XPD gene polymorphism and host characteristics in the association with cutaneous malignant melanoma risk

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We recently reported an association between low DNA repair capacity, measured through the host-cell reactivation assay, and melanoma risk in subjects with dysplastic naevi or low tanning ability. We investigated the genetic basis for these findings by analysing the Asp312Asn and Lys751Gln polymorphisms of the XPD (ERCC2) DNA repair gene in the same subjects. Similar to our previous report, no significant association between XPD polymorphisms and melanoma risk was found in 176 melanoma cases and 177 controls (odds ratio (OR) = 1.5, 95% confidence interval (CI) = 0.9–2.5 for 312Asn; OR = 1.3, 95% CI = 0.8–2.1 for 751Gln, adjusted for age, gender, dysplastic naevi and pigmentation characteristics). However, XPD variants were associated with increased risk in older (> 50 years) subjects (OR = 3.4, 95% CI = 1.6–7.3 for 312Asn; OR = 2.3, 95% CI = 1.1–4.9 for 751Gln). The 751Gln allele was associated with elevated melanoma risk among subjects without dysplastic naevi (OR = 2.6, 95% CI = 1.1–6.4). Subjects with low tanning ability and XPD variants exhibited a nonsignificant increase of melanoma risk (OR = 2.3, 95% CI = 0.7–7.0 for 312Asn; OR = 3.0, 95% CI = 1.0–8.8 for 751Gln). DNA repair capacity was slightly decreased in subjects carrying 751Gln alleles. XPD variants may modify melanoma risk in subjects with specific host characteristics, such as older age, lack of dysplastic naevi or low tanning ability.

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Between 1940 and 2000, descriptive studies documented a progressive worldwide rise in the incidence of cutaneous malignant melanoma. Melanoma risk increases with age (Kosary et al, 1996; Gilchrest et al, 1999) and other risk factors, such as sunlight exposure (IARC, 1992), particularly intermittent (Gilchrest et al, 1999), family history of melanoma, dysplastic naevi or atypical naevi, number of naevi, skin sensitivity to sun, freckling, fair hair, eye and skin colour (Tucker et al, 1997; Landi et al, 2001). Individuals with inherited defects in nucleotide excision repair (eg, patients with xeroderma pigmentosum, XP) have low repair of UV-induced DNA lesions and are at extremely high risk of skin cancers, including melanoma (Berneburg and Lehmann, 2001). In healthy subjects, polymorphisms in DNA repair genes may be associated with altered DNA repair capacity and susceptibility to cancer (Benhamou and Sarasin, 2002).

The XP complementation group D (XPD, also known as ERCC2) protein, a subunit of the Transcription Factor IIH (TFIIH), is involved in DNA unwinding during DNA repair and initiation of basal transcription (Berneburg and Lehmann, 2001). Several single nucleotide polymorphisms (SNPs) in XPD gene exons have been identified (Benhamou and Sarasin, 2002). The nonsilent variants at codons 312 (exon10, G>G, Asp312Asn) and 751 (Exon23, A>C, Lys751Gln) are amenable to investigation in epidemiological studies in Caucasians, given their high frequency (Benhamou and Sarasin, 2002). The XPD 751Gln variant substantially modifies the amino-acid electronic configuration in a domain important for the interaction with the helicase activator p44 and may produce the most relevant change in XPD function (Goñi et al, 1998; Benhamou and Sarasin, 2002). Aging has been associated with depressed in situ repair of UV-specific DNA lesions in the skin of subjects carrying the XPD variants 312Asn and 751Gln (Hemminki et al, 2001), who may therefore be at higher risk for cancer at an older age (Dybdahl et al, 1999; Sturgis et al, 2000). We recently showed that specific host characteristics, such as presence of dysplastic naevi and low tanning ability, modify the risk of melanoma associated with low DNA repair capacity (Landi et al, 2002). We report here on (i) the association between the Asp312Asn and Lys751Gln XPD polymorphisms and cutaneous malignant melanoma risk, (ii) the effect modification of age, presence of dysplastic naevi and tanning ability, and (iii) the association between XPD polymorphisms and DNA repair capacity, in the same subjects.
MATERIALS AND METHODS

