[Dermato-Endocrinology 1:1, 54-57; January/February 2009]; ©2009 Landes Bioscience

Report

The unfavorable effect of the A allele of the vitamin D receptor promoter polymorphism A-1012G has different mechanisms related to susceptibility and outcome of malignant melanoma

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Key words: malignant melanoma, vitamin D receptor, promoter polymorphism haplotypes

The A allele of the A-1012G (rs4516035) *vitamin D receptor* (*VDR*) promoter polymorphism is associated with increased susceptibility and worsened outcome in malignant melanoma (MM). The A allele contains a GATA-3 binding site. There is a second polymorphism in the same promoter region, G-1520C (rs7139166), and there is potential for another GATA binding site in the G allele. Here, we tested the hypothesis that the $G^{-1520}A^{-1012}$ haplotype might be a greater risk factor for MM than A-1012 alone. The A allele of A-1012G was preferentially linked to G of G-1520C and was more frequent in MM patients (p = 0.011) but G of G-1520C was not (p = 0.756). The CA haplotype was a very significant risk factor for MM (p = 0.0001) while the CG haplotype was protective (p = 0.014, combined model p = 0.00002). There was no effect of GA haplotype (p = 0.931), suggesting that that the difference in frequencies of the A allele between patients and controls was accounted for by the differences in frequencies of the CA haplotype. The A allele of A-1012G was more frequent in patients with metastasis (p = 0.054) than MM patients without metastasis, as was the G allele of G-1520C (p = 0.028). The GA haplotype was more frequent in patients with metastasis (p = 0.015), while frequencies of CA were similar. We suggest that the different roles of the A allele of A-1012G in susceptibility and metastasis risk may be a function of the availability of transcription factors in the differing cellular backgrounds related to susceptibility and progression of MM.

Introduction

We have recently described a novel polymorphism, A-1012G, of the *vitamin D receptor* (*VDR*) gene which is a G to A substitution at position -1012 (rs4516035) in the 1A promoter region. We reported that the A allele is associated with increased susceptibility and worsened outcome in malignant melanoma (MM).[1] We have also reported a protective effect of the A allele on susceptibility to non-familial psoriasis and an enhanced effect on vitamin D analogue treatment.[2] The A but not the G allele contains a potential binding site for the T-helper 2 (Th2) signalling factor, GATA-3, which might suggest a Th1 to Th2 T-cell switching effect of the A allele, particularly since the active form of vitamin D, $1,25(OH)_2D_3$, is also known to polarize T-cells to Th2.[3] This would be fully compatible with the observed clinical associations, given that Th1 responses are more effective than Th2 against MM,[4] while the inflammatory response in psoriasis is Th1-mediated[5,6] and thus a suppression of the Th1 effect would be beneficial.

There is a second polymorphism in the 1A promoter region, G-1520C (rs7139166), in which there is a G to C substitution 508 base pairs upstream of A-1012G. Analysis with the TESS[7] database suggests a further GATA binding site in the G allele but not the C allele. The aim of the present study was to test the hypothesis that the A allele of A-1012G might be correlated with this G allele of G-1520C and the resulting $G^{-1520}A^{-1012}$ haplotype, producing a pair of GATA-3 binding sites, might be a greater risk factor for MM than the A allele alone.

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Submitted: 12/15/08; Accepted: 12/15/08

Previously published online as a *Dermato-Endocrinology* E-publication: http://www.landesbioscience.com/journals/dermatoendocrinology/article/7674

Results

Both loci were in HWE in patients and controls except for A-1012G in MM patients (p = 0.02) and there was marked linkage disequilibrium ($p < 1.10^{-9}$) between the loci in both controls and MM patients. The G alle of G-1520C was preferentially linked to A of A-1012G and the C of G-1520C to G of A-1012G.

MM susceptibility. There were 176 MM patients (mean age 54.3 years, 110 females), including 19 patients with metastasis, and 80 controls (mean age 56.2 years, 40 females). A greater proportion of the MM patients were therefore female. There was no allelic association with gender for either polymorphism in the control or combined populations (p > 0.25 in all cases). Allele and haplotype frequencies in MM patients and controls are shown in Table 1.

Allele and haplotype frequencies. The A allele of A-1012G was more frequent in MM patients (p = 0.011) while frequencies of G-1520C alleles were very similar between patients and controls (p = 0.756). The CA haplotype was a very significant risk factor for MM (p = 0.0001) while the CG haplotype was protective (p = 0.014).

