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Genotype <21CAs/≥21CAs and allele <21CAs of the MANBA gene in melanoma risk and progression in a Swedish population

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Abstract. Cutaneous melanoma is characterized by poor patient outcome in its later stages. The search for genetic markers is therefore crucial for the identification of populations at risk for melanoma. Highly polymorphic CA repeats in 3' proximity in the MANBA gene were examined by PCR-capillary electrophoresis in 185 Swedish melanoma patients and 441 tumor-free age- and gender-matched individuals. The associations of the polymorphisms with melanoma risk, the pigment phenotypes of the patients and tumor characteristics were analyzed. A significant difference in allelic distribution between melanoma patients and tumor-free individuals was observed. The frequency of the MANBA genotype <21CAs/≥21CAs was significantly higher in melanoma patients than in the controls. When comparing allele distribution in patients and their matched controls, the allele <21CAs was found to be associated with the female gender (39.8 vs. 31.2%, P=0.041, OR=1.46, 95% CI 1.02-2.10), but not with male gender (34.4 vs. 30.9%, P=0.39). Within the melanoma group, there were no differences in the distribution of the MANBA alleles associated with patient gender or age before or after 55 years at diagnosis, nor was there any association between the MANBA genotype and pigment phenotype or tumor sites. The MANBA allele <21CAs was, however, associated with thin melanomas at diagnosis (Breslow thickness ≤1.5 mm and Clark levels I and II). In conclusion, these data suggest that MANBA polymorphisms might be an indicator of tumor growth and progression and, together with other markers, could be used to identify individuals at increased risk of melanoma.

Introduction

Cutaneous melanoma has become a serious public health problem due to its increasing incidence over the last decades (1) and its resistance to most current therapies in the later stages of its progression (2). Genetic predispositions, pigment phenotype and environmental stimuli such as sun exposure, particularly during childhood, have been identified as risk factors. However, the exact mechanisms behind tumor initiation, development and progression remain far from being fully understood. Based on recent genetic investigations, several high-risk susceptibility genes for melanoma, such as the oncogene BRAF (3), suppressor gene CDKN2A (4) and progression genes PTEN (5) and CDK4 (6), have been identified in melanoma cell lines, melanoma patients and melanoma high risk families. In case-control studies, various low-risk genes, such as MC1R (7) and EGF variants (8), were also found to be involved. Additional unknown susceptibility genes for melanoma risk and progression have yet to be identified (9,10).

MANBA is a gene coding for ß-mannosidase, a lysosomal exoglycosidase in the pathway for N-linked glycoprotein oligosaccharide catabolism. Deficiency of ß-mannosidase has been reported to result in the lysosomal storage disease called ß-mannosidosis, which has a wide spectrum of neurological symptoms (11). In animal experiments, it has been shown that rats with hepatoma or leukemia exhibit high levels of ß-mannosidase in serum (12). In several studies, high levels and activity of ß-mannosidase have been described in human tumors, such as glioma, adenocarcinoma and squamous cell carcinoma of the upper gastrointestinal tract (13-15). Inhibitors of carbohydrates have been identified as a new class of potential anticancer drugs (16,17). Such drugs have been reported to inhibit ß-mannosidase activity in a metastatic model of mouse B16 melanoma (18). However, to our knowledge, MANBA polymorphisms in melanoma risk and tumor progression have not been studied.

In the present case-control study, we examined dinucleotide CA repeats of the MANBA gene in Swedish melanoma patients and in age- and gender-matched tumor-free individuals. The associations of the polymorphisms with patient age at diagnosis, gender, pigment phenotype and tumor characteristics were also analyzed.

Materials and methods

Patient selection. This research project was approved by the Ethics Committee, Linköping University, Linköping, Sweden. Participating melanoma patients were informed of the study