Study subjects

From December 1994 to January 1999, we recruited 183 (87 male and 96 female) cases with incident nonmelanoma cutaneous malignant melanoma (of any stage) and 179 (89 male and 90 female) controls between the ages of 17 and 77 years at the Dermatology Unit of the Bufalini Hospital in Cesena, Italy, as previously described (Landi et al., 2001, 2002). The Hospital examined approximately 85% of all the melanoma patients of the Northern Marche and Southern Romagna areas, from which all study subjects came from (Landi et al., 2002). Approximately 95% of the cases and 83% of the controls agreed to participate in the study. Control subjects, frequency-matched to the cases by decade (5) has absolutely no change'.

Because no one selected choice 4 and only three selected choice 5, the answers were categorised into three groups: high tanning response (choice 1), medium tanning response (choice 2), and low tanning response (choices 3 and 5) (Landi et al., 2002). An expert oncologist, blinded to melanoma status, assessed dysplastic naevus diagnoses and naevus counts in standardised photographs of the subjects' backs, as previously described (Landi et al., 2002). In total, 15 cases and 15 controls had either no photographs or uncertain dysplastic naevus diagnosis. A three-category variable (dysplastic naevi, no dysplastic naevi, unknown/uncertain dysplastic naevi) was used in the logistical models. Naevi and dysplastic naevi tend to disappear with age, making their diagnosis difficult to assess in older individuals (Tucker et al., 1997). We, therefore, classified all subjects (47 cases and 28 controls) older than 60 years of age as with uncertain dysplastic naevus status (Landi et al., 2002). However, the use in the analysis of dysplastic naevus data from these older subjects did not change the results.

DNA extraction and XPD genotyping

Genomic DNA was extracted from 1–2 × 10^6 cryopreserved lymphocytes. We used the Nucleon Extraction and Purification kit (Amersham LIFE SCIENCE, UK), following the manufacturer's instructions. Using lymphocyte DNA as template, we genotyped the Asp312Asn SNP using a Taqman 5'-ATCTTGTCCTCTACCgCCATTCT-3' and 5'-TgTgAgAcgTgAAtTGa-gAAAT-3' in 50 µl polymerase chain reaction (PCR) reactions containing: 10 mM Tris-HCl (pH 8.8 at 25°C), 1.5 mM MgCl2, 50 mM KCl, 0.1% Triton X-100, 0.2 mM each deoxynucleotidetriphosphate, 20 pmoles of each primer, 2 U of Taq DNA Polymerase Dy-nyzynase (Finzymes, ESPOO, Finland) and 50–100 ng of genomic DNA. The cycling conditions were: initial denaturation at 95°C for 3 min, 30 cycles of denaturation at 94°C for 45 s, primer annealing at 60°C for 45 s, primer extension at 72°C for 1 min and final extension at 72°C for 5 min. The PCR product was digested with 10-15 U of PstI enzyme (Promega, Madison, WI, USA) in a 25-µl-reaction mixture for 2 h at 37°C, as suggested by the manufacturer, and separated on a 2% agarose gel. In addition to a PstI site away from the polymorphism that serves as internal control for digestion, the Lys(A) allele has a second PstI restriction site. The three possible genotypes are defined by three distinct banding patterns: AA (100- and 224-bp fragments), AC (66-, 100-, 158- and 224-bp fragments) and CC (66-, 100- and 158-bp fragments). Genotypes for Lys751Gln were successfully determined in 176 cases and 177 controls.

Hand-cell reactivation (HCR) assay

We measured DNA repair capacity in cryopreserved lymphocytes by the CAT-gene based HCR assay, as previously described (Athas et al., 1991; Landi et al., 2002). CAT activity >1000 c.p.m. in unirradiated cells is required to measure accurately the DNA repair capacity with a signal-to-noise ratio at least two-fold higher than the background level (200 c.p.m.) in cells with irradiated plasmids (Landi et al., 2002). DNA repair capacity measured in lymphocytes with baseline CAT activity <1000 c.p.m. (44 cases and 32 controls) was not considered in the analysis.

