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Importance of polymorphisms at NF- κ B1 and NF- κ BI α genes in melanoma risk, clinicopathological features and tumor progression in Swedish melanoma patients

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Running title: NF- κ B1 and NF- κ BI α in melanoma patients

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Abstract

In this study, functional polymorphisms of NF- κ B1 and NF- κ BI α genes were examined in 185 melanoma patients and 438 tumor-free individuals. Associations of the polymorphisms with melanoma risk, age and pigment phenotypes of the patients and clinico-pathological tumor characteristics were analyzed. DNAs were isolated from mononuclear cells of venous blood. Polymorphisms of the genes were genotyped by a PCR-RFLP technique, and transcription level of NF- κ BI α was examined by a quantitative real-time reverse transcription PCR. Results showed that both ATTG insertion polymorphism of NF- κ B1 and A to G polymorphism of NF- κ BI α genes were correlated with melanoma risk, especially, in a combination of ATTG₂/ATTGT₂ and GG. NF- κ B1 ATTG₂/ATTG₂ and NF- κ BI α GG genotypes were associated with male gender and age > 65 years (at diagnosis). Patients with ATTG₁/ATTG₁ genotype had thinner tumors and lower Clark levels at diagnosis. Frequency of ATTG₁/ATTG₁ genotype was higher in patients with melanomas on intermittently sun-exposed pattern of the body and NF- κ BI α GG was more frequent in the patients with melanomas at rarely exposed sites. There were no differences in the gene transcription level between patients with different NF- κ BI α genotypes. These data suggest that NF- κ B1 and NF- κ BI α genes might be susceptible genes for melanoma risk and functional polymorphisms of these genes might be biological predictors for melanoma progression.

Keywords: polymorphisms, NF- κ B1, NF- κ BI α , melanoma risk, clinicopathological features, phenotypes

INTRODUCTION

Melanoma is the most aggressive cancer in the skin. Incidence of melanoma has dramatically increased during the last decades (1) and prognosis for the later stages of melanomas is usually very poor due to resistance to current therapy (2). The genetic predisposition for melanoma is highly important (3,4) although exposure of ultraviolet (UV) radiation from the sunlight is a major environmental risk factor for melanoma (5). The molecular mechanism behind melanoma formation and progression is still not clarified although a large number of candidate genes have been speculated to be involved in initiation, development and progression of melanoma (6-8).

In melanoma, upregulation of nuclear factor-kappa B (NF- κ B) switch from pro- to antiapoptotic functions, and NF- κ B is constantly activated in metastatic melanoma (9). Further, UV exposure from the sunlight has been shown to activate NF- κ B independently from the DNA damaging effect (10). Activity of NF- κ B has been observed in various cancer types (11), including multiple myeloma (12), breast cancer (13) and melanoma (14,15) to contribute to tumor angiogenesis, progression, and metastasis (16-19). The most common gene with high frequency of mutations, deletions, and expression in melanoma is the melanoma-associated p16^{INK4a} gene. Deficiency of the p16^{INK4a} results in overexpression of cyclin D1, leading to enhancement of cell proliferation (20). Moreover, since wild type of p16^{INK4a} inhibits NF- κ B transcriptional activity by binding to the NF- κ B subunit p65 the dysfunction of p16^{INK4a} results in increased activity of NF- κ B gene (21).

NF- κ B is a heterodimer in Rel family with five members: RelA, RelB, c-Rel, p50/105 (NF- κ B1) and p52/p100, and the dimeric form of NF- κ B p50/RelA is the most common form. In normal cells, NF- κ B is inactivated in the cytoplasm by binding to their inhibitors, I κ B α , β or γ . When the inhibitory proteins are phosphorylated and degraded, NF- κ B is subsequently released and further translocated into the nucleus, where the gene transcription is initiated (22). NF- κ B is known to coordinate expression of more than 150 genes and contribute to the balance between apoptosis and survival (11). In the melanocytes and early stages of melanoma, NF- κ B upregulates the major pro-apoptotic pathways, leading to caspase activation and apoptosis. However, in late stages of melanoma, these pro-apoptotic pathways are inhibited by activation of the NF- κ B, which results in tumor growth (23).