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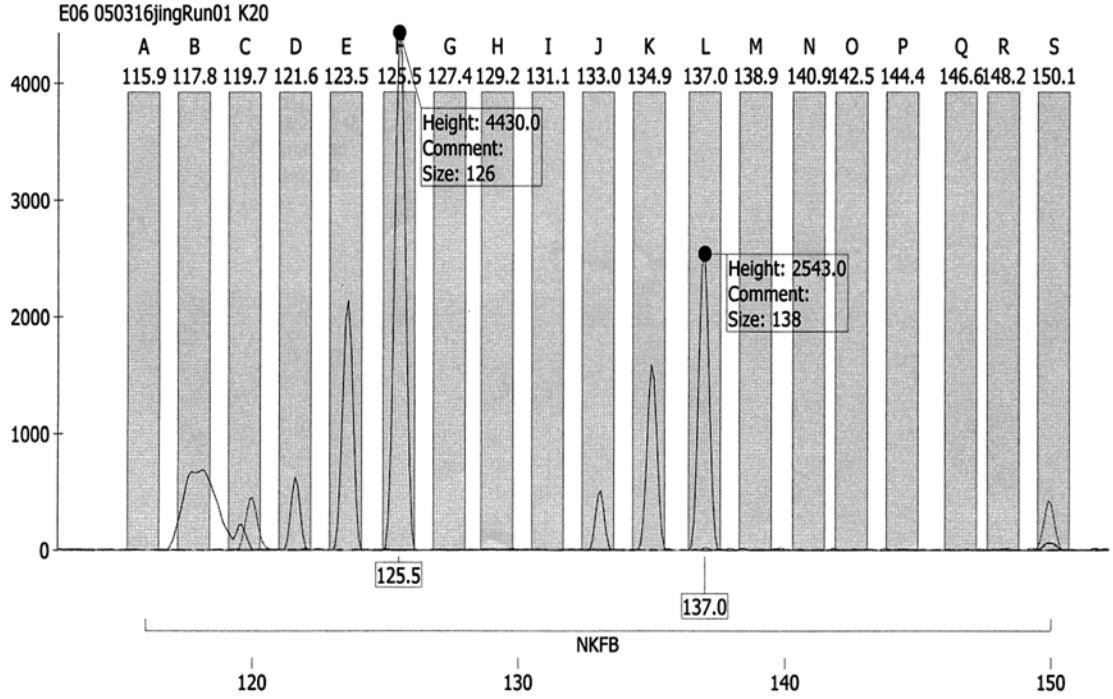

Figure 1. An individual with an allele length of 126 and 38 bps, corresponding to 18CAs/24CAs, was determined by MegaBACE using the PCR-capillary electrophoresis technique.

protocol and gave gave their signed consent. Participants were all from the southeastern region of Sweden and comprised 185 melanoma patients, as well as 441 age- and gender-matched tumor-free individuals chosen at random from our regional DNA biobank.

All melanoma patients were Caucasian, with an average age of 54 years (range 19-80 years) at diagnosis. Histopathological melanoma subtypes, Breslow thickness (20 melanomas *in situ*, 52 tumors with a thickness <0.75 mm, 50 tumors of 0.75 to ≤1.5 mm and 54 tumors >1.5 mm) and Clark levels were reviewed from the original patient pathology reports. As previously described, tumor locations were registered on a schematic body chart divided into chronically, intermittently and rarely sun-exposed skin areas (19). Patients were classified into one of two groups for each phenotype parameter: eye color, blue + green or brown + mixed; hair color, blond + red or brown + black; and skin type, I+II or III+IV.

Genomic DNA extraction. After a short interview, 5-7 ml of venous blood from each patient was collected into an anti-coagulant tube for further genomic DNA extraction. The genomic DNAs were isolated from the mononuclear cells of the venous blood according to the manufacturer's instructions using the DNA Blood Maxi Kit from Qiagen (Germany). In brief, blood samples were incubated with buffered detergent at room temperature and then mixed with absolute alcohol at 70˚C for 10 min. The samples were put through spin silicon columns, then the columns were washed with buffers and solvent in the store buffer provided by Qiagen. The DNA concentration of each sample was measured by a Nanodrop ND-1000 Spectrophotometer (Wilmington, DE, USA). DNA working solution (50 ng/μl) was prepared from the original DNA stocks in Milli-Q water and maintained at 4˚C for further analysis. Identical procedures were used to extract genomic DNA from the blood of tumor-free individuals.

Genotyping of the MANBA gene. The PCR-capillary electrophoresis technique was used to genotype the polymorphic CA repeats of the MANBA gene as previously described by Curran *et al* (20). Primers for MANBA were FAM-labeled forward primer 5'-CTTCAGTATCTAAGAGTATCCT-3' and an unlabeled reverse primer 5'-CAAGTAAGACTCTA CGGAGTC-3', which amplified a fragment ranging from 116 to 146 bps. In brief, PCR was performed in a 20-μl mixture containing 50 ng genomic DNA, 1X PCR buffer (Promega, Madison, WI), 0.5 μM of each primer (Invitrogen, Scotland), 3.75 mM $MgCl_2$ (Promega), 0.5 U DNA polymerase (Promega) and 0.25 mM dNTP (Invitrogen). Amplification was performed with an annealing temperature of 58˚C for 30 sec for 40 cycles. The PCR products were checked in 1.5% agarose gel with 0.2 μg/ml ethidium bromide for a specific band. PCR product (μl) was mixed with 25 μl Milli-Q water and 0.25 μl MegaBACE ET 400-R Size Standard (Amersham Biosciences, Buckinghamshire, UK), and the mixture was injected into the capillary of the MegaBACE instrument (Amersham Biosciences). Data were analyzed with the software MegaBACE Fragment Profiler to determine the allele length of each sample.