All laboratory analyses were performed blinded to the case status.

Statistical analysis

We calculated adjusted odds ratios (ORs) and 95% confidence intervals (CIs) by unconditional multiple logistic regression models that included the matching variables (i.e., age and gender), presence of dysplastic naevi, skin colour, tanning ability and eye colour as independent variables. Because of the relatively small number of subjects with the homozygous variant genotypes, we used two-category variables (consensus vs heterozygous/homozygous) in logistic regression analysis. The models with three-category variables were not substantially different from the models with two-category variables (P≥0.17, likelihood ratio tests). We used the likelihood ratio test to test for interactions, which were also evaluated using a case-only analysis (Yang et al., 1997). The case-only approach confirmed the results of the case–control analysis. Here, we report the analysis based on cases and controls. Age was a matching variable and, therefore, we could not estimate its main effect on melanoma risk in the logistical models including the interaction between age and the XPD polymorphisms.

Harley–Weinberg equilibrium was tested using the asymptotic Pearson's χ²-test. We used the Fisher's exact test to compare proportions and the Spearman's rank correlation statistics to assess linear correlation between variables. All statistical tests were two-sided. The Stata 7.0 statistical package was used for all analyses (Stata Corporation, College Station, TX, USA).
RESULTS

Distribution of the XPD polymorphisms

Frequencies of XPD 312Asn and 751Gln alleles were 0.396 and 0.403, respectively, in cases, and 0.398 and 0.427, respectively, in controls. The distribution of both polymorphisms among controls was consistent with the Hardy–Weinberg equilibrium ($P = 0.30$ for Asp312Asn and $P = 0.81$ for Lys751Gln), also when the subjects were subdivided in age, dysplastic naevus status or tanning ability categories. Consistently with previous reports (Butkiewicz et al., 2001; Zhou et al., 2002), carriers of the 312Asp/Asp genotype tended to have the 751Lys/Lys genotype (test for the association between Asp312Asn and Lys751Gln genotypes: $P = 2.1 \times 10^{-24}$ in controls, and $P = 1.1 \times 10^{-22}$ in cases).

In controls, XPD genotypes were not associated with age, gender or the strongest risk factors for melanoma in this population (Landi et al., 2001), that is, eye colour, skin colour, tanning ability to prolonged and repeated sun exposure, and presence of dysplastic naevi (Table 1). In addition, XPD genotypes were not associated with hair colour, freckling, skin response to 30 min of sun exposure, naevus number, lifetime number of severe sunburns, and lifetime or childhood cumulative hours of sun exposure during vacation (data not shown).

XPD polymorphisms and melanoma risk

Overall, the XPD polymorphisms were not significantly associated with melanoma risk (Table 2). Subjects with at least one 312Asn variant allele had an OR for melanoma of 1.5 (95% CI 0.9–2.5), adjusted for age, gender, presence of dysplastic naevi, skin colour, tanning ability, and eye colour) compared with individuals with the 312Asp/Asp genotype. Subjects with at least one 751Gln variant allele had an OR of 1.3 (95% CI 0.8–2.1) in comparison with subjects carrying the 751Lys/Lys genotype.

Upon dividing the subjects into two age groups, we found an increased melanoma risk for older subjects carrying variant alleles (Table 2). Among subjects with age ≥50 years, those with at least one 312Asn allele had an OR of 3.4 (95% CI 1.6–7.3). Subjects with the 751Gln variant had OR = 2.3 (95% CI 1.1–4.9). In subjects <50 years of age, the XPD variant alleles were not associated with melanoma (OR = 0.7, 95% CI = 0.4–1.5 for 312Asn; OR = 0.7, 95% CI = 0.4–1.5 for 751Gln). The relative odds due to the XPD variants were significantly higher in older subjects ($P = 0.004$ for 312Asn; $P = 0.03$ for 751Gln). We used the 50-years cutoff to define the age categories in order to compare our results with those reported in previous studies (Sturgis et al., 2000; Hemminki et al., 2001). We found similar results using the actual median age of all the subjects (48 years) or the median age of the controls (45 years) (data not shown).

