OR for melanoma diagnosed at aged 70+ years was also increased for *HR23B*, but there was no consistent trend across age groups. **ORs were unchanged after adjustment for family history, and** none of the LRTs for age at diagnosis and NER genotypes were statistically significant (data not shown). ORs stratified on phenotypic index showed no difference across categories and none of the LRTs were statistically significant (data not shown).

The total number of variant alleles combining all NER genes is presented for cases and controls in Table 6. Although the distributions were similar, there was a statistically significant trend towards more variant alleles in cases compared with controls. **ORs for melanoma are presented using the common category (n = 4 variant alleles) as the referent group. Weak positive associations were observed for 5, 7 and 10 or more variant alleles.**

## Discussion

Previous studies of NER genotypes and melanoma are few and do not provide consistent results. Winsey et al. [14] reported a higher frequency of the more common *XPF* nt 2063 T/T genotype in melanoma cases compared to controls. The OR was 1.65 (95% CI 1.03-1.66) comparing T/T with A/A genotypes. The authors also reported a slightly higher frequency of the *XPD* codon 312 Asn/Asn and codon 751 Gln/Gln genotypes in cases compared to controls, but ORs were not calculated. In this study, cases were 211 patients referred to a regional cancer center in the United Kingdom for treatment of late stage melanoma, and controls were cadaveric renal transplant donors. Tomescu et al. [15] found a higher frequency of the *XPD* codon 751 Lys/Lys genotype in melanoma cases versus controls (OR = 2.8, 95% CI 1.2-7.0). Cases were patients attending a cancer referral center in Scotland who were under age 50 with localized disease and no history of excessive sun exposure. The authors also reported positive associations for *XPD* markers in exon 6 and exon 22, but did not evaluate codon 312. Controls were donors of blood samples to the Scottish Blood Transfusion Service. Baccarelli et al. [16] reported no overall association between *XPD* codon 312 and 751 genotypes and melanoma, but noted stronger associations for both variant *XPD* genotypes and melanoma at older ages. The authors also reported that DNA repair capacity, as measured by the host cell reactivation assay, was lower in participants carrying the *XPD* codon 751Gln allele. Cases were patients from a referral hospital in Italy, and controls were outpatients with minor accidental trauma or personnel from the same hospital. **Blankenburg et al. [17] reported no association for *XPG* codon 1104 genotype and melanoma, and no association for three markers in *XPC*: T1601C, G2166A, and C3507G. In the same study population, the authors found positive associations for three other *XPC* markers: intron 9 PAT, intron 11 C-6A, and codon 939 Lys/Gln [19].** The

**adjusted OR for *XPC* codon 939 comparing Gln/Gln versus Any Lys genotype was 1.82 (95% CI 1.07-3.08). In a subgroup of cases with multiple primary melanomas, the authors found slightly stronger associations for each of the three markers versus controls: the OR for codon 939 was 1.89 (95% CI 1.18-3.03) [19].** Cases were recruited from dermatology clinics in Germany, and controls were local blood donors or health care personnel. None of the aforementioned case-control studies were population-based. In the Nurses' Health Study Cohort, Han et al. [18] reported modest inverse associations between *XPD* codon 312 Asn/Asn and codon 751 Gln/Gln genotypes and risk of melanoma, but the results were not statistically significant. Interactions were observed between *XPD* genotypes and sunlight exposure [18]. Taken together, the results of previous studies and the GEM Study suggest that *XPD* genotypes play a role in the etiology of melanoma, but further study is needed to determine which alleles are associated with increased risk.

The available data suggest lack of association for polymorphisms in the remaining NER genes and melanoma when NER genes are evaluated singly. In the GEM Study, we found that when we combined genotypes across six NER genes (*XPD*, *HR23B*, *XPG*, *XPC*, *XPF* and *ERCC6*), cases showed a statistically significant trend towards more variant alleles compared with controls, although the magnitude of the trend was very small. Several previous studies used a similar approach to build "multigenic models" for breast cancer. The authors demonstrated increased risk of breast cancer among women with a higher number of variant alleles for DNA repair genes [32] and hormone metabolism genes [33-34]. The statistical approach of counting the number of variant alleles across genes involved in a biochemical pathway was advocated by Mohrenweiser et al. [1] as a method for creating "pathway genotypes." Our data provide preliminary evidence in support of a multigenic model for

melanoma that includes NER genes. However, additional studies that incorporate a large number of NER genes are needed.