Statistical analysis. The χ^2 test was used to estimate the frequency differences in alleles and genotypes between melanoma patients and tumor-free controls and between subgroups of melanoma patients. All P-values shown were two-sided and P<0.05 was determined to be statistically significant. Risk of the genotypes was evaluated by odds ratio (OR) and 95% confidence intervals (CI).

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Polymorphic CA repeats of the MANBA gene were examined by a PCR-capillary electrophoresis technique. A typical curve is shown in Fig. 1. There were 13 polymorphic alleles identified in melanoma patients (16-28CA), and 13 in tumor-free individuals (13CAs, 16-27CAs), respectively (Table I). No patients had the 13CAs allele and only one had 28CAs, as in the melanoma group. The patients had allelic fragments from 122 to 146 bps, corresponding to 16-28 CA repeats, while the controls had allelic fragments of 116 and 122-144 bps, which contained 13 16-27 CA repeats respectively. The alleles with 18 and 24 CA repeats were the most common alleles in both groups. We did not find a significant difference in allelic distribution between melanoma patients and controls (P=0.46).

There were 36 genotypes found in the melanoma patients and 44 in the tumor-free controls. Among these genotypes, only distribution of the genotype 19CAs/24CAs differed significantly between the patients and controls (8.1 vs. 4.1%, OR=2.07, 95% CI 1.02-4.21, P=0.04). As shown in Table II, whole genotypes were analyzed for melanoma risk divided into three separate groups: i) individuals with both MANBA alleles shorter than 21 CA repeats (<21CAs/ <21CAs), ii) individuals with one allele shorter than 21 CA repeats and the other equal to or longer than 21 CA repeats (<21CAs/ ≥21CAs), and iii) individuals with both alleles equal to or longer than 21 CA repeats (≥21CAs/≥21CAs). An increase in melanoma risk was found to be associated with genotypes <21CAs/≥21CAs compared with genotypes ≥21CAs/≥21CAs (OR=1.48, 95% CI 1.02-2.13, P=0.037). Further analysis revealed that there were more melanoma patients than tumor-free individuals with allele <21CAs (37 vs. 31%, OR=1.31, 95% CI 1.02-1.69, P=0.037).

Table I. Distribution of MANBA alleles in melanoma patients and healthy controls.

Allele types	No. patients (%)		No. controls (%)	
1. 13CAs	0	(0.00)	1	(0.00)
2. 16CAs	2	(0.54)	4	(0.45)
3. 17CAs	1	(0.27)	2	(0.23)
4. 18CAs	94	(25.41)	202	(22.90)
5. 19CAs	35	(9.46)	60	(6.80)
6. 20CAs	5	(1.35)	4	(0.45)
7. 21CAs	31	(8.39)	85	(9.64)
8. 22CAs	11	(2.97)	32	(3.63)
9. 23CAs	41	(11.08)	108	(12.24)
10. 24CAs	114	(30.81)	304	(34.47)
11. 25CAs	11	(2.97)	34	(3.85)
12. 26CAs	22	(5.95)	44	(4.99)
13. 27CAs	2	(0.54)	2	(0.23)
14. 28CAs	1	(0.27)	0	(0.00)

No significant association was found between allele distribution, tumor site and histological types. Allele <21CAs was significantly less frequent in patients with thicker melanomas (>1.5 mm according to Breslow) compared to those with thinner (≤1.5 mm) melanomas (25.9 vs. 40.2, 95% CI 0.31-0.87, P=0.012, P=0.012, Table III). The association between MANBA genotype and Clark level was analyzed in

Table II. Distributions of MANBA genotypes and alleles in melanoma patients and healthy controls.