Several polymorphic sites in NF- κ B and its inhibitor genes have been identified (24, 25). Recently, a four nucleotides insertion/deletion, -94ins/delATTG polymorphism located between two putative key promoter regulatory elements was described in the promoter region of NF- κ B1 gene which is potentially functional polymorphism in NF- κ B1 associated with human diseases (26), and cancers (24,27). In addition, there are reports concerning polymorphism in NF- κ B1 α gene and various cancer types (28,29). In the present study, we examined polymorphisms in both NF- κ B1 and NF- κ B1 α genes in Swedish melanoma patients and tumor-free individuals, and further analyzed associations of the polymorphisms with melanoma risk, patient's age at diagnosis, gender, tumor characteristics, and pigment phenotypes.

MATERIALS AND METHODS

Melanoma patients and tumor-free population. This study was approved by the Regional Ethical Committee at Linköping University, Sweden. The study details were given to all melanoma patients participating and written consents were signed by the patients.

One hundreds and eighty-five melanoma patients (97 males, 88 females) from South-Eastern region of Sweden were diagnosed in Linköping University Hospital and Kalmar Hospitals within the same geographical region, Sweden. Four hundred and thirty-eight age and gender matched tumor-free individuals from the same region were randomly chosen from our DNA bank. All patients were Caucasian with an average age of 54 years old (range 19-80 years) at diagnosis (Fig. 1), and 7/185 patients had multiple primary melanomas. Tumor location was registered on a schematic body chart, as previously described, divided into chronically, intermittently and rarely sun-exposed areas according to clothing habits and general sun-exposure pattern (30). Tumor characteristics as histopathological subtypes, Breslow thickness, and Clark levels were reviewed from patient's surgery and pathology records (Tab. 1). Gender and pigment phenotypes were recorded (Tab. 2).

DNA isolation. After the interview each patient donated 5 ml of venous blood in a heparinized tube for genomic DNA extraction. According to the manufacturer's instruction, genomic DNA was extracted from mononuclear cells by a DNA Blood Maxi Kit from Qiagen (Germany). In brief, blood samples were incubated with buffered detergent at room temperature and then

with absolute alcohol at 70°C for at least 10min. The samples were then taken through spin silicon columns. The columns were washed with buffers and then solved in store buffer provided by Qiagen. DNA concentration was measured by a Nanodrop, ND-1000 Spectrophotometer (Wilmington, DE, USA). DNA working solution of 50ng/μl in Milli-Q water was prepared from the original DNA stocks and kept at 4°C for further analysis.

Genotyping of NF-κB1 and NF-κBIα by PCR-RFLP.

Polymorphisms in NF-κB1 and NF-κBIα genes were determined by PCR-RFLP. Primers for NF-κB1 were 5'-TGGGCACAAGTCGTTTATGA-3' and 5'-CTGGAGCCGGTAGGGAAG-3', amplifying a fragment of 281 or 285bp. Primers for NF-κBIα were 5'-GGCTGAAAGAACATGGACTTG -3' and 5'-GTACACCATTTACAGGAGGG-3' which amplify a fragment of 424bps. In brief, the PCR was performed in 20μl mixture containing 50ng DNA, 1×PCR buffer (Promega, Madison, WI, USA), 0.5μM each primer (Invitrogen, Scotland), 1.5mM (NF-κB1) or 3.75mM (NF-κBIα) MgCl₂, 0.5U DNA polymerase (Promega), 0.25mM dNTP (Invitrogen). The amplification was performed with annealing temperature of 60°C for 30 seconds for 35 cycles. The PCR product of NF-κB1 was digested with PflM I (8U/μl) from Biolabs (New England, UK), in a 20μl reaction system (5μl PCR product + 6μl PFIM I + 6.8μl H₂O + 2μl 10 x buffer + 0.2 μl 100xBSA) at 37°C for 18 hours. The ATTG insertion allele had two ATTG at its promoter, named as ATTG₂, which could be cleaved by PflM I into two lengths of 45 and 240bp, while the wild type (WT) allele has onlyh one ATTG at its promoter, named as ATTG₁, kept

