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Association of the 609 C/T NAD(P)H: Quinone Oxidoreductase (*NQO1*) Polymorphism with Development of Cutaneous Malignant Melanoma

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ABSTRACT

Cutaneous Malignant Melanoma (CMM) is a life threatening disease whose incidence and mortality rates have risen rapidly in the White Caucasian population in recent decades. The aim of the current study was to investigate the association between polymorphisms in genes involved in DNA-repair and detoxification of reactive metabolites and the development of CMM. The patient cohort consisted of 69 individuals while the control population consisted of 100 individuals. We found a statistically significant association between the presence of the wild type *NQO1* C allele, MDHFR $C^{667}T$, TS ^{1494}del6, TSER polymorphisms and development of CMM (P=0.04; odds ratio=2.35). The *NQO1* CC genotype was more strongly associated with CMM development (P=0.016; odds ratio=2.92). The *NQO1* gene codes for a protein that has been widely considered to be protective through its ability to detoxify quinones. However recent studies have also linked it to an important source of reactive oxygen and to NF-κB-dependent proliferation of cultured melanoma cells. In conclusion these results link molecular epidemiology and experimental evidence for the role of the *NQO1* gene product in development of CMM. MDHFR and TS in Folic acid metabolism are responsible for methylation of methyl group. Two important roles of folate 'related to this study' are the conversion of homocysteine to methionine and the generation of Thymidylate (dTMP) which is required for DNA synthesis. According to many studies done at this area, folate deficiency has been associated with chromosome strand breaks, impaired DNA repair, DNA hypomethylation and hypermethylation all of which have been associated with cancer cell formation. The result of study shows, MTHFR $C^{677}T$ and TS 6bp deletion/insertion are not related to increased risk of CMM and therefore have no effect on an individual's susceptibility.

Keywords: Cutaneous Malignant Melanoma (CMM); Polymorphism; Quinone Oxidoreductase (NQOI)

Abbreviations: CMM: Cutaneous Malignant Melanoma; UV: Ultraviolet; CDK: Cyclin Dependent Kinase; CDKI: Cyclin Dependent Kinase Inhibitor; ROS: Reactive Oxygen Species; 8-oxo dG: 8-oxo 7,8-dihydro-2'-deoxyguanosine; *OGG1*: 8 oxo deoxyguanosine DNA glycosylase 1; NAT: N-acetyl Transferase; NAD(P)H: Nicotinamide Adenine Dinucleotide Phosphate; *NQO1*: Quinone Oxidoreductase; GST: Glutathione-S-Transferase; *XRCC1*: X-ray Repair Complementing Defective Repair in Chinese Hamster Cells 1.

INTRODUCTION

Cutaneous Malignant Melanoma (CMM) is a life threatening disease whose incidence and mortality rates have risen rapidly in the White Caucasian population in recent decades. The consistent stimulation of cutaneous skin cells 'melanocytes' by weapons such as Biological Mass Destructions which cause the change of skin colour to brown and hypertrophy, it is known as cutaneous Malignant Melanoma (CMM) in some countries such as United kingdom. However in some countries like Iran it is known as Acne, it is removable by surgery and it is non-lethal. It can be seen at the skin of face, neck, around the body and feet nail. There is considerable epidemiological evidence supporting a role of exposure to UV (Ultra Violet) radiation as an important environmental factor in the aetiology of CMM. Interestingly, intermittent sun exposure early in life rather than cumulative sun exposure appears to be a better indicator of disease risk [1,2]. The strong aetiological role for UV exposure is clearly supported by the observation that childhood immigrants to Australia where ambient

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UV levels are amongst the highest in the world is associated with an increased lifetime risk of CMM. Individuals with light skin colour, freckles and a tendency to burn appear to be especially susceptible [3-5]. The observation that only a subset of individuals exposed to sunlight progress to develop CMM indicates that other factors including genetic variation may also play a role in the aetiology of CMM. In support of this, several high penetrance genes have being identified that are associated with risk of familial CMM these include mutations in genes encoding proteins involved in regulation of the cell cycle including CDK4 and the CDKIs p16 and p14ARF [6]. However these mutations are rare and are only able to account for a relatively small percentage of cases of CMM.