ORs for *XPD* genotypes were slightly elevated among GEM Study patients with age at onset of melanoma prior to age 30. Younger age at onset of skin cancer is a feature of patients with Xeroderma Pigmentosum [XP], a familial syndrome caused by mutations in *XPD* and other XP-related genes [12]. *XPD* is a multi-functional protein that plays a role in transcription, DNA repair, and tissue aging [35]. *XPD* codon 312 and 751 variants could affect one more of these biologic functions. Identifying risk factors for early-onset melanoma is an important public health issue for the entire population, not solely among high-risk families. A significant proportion of melanoma cases are diagnosed before the age of 30, and the incidence appears to be increasing among younger persons in at least some parts of the world [23].

There are several limitations to the present study. **Participation rates were slightly above 50% for cases and controls, in the range commonly observed for epidemiologic studies that require biologic samples. The similarity of response rates for case and controls and the lack of strong differences between participants and non-participants reduce the possibility of selection bias. Furthermore, participation in the study was unlikely to be related to NER genotypes, and observed genotype frequencies were in the range of previous studies and databases.**

**We evaluated a limited number of NER gene polymorphisms. The SNPs chosen for evaluation are common and represent** non-conservative amino acid substitutions within conserved regions of the encoded proteins [1]. Conserved regions often mediate protein-protein interactions and regulate enzymatic activity [36-37]. Compared to phenotypic assays for DNA repair, genotyping assays have lower coefficients of variation, are stable over time, and are

readily performed in large, population-based studies [2, 13]. However, the functional significance of most DNA repair gene polymorphisms is unknown or poorly characterized. The *XPD* codon 312, *XPD* codon 751, *XPG* codon 1104, *XPC* codon 939 and *ERCC6* codon 1213 polymorphisms are predicted to have significant functional impact, based upon the high degree of amino acid conservation across species and changes in polarity, charge and computer simulations of altered protein structure [1,37]. Functional assays for the *XPD* codon 312 and 751 polymorphisms do not provide consistent results regarding which alleles would be predicted to increase cancer risk [26]. Different *XPD* alleles have been associated with chromosomal aberrations [28, 38-39], DNA adducts [40-42], reduced efficiency of DNA repair as measured by the host cell reactivation assay [43-44], increased UV-induced DNA strand breaks detected by the alkaline comet assay [45], reduced efficiency of p53-mediated apoptosis [46] and reduced repair of UV-specific cyclobutane pyrimidine dimers in human skin [47]. Most functional studies suggest that the *XPD* codon 312 Asn and codon 751Gln alleles are associated with decreased DNA repair, and several case-control studies of lung cancer demonstrated increased risk among carriers of these variant alleles [26]. The *XPC* codon 939 polymorphism may lead to reduced repair of radiation-induced DNA damage [28]. There are no published functional studies of the remaining NER gene polymorphisms in our study, and no functional studies have evaluated combinations of NER genotypes and DNA repair capability.

The NER gene polymorphisms measured in the present study could lie in linkage disequilibrium with causal variants nearby in the genome. For example, the *XPC* codon 939 Gln variant is in strong linkage disequilibrium with a poly-AT insertion that causes reduced *XPC* expression and lower DNA repair activity [19, 44, 48-49]. **The combination of variant alleles at *XPD* codons 312 and 751 showed a stronger case-control difference than either variant**

alone. The *XPD* codon 312 Asn (A) and 751 Gln (C) alleles could encode a functionally important combination of *cis* amino acids, or other alleles could lie upstream, downstream, or within the *XPD* 312 A + 751 C haplotype that increase risk of melanoma. So far, no causal alleles have been found within or nearby the *XPD* gene that lie in linkage disequilibrium with the variant haplotype [26].