intact after PflM I digestion. The PCR product of NF- κ BI α fragment was digested with a restriction enzyme of HaeIII (10U/ μ l) from Promega in a 25 μ l reaction mixture (10 μ l PCR product + 2 μ l HaeIII + 13 μ l 10 x buffer) at 37°C for 18 hours. The G allele had a HaeIII restriction site and was cleaved into two lengths of 108 and 316bp, and the A allele kept intact after HaeIII digestion. Both the PCR and restriction products were visualized in 2% agarose gel with 0.2 μ g/ml ethidium bromide. DNA ladder (100bp) was included in each run to determine sizes of the fragments. PCRs without DNA template added were used as negative controls. Genotypes of NF- κ B1 polymorphisms were distinguished as WT (ATTG₁/ATTG₁), heterozygote (ATTG₁/ATTG₂) and polymorphic homozygote (ATTG₂/ATTG₂). Genotypes of NF- κ BI α polymorphisms were determined as WT (AA), heterozygote (AG) and polymorphic homozygote (108 and 316bps, GG).

RNA reverse transcription of NF- κ BI α gene. Peripheral blood (2.5ml) from the selected patients with NF- κ BI α AA, AG or GG genotype was collected into the PAXgene Blood RNA Tube (DB and Qiagen, Germany) and kept at -20°C for RNA isolation. Total RNA was extracted from the peripheral blood mononuclear cells by using PAXgene Blood RNA Kit (DB and Qiagen) and Rnase-FreeDNase set (Qiagen) according to the protocol provided by the manufacturer. In brief, first strand cDNA was reversely transcribed from RNA by using 1st Strand cDNA Synthesis Kit for RT-PCR (Roche applied science, Germany). The cDNA was diluted 20 times in Milli-Q water for Quantitative real-time PCR.

Quantitative real-time PCR. SYBR Green was used as fluorescent dye for real-time PCR. Primers for NF- κ B α were 5'-GAGCAGATGGTCAAGGAGCTG-3' and 5'-GGCCAAGTGCAGGAACGAG-3', which amplify a fragment of 117bps. ACTB gene was used as endogenous control with primers 5'-GAAAATCTGGCACCACACCT-3' and 5'-GATCTGGGTCATCTTCTCG-3', produced a fragment of 119bps. The PCR was performed in 25 μ l mixture containing 1 μ l 20-time diluted cDNA, 12.5 μ l SYBR Green JumpStart Taq ReadyMix (Sigma, Germany), 0.5 μ l each primers (Invitrogen) and 10.5 μ l water. Amplification was carried out in 96-well optical Reaction plate from ABI applied Biosystem (Foster City, CA, USA) in ABI PRISM 7700 Sequence Detector (ABI applied Biosystem) with Sequence Detector V1.7 program. The reaction was initiated by a denaturation step at 94°C for 2 minutes, followed by 40 cycles of 94°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds.

To create a standard curve for relative quantification, a representative cDNA sample with the NF- κ B α GG genotype was chosen as standard, serially diluted in water (2^{-1} , 2^{-2} , 2^{-3} , 2^{-4} , 2^{-5} , 2^{-6} , 2^{-7}). All cDNA samples including the standards were subjected to quantitative real-time PCR in triplicate. The quantity of the standard was plotted against threshold cycle number, at which the fluorescence increased above background. The expression of the target gene was evaluated by this standard and shown as value relative to expression in the standard curve. The relative NF- κ B α expression value was corrected by relative value of ACTB from the same DNA samples. Triplicate NF- κ B α

expression values were individually divided by the relative ACTB values and the mean was named as NF- κ BI α /ACTB normalized.