UVB (290-320 nm) is strongly absorbed by DNA and is known to induce a number of mutagenic DNA lesions including cyclobutane pyrimidine dimers and pyrimidine (6-4) photoproducts that are substrates for the nucleotide excision repair (NER) pathway in cells. In contrast the ability of UVA (320-400 nm) to damage DNA has being largely ascribed to the generation of intracellular Reactive Oxygen Species (ROS). For example UVA has being shown to induce single stranded DNA breaks as well as 8-oxo7,8-dihydro-2'-deoxyguanosine (8-oxo dG) [7-13]. These DNA lesions are repaired predominantly by the Base Excision Repair Pathway (BER).

Polymorphic genes involved in the NER and BER pathways and in the detoxification of ROS are therefore attractive candidates for genetic markers of individual susceptibility to CMM. Two key candidate genes in the BER pathway are *OGG1*, the major DNA glycosylase involved in the removal of 8-oxo dG from genomic DNA, and *XRCC1*. Interestingly, several studies have provided evidence that the cys^{326} variant of *OGG1* has a reduced ability to restore repair activity in *E.Coli* deficient in the repair of 8-oxo dG [14]. In addition, the catalytic efficiency (kcat/kM) of excision of 8-oxo dG from γ-irradiated DNA by ser^{326}*OGG1* protein is twice that of cys^{326} *OGG1* [15,16]. Moreover cys/cys genotype has being found to be a repair deficient phenotype [17-19]. Although the precise role of *XRCC1* remains unclear it is thought to play a structural role in the organisation of other BER repair factors [20]. Recent studies have shown that the arg^{399}gln polymorphism of *XRCC1* is associated with reduced DNA repair capacity as assessed by the persistence of DNA adducts and strand breaks [18, 21].

In addition, many of the enzymes believed to be involved in the detoxification of UVA-induced ROS (including *GSTT1*, *GSTM1* and *NQO1*) are also polymorphic in the human population and as such are also attractive candidates for influencing individual cancer susceptibility. *GSTM1* and *GSTT1* null genotypes have been consistently associated with individual susceptibility to Basal Cell Carcinoma (BCC) but their role in the aetiology of CMM remains far less clear [22]. *NQO1* is an obligate two-electron reductase involved in detoxification reactions converting quinones to hydroquinines preventing single-electron redox cycling reactions [23]. In addition *NQO1* is believed to play a direct role in antioxidant defense by catalysing the reduction of alpha-tocopherol quinone to the antioxidant α-tocopherol hydroquinone [23]. Interestingly the *NQO1**0 allele which codes for an essentially non-functional protein has also been identified as a candidate loci that is associated with individual susceptibility to BCC [24,25]. However any possible role for this *NQO1* polymorphism in susceptibility to CMM remains unexplored.

The aim of the current study was to investigate the possible association of a range of polymorphic genes involved in BER and the detoxification of ROS and individual susceptibility to CMM.

MATERIALS AND METHODS

Study population

The patient cohort comprised 69 individuals with histologically confirmed malignant melanoma who were referred to the Skin Oncology Clinic, University Hospital, Birmingham NHS Trust. Clinical data for these individuals are presented in Table 1. The control population consisted of 100 healthy volunteers with no family history of malignant melanoma. Because of the possible confounding effect of ethnicity all patients and volunteers were White Caucasians.

Table 1: Clinical Data for malignant melanoma population.

	No. of Patients
Age	
Median (range)	69
Sex	
Male	30
Female	39
Anatomical site of primary lesion	
Head and neck	10
Upper extremities	10
Trunk	15
Lower extremities	14
Unknown	20
Thickness of Tumour	
<0.75	6
0.75-1.5	15
1.6-3.9	29

>4.0	5
Unknown	15
Nodes involved as primary	
Positive	27
Negative	22
Unknown	20
Stage of disease	
I	10
II	15
III	14
IV	10

Blood sample collection

Ethical approval was granted from South Birmingham (United Kingdom) Local Research Ethics Committee (reference number 0534) before commencement of the study. After written consent, blood samples (2 ml) were obtained by venepuncture and collected in vacutainer tubes (Haemograd, Becton-Dickinson, United Kingdom) containing EDTA. All samples were stored on ice until required and were processed within 3 hours of sample collection.