After the subjects were stratified according to the presence of dysplastic naevi (Table 2), individuals without dysplastic naevi and with the 751Gln/Lys or 751Gln/Gln genotypes showed an increased melanoma risk (OR = 2.6, 95% CI = 1.1–6.4) compared to those without dysplastic naevi and with the 751Lys/Lys genotype. Among subjects without dysplastic naevi, the association between the XPD polymorphism and melanoma risk appeared limited to older subjects. When we repeated the analysis in subjects who were 50 years of age or older, having variant alleles at codon 751 was associated with the highest risk of melanoma among individuals without dysplastic naevi (OR = 13.9, 95% CI = 1.4–136.3). Among

*Table 1* Characteristics of cases and controls by XPD Asp312Asn and Lys751Gln polymorphisms

| Age (years) | XPD 312 | | XPD 751 | |
|-------------|---------|---|---------|---|
|             | Case     | Control | Case     | Control |
|             | Asp/Asp  | Asp/Asn | Asn/Asn | Lys/Lys  | Lys/Gln | Gin/Gln |
|             | (G/G)    | (G/A)   | (A/A)   | (A/A)    | (A/C)   | (C/C)   |
| 17–49       | n = 52   | n = 94  | n = 18  | n = 59   | n = 89  | n = 24  |
| 50–77       | 31       | 43      | 6       | 28       | 58      | 16      |
| Gender      |          |         |         |          |         |         |
| Male        | 28       | 41      | 9       | 30       | 43      | 12      |
| Female      | 24       | 53      | 9       | 29       | 46      | 12      |
| Eye coloura |          |         |         |          |         |         |
| Dark        | 13       | 27      | 2       | 19       | 38      | 11      |
| Medium      | 24       | 54      | 12      | 33       | 42      | 10      |
| Light       | 15       | 13      | 4       | 7        | 8       | 3       |
| Skin colour |          |         |         |          |         |         |
| Dark/medium | 22       | 43      | 6       | 37       | 62      | 16      |
| Light       | 29       | 51      | 12      | 22       | 27      | 8       |
| Tanning ability to prolonged sun exposure | | | | | | |
| High/medium | 36       | 57      | 10      | 51       | 71      | 21      |
| Low         | 15       | 33      | 8       | 8        | 15      | 2       |
| Dysplastic naevi | | | | | | |
| No          | 12       | 32      | 8       | 29       | 60      | 14      |
| Yes         | 23       | 27      | 5       | 11       | 11      | 6       |

aDark – black or dark brown. Medium – light brown, brown-green, green or blue-green. Light – light blue, dark blue or grey. bSubjects older than 60 years of age were considered as with uncertain dysplastic naevus status. The total number of subjects may vary across variables due to missing values.
Table 2. XPD Asp312Asn and Lys751Gln polymorphisms and risk of cutaneous malignant melanoma in the overall analysis and by age, presence of dysplastic naevi or tanning ability.

| XPD 312 | Genotype | Case | Control | OR* (95% CI)* |
|---------|----------|------|---------|--------------|
| Overall analysis | Asp/Asp (G/G) | 52 | 59 | 1.0 Ref. |
| | Asp/Asn (G/A) | 112 | 113 | 1.5 |
| | Asn/Asn (A/A) | 63 | 39 | 3.4 |
| By age (years) | 17–49 | 31 | 28 | 1.0 Ref. |
| | 17–49 | 49 | 74 | 0.7 |
| | 50–77 | 21 | 31 | 1.0 Ref. |
| | 50–77 | 63 | 39 | 3.4 |
| Test of heterogeneity of ORs | P = 0.004 |
| By presence of dysplastic naevi | No | 12 | 29 | 1.0 Ref. |
| | No | 40 | 74 | 1.4 |
| | Yes | 23 | 11 | 1.0 Ref. |
| | Yes | 32 | 17 | 0.9 |
| Test of heterogeneity of ORs | P = 0.52 |
| By tanning ability | High/medium | 36 | 51 | 1.0 Ref. |
| | High/medium | 67 | 92 | 1.3 |
| | Low | 15 | 8 | 1.0 Ref. |
| | Low | 41 | 17 | 2.3 |
| Test of heterogeneity of ORs | P = 0.39 |