We did not adjust P-values for multiple comparisons. We conducted a "candidate gene" study, rather than an exploratory genome-wide search. Our goal was to estimate effects for SNPs that had been previously examined by other investigators, particularly *XPD* 312 and 751. Furthermore, the SNPs were related to each other via a common biochemical pathway and some were within the same genes, and thus do not represent independent statistical tests. Departures from Hardy-Weinberg equilibrium were observed for *HR23B*, *XPC*, and *XPG*. Laboratory error is an unlikely explanation, since positive controls were incorporated into each genotyping assay and allele frequencies were similar to previous studies. The differences between observed and expected genotype frequencies were small, and may have arisen due to chance. The significant departures from Hardy-Weinberg equilibrium occurred for variants that were not associated with melanoma, and thus do not affect the results for *XPD* 312 and 751.

ORs in the GEM Study were estimated comparing cases of higher order primary cancers to controls with single primaries. The GEM Study is essentially a case-control study in a "high risk" subset of the population, in the sense that the patients at risk of becoming a "case" are those who already have a diagnosis of melanoma. This study design has been used extensively to identify risk factors for second primaries, but has only recently been utilized in genetic epidemiology [20]. Although the GEM study design has not been extensively validated, our

results for *XPD* are consistent with several other lines of evidence. Polymorphisms in *XPD* have been associated with increased risk of basal cell carcinoma [50-52]. A cohort study of patients with a first diagnosis of non-melanoma skin cancer showed an elevated risk of second primary cancers (all sites) for carriers of the *XPD* 751 Gln allele [53]. Finally, patients with triple primary cancers, including melanoma, showed increased sensitivity to UV-induced DNA damage [54].

There are several strengths of our study. Compared with previous studies of NER genes and melanoma, the GEM Study included a larger number of patients and was based upon population-based ascertainment of incident cases. Study participants were drawn from nine geographic regions in Australia, Canada, Italy and the United States, and thus the results may be generalizable to persons of European ancestry living in a variety of climates and latitudes. The GEM Study is efficient for rare risk factors, including rare inherited mutations that may be more common in cancer patients compared with population controls. In the present study, we did not observe a notably increased prevalence of variant genotypes in NER genes among incident cases of first primary melanoma (GEM controls) compared with previous studies. Thus, the chief advantage of the GEM study design for common genetic factors is the fact that cases and controls are drawn from population-based rosters and are patients who are being treated within the medical system. Thus, the response rate for DNA samples is high, and the problem of identifying a suitable choice of population controls is avoided. The latter issue is especially relevant to large, international collaborative studies.

In conclusion, the results of the present study provide further evidence for a role of the NER pathway for DNA repair in the etiology of melanoma. The NER pathway repairs *cis-syn*-cyclobutane dimers and pyrimidine (6-4) pyrimidone photoproducts, the two major classes of

DNA lesions induced by solar ultraviolet radiation [12]. Patients with mutations in *XPD* have an increased risk of skin cancer at younger ages, including basal cell carcinoma, squamous cell carcinoma, and melanoma. However, highly penetrant mutations in *XPD* are rare, and limited to a small proportion of skin cancer patients [26, 35]. Common polymorphisms, such as *XPD* codons 312 and 751, could contribute to a large number of melanoma cases. If confirmed in other populations, the association of *XPD* polymorphisms with increased risk of first and/or higher primary melanoma could contribute to greater understanding of the role of ultraviolet radiation and other risk factors in etiology of the most malignant form of skin cancer. In addition, studies of the interaction of *XPD* polymorphisms with sunlight exposure might help identify a high risk subset of people who could benefit from more rigorous control of sun exposure, skin surveillance and, possibly, use of topical applications that promote repair of pyrimidine dimers following sun exposure [55].