Genotyping analyses

Genomic DNA was isolated from whole blood (approximately 1 ml) using a QIAamp midi kit (Qiagen, United Kingdom), according to the manufacturer's instructions. All primers were synthesised by Alta Bioscience (The University of Birmingham, United Kingdom). PCR (Polymerse Chain Reaction) genotyping reactions were carried out as previously reported to detect deletions in *GSTM1* and *GSTT1*, with the exception that GST and albumin (internal control) primers sequences were as detailed previously [26,27]. In addition, previously reported RFLP-PCR methods were employed to detect common single nucleotide polymorphisms in *NQO1*, *OGG1*, *XRCC1*, *XRCC3*, *ERCC2* (XPD), *CYP2D6* and *NAT2* (Table 2).

Statistical analysis

Phenotype frequencies were calculated by counting the number of individuals in a population positive for an allele (∑ (homozygotes+heterozygotes)). Allele frequencies were obtained directly by counting the number of chromosomes bearing an allele (∑ (homozygotes+0.5 × heterozygotes)). Associations were assessed using contingency table analysis and Fisher's exact test. The OR and relative risk were calculated and a Bonferroni correction was applied to correct for multiple comparisons.

RESULTS

Phenotype, allele and genotype frequencies were calculated for the eleven Single Nucleotide Polymorphisms (SNPs) examined (*XRCC1* Arg^{194}Trp, *XRCC1* Arg^{399}Gln, *XRCC3* Thr^{241}Met, *XRCC3* IVS5-14, *ERCC2* Lys^{751}Gln, *NQO1* Pro^{187}Ser, *OGG1* Ser^{326}Cys, *CYP2D6* G→A and *NAT2*). In the case of *NAT2* Ile^{114}Thr, Arg^{197}Gln and Gly^{286}Glu all represent alleles encoding a slow acetylator protein. These alleles were not distinguished in the analysis and were classified as Slow (S) acetylator alleles. The wild type allele encodes a fast acetylator protein and was classified as a Fast (F) allele. For analysis of GSTM and GSTT because our PCR test was not able to distinguish homozygous positive individuals from heterozygotes individuals were classified as either GST positive (wild type and heterozygous combined) or GST null. Analysis of the data revealed a statistically significant association with the C/T polymorphism at position 609 of *NQO1* and the occurrence of CMM. Individuals with a CC genotype had a significantly increased risk of developing CMM (OR=2.92(1.38-6.1); P=0.016 (Table 3). Presence of the C allele was also associated with development of CMM (OR=2.35(1.23-4.5); P=0.04. The association was further reduced in significance once the heterozygous genotype frequency was included and was not significantly different following application of the Bonferroni correction factor (P=0.152). No associations with melanoma development were found with any of the other genetic polymorphisms studied (Tables 3 and 4).

Table 2: Genes selected for PCR-RFLP analysis with position of single nucleotide polymorphisms and resulting amino acid change indicated.

Gene (Methodology reference)	Position of polymorphism	Nucleotide substitution	Amino acid change
NAT2 Hickman and Sim et al. [28]	341	T→C	Ile^{114}Thr (NAT*5)
	590	G→A	Arg^{197}Gln (*NAT2**6)
	857	G→A	Gly^{286}Glu (*NAT2**7)
	1846	G→A	Splicing defect (*CYP2D6**4)
	1245	C→T	Ser^{326}Cys
CYP2D6 Smith et al. [29]	1846	G→A	Splicing defect (CYP2D6*4)
OGG1 Hardie et al. [30]	1245	C→T	Ser^{326}Cys

NQO1 Traver et al. [24]	609	C→T	Pro[187]Ser (*NQO1**0)
XRCC1 Lunn et al. [21]	26304	C→T	Arg[194]Trp
	28152	G→A	Arg[399]Gln
XRCC3 De Ruyck et al. [31]	18067	C→T	Thr[241]Met
	17 893	A→G	IVS5-14
ERCC2 (XPD) Yu et al. [32]	35931	C→A	Lys[751]Gln

Table 3: Analysis of *GSTM1* and *GSTT1* gene deletion frequencies in control and malignant melanoma cohorts.

			Genotype frequency	
Gene	**Polymorphism**		**Controls**	**Melanoma**
GSTM1	M1 deletion	M1	0.54	0.63
		M0	0.46	0.37
GSTT1	T1 deletion	T1	0.81	0.9
		T0	0.19	0.1

Table 4: Phenotypic, allelic and genotype frequencies of gene polymorphisms in controls and malignant melanoma cohorts.