Analysis of a model containing both CA and CG haplotypes showed a lower Akaike information criterion than either alone and was highly significant (p = 0.00002). There was virtually no difference between the frequencies of the GA haplotype between cases and controls (p = 0.931) suggesting that that the difference in frequencies of the A allele between patients and controls was accounted for by the differences in frequencies of the CA haplotype.

MM metastasis. There were 42 patients who were known to have developed metastasis to lymph node and/or internal organs and 80 MM patients, with at least five years follow up, who had not developed recurrence of any type and who were genotyped for both polymorphisms.

Allele and haplotype frequencies. Allele and haplotype frequencies in patients with and without metastases are shown in Table 2. The A allele of A-1012G was more frequent in patients with metastasis (p = 0.054) than patients without metastasis, as was the G allele of G-1520C (p = 0.028). The GA haplotype was more frequent in patients with metastasis (p = 0.015), while frequencies of CA were similar between the two samples.

Table 1 Allele and haplotype frequencies in MM patients and controls

Loci	Haplotype/Allele	Frequencies (%) Control	MM	p value
G-1520C/A-1012G				
	CG	40.8	27.9	0.014
	GG	6.0	7.7	0.719
	CA	0.7	11.8	0.0001
	GA	52.6	52.7	0.931
A-1012G only				
	G	46.7	35.2	0.011
	A	53.3	64.8	
G-1520C only				
	C	41.4	40.1	0.756
	G	58.6	59.9	

Table 2 Allele and haplotype frequencies in MM patients with and without metastasis

Loci	Haplotype/Allele	Frequencies (%) No Mets	Mets	p value
G-1520C/A-1012G				
	CG	34.7	25.8	0.103
	GG	7.4	5.2	0.506
	CA	11.1	6.3	0.388
	GA	46.8	62.7	0.015
A-1012G only				
	G	41.9	31.0	0.054
	A	58.1	69.1	
G-1520C only				
	C	46.3	32.1	0.028
	G	53.8	67.9	

Discussion

Unlike the A-1012G polymorphism there was very little effect of G-1520C on MM susceptibility, which is surprising in view of the tight linkage with A-1012G. In addition there was no effect of the GA haplotype which discounts our hypothesis that the GA haplotype might be a greater risk factor for MM than the A allele alone, based on the possibility of paired GATA response elements in G^{-1520} and A^{-1012}. In fact, the CA haplotype was a powerful risk factor and may well be responsible for the entire effect of the A allele of A-1012G. The evidence for this is that the frequency difference for CA between MM patients and controls (10.4%) was similar to the frequency difference of the A allele (11.8%) but both differed from that of GA (1.3%). The control genotype frequencies found in this study at both A-1012G and G-1520C are very similar to those reported in the dbSNP database at the NCBI.[8]

In addition to the association with MM susceptibility, the A allele of A-1012G was associated with MM metastasis. The G allele of G-1520C was also associated with MM metastasis while having no relationship with susceptibility. Similarly, the GA haplotype was significantly associated with MM metastasis, again in marked contrast to the situation in MM susceptibility.

The molecular basis for these effects is unclear. We have shown in gel shift assays that GATA-3 binds preferentially to the A allele of A-1012G (unpublished results) and d'Alesio et al.[9] and Fang et al.[10] have also shown that GATA binding occurs preferentially at the A allele. Both also showed increased promoter activity of the A allele of A-1012G in reporter assays. Fang et al. also compared the CA and GA haplotypes separately and both were significantly more active compared to the CG haplotype but CA was not significantly more active than GA. The association of a more active promoter haplotype with increased cancer risk and poorer prognosis is at odds with the accepted understanding that vitamin D is beneficial in cancer.

The difference in impact of the CA and GA haplotype in terms of MM susceptibility and outcome would suggest separate mechanisms of actions in the alleles of the two polymorphisms in the two situations. A likely explanation is that the effects of the haplotypes reflect vitamin D response in the various cell types involved in MM aetiology. The availability of transcription factors is limited by the cellular background and this may well alter the importance of a given promoter SNP which depends on the presence of particular transcription factor for its effect. The C allele of G-1520C is within a predicted binding site for the CCAAT/enhancer-binding protein (C/EBP). Several interactions between the VDR and C/EBP family have been previously reported. VDR has been reported to upregulate C/EBP expression[11] and C/EBP enhances the VDR-mediated activation of the $25(OH)D_3$-24-hydroxylase.[12] Dhawan et al.[12] reported two further putative C/EBP binding sites in the *VDR* promoter, one of which begins at -1,490 bp relative to exon 1a, only 30 bases downstream of G-1520C. Tong et al.[13] showed that GATA-3 and C/EBP form protein complexes to mediated target gene expression while Cousins et al.[14] reported GATA-3 and C/EBP binding sites in the promoter of the IL-5 gene. In addition to the malignant characteristics of the MM cells themselves, several other cell types are likely to affect MM development and progression. Indeed, as vitamin D resistance is a common event in MM,[15] the effect of $1,25(OH)_2D_3$ on these other cell types might be pivotal. As previously described,