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**Table 1. Assay conditions for NER gene polymorphisms**

Locus	SNP	dbSNP ID number	Forward primer Reverse primer	Probe sequences <sup>a</sup> VIC probe FAM probe	Annealing temp.
<i>XPD</i> ( <i>ERCC2</i> )	nt 23529 G / A Asp 312 Asn	rs 1799793	GAGACGGACGCCACCT GGAGGCGGGAAAGGGACT	Asp 312 (G) FAM CTGCCCgACGAAG  Asn 312 (A) VIC CTGCCCaACGAAGT	56.0°C
<i>XPD</i> ( <i>ERCC2</i> )	nt 35931 A / C Lys 751 Gln	rs 13181	GCCTGGAGCAGCTAGAATCAG ACCCGCCCACTCAGA	Lys 751 (A) VIC CTCTATCCTCTtCAGCGTC  Gln 751 (C) FAM TATCCTCTgCAGCGTC	60.0°C
<i>HR23B</i> ( <i>RAD23B</i> , <i>RD23B</i> )	nt 1097 C / T Ala 249 Val	rs 1805329	AGAGAAAGTCAGGCTGTGGTTGA TGTAGTTGTTGCTGTCGTAGTTGCT	Ala 249 (C) VIC CTGAGGAgCCCCAGTA  Val 249 (T) FAM CTGAGGAaCCCCAGTAC	62.0°C
<i>XPG</i> ( <i>ERCC5</i> )	nt 3507 G / C Asp 1104 His	rs 17655	GCCTCTCAGAATCATCTGATGGAT GTTCTCCTTTGTACATTCATTAAAGATGAA	His 1104 (C) VIC CTTTCAGCATgTTCAC  Asp 1104 (G) FAM CAGCATcTTCACCTTGA	60.0°C
<i>XPC</i>	nt 2920 A / C Lys 939 Gln	rs 2228001	CAGCAGCTTCCCACCTGTTC CTGGTGGGTGCCCTCTAGT	Lys 939 (A) VIC CTCACAGCTtCTCAA  Gln 939 (C) FAM CACAGCTgCTCAA	62.0°C
<i>XPF</i> ( <i>ERCC4</i> )	nt 17103 G / A Arg 415 Gln	rs 1800067	TCAGGTCAAGTACTGATTTGTGCAA GCGTCCAAGAGTGATATAGTCTCTCA	Arg 415 (G) VIC ACATGTTcGGTCATCA  Gln 415 (A) FAM AACATGTTtGGTCATCAC	60.0°C

<i>XPF</i> ( <i>ERCC4</i> )	nt 2063 T / A	rs 1799797	CTACTCTCCACTAGGAGTCGGCTT  CCTGACTCCATGGAAGCTCTTC	(T) VIC CCTTCGGCtGCGTTCGGCT  (A) FAM CCTTCGGCaGCGTTCGGCT	66.0° C
<i>ERCC6</i> ( <i>CSB</i> )	nt 3716 A / G Arg 1213 Gly	rs 2228527	GAGACCAAAGCAAAAGCCTAAGAAC  ACCAGGTGTGGAATTCGAGTTC	Arg 1213 (A) VIC TCTAAGCATTGCaGAGAC  Gly 1213 (G) FAM CTAAGCATTGCgGAGAC	60.0° C
<i>ERCC6</i> ( <i>CSB</i> )	nt 3768 G / C Arg 1230 Pro	rs 4253211	TCGAATTCCACACCTGGTGAAG  CCTCACTCTTGTTTTCACTGTCTTG	Arg 1230 (G) FAM CTGGTAAcGCCTTTT  Pro 1230 (C) VIC TTCTGGTAAgGCCTTTT	60.0° C

<sup>a</sup>Lower case indicates sequence variant. All probes except *XPF662* are minor groove binding (MGB). *XPD 312*, *XPF 662*, *XPF 2063* and *ERCC6 1213* probes were designed for the anti-sense strand. All DNA sequences are listed 5'-3'.

