The Relationship Between the Epidermal Growth Factor (EGF) 5′UTR Variant A61G and Melanoma/Nevus Susceptibility

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The inheritance of a G allele in position 61 in the 5′UTR of the epidermal growth factor (EGF) gene has been reported to increase melanoma susceptibility, a finding we have investigated in this study. The most potent phenotypic risk factor for melanoma is the atypical mole syndrome (AMS) phenotype. Our hypothesis is that the AMS is genetically determined and that nevus genes are also low penetrance melanoma susceptibility genes. We report that the G allele frequencies were the same in 697 healthy women and 380 melanoma cases (OR 0.97, 95% CI 0.8–1.2 p = 0.76). We therefore found no evidence that this polymorphism is a melanoma susceptibility gene. Furthermore, we found no evidence that the polymorphism controls the nevus phenotype (nevus number, number atypical nevi or AMS phenotype). We did find some evidence that the G allele may be associated with decreased tumor Breslow thickness (OR 0.5, 95% CI 0.3–0.9) for the A/A genotype *versus* A/G and G/G combined in tumors of thickness >3.5 *vs* $\leqslant 3.5$ mm and may therefore act as a predictor of survival, although this finding is not in accord with the original report. This is the second study to find no association between EGF +61 and melanoma susceptibility.

Key words: EGF/polymorphism/breslow thickness/melanoma/nevus
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Although the identification of high penetrance melanoma susceptibility genes, such as CDKN2A (Goldstein and Tucker, 1997) CDK4 (Zuo *et al*, 1996), and p14ARF (Randerson-Moor *et al*, 2001) has been fruitful in recent years, the identification of low penetrance melanoma susceptibility genes has to date proved challenging. MC1R variants have certainly been established as susceptibility genes for melanoma (Valverde *et al*, 1996) and these variants have furthermore been shown to modify the penetrance of the high penetrance gene CDKN2A (Box *et al*, 2001; van der Velden *et al*, 2001). Candidate gene approaches to identifying others have so far been less persuasive. There is evidence for (Aitken *et al*, 1999; Kumar *et al*, 2001) and against (Bertram *et al*, 2002) different polymorphisms in CDKN2A as a melanoma or nevus susceptibility gene. There are conflicting data on the role of genes coding for detoxifying enzymes GSTM1 and CYP2D6 (Wolf *et al*, 1992; Dolzan *et al*, 1995; Lafuente *et al*, 1995; Strange *et al*, 1999; Kanetsky *et al*, 2001), and an unconfirmed small effect of the vitamin D receptor gene (Hutchinson *et al*, 2000). Suggestions that a polymorphism in the DNA repair gene XRCC3 predisposed to melanoma (Winsey *et al*, 2000) were not confirmed (Duan *et al*, 2002; Bertram *et al*, 2004) and there is a single report of the nucleotide excision repair gene XPD in association with melanoma (Tomescu *et al*, 2001). A report of a p53 polymorphism in association with melanoma remains to be repeated (Shen *et al*, 2003). Most recently a study was published which suggested that polymorphisms in the BRAF gene might predispose to melanoma (Meyer *et al*, 2003). The observation, however, was made for males but was not present for females and therefore clearly needs to be assessed in another population.

The epidermal growth factor (EGF) gene, located at 4q25–27, has recently been proposed to be a melanoma susceptibility gene (Shahbazi *et al*, 2002) although the findings reported were not confirmed recently by another UK group (McCarron *et al*, 2003). The original study identified an A to G polymorphism in the 5′UTR (position 61) of the EGF gene, which appeared to be associated with an increase in EGF production *in vitro*, an increased risk of melanoma, and an increase in tumor Breslow thickness (Shahbazi *et al*, 2002). Consequently, the authors suggested that the EGF 5′UTR A61G polymorphism might act as a marker for both melanoma risk and outcome. We conducted a similar case–control study, using a substantially larger series of incident melanoma cases, in order to investigate the findings of Shahbazi *et al*.

The most potent phenotypic risk factor for melanoma is the atypical nevus phenotype (Swerdlow *et al*, 1986; Bataille *et al*, 1996). Twin studies have shown that nevi are primarily genetically determined (Easton *et al*, 1991; Zhu *et al*, 1999; Wachsmuth *et al*, 2001) and our hypothesis is therefore that genes controlling nevus phenotype may act as melanoma susceptibility genes. In addition, we also therefore assessed the possible role for the EGF polymorphism as a nevus susceptibility gene.