Statistical analysis. Chi-square test was used to estimate differences in frequency distributions of genotypes and alleles in NF- κ B1 and NF- κ BI α genes between melanoma patients and tumor-free individuals, as well as within subgroups of melanoma patients. Multivariate logistic regression analysis was performed to obtain the adjusted odds ratios (OR) for melanoma risk of polymorphisms and their 95% confidence intervals (CI). Mann-Whitney test was used to estimate differences in gene expression levels between subgroups of patients with various genotypes. All p-values shown were two-sided and $p < 0.05$ was judged as statistical significance.

RESULTS

Tumor characteristics in 185 melanoma patients were examined.

Histopathological types of melanomas were divided into melanoma in situ, superficial spreading melanoma, nodular melanoma, lentigo malignant melanoma, and acro-lentiginous melanoma (Tab. 1). Breslow thickness was analyzed in three groups (<0.75, 0.75-1.5 and >1.5 mm), and Clark level in two groups (I + II and III + IV). Tumor location was registered as the first tumor diagnosed, according to sun-exposed body areas and sun-exposure behavior. Pigment phenotypes of melanoma patients were registered with the eye and hair color as well as skin types (Tab. 2).

Promoter nucleotide insertion polymorphism in NF- κ B1 gene and melanoma risk. Polymorphism of four nucleotide insertion (-94insATTG) at promoter region of NF- κ B1 gene was determined according to the sizes of PCR fragments. NF- κ B1 ATTG₁/ATTG₁ genotype was revealed by the presence of a single 281bp fragment, and heterozygote ATTG₁/ATTG₂ genotype by the presence of 240 and 285bp fragments, and polymorphic homozygote ATTG₂/ATTG₂ genotype with the presence of 240bp fragment (Fig. 2). Distribution of the genotypes was found to be significantly different between melanoma patients and tumor-free individuals ($P \leq 0.025$). Frequency of ATTG₂/ATTG₂ genotype was higher in the patients than the controls (36.2 versus 26.5%, OR=1.58, 95%CI: 1.09-2.28). Moreover, the frequency of the ATTG₂/ATTG₂ genotype was higher in male patients as compared with the controls (44.8 vs 26.8%, $P \leq 0.001$, OR=2.22, 95%CI: 1.36-3.65). However,

female patients did not differ from controls in the frequency of ATTG₂/ATTG₂ (P=0.42). Analysis with a cut-off points of 45 and 65 years old at diagnosis showed that ATTG₂/ATTG₂ genotype was associated with the patients over 65 years old compared with the controls (46.9 vs 29.4%, P≤0.01, OR=2.13, 95%CI: 1.10-4.10), but not in the younger patients (Table 3).

Promoter nucleotide insertion polymorphism of NF-κB1 gene in melanoma patients. In case-case study, when frequency of the polymorphism distribution was compared between male and female patients ATTG₂/ATTG₂ was higher in the males than females (44.8 vs 27.2%, P≤0.01, OR=2.16, 95%CI: 1.17-4.01). However, we did not find the difference between various age groups of patients (<45, 45-65 and >65 years old, P≤1, data not shown). As shown in Tab. 4, frequency of ATTG₁/ATTG₁ genotype was decreased by increase in tumor thickness (26.9% in category <0.75mm, 16% in category 0.75-1.5 mm, 9.3% in category >1.5 mm). The frequency of ATTG₁/ATTG₁ genotype was significantly lower in patients with the tumor thicker than 0.75mm compared with those with tumors thinner than 0.75mm (P=0.025, OR=0.388, 95% CI 0.167-0.902). Odds ratio was even lower when comparing patients with tumors thicker than 1.5mm or thinner than 0.75mm (P=0.018, OR=0.277, 95% CI 0.0917-0.837). Frequency of the ATTG₁/ATTG₁ genotype was significantly lower in patients with tumors at Clark stages III and IV (10.3 vs 28%, P=0.028, OR=0.30, 95%CI: 0.13-0.68). Moreover, the ATTG₁/ATTG₁ genotype was significantly associated with melanomas located on intermittently exposed sites compared with those with the tumor located in chronically exposed areas of the

bodies (20.8 vs 7.0%, $P=0.037$, $OR=3.51$, 95%CI: 1.02-12.13). Further analysis showed that the polymorphisms were more common in patients with tumor located in intermittently exposed locations than other sites in superficial spreading melanoma ($P=0.008$) although there was few cases. There were no statistical differences between different histopathological types of melanomas and between the pigment phenotypes (data not shown).