**Table 3. Genotype frequencies, allele frequencies, and odds ratios for NER gene polymorphisms and melanoma**

Locus	Cases	Controls	OR <sup>a</sup> (95% CI)	OR <sup>b</sup> (95% CI)
<i>XPD Codon 312</i> <sup>c</sup>				
Asp/Asp	482 (41%)	1039 (43%)	Referent	Referent
Asp/Asn	532 (45%)	1098 (46%)	1.0 (0.9-1.2)	1.1 (0.9-1.3)
Asn/Asn	162(14%)	260 (11%)	1.3 (1.1-1.7)	1.5 (1.2-1.9)
Trend test			P = 0.03	P = 0.004
Asp <sup>d</sup>	0.64	0.66		
Asn	0.36	0.34		
Chi square test <sup>e</sup>		P = 0.03		
<i>XPD Codon 751</i> <sup>c</sup>				
Lys/Lys	441 (36%)	981 (40%)	Referent	Referent
Lys/Gln	576 (48%)	1128 (46%)	1.1 (1.0-1.3)	1.2 (1.0-1.4)
Gln/Gln	195 (16%)	327 (13%)	1.3 (1.1-1.6)	1.4 (1.1-1.7)
Trend test			P = 0.007	P= 0.004
Lys <sup>d</sup>	0.60	0.63		
Gln	0.40	0.37		
Chi square test <sup>e</sup>		P = 0.007		
<i>HR23B Codon 249</i> <sup>c</sup>				
Ala/Ala	781 (65%)	1530 (64%)	Referent	Referent
Ala/Val	369 (31%)	804 (33%)	0.9 (0.8-1.0)	0.9 (0.8-1.1)
Val/Val	50 (4%)	71 (3%)	1.4 (1.0-2.0)	1.3 (0.9-2.0)
Trend test			P = 0.90	P=0.83
Ala <sup>d</sup>	0.80	0.80		
Val	0.20	0.20		
Chi square test <sup>e</sup>		P = 0.92		
<i>XPG Codon 1104</i> <sup>c</sup>				
Asp/Asp	731 (61%)	1513 (63%)	Referent	Referent
Asp/His	389 (33%)	780 (32%)	1.0 (0.9-1.2)	1.0 (0.9-1.2)
His/His	73 (6%)	115 (5%)	1.3 (1.0-1.8)	1.4 (1.0-2.0)
Trend test			P = 0.17	P = 0.18
Asp <sup>d</sup>	0.78	0.79		
His	0.22	0.21		
Chi square test <sup>e</sup>		P = 0.16		
<i>XPC Codon 939</i> <sup>c</sup>				
Lys/Lys	409 (34%)	785 (32%)	Referent	Referent
Lys/Gln	580 (48%)	1252 (51%)	0.9 (0.8-1.0)	0.9 (0.7-1.0)
Gln/Gln	220 (18%)	402 (16%)	1.1 (0.9-1.3)	1.1 (0.9-1.3)
Trend test			P = 0.98	P = 0.99
Lys <sup>d</sup>	0.58	0.58		
Gln	0.42	0.42		
Chi square test <sup>e</sup>		P = 0.98		

<i>XPF Codon 415<sup>c</sup></i>				
Arg/Arg	1026 (85%)	2073 (85%)	Referent	Referent
Arg/Gln	173 (14%)	360 (15%)	1.0 (0.8-1.2)	1.0 (0.8-1.2)
Gln/Gln	9 (1%)	12 (0%)	1.5 (0.6-3.6)	1.9 (0.7-5.2)
Trend test			P = 0.94	P = 0.75
Arg <sup>d</sup>	0.92	0.92		
Gln	0.08	0.08		
Chi square test <sup>e</sup>		P = 0.96		
<i>XPF Nt 2063<sup>c</sup></i>				
T / T	626 (52%)	1236 (51%)	Referent	Referent
T / A	504 (41%)	1013 (41%)	1.0 (0.9-1.1)	1.0 (0.8-1.1)
A / A	85 (7%)	196 (8%)	0.9 (0.7-1.1)	0.9 (0.6-1.2)
Trend test			P = 0.37	P = 0.46
T <sup>d</sup>	0.72	0.71		
A	0.28	0.29		
Chi square test <sup>e</sup>		P = 0.38		
<i>ERCC6 Codon 1213<sup>c</sup></i>				
Arg/Arg	769 (64%)	1479 (61%)	Referent	Referent
Arg/Gly	383 (32%)	807 (34%)	0.9 (0.8-1.1)	0.9 (0.8-1.1)
Gly/Gly	55 (5%)	122 (5%)	0.9 (0.6-1.2)	0.9 (0.6-1.3)
Trend test			P = 0.17	P = 0.32
Arg <sup>d</sup>	0.80	0.78		
Gly	0.20	0.22		
Chi square test <sup>e</sup>		P = 0.18		
<i>ERCC6 Codon 1230<sup>c</sup></i>				
Arg/Arg	976 (80%)	2018 (83%)	Referent	Referent
Arg/Pro	225 (19%)	392 (16%)	1.2 (1.0-1.4)	1.2 (1.0-1.4)
Pro/Pro	14 (1%)	26 (1%)	1.1 (0.6-2.1)	1.2 (0.6-2.5)
Trend test			P = 0.08	P = 0.10
Arg <sup>d</sup>	0.90	0.91		
Pro	0.10	0.09		
Chi square test <sup>e</sup>		P = 0.08		