Abbreviations: AMS, atypical mole syndrome; EGF, epidermal growth factor

Results

Genotyping was successful in 669 (96%) controls and 380 (100%) cases (summarized in Table I). The genotypes observed in the Yorkshire, St Albans and the total control groups were all found to be in Hardy–Weinberg equilibrium ($p = 0.62$, 0.37, and 0.34, respectively). The EGF genotype and allele frequencies of the incident melanoma cases ($n = 380$) were not significantly different from the controls ($n = 669$, $p = 0.80$ and 0.76 respectively). Furthermore, the genotype and allele frequencies of the female incident melanoma cases did not significantly differ from the controls ($p = 0.94$, 0.94). Genotype analysis carried out only on controls recruited from the Yorkshire region ($n = 389$) showed no significant difference from that observed in the incident melanoma cases ($p = 0.79$). The frequency of the G allele (0.42) was comparable to that reported by Shahbazi *et al* for their 99 controls (0.44). There was no evidence of an association between heterozygous (AG) or homozygous (GG) genotype and melanoma status when comparing the incident melanoma cases with the controls (OR = 1.03, [95% CI 0.77–1.39]; OR = 0.91, [95% CI 0.61–1.37], respectively) or with just the Yorkshire controls (OR = 1.10, 95% CI (0.79–1.53); OR = 0.99, 95% CI (0.63–1.56), respectively, Table I).

An overall test of association between genotype and Breslow thickness gave non-significant evidence of association (Fisher's exact test, $p = 0.08$). In contrast to Shahbazi *et al*, however, we observed that the A/A genotype appeared more frequently and the G/G genotype less frequently in patients with thick tumors (>3.5 mm) at presentation (Table II). Shahbazi *et al* reported a positive association between the G/G genotype and thick tumors (OR 3.7 (95% CI 1.0–13.2), $p = 0.045$), whereas the corresponding estimated OR from our data is 0.58 (95% CI 0.17–1.57). Performing a *post hoc* contrast between the A/G and G/G genotypes combined and the A/A genotype we find an OR of 0.47 (95% CI 0.25–0.91, $p = 0.014$, Table II) for thicker tumors and a negative association between the presence of the G allele and a Breslow thickness of >3.5 mm (OR 0.60 (95% CI 0.37–0.96), $p = 0.026$).

There was no association between genotype and the mean total number of banal or atypical nevi in either the healthy women ($p = 0.90$ and 0.65, respectively) or in cases ($p = 0.43$ and $p = 0.79$, Table III). There was no association between the polymorphism and AMS score in either the cases or healthy women ($p = 0.66$ and 0.83, respectively).

Discussion

We have found no evidence to support the findings of Shahbazi *et al* (2002), that the 5′UTR A61G polymorphism of the EGF is associated with melanoma risk. Indeed, the CI for the GG genotype (which was the only group in which the previous study found an association with melanoma) in our study did not overlap with that of Shahbazi *et al*, showing that the two studies are formally inconsistent. This discrepancy is not easily explainable. The cases for both studies were recruited from similar Caucasian UK populations, although 43% of those recruited by Shahbazi *et al* were not incident cases. The genotype frequencies in the controls in

Table I. Genotype and allele frequency by case/control status

	Present study						McCarron *et al* (2003)		Shahbazi *et al* (2002)			
	Incident cases	All GP controls	Yorkshire GP controls	Incident cases *versus* GP controls		Incident cases *versus* Yorkshire GP controls	Cases	Controls	Cases	Controls	Cases *versus* controls	
	(n = 380)	(n = 669)	(n = 389)	OR (95% CI)		OR (95% CI)	(n = 159)	(n = 310)	(n = 135)	(n = 99)	OR (95% CI)	
Genotype												
A/A	124 (32.6%)	219 (32.8%)	133 (34.2%)	1.00		1.00	56 (35.2%)	121 (39.0%)	21 (15.6%)	32 (32.3%)	1.00	
A/G	198 (52.1%)	338 (50.5%)	193 (49.6%)	1.03 (0.8–1.4)	$p = 0.81$	1.10 (0.8–1.5)	$p = 0.55$	82 (51.6%)	131 (42.3%)	50 (37.0%)	47 (47.5%)	1.6 (0.8–3.2)	$p = 0.16$
G/G	58 (15.3%)	112 (16.7%)	63 (16.2%)	0.91 (0.6–1.3)	$p = 0.65$	0.99 (0.6–1.5)	$p = 0.95$	21 (13.2%)	58 (18.7%)	64 (47.4%)	20 (20.2%)	4.9 (2.3–10.2)	$p = <0.0001$
Allele												
A	446 (58.7%)	776 (58.0%)	459 (59.0%)	1.00		1.0		194 (61.0%)	273 (60.2%)	92 (34.1%)	111 (56.1%)	1.0	
G	314 (41.3%)	562 (42.0%)	319 (41.0%)	0.97 (0.8–1.2)	$p = 0.76$	1.01 (0.8–1.2)	$p = 0.90$	124 (39.0%)	247 (39.8%)	178 (65.9%)	87 (43.9%)	2.7 (1.9–4.0)	$p = <0.0001$

GP, general practitioner.