			Phenotype and allele frequencies					Genotype frequencies	
				Phenotype	Allele			Genotype	
Gene	Polymorphism	Controls	Control values	Melanoma	Controls	Melanoma	Allele	Controls	Melanoma
XRCC1	Arg[194]Trp	C	0.98	1	0.93	0.97	cc	0.87	0.93
		T	0.13	0.07	0.07	0.03	tt	0.02	0
							ct	0.11	0.07
	Arg[399]Gln	G	0.85	0.86	0.66	0.66	gg	0.44	0.45
		A	0.56	0.55	0.34	0.34	aa	0.15	0.14
							ga	0.41	0.41
XRCC3	Thr[241]Met	C	0.89	0.86	0.72	0.71	cc	0.56	0.55
		T	0.44	0.45	0.28	0.29	tt	0.11	0.14
							ct	0.33	0.3
	IVS5-14	A	0.88	0.92	0.66	0.65	aa	0.43	0.38
		G	0.57	0.62	0.34	0.35	gg	0.12	0.08
							ag	0.44	0.54
ERCC2	Lys[751]Gln	C	0.91	0.9	0.69	0.67	cc	0.46	0.43
		A	0.54	0.57	0.32	0.33	aa	0.09	0.1
							ca	0.45	0.47
NQO1	Pro[187]Ser	C	0.96	0.97 [a]	0.79	0.90 [b]	cc	0.62	0.83 [c]
		T	0.38	0.17	0.21	0.1	tt	0.04	0.03
							ct	0.34	0.14
OGG1	Ser[326]Cys	C	0.89	0.94	0.74	0.78	cc	0.58	0.61
		T	0.42	0.39	0.26	0.22	tt	0.11	0.06
							ct	0.31	0.33
CYP2D6	*CYP2D6**4	G	0.94	0.95	0.84	0.84	gg	0.73	0.72
		A	0.27	0.28	0.16	0.16	aa	0.06	0.05
							ga	0.21	0.23
NAT2	Ile[114]Thr	Fast	0.45	0.34	0.26	0.19	FF	0.07	0.04
	Arg[197]Gln	Slow	0.93	0.96	0.74	0.81	SS	0.55	0.66
	Gly[286]Glu						FS	0.38	0.3
MTHFR	Cyt[677]Thy	C	0.78	1	0.75	0.24		0.6	0.48

					CC		
	T	0.75	0.36	0.68	0.31	ct	0.3	0.39
						tt	0.91	0.11
TS[1494]del	+6bp	1	0.79	0.65	0.34	+6bp/+6bp	0.45	0.38
	-6bp	0.56	0.84	0.63	0.36	+6bp/-6bp	0.4	0.5
						-6bp/-6bp	0.13	0.11

Note: [a]: Presence of C allele was significantly associated with melanoma development until adjusted by a Bonferroni correction factor of eight; [b]: Presence of C allele was significantly associated with melanoma development (P=0.04); [c]: Presence of CC genotype was significantly associated with melanoma development (P=0.016).

DISCUSSION

The aim of this study was to investigate whether genetic polymorphisms in a sample of genes whose products are involved in DNA repair and detoxification of reactive metabolites were associated with the development of CMM.

We found a statistically significant inverse association between an *NQO1* polymorphic variant and risk of development of CMM (Table 3). *NQO1* is believed to play a role in antioxidant defence and is able to catalyse the reduction of α-tocopherol quinone to the antioxidant α-tocopherol hydroquinone [23]. The 609 C→T (Pro[187]Ser) polymorphism was initially characterised in humans [24]. The Ser187 variant of *NQO1* encodes a protein with essentially zero biological activity. This is believed to be related to a greatly enhanced rate of protein degradation of the variant form by the ubiquitin/proteosomal system [23]. The frequency of the homozygous TT genotype has previously been reported to be approximately 5% in the Caucasian population [28-33]. In agreement with this we observed a TT genotype frequency of 4.0% in our control population. Previous studies have associated the homozygous TT genotype with an elevated risk of a number of cancers [34-38]. Interestingly, Clairmont et al. reported that the *NQO1**0 allele was a predictor of number of basal cell carcinomas in a multivariate model of patients of known *GSTM1*, *GSTT1* and *CYP2D6* EM genotype [25]. However any possible role for this *NQO1* polymorphism in susceptibility to CMM remains unexplored.