<sup>a</sup> Unadjusted

<sup>b</sup> Adjusted for age at diagnosis, sex, age-sex interaction and center

<sup>c</sup> Genotype frequencies N (%)

<sup>d</sup> Allele frequencies

<sup>e</sup> Comparing cases and controls.

**Table 4. *XPD* haplotype frequencies and odds ratios for combined *XPD* genotypes and melanoma.**

a. Estimated haplotype frequencies in cases and controls

<i>XPD</i> 312	<i>XPD</i> 751	Cases	Controls	P-value <sup>a</sup>
Asp (G)	Lys (A)	55%	58%	0.02
Asp (G)	Gln (C)	8%	8%	0.86
Asn (A)	Lys (A)	5%	5%	0.19
Asn (A)	Gln (C)	32%	29%	0.003
Lewontin's D'		0.792	0.752	
Linkage Disequilibrium Test		P < 0.001	P < 0.001	

<sup>a</sup>Comparing cases and controls

b. Odds ratios for *XPD* 312 + 751 haplotypes and melanoma

Number of copies of A + C Haplotype	Controls (Number imputed)	Cases (Number imputed)	OR <sup>a</sup> (95% CI)	OR <sup>b</sup> (95% CI)
0	1213 (21)	545 (9)	Referent	Referent
1	998 (804)	507 (404)	1.1 (1.0-1.3)	1.2 (1.0-1.4)
2	182	119	1.5 (1.1-1.9)	1.6 (1.2-2.0)
Trend test			P=0.004	P=0.002