| XPD 751 | Genotype | Case | Control | OR* (95% CI)* |
|---------|----------|------|---------|--------------|
| Overall analysis | Lys/Lys (A/A) | 58 | 59 | 1.0 Ref. |
| | Lys/Gln (A/C) | 118 | 118 | 1.3 |
| By age (years) | 17–49 | 34 | 30 | 1.0 Ref. |
| | 17–49 | 51 | 75 | 0.7 |
| | 50–77 | 24 | 29 | 1.0 Ref. |
| | 50–77 | 67 | 43 | 2.3 |
| Test of heterogeneity of ORs | P = 0.03 |

*Odds ratios (ORs) and 95% confidence intervals (95% CIs) adjusted for age, gender, dysplastic naevus status, skin colour, tanning ability and eye colour.

In subjects with medium or high tanning ability, we observed no association of melanoma risk with the 312Asn (OR = 1.3, 95% CI = 0.7–2.4) or 751Gln (OR = 1.0, 95% CI = 0.6–1.8) variant. However, the risk modification by tanning ability was not statistically significant (P ≥ 0.08). Even though based on small number of subjects, we found similar results after dividing the subjects with low tanning ability in two groups with high or low lifetime recreational sun exposure (data not shown).
DISCUSSION

Previous investigations in noncancer subjects with different ethnic backgrounds reported variable frequencies of the 312Asn and 751Gln alleles (Goode et al, 2002). We found a high frequency of the two XPD variants in this Italian population, as previously described by other investigators (Matullo et al, 2001) for the 751Gln polymorphism.

Similar to our previous report on DNA repair capacity and melanoma (Landi et al, 2002), we did not find a significant overall association between melanoma risk and the XPD polymorphisms. However, both 312Asn and 751Gln alleles were associated with increased melanoma risk in subjects who were 50 years of age or older. Recently, Hemminki et al (2001) showed that the Asp312Asn and Lys751Gln polymorphisms might interact with age in their association with repair rates of UV-induced lesions in the skin. In the Hemminki’s study, subjects ≥50 years of age carrying the XPD variants exhibited lower DNA repair.

To date, only two studies have investigated the association between melanoma risk and XPD polymorphisms. A small study that compared the results from 28 Stage I melanoma patients with 33 samples from healthy blood donors found an overrepresentation of the 751Lys allele among the melanoma cases (Tomescu et al, 2001). This first investigation that did not analyse the Asp312Asn polymorphism was restricted to subjects younger than 50 years of age. In our study, the proportion of the 751Lys/Lys genotype was moderately higher among cases than controls in younger subjects (Table 2). A second investigation conducted in 125 melanoma cases and 211 cadaveric renal transplant donors failed to find an association between Asp312Asn or Lys751Gln and melanoma (Winsey et al, 2000). The median age of the cases was 52 years; the age of the control group was not reported. No data on the interaction between XPD variants and age or other risk factors were provided in these two studies. Similar to our results, two additional investigations on basal cell carcinoma (Dybdahl et al, 1999) and head and neck cancer (Sturgis et al, 2000) suggested that carriers of the XPD 751Gln allele may be at risk for cancer at an older age. None of the two studies evaluated the Asp312Asn polymorphism.

Gilchrest et al (1999) suggested that age plays a major role in vulnerability to photocarcinogens. Melanoma risk exhibits a steep increase with age. In the study area, melanoma incidence rates, which we calculated from available data (Parkin et al, 1997), were equal to 6.1/100 000 person-years in subjects between 15 and 49 years of age and 17.5/100 000 person-years between 50 and 74 years. Cumulative DNA damage during aging is the result of errors in DNA replication occurring at low but finite rate or of incompletely repaired DNA damage (Yaar and Gilchrest, 2001). As age increases, subjects accumulate more opportunities for DNA damage due to prolonged exposure to UV radiation and other carcinogens. Furthermore, defences against sunlight exposure, such as skin pigmentation (Yaar and Gilchrest, 2001), epidermal thickness (Yaar and Gilchrest, 2001), and DNA repair efficiency (Wei et al, 1993; Yaar and Gilchrest, 1998) may decrease with age.