Table II. Association between epidermal growth factor polymorphism and Breslow thickness

	Present study N (%)				McCarron *et al* (2003) N (%)				Shahbazi *et al* (2002) N (%)		
	<1.5 mm (n = 197)	1.5–3.5 mm (n = 116)	>3.5 mm (n = 50)	*In situ* (n = 26)	0.1–1.5 mm (n = 83)	1.5–3.5 mm (n = 29)	>3.5 mm (n = 20)	*In situ* (n = 24)	0.1–1.5 mm (n = 74)	1.5–3.5 mm (n = 18)	>3.5 mm (n = 12)
Genotype											
A/A	59 (30.0%)	36 (31.0%)	24 (48.0%)[a]	11 (16.5%)	31 (37.3%)	11 (37.9%)	5 (25.0%)	6 (25.0%)	10 (13.5%)	4 (22.2%)	1 (8.3%)
A/G	111 (56.3%)	57 (49.2%)	21 (42.0%)	12 (46.2%)	42 (50.6%)	17 (58.6%)	9 (45.0%)	8 (33.3%)	31 (41.9%)	5 (27.8%)	2 (16.7%)
G/G	27 (13.7%)	23 (19.8%)	5 (10.0%)[b]	3 (11.5%)	10 (21.1%)	1 (3.5%)	6 (30.0%)	10 (41.7%)	33 (44.6%)	9 (50.0%)	9 (75.0%)
Allele											
A	229 (58.1%)	129 (55.6%)	69 (69.0%)	66 (64.7%)	104 (62.7%)	39 (67.2%)	19 (47.5%)	20 (41.7%)	51 (34.5%)	13 (36.1%)	4 (16.7%)
G	165 (41.9%)	103 (44.4%)	31 (31.0%)[c]	36 (35.2%)	62 (37.3%)	19 (32.8%)	21 (52.5%)	28 (58.3%)	97 (65.5%)	23 (63.9%)	20 (83.3%)

[a]p = 0.014 (OR 0.5 [95% CI 0.3–0.9]) for A/A genotype *versus* A/G and G/G combined in tumors with a Breslow thickness of >3.5mm *vs* ⩽3.5 mm.
[b]p = 0.27 (o 0.6 [95% CI 0.2–1.6]) for G/G genotype *versus* A/A and A/G combined in tumors with a Breslow thickness of >3.5mm *vs* ⩽3.5 mm.
[c]p = 0.026 (odds ratio 0.6 [95% CI 0.4–1.0]) for G allele *versus* A allele in tumors with a Breslow thickness of >3.5 mm *vs* ⩽3.5 mm.

Table III. Association between epidermal growth factor polymorphism and nevus number and atypical mole syndrome (AMS) score

	GP controls (n = 627)				Incident cases (n = 343)				Female incident cases (n = 201)			
	A/A	A/G	G/G		A/A	A/G	G/G		A/A	A/G	G/G	
Mean total no. nevi	56.51	55.76	57.91	p = 0.90	69.25	73.82	63.33	p = 0.43	68.45	80.16	55.88	p = 0.38
Mean no. atypical nevi	0.28	0.34	0.26	p = 0.65	0.89	1.30	0.83	p = 0.79	0.88	1.68	0.56	p = 0.78
AMS score												
0	91 (32.4%)	143 (50.9%)	47 (16.7%)		31 (33.3%)	51 (54.8%)	11 (11.8%)		18 (34.6%)	29 (55.8%)	5 (9.6%)	
1	76 (34.0%)	114 (50.0%)	36 (16.0%)		39 (35.5%)	50 (45.5%)	21 (19.1%)		23 (31.9%)	33 (45.8%)	16 (22.2%)	
2	28 (33.0%)	44 (52.0%)	12 (14.0%)	p = 0.83	21 (27.3%)	41 (53.2%)	15 (19.5%)	p = 0.66	16 (37.2%)	18 (41.9%)	9 (20.9%)	p = 0.67
⩾3[a]	8 (22.2%)	20 (55.6%)	8 (22.2%)		21 (33.3%)	35 (55.6%)	7 (11.1%)		8 (23.5%)	24 (70.6%)	2 (5.9%)	

[a]Individuals with an AMS score of 3, 4, or 5.

this study were not significantly different from those seen in controls in Shahbazi *et al* ($p = 0.68$). Our study had over 99% power to detect an association of the size reported by Shahbazi *et al* and a power of 89% to detect a more modest OR of melanoma of 1.6 to carriers of a G allele compared with non-carriers.