A to G polymorphism in NF- κ BI α gene and melanoma risk.

Genotypes of NF- κ BI α were determined according to the sizes of PCR fragments in melanoma patients and tumor-free individuals. As shown in figure 3, NF- κ BI α AA genotype was represented by the presence of a single 424bp band, and heterozygote AG genotype by the presence of 108, 316 and 424bp fragments, and polymorphic homozygote GG genotype with the presence of 108 and 316bp fragments.

Frequencies of the NF- κ BI α genotypes were significantly different between melanoma patients and the controls ($P\leq 0.01$). The GG genotype of NF- κ BI α was significantly higher in the patients (45.4 vs 32.6%, $OR=1.72$, 95%CI: 1.21-2.44). Moreover, frequency of the genotype was even higher in female patients (53.4 vs 32.0%, $OR=2.44$, 95% CI: 1.47-4.04, $P\leq 0.001$), and in the patients of 45-65 years old ($P\leq 0.05$) compared with the controls. However, there was no difference in the male patients (38.5 vs 33.5% in male controls, $P\leq 1$, Tab. 5). Further, we showed that the G allele was markedly higher in melanoma patients (65.1 vs 57.9%, $P\leq 0.025$ $OR=1.36$, 95%CI: 1.06-1.75) and

in female patients (69.3 vs 56.8% in female control, $P \leq 0.001$, OR=1.72, 95%CI: 1.19-2.50), but not in male patients (61.5 vs 59.1% in male control, $P \leq 1$, Tab. 6).

When combinations in distributions of NF- κ B1 and NF- κ BI α polymorphisms was analyzed in the patients and control individuals, the combination of ATTG2/ATTGT2 and GG revealed even more statistical significance ($P < 0.001$).

A to G polymorphism of NF- κ BI α gene in melanoma patients.

There was no different in distributions of A to G polymorphism between male and females patients (53.4 vs 38.5, OR=1.83, 95%CI: 1.02-3.29). When distribution of NF- κ BI α A to G polymorphism was analyzed in patients with five-year intervals significance was only found in the patients with age above 60 years at diagnosis. In whole group of the patients, as shown in Tab. 7, frequency of the NF- κ BI α GG genotype was not different between the patients > 60 and < 60 years old (53.2 vs 39.8%, $P=0.13$). However, if the patients were further divided by gender frequency of the GG genotype was found significantly higher in the male patients who were over 60 years old (51.1 vs 26.5%, $P=0.02$ OR=2.89, 95%CI: 1.23-6.79), but not in the female patients ($P=0.78$).

Association of NF- κ BI α polymorphism with tumor characteristics and patients' pigment phenotypes was also analyzed. Frequency of NF- κ BI α GG genotype was significant different between patients with tumors located at rarely exposed site and tumors in the other sites (69.6 vs 43.3%, $P=0.02$, OR=3.0, 95% CI: 1.17 - 7.67).

NF- κ BI α mRNA expression with A to G polymorphism. In order to investigate influence of A to G polymorphism in NF- κ BI α on gene expression, quantitative real-time reverse transcription-PCR was used to quantitate NF- κ BI α mRNA in melanoma patients. Twelve randomly chosen samples (1 with AA, 8 with AG and 3 with GG genotype) were examined. The relative expression of NF- κ BI α mRNA with AA, AG and GG genotype was 1.29, 1.39, and 1.40, respectively. The expression of NF- κ BI α at transcription levels was not different in the melanoma patients with these different genotypes.