In the present study we observed that individuals with a "wild type" CC *NQO1* genotype had a statistically significant increased risk of developing CMM (OR=2.92 (1.38-6.1); P=0.016. Furthermore, presence of the C allele was also associated with development of CMM (OR=2.35 (1.23-4.5); P=0.04.

Our findings are at first surprising in light of the apparent protective role of functionally active *NQO1* protein that has previously been reported. However, *NQO1* has also being reported to be greatly up regulated in tumours of the liver, lung, colon and breast [39]. In particular, elevated levels of *NQO1* enzyme activity in darkly pigmented congenital nevus cells and cultured melanoma cell lines has also being observed previously [40]. It is possible that *NQO1* activity may confer a possible growth/survival advantage to at least a subset of certain tumours.

Interestingly, Brar reported that constitutive activation of NF-B is important for proliferation of the malignant melanoma cell lines CRL[1585] and CRL[1619] [41]. NF-κB activation has also being reported to be important in supporting the survival and proliferation of a number of cancers including carcinomas of the breast, ovary, colon, lung, head and neck and pancreas [42-45]. NF-κB is constitutively activated in melanoma cell lines including the Hs[294]T line, where activation was observed as a result of enhanced degradation of the inhibitory partner inhibitory κB alpha (IKκBα) [46,47]. No such activation is observed in normal melanocytes [48]. In the study by Brar, NF-κB activation and cell growth was inhibited by antioxidants suggesting an intermediary role of ROS. In addition, an inhibitor of *NQO1* (dicumarol) inhibited NF-κB activation and cellular growth indicating a possible role of *NQO1* in ROS generation in these cell lines [41]. Based on results using a quinone analogue (capsaicin) the authors speculated that the source of ROS may involve a *NQO1*/quinone redox couple. An elevated level of ROS has also been observed in dysplastic nevi compared to normal skin melanocytes [49]. Dysplastic nevi are widely considered to be melanoma precursors further supporting the hypothesis that aberrant redox homeostasis is important in melanoma development.

It must be noted however, that other sources of ROS are also considered to contribute to NF-κB activation in melanoma cell lines. These include NAD(P)H oxidase activity and Gamma-Glutamyl Transferase (GGT) [50-52]. Indeed the inhibitory effects of dicumarol on NF-κB activation in melanoma cells appears to be at least in part, to be due to inhibition of NAD(P)H oxidase [50]. Morre have also shown a similar inhibition of growth of human and mouse tumour lines including those derived from melanoma by capsaicin but this was considered to be related to inhibition of NAD(P)H oxidase activity [53,54]. *NQO1* may nevertheless contribute to the survival and proliferation advantage as discussed above. A pro-oxidant role of melanin has been proposed in the pathogenesis of melanoma and it is possible that *NQO1* could also generate ROS by promoting redox cycling of melanin [55].

Together these studies indicate that intracellular ROS from multiple sources contribute to melanoma cell survival and growth. We hypothesise that under certain circumstances the wild type *NQO1* gene product contributes to the promotion of growth of cutaneous malignant melanomas *in vivo* either by the mechanism proposed by Brar or by promoting redox cycling of melanin [41]. This biologically plausible mechanism may also explain our observation that the wild type *NQO1* C allele and *NQO1* CC genotype is significantly associated with melanoma development.

This study used a candidate gene approach to analyse genetic factors involved in disease susceptibility. A major potential problem in this approach is the possibility of finding associations by chance. We have addressed this by applying the conservative Bonferroni correction for multiple comparisons and by only considering an association significant if it maintained a P<0.05 following correction. Although the data presented in this study indicate a role for *NQO1* in individual susceptibility to CMM further work is required with larger sample numbers to confirm this association. In addition, work to investigate levels of ROS in *NQO1* wild type and mutant melanoma cells is planned [56-61].

CONCLUSION

This study has identified a genetic factor that may influence an individual's susceptibility to CMM. The data presented also provide further insight into the possible biological role of *NQO1* in CMM tumour progression. Antioxidants have been shown to inhibit both growth in both melanoma cell lines and tumours in animal models. Therefore, *NQO1* wild type individuals and those with elevated NAD(P)H oxidase may represent prime candidates for the future testing of novel therapeutic strategies involving the use of antioxidants for interruption of oxidant signalling pathways involved in melanoma growth.

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