Th1 cells and dendritic cells make up the primary immune defence to MM[4] and $1,25(OH)_2D_3$ has been shown to influence both cell types, suppressing the Th1 response[3] and freezing dendritic cells in an immature state.[16] As MM progresses from radial to vertical growth phase the stromal partner changes from epidermal keratinocytes to dermal fibroblasts. Vitamin D responses have been well documented in both of these cell types.[17,18] D'Alesio et al.[9] linked an effect of VDR genotype on the height of adolescent girls with an increased level of insulin-like growth factor (IGF-1) in haplotypes containing A^{-1012}. During melanoma progression, IGF-1 has been reported to be produced by the stromal fibroblasts and induce survival, growth and motility in melanoma cells.[19] Furthermore, $1,25(OH)_2D_3$ is known to upregulate the IGF-1 receptor and IGF-binding protein 3.[20]

Our study demonstrates further the complexity of the role of $1,25(OH)_2D_3$ in cancer. There is abundant evidence that overall $1,25(OH)_2D_3$ has a protective effect and there are multiple facets to this protection, e.g., anti-proliferation, pro-differentiation, anti-vascular etc. However, some of the demonstrated effects of $1,25(OH)_2D_3$ do not appear, intuitively, to be protective e.g., downregulation of Th1 immunity. As $1,25(OH)_2D_3$ resistance appears to be relatively common in MM cells, these negative actions affecting other cell types may predominate. The overall benefit of $1,25(OH)_2D_3$ would depend on the balance of these effects. The vitamin D system in MM may be complicated by the relative abundance of vitamin D in the skin when compared to internal cancers; it may be that the ability to evade vitamin D signalling is unusually important for the development of skin cancers. Given this, then $1,25(OH)_2D_3$ treatment of MM could be deleterious. It is important to take these factors into account when considering the use of $1,25(OH)_2D_3$ and its analogues as adjuvant therapies in malignant melanoma.

Methods

We performed genetic analyses under a case-control design to test for association between polymorphisms/haplotypes and MM susceptibility. In addition, we examined whether these polymorphisms/haplotypes were associated with specific MM outcomes. To avoid bias from any relationship of genotype and MM outcome, patients for the susceptibility study were stratified by Breslow (tumor) thickness, which is the single most sensitive predictor of MM outcome, so that the proportions of lesions in each thickness group reflected the proportions of the whole MM population in our geographical locality. Patients were randomly selected from each Breslow thickness groups of the 191 patients presenting sequentially to our hospital. This is the population used in a previous study of the effects of A-1012G and MM.[1] This study was approved by the local Research Ethics Committee and written informed consent was given by the patients.

MM outcome was assessed by comparing patients with metastasis, to lymph nodes or internal organs, to patients without recurrence of any type, having at least five years follow up (90% of metastases in the study presented within five years of diagnosis). For the outcome study the metastasis population was enriched with further patients, known to have developed metastasis, and the control MM patients were patients with five year recurrence free years follow up.

Genotyping. The A-1012G polymorphism was genotyped as previously described.[1] The G-1520C polymorphism is within a natural restriction site for the NlaIII restriction enzyme, such that the C allele is restricted and the G allele remains uncut. To determine genotype, PCR was carried out using primers forward 5'-TGC AGA GAA TGT CCC AAG GT-3' and reverse 5'-GTC CTG CCA GTC TGA TGG AT-3' followed by NlaIII (NEB, USA) digestion and agarose gel electrophoresis.

Statistics. Hardy Weinberg and linkage disequilibrium were analysed in the control population and the stratified MM population. The significance of the Hardy Weinberg disequilibrium coefficient was tested by the goodness of fit (χ^2) test. Linkage disequilibrium between the G-1520C and A-1012G loci was assessed by the composite disequilibrium coefficient in view of divergence from Hardy Weinberg equilibrium (HWE) in the melanoma patients (see below). We estimated haplotype frequencies in MM patients and controls and conducted allele-based and haplotype-based inference using the CHAPLIN Programme[21,22] under a multiplicative model of haplotype effect. Rather than rely on asymptotic inference, we established empirical significance of both allelic and haplotype effects on outcome using 10,000 permutations of the data.

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