DISCUSSION

To our knowledge this is the first report concerning association of polymorphisms in NF- κ B1 and NF- κ BI α genes with melanoma in a Swedish population. In this study, we found that four nucleotide insertion polymorphism -94insATTG at promoter region of NF- κ B1 gene and A to G polymorphism in 3' UTR region of NF- κ BI α predisposed to melanoma risk, which indicated that the individuals with ATTG₂/ATTG₂ genotype had usually higher levels of NF- κ B1 transcript and further increase in production of NF- κ B protein. NF- κ B signaling pathway plays an important role in formation, progression, angiogenesis and metastasis of melanoma (16,31). Moreover, single nucleotide polymorphism at 3' UTR of NF- κ BI α has effects on the gene expression by enhancing gene expression (32). However, we didn't find any effect of this polymorphism on gene expression, suggesting that other genes in linkage with the G allele might be involved increasing melanoma risk.

We found that -94insATTG in NF- κ B1 gene, ATTG₂/ATTG₂ genotype was a susceptibility factor in the male patients. ATTG₂/ATTG₂ genotype was correlated with the patients over the age of 65 years suggesting that the polymorphisms of NF- κ B1 and BI α might function differently in the individuals with different age at tumor onset and gender. Since NF- κ B and their inhibitors are regulated by a variety of factors, including hormones, mitogens and cytokines (11), these factors might be involved in the alternative expression of NF- κ BI α polymorphism in melanoma patients. Both -94insATTG in NF- κ B and A to G polymorphism of NF- κ BI α gene were related to melanoma risk in

patient's gender and tumor onset dependent manner. In addition, NF- κ BI α GG genotype was significantly higher in the female patients, but not in males, suggesting a strong predisposing function of GG genotype only in the female patients. Moreover, this genotype was significantly associated with male patients with late melanoma onset >60 years.

G₁/ATTG₁ and NF- κ BI α GG genotypes were related to tumor location. The GG genotype was significantly associated with the melanomas at rarely exposed site, while frequency of ATTG₁/ATTG₁ genotype was higher in the patients with melanomas at intermittently exposed sites, indicating that different susceptible polymorphisms or genomic changes were associated with sun-exposure pattern of the body.

We found that NF- κ B polymorphism was related to Breslow vertical thickness in melanoma. The patients with thinner melanomas were usually those with ATTG₁/ATTG₁ genotype, and patients with thicker melanomas had less frequency of the genotype, suggesting that NF- κ B1 might be an important gene for tumor progression. Breslow thickness is an important prognostic factor in melanoma and patients with thinner tumor have a longer survival than those with thicker tumors (33). Further, frequency of ATTG₁/ATTG₁ genotype was significantly correlated with the patients with early stage of melanoma (Clark I and II). Taken together, we proposed that NF- κ B1 ATTG₁/ATTG₁ genotype might serve as a tumor growth indicator, together with Breslow thickness and Clark level to predict the outcome of melanoma patients.

In conclusion, our case-control study supported the hypothesis that polymorphisms in NF- κ B1 and NF- κ BI α genes act as susceptible genetic markers to predict melanoma risk, especially, in the combination of ATTG2/ATTGT2 and GG. In melanoma patients, the genotypes of these polymorphisms were correlated with patient's gender, tumor onset and location. NF- κ B1 ATTG₁/ATTG₁ genotype might be a biological marker for melanoma progression.

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Figure legends

Fig. 1. Age distribution of melanoma patients at diagnosis (n=185), mean age 54 years (range 19-80).

Fig. 2. Genotypes of NF- κ B1 in melanoma patients and tumor-free individuals by PCR-RFLP. Lane 1: 100bp DNA ladder. Lanes 2 and 4: NF- κ B1 WT ATTG₁/ATTG₁ genotype. Lane 3, 5 and 6: NF- κ B1 heterozygote ATTG₁/ATTG₂ genotype. Lane 7: NF- κ B1 polymorphic homozygote ATTG₂/ATTG₂ genotype, and lane 8 was negative control without DNA template added.

Fig. 3. Genotypes of NF- κ BI α in melanoma patients and tumor-free individuals by PCR-RFLP. Lane 1: 100bp DNA ladder. Lane 2: NF- κ BI α WT AA genotype. Lane 3 and 5: NF- κ BI α heterozygote AG genotype. Lane 4 and 6: NF- κ BI α polymorphic homozygote GG genotype, and lane 7: negative control without DNA template added.