Genotype/alleles	Patient (%)		Control (%)		OR (95% Cl)		P-value
≥21CAs/≥21CAs	70	(37.8)	210	(47.6)	1.00		
<21CAs/≥21CAs	93	(50.3)	189	(42.9)	1.48	(1.02-2.13)	0.037
<21CAs/<21CAs	22	(11.9)	42	(9.5)	1.57	(0.88-2.81)	0.130
≥21CAs	233	(63.0)	609	(69.0)	1.00		
<21CAs	137	(37.0)	273	(31.0)	1.31	(1.02-1.69)	0.037

Table III. Association of MANBA alleles with melanoma thickness and stage.

Variables	Allele type (%) ≥21CAs		Allele type (%) <21CAs		OR (95% Cl)		P-value
Breslow thickness							
≤1.5 mm	122	(59.8)	82	(40.2)	1.00		
>1.5 mm	80	(74.1)	28	(25.9)	0.52	(0.31-0.87)	0.0120
Clark level							
I/II	83	(55.3)	67	(44.7)	1.00		
III/IV/V	134	(69.1)	60	(30.9)	0.55	(0.36-0.86)	0.0088

Table IV. Distribution of MANBA alleles in melanoma patients with different gender and age at diagnosis.

Variables	Allele type [no. (%)]		P-value
	≥21CAs	<21CAs	
Male	126 (65.6)	66 (34.4)	
Female	106 (66.2)	70 (39.8)	>0.05
≤55 years	111 (63.8)	63 (36.2)	
>55 years	122 (62.2)	74 (37.8)	>0.05

the Clark level I/II and III/IV/V groups. In patients with thick melanomas (Clark levels III, IV and V), the frequency of the allele <21CAs was found to be lower (30.9 vs. 44.7%, OR=0.55, 95% CI 0.36-0.86, P=0.0088). Using a cut-off of 55 years of age, we did not find any difference in the distribution of the MANBA alleles associated with patient gender or age at melanoma diagnosis, (Table IV), nor did we find any association between the genotypes and the skin type, hair or eye color of the patients (data not shown).

Discussion

Cutaneous melanoma, the most aggressive malignancy of the skin, is a multifactor disease in which genetics and environmental stimuli such as UV irradiation interact in tumor genesis (1,21). The MANBA gene, which codes an enzyme catalyzing the degradation of glycoprotein, has been shown to be involved in gene-environmental interactions in the development and progression of certain tumors, including tumors of the nervous system (13,14). To our knowledge, this is the first study on polymorphisms of the MANBA gene in melanoma patients. In this case-control study, we found that genotypes <21CAs/≥21CAs in the MANBA gene were associated with melanoma risk, indicating that the highly polymorphic CA repeat in the MANBA gene might be involved in the initiation and development of melanoma, and that these polymorphisms might be used in melanoma risk profiling. Additionally, allele <21CAs was also significantly associated with melanoma risk. However, in order to confirm these findings, more accurate molecular studies on gene-gene and gene-protein interactions are needed. In the present study, we further analyzed correlations of the MANBA polymorphisms with tumor characteristics, such as histopathological type, Breslow thickness and Clark level. The distribution of the <21 allele was found to be related to Breslow vertical tumor thickness, an important prognostic factor of melanoma. Patients with thinner tumors have a longer survival than patients with thicker tumors (22). Patients with thinner melanomas were usually those with the <21 allele, and patients with thicker melanomas had a lower frequency of the alleles. The frequency of the <21 allele was also found to be significantly associated with patients diagnosed with melanomas of Clark levels I and II compared with those of Clark levels III, IV and V. Clark level has been widely accepted as an important clinical predictor for melanoma progression and prognosis. Taken together, the findings indicate that these alleles may play a role in protecting the tumors from progression, further suggesting that they might be useful as additional predictors for melanoma progression or even prognosis. However, in order to draw this conclusion, long-term survival data is needed by continued follow-up of our patients.

Although the exact mechanism behind the differing distributions of the <21 allele is unknown, we speculate, based on the general functions of the MANBA gene, that the <21 allele might affect its transcription and expression, resulting in a lower protein level of ß-mannosidase. This in turn decreases the turnover of metabolic substances for tumor growth and progression. However, the interaction of MANBA with other 'melanoma-related' genes or proteins is likely. Functional investigations on polymorphisms in MANBA genetic expression are necessary in order to further examine the role of MANBA polymorphisms in melanoma susceptibility.

In this case-control study, MANBA polymorphisms were found to be associated with melanoma risk and, together with other markers, might be utilized to identify individuals at high risk of melanoma. The MANBA allele pattern might further be used as a tumor growth indicator.

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