In older subjects, the equilibrium between DNA damage and repair may be altered and further destabilised in presence of variant alleles in DNA repair genes, which may be otherwise negligible. The genetic alterations and the possible sequence of mutations needed for the progression to melanoma are still to be defined. Bennet (2003) recently proposed a speculative model for the genetic basis of primary melanoma in relation to cell senescence. In this model, early gene mutations leading to melanocyte proliferation, such as those in the RAS, protein tyrosine kinase or BRAF genes, are followed by lesions in the p16/TP53 pathway. These alterations may be associated with the development of common or dysplastic naevi and, eventually, with the radial growth phase of malignant melanoma. Additional molecular events possibly linked to apoptosis suppression, such as PTEN loss, overexpression of a number of protein tyrosine kinases and RAS or beta-catenin activation, could be required for the progression to invasive melanoma. Further research is needed to assess how variations in XPD and, in general, DNA repair function affect the molecular events involved in melanoma development.

In a previous study on the same population, we showed an interaction in the association with melanoma risk between the overall DNA repair capacity, measured using the HCR assay, and presence of dysplastic naevi or low tanning ability (Landi et al, 2002). In the present study, the effect of the XPD 751Gln allele was evident in subjects without dysplastic naevi, particularly at older age. Presence of dysplastic naevi did not appear to modify the association between XPD Lys751Gln polymorphism and melanoma risk in subjects younger than 50 years of age. The presence of dysplastic naevi is one of the strongest risk factors for melanoma. The association between dysplastic naevi and melanoma in our population was also very strong (Landi et al, 2001) and this could have overwhelmed the effect of XPD variants in older subjects with dysplastic naevi. Caution is needed in the appraisal of these results because the relatively small sample size limited the assessment of the association between melanoma and XPD genotype when subjects were categorised at the same time by dysplastic naevus status and age. Naevi and dysplastic naevi tend to disappear with age, making their diagnosis difficult to assess in older individuals (Tucker et al, 1997). To be conservative, we classified all subjects older than 60 years of age as of uncertain dysplastic naevus status. However, the inclusion in the statistical analysis of the dysplastic naevus diagnoses in older subjects did not change the results.

As for tanning ability, similar to what we found in the association between DNA repair capacity and melanoma (Landi et al, 2002), there was a nonsignificantly increased risk of melanoma in subjects with both low tanning ability and a variant genotype. Subjects with a low tanning ability may be at increased risk of DNA damage, because they have lower protection against UV-exposure. A less efficient DNA repair may increase melanoma risk in these subjects.

We found a nonsignificant association between the overall DNA repair capacity and Lys751Gln polymorphism. This finding is consistent with previous investigations that measured DNA repair capacity by the HCR assay on peripheral lymphocytes of healthy subjects (Seker et al, 2001; Spitz et al, 2001) or on lymphoblastoid cell lines (Qiao et al, 2002). The sensitivity of the assay measuring DNA repair capacity may have been a limiting factor in the assessment of the association. A recent study using the HCR assay based on the luciferase reporter gene found, as we did, significantly lower levels of DNA repair capacity in 751Gln/Gln than in 751Lys/Lys subjects, but in this case the association was statistically significant (Qiao et al, 2002a).

In conclusion, the XPD 312Asn and 751Gln variant alleles were associated with an increased risk for melanoma in subjects older than 50 years of age. The presence of dysplastic naevi and low tanning ability may also affect the association between XPD polymorphisms and melanoma risk. As with all statistical interactions that are identified for the first time, replication in an independent study is necessary. Future studies, assessing multiple genes involved in nucleotide excision repair, may help evaluate the genetic basis of the interplay between DNA repair and age or other host characteristics.

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