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Epidermal Growth Factor Gene (*EGF*) Polymorphism and Risk of Melanocytic Neoplasia

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A common single nucleotide polymorphism (SNP) in the 5′ untranslated region (5′UTR) of the epidermal growth factor (*EGF*) gene modulates the level of transcription of this gene and hence is associated with serum levels of EGF. This variant may be associated with melanoma risk, but conflicting findings have been reported. An Australian melanoma case–control sample was typed for the EGF + 61A > G transversion (rs4444903). The sample comprised 753 melanoma cases from 738 families stratified by family history of melanoma and 2387 controls from 645 unselected twin families. Ancestry of the cases and controls was recorded, and the twins had undergone skin examination to assess total body nevus count, degree of freckling and pigmentation phenotype. SNP genotyping was carried out via primer extension followed by matrix-assisted laser desorption time of flight (MALDI-TOF) mass spectroscopy. The EGF + 61 SNP was not found to be significantly associated with melanoma status or with development of nevi or freckles. Among melanoma cases, however, G homozygotes had thicker tumors ($p = 0.05$), in keeping with two previous studies. The *EGF* polymorphism does not appear to predispose to melanoma or nevus development, but its significant association with tumor thickness implies that it may be a useful marker of prognosis.

Key words: epidermal growth factor/genetic variation/melanoma/nevi/predisposition
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The mitogen-activated protein kinase (MAPK) pathway is a key signal transduction pathway in which extracellular growth factor signals are transmitted from cell surface receptors through a cascade of protein kinases to nuclear transcription factors, which regulate cell proliferation or differentiation by altering gene expression. Constitutive activation of this pathway frequently occurs in malignant melanoma and appears crucial for autonomous growth of these tumors (Cohen *et al*, 2002; Dong *et al*, 2003; Satyamoorthy *et al*, 2003). Recently, somatic mutations of the *BRAF* gene have been shown to be the principal mechanism by which the MAPK pathway is activated in (20%–83%) melanomas (Brose *et al*, 2002; Davies *et al*, 2002; Dong *et al*, 2003; Maldonado *et al*, 2003; Pollock *et al*, 2003; Satyamoorthy *et al*, 2003) and (74%–82%) benign melanocytic nevi (Dong *et al*, 2003; Pollock *et al*, 2003; Uribe *et al*, 2003; Yazdi *et al*, 2003). In the majority of melanomas without *BRAF* mutations, the MAPK pathway has been found to be activated through mutation of members of the *RAS* proto-oncogene family, particularly *NRAS* (van Elsas *et al*, 1996). Further support for this notion comes from the finding that we James *et al*,[1] and others (Meyer *et al*, 2003), have shown that germline polymorphisms in the *BRAF* gene are significantly associated with melanoma predisposition. It is reasonable to expect that constitutive natural variation or somatic mutations in genes encoding other members of the MAPK pathway could confer inherited susceptibilty to melanoma, or play a role in melanoma development.

The epidermal growth factor gene (*EGF*) encodes a ligand for the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase (RTK) that transduces growth signals to MAPK via RAS and BRAF. Shahbazi *et al* (2002) reported a striking association between melanoma risk and G/G genotype at the +61 A > G polymorphism in the 5′ untranslated region (5′UTR) of *EGF* ($p < 0.0001$). This was backed up by the finding that peripheral blood mononuclear cell cultures from individuals homozygous for the G allele produced significantly more EGF than cells from homozygotes for the A allele, while heterozygotes were found to produce intermediate levels (Shahbazi *et al*, 2002). The Breslow thickness of melanomas at time of presentation was also greater for G/G homozygotes ($p = 0.045$). A follow-up report failed to confirm the association with melanoma risk, although it did support ($p = 0.03$) an association between G/G genotype and thicker tumors (McCarron *et al*, 2003). In the present study, we sought to extend this work and to test the hypothesis that the nucleotide 61 polymorphism of the *EGF* gene predisposes to the development of nevi and freckles as well as melanoma.

Results

There were 3391 individuals genotyped where melanoma status was also known. Case and control genotype

Abbreviations: EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; SNP, single nucleotide polymorphism

[1] James MR, Roth RB, Shimm, *et al*: BRAF polymorphisms and risk of melanocytic neoplasia. Manuscript in preparation.

Table I. CMM and EGF + 61 genotype in three studies

	Shahbazi *et al* (2002)		McCarron *et al* (2003)		Present study	
	Controls	CMM[a]	Controls	CMM	Controls	CMM
A/A	32 (32.3)	21 (15.6)	121 (39.0)	56 (35.2)	883 (33.4)	274 (36.8)
A/G	47 (47.5)	50 (37.0)	131 (42.3)	82 (51.6)	1317 (49.8)	354 (47.5)
G/G	20 (20.2)	64 (47.4)	58 (18.7)	21 (13.2)	446 (16.9)	117 (15.7)
HWE-P	0.718	0.043	0.037	0.288	0.238	0.881
Assoc LOD	3.84		0.53		0.48	
p-value	0.00003		0.120		0.14	

[a]CMM, cutaneous malignant melanoma. Count (percent) of each genotype. HWE-P represents p-value from exact test for Hardy–Weinberg equilibrium. Assoc LOD represents the contingency χ^2 for each 2×3 table coverted into a LOD score (via inversion of the p-value).

frequencies were in Hardy–Weinberg equilibrium. Allele and genotype frequencies did not differ significantly between melanoma cases and controls (Table I). Comparing our sample to those previously studied found that all the control genotype frequencies could be equated ($p = 0.133$). The case genotype frequencies observed in our Australian sample did not differ significantly from those in a UK sample described by McCarron *et al* (2003) (homogeneity of genotype frequencies $p = 0.25$; homogeneity of association with melanoma, $p = 0.09$), but were significantly different from that in the UK cases described by Shahbazi *et al* (2002) (overall homogeneity $p = 0.00001$).