<sup>a</sup> Unadjusted

<sup>b</sup> Adjusted for age at diagnosis, sex, age-sex interaction and center

**Table 5. Odds ratios for NER genotypes and melanoma according to age at diagnosis.**

Locus	Age at Diagnosis			
	Less than 30 OR <sup>a</sup> (95% CI)	30-49 OR <sup>a</sup> (95% CI)	50-69 OR <sup>a</sup> (95% CI)	70 and over OR <sup>a</sup> (95% CI)
<i>XPD</i> 312				
Asp/Asp	Referent	Referent	Referent	Referent
Asp/Asn	1.2 (0.4-3.6)	1.2 (0.8-1.8)	1.1 (0.9-1.4)	1.0 (0.8-1.3)
Asn/Asn	3.9 (0.9-17.0)	2.1 (1.2-3.7)	1.3 (0.9-1.9)	1.3 (0.9-2.0)
<i>XPD</i> 751				
Lys/Lys	Referent	Referent	Referent	Referent
Lys/Gln	1.8 (0.5-6.4)	1.3 (0.9-2.0)	1.2 (0.9-1.5)	1.0 (0.7-1.3)
Gln/Gln	6.3 (1.4-28.6)	1.7 (1.0-2.8)	1.3 (0.9-1.8)	1.4 (0.9-2.0)
<i>HR23B</i>				
Ala/Ala	Referent	Referent	Referent	Referent
Ala/Val	0.3 (0.1-1.0)	0.9 (0.6-1.3)	0.9 (0.7-1.2)	0.9 (0.7-1.2)
Val/Val	1.0 (0.2-6.8)	1.9 (0.6-5.4)	0.9 (0.5-1.7)	2.1 (1.0-4.3)
<i>XPG</i> 1104				
Asp/Asp	Referent	Referent	Referent	Referent
Any His	1.9 (0.7-5.4)	1.1 (0.7-1.6)	0.9 (0.7-1.1)	1.2 (0.9-1.5)
<i>XPC</i> 939				
Lys/Lys	Referent	Referent	Referent	Referent
Lys/Gln	0.5 (0.2-1.7)	1.1 (0.7-1.6)	0.9 (0.7-1.2)	0.7 (0.5-1.0)
Gln/Gln	2.9 (0.8-10.1)	1.3 (0.7-2.1)	1.0 (0.7-1.5)	1.0 (0.7-1.5)
<i>XPF</i> 415				
Arg/Arg	Referent	Referent	Referent	Referent
Any Gln	0.8 (0.2-3.1)	0.9 (0.6-1.5)	1.1 (0.8-1.5)	1.0 (0.7-1.4)
<i>XPF</i> 2063				
T / T	Referent	Referent	Referent	Referent
T / A	0.7 (0.3-2.0)	1.0 (0.7-1.5)	0.9 (0.7-1.1)	1.1 (0.9-1.5)
A / A	0.8 (0.1-4.7)	0.6 (0.2-1.3)	1.2 (0.8-1.9)	0.6 (0.4-1.1)
<i>ERCC6</i> 1213				
Arg/Arg	Referent	Referent	Referent	Referent
Any Gly	1.4 (0.5-3.7)	0.8 (0.5-1.1)	1.0 (0.8-1.2)	0.9 (0.7-1.2)
<i>ERCC6</i> 1230				
Arg/Arg	Referent	Referent	Referent	Referent
Any Pro	1.1 (0.3-3.7)	1.2 (0.7-1.9)	1.1 (0.8-1.5)	1.2 (0.9-1.7)

<sup>a</sup> Adjusted for sex and center.

**Table 6. Total number of variant alleles for all NER genes combined in cases and controls, and ORs for melanoma.**

Number of variant alleles	Cases (%)	Controls (%)	OR <sup>a</sup> (95% CI)	OR <sup>b</sup> (95% CI)
0	11 (1.0%)	26 (1.1%)	<b>0.9 (0.4-1.9)</b>	<b>1.0 (0.5-2.2)</b>
1	56 (4.9%)	113 (4.9%)	<b>1.1 (0.7-1.5)</b>	<b>1.0 (0.7-1.5)</b>
2	113 (10%)	260 (11%)	<b>0.9 (0.7-1.2)</b>	<b>0.9 (0.7-1.2)</b>
3	165 (15%)	391 (17%)	<b>0.9 (0.7-1.2)</b>	<b>0.9 (0.7-1.2)</b>
4	218 (19%)	469 (20%)	<b>Referent</b>	<b>Referent</b>
5	216 (19%)	392 (17%)	<b>1.2 (0.9-1.5)</b>	<b>1.2 (0.9-1.5)</b>
6	155 (14%)	305 (13%)	<b>1.1 (0.9-1.4)</b>	<b>1.1 (0.9-1.5)</b>
7	114 (10%)	188 (8.1%)	<b>1.3 (1.0-1.7)</b>	<b>1.4 (1.0-1.9)</b>
8	56 (4.9%)	106 (4.6%)	<b>1.1 (0.8-1.6)</b>	<b>1.1 (0.8-1.7)</b>
9	19 (1.7%)	47 (2.0%)	<b>0.9 (0.5-1.5)</b>	<b>0.8 (0.5-1.5)</b>
10 +	10 (0.9%)	15 (0.7%)	<b>1.4 (0.6-3.2)</b>	<b>1.8 (0.7-4.6)</b>

<sup>a</sup> Unadjusted

<sup>b</sup> Adjusted for age at diagnosis, sex, age-sex interaction and center

Test for trend: P = 0.03 (unadjusted), P = 0.02 (adjusted for age, sex, center and age-sex interaction).