A possible explanation for this discrepancy is the composition of the Shahbazi *et al* case set (77 new cases and 58 undergoing follow-up) leading to an underrepresentation of tumors with a Breslow thickness >3.5 mm ($n = 12/135$; 9.4% *vs* an expected 17.1%). (Patients with tumors >3.5 mm have a poorer prognosis and are therefore less likely to be seen in follow-up clinics). An under-representation of thicker tumors could bias the results if the G allele was correlated with Breslow thickness as was suggested by Shabhazi *et al*. These authors were aware of the possible bias by Breslow thickness but were reassured that the bias was unlikely to be problematic as their results suggested that the G allele was associated with greater Breslow thickness, which would have reduced the relative risk for melanoma rather than increasing it. In our study this relationship with Breslow thickness, however, was not confirmed. This under representation of thick tumors was not observed in our sample set of cases recruited at first presentation ($n = 50/363$; 13.8%).

Shahbazi *et al* also reported that the 61*G allele is associated with increased EGF production. Whilst the issue of EGF production has not been addressed in our study, our findings with regard to Breslow thickness are discrepant to those reported by Shahbazi *et al* and by McCarron *et al*, although the correlation in the latter study was more modest than in the original report. Our study would suggest if anything, that the relationship between the G allele and Breslow thickness was the reverse of that suggested by the previous study. Overall then, the evidence that this EGF polymorphism may have an effect on tumor thickness is weak and much larger studies will be necessary to resolve this question.

We and another UK group (McCarron *et al*, 2003) have found no evidence to confirm a polymorphic form of the EGF gene as a low penetrance melanoma susceptibility or a nevus gene in a similar population.

Materials and Methods

Study population Three hundred and eighty population-based incident melanoma cases were recruited in Yorkshire, UK in the period since September 2001 till December 2002. Six hundred and ninety-seven female healthy controls aged 19–46 years were recruited via GPs from Yorkshire ($n = 396$) and an area of the UK approximately 200 miles south of Yorkshire (around St Albans, Hertfordshire) ($n = 301$), in a study to identify nevus genes (Bertram *et al*, 2004). These women formed the comparison group. As cases were both male and female but controls were female, case–control comparisons were carried out additionally in females alone, although there was no *a priori* reason to suppose that sex would have an effect. Written informed consent was obtained from all participating individuals and institutional and regional ethical committee approval was obtained.

All subjects were examined by nurse examiners, and their nevus phenotype was determined as described in previous studies (Bertram *et al*, 2002). Nevi 2mm or greater in number were counted and the number of atypical nevi recorded. The AMS score, as a measure of the overall phenotype, was calculated as reported previously (Newton Bishop *et al*, 1994). Hair and eye color were recorded. A blood or buccal cell sample was taken for the isolation of DNA.

Genotyping We designed an ARMS test for the allele-specific amplification of the EGF A61G variant. A common forward primer 5′-CAT TTG CAA ACA GAG GCT CA-3′ and an allele specific reverse primer, 5′-GAA CTG ATG GAA AGT TCC ACC C-3′ (G allele) or 5′-GAA CTG ATG GAA AGT TCC ACC T-3′ (A allele) were used to amplify a 186 bp band. A second internal mismatch (wobble base) was included in the reverse primers (third base from 3′ end) to minimize non-specific binding of the primers. A second product of 236 bp (human growth hormone gene, hGH) was co-amplified with the test product to provide an internal amplification control (forward, 5′-GAG TTT GTA AGC TCT TGG GGA AT-3′; reverse, 5′-TCC TTT GGG ATA TAG GCT TCT TC-3′). All gels were scored blind independently by two individuals. Over 15% of samples were amplified in duplicate and the results were $>99\%$ concordant.

Statistical analyses Hardy–Weinberg equilibrium was tested for in the control samples using a χ^2 goodness-of-fit test. Genotype and allele frequencies were compared using χ^2 tests. ORs and exact 95% CI were calculated to estimate the association between genotype and melanoma status. The association between genotype and Breslow thickness was analyzed using χ^2 tests using the same categories of tumor thickness as Shahbazi *et al* (Shahbazi *et al*, 2002) (<1.5 mm, 1.5–3.5 mm, and >3.5 mm), except that we did not include patients with *in situ* tumors in our study. Differences in numbers of benign and atypical nevi between groups were investigated using *t*-tests or analysis of variance on log-transformed values. Dependence of the AMS score on genotype was analyzed using ordinal logistic regression. Analyses were carried out in STATA (StataCorp, 2001).

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