We did detect a weak association between genotype and tumor thickness (Table II; Kruskal–Wallis $p = 0.049$), which was similar in magnitude and direction to that found in both previous studies. The effect is small: only a fall from 21.4% *in situ* melanomas in the A carriers to 16.8% in the G homozygotes. Summarizing the data from the present study using the same four thickness categories as the two previous studies led to a small loss of information: the ordinal logistic regression model obtained $p = 0.06$. Combining the present study with the previous two found no evidence for heterogeneity of association, with the overall association $p = 0.02$.

Within the control twin families, there was no association between EGF + 61 genotype and total body nevus count (geometric mean count A/A, 98.6; A/G, 100.8; G/G 101.9; $p = 0.56$). This was also the case for subtotals of papular, macular or atypical nevi and for freckling score (data not shown).

Discussion

We have confirmed a small effect of EGF + 61 genotype on tumor thickness within a total population sample of melanoma cases from the state of Queensland, Australia. Since melanoma is so common in our population, surveillance is intense, with the result that the average tumor thickness in our sample is approximately half that seen in the two British studies. Despite this difference, roughly the same pattern of association is seen in the three studies, with the thickest tumors being from the G/G homozygotes.

We did not see the extreme association with melanoma risk observed by Shahbazi and coworkers (2002), but our findings are very close to the study of McCarron *et al* (2003). It is difficult to imagine ethnic differences leading to such a large discrepancy between study findings among cases only. As noted earlier, control genotype frequencies were very comparable across all three studies, so one plausible explanation is Type I error in the initial publication.

There are several lines of evidence that make an association between *EGF* genotype and melanoma behavior plausible, in keeping with the functional results presented by Shahbazi *et al* (2002). As noted earlier, *BRAF* somatic mutations are extremely common in cutaneous melanomas and benign melanocytic nevi, and, along with *NRAS* mutation, lead to the constitutive activation of the MAPK pathway that appears crucial to autonomous growth of these tumors. Animal models further implicate this pathway in melanocytic and melanoma pathogenesis. The *Tu* melanoma locus in Xiphophorus is known to be Xmrk, an RTK homolog of the EGFR (e.g. Wittbrodt *et al*, 1992; Winkler *et al*, 1994). Overexpression of this gene in medakafish along with injection of TGFα leads to a similar phenotype. Similarly, Sutton *et al* (2002) have demonstrated that TGFα overexpression in mice leads to ocular melanocytosis.

We conclude that *EGF* polymorphism does not appear to predispose to melanoma or nevus development, but its significant association with tumor thickness implies that it may modify tumor progression.

Table II. Breslow thickness of Queensland melanomas *versus* EGF + 61 genotype

	EGF + 61 genotype		
Breslow thickness	A/A	A/G	G/G
In situ	41 (36.6)	58 (51.8)	13 (11.6)
0.1–1.5 mm	145 (35.3)	201 (48.9)	65 (15.8)
1.5–3.5 mm	16 (35.6)	17 (37.8)	12 (26.7)
>3.5 mm	4 (50.0)	2 (25.0)	2 (25.0)

Count (percent) of each genotype.
EGF, epidermal growth factor.

Materials and Methods

Participants An Australian case–control panel was made up of 753 melanoma cases from 738 families participating in the Queensland Familial Melanoma Project (QFMP) (Aitken *et al*, 1994), and 2387 controls from 645 twin families enrolled in the Brisbane Twin Nevus Study (BTNS) (Zhu *et al*, 1999). Ancestry of the cases and controls was recorded (grandparental country of birth and ethnicity), along with phenotypic risk factors such as hair and eye color, and tanning type. Tumor thickness and level were recorded for the cases.

The BTNS twins and their siblings closest in age have all undergone total body skin examination at age 12 years by a trained nurse who assessed nevus count, degree of freckling and pigmentary phenotypes (Zhu *et al*, 1999). Parents of the twins were genotyped, but only self-reported pigmentary characteristics are available for them. No members of the BTNS have been diagnosed with melanoma. Approval to undertake this study was granted by the Human Research Ethics Committee of the Queensland Institute of Medical Research, and all participants gave their signed informed consent.

Genotyping The functional *EGF* promoter SNP (rs4444903) was typed by primer extension and MALDI-TOF mass spectroscopy (MassARRAY, Sequenom, San Diego, California) as previously described (Bansal *et al*, 2002). SNP identity and other linked information may be found in the public databases by using the unique "rs" accession number (NCBI dbSNP, 2003). This SNP had a dropout rate of $<0.5\%$ and few Mendelian errors. Where the latter were encountered, the entire family was dropped from analysis. To estimate error rates due to genotyping technical causes, the SNP was typed twice on 3268 DNAs independently at different times. Of the 6536 genotypes, there were only seven unresolved errors (not attributable to Mendelian, DNA, or other non-technical causes), which is an error frequency of 0.11%. Of 159 pairs of monozygotic (MZ) twins in the sample set, none were discordant at this SNP, further confirming the low genotyping error rate (one member of each MZ twin pair was set to missing for subsequent association analysis).

Statistical analysis Allelic association between melanoma and the SNP was tested using MENDEL 5.0 (Lange *et al*, 2001), which implements a measured genotype approach allowing use of family data as well as unrelated individuals. Combined analysis of the published studies was performed via log-linear and ordinal logistic regression analysis using the R statistical package (R Development Core Team, 2003).

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