Original Research

A polymorphic GGC repeat in the *NPAS2* gene and its association with melanoma

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Impact statement

This report describes a variable microsatellite repeat sequence located in the 5′ untranslated exon of *NSPAS2*, a gene encoding a clock transcription factor. Significantly, this study is the first to show that a variant copy number GGC repeat sequence in the *NPAS2* clock gene associates with melanoma risk and which may be useful in the assessment of melanoma predisposition.

Abstract

Circadian clock regulation in mammals is controlled by feedback loops of a set of circadian genes. One of these circadian genes, *NPAS2*, encodes for a member of the bHLH-PAS class of transcription factors and is expressed in the forebrain and in some peripheral organs such as liver and skin. Other biological processes are also regulated by circadian genes. For example, *NPAS2* is involved in cell proliferation, DNA damage repair and malignant transformation. Aberrant expression of clock genes has been previously observed in melanoma which led to our effort to sequence the *NPAS2* promoter region in this cancer type. The *NPAS2* putative promoter and 5′ untranslated region of ninety-three melanoma patients and ninety-six control subjects were sequenced and several variants were identified. Among these is a novel microsatellite comprising a GGC repeat with different alleles ranging from 7 to 13 repeats located in the 5′ untranslated exon. Homozygosity of an allele with nine repeats (9/9) was more prevalent in melanoma than in control subjects (22.6% and 13.5%, respectively, *P*: 0.0206) suggesting that some *NPAS2* variants might contribute to melanoma susceptibility.

Keywords: *NPAS2*, clock genes, microsatellite repeats, melanoma

***Experimental Biology and Medicine* 2017; 242: 1553–1558. DOI: 10.1177/1535370217724093**

Introduction

The circadian 24-h rhythms are endogenous self-sustained and cell-autonomous oscillations which are a fundamental physiological feature responsible for physiological homeostasis. Circadian rhythmicity is observed in almost all organisms from cyanobacteria to humans. The circadian clock acts as a multifunctional timer that provides temporal structures to a vast variety of behavioral and physiological processes.[1] Indeed, disruption of these rhythms in mammals is associated with a several pathological conditions including depression, diabetes, metabolic syndrome, and cancer.[2] At the molecular level, the mammalian circadian clock is controlled by transcriptional/translational feedback loops involving several key elements.[3] These elements are ''clock genes'' and are controlled by a wide range of circadian rhythms in physiological processes and behavior. Positive regulators are basic helix-loop-helix-PAS (bHLH-PAS) transcriptional factors such as BMAL1 (brain and muscle ARNT-like protein 1) and CLOCK (circadian locomotor output cycles kaput) or NPAS2 (neuronal PAS domain protein). When the signal triggers this mechanism, BMAL1 and CLOCK/NPAS2 heterodimerise and initiate the transcription of *Period* (*Per1,2,3*) and *Cryptochrome* (*Cry1,2*) genes through binding to E-box sequences in the promoters of *Per* and *Cry* genes.[4–6] After reaching a critical concentration in the cytoplasm, PER and CRY proteins form heterotypic complexes, translocate back into the nucleus and specifically inhibit BMAL1-CLOCK/NPAS2-mediated transcription by shutting off their own transcription.[7] In the second loop, BMAL1-CLOCK/NPAS2 heterodimers bind to E-boxes in the promoters of the retinoic acid-related orphan nuclear receptors *Rev-erbα* and *Rorα* genes and activate their

expression. RORα and REV-ERBα compete for the ROR element in the *Bmal1* promoter. In turn, RORα promotes and REV-ERBα represses *Bmal1* expression.[8,9]

NPAS2, also known as *Mop4*, is the largest circadian clock gene. It is located on chromosome 2 (2q11.2) and is expressed primarily in the forebrain. However, it is also expressed in some peripheral organs such as liver and skin.[10,11] The *NPAS2* gene encodes a member of the bHLH-PAS class of transcription factors and is composed of 21 exons. The first exon, however, is a not-coding exon, and the length of the main transcript is about 4007 bp. As NPAS2 forms heterodimers with BMAL1, the circadian mRNA pattern of *NPAS2* and *Bmal1* is synchronized in various tissues.[12] *NPAS2* is also a RORα and REV-ERBα target gene, and possesses ROREs (*R*etinoid related *O*rphan *R*eceptor response *E*lements) within its promoter region allowing for coordinated expression of the positive arm of the circadian feedback loop.[13]

Several lines of evidence suggest that clock genes may contribute to neoplastic transformation.[14] Indeed, *NPAS2* is involved in the cell cycle regulation, specifically in the mechanisms relating to DNA repair and as such may have a putative role as tumor suppressor.[10] Specifically, the BMAL1/NPAS2 heterodimer negatively regulates the expression of the c-*Myc* oncogene.[15] Moreover, *NPAS2* influences the activity of other genes involved in tumorigenesis.[16] For instance, *CDKN2AIP* (*p16IP*) gene is a transcriptional target of NPAS2, is involved in cell cycle regulation[16] and can activate *p53/TP53* through *CDKN2A*-dependent pathways. This activity, for example, is relevant in melanoma since *CDKN2A* (*p16*) is the major known high-risk melanoma susceptibility gene and is mutant in almost all melanomas and mutant alleles are present in the 40% of hereditary melanomas.[16,17] In addition, epidemiological evidence suggests an association between polymorphisms in *NPAS2* and the risk for cancer.[16,18] Among all skin cancer types, malignant melanoma is recognized as one of the most aggressive type, with a high incidence frequency in Caucasian population, estimated as about 10 cases per 10^5 people/year. [19] This cancer can metastasize very rapidly and accounts for the majority of deaths from all skin cancers, about 2–3 per 10^5 people/year in North West Europe. The malignant melanoma develops from the melanocyte, the cell type that is responsible for the protection of keratinocytes by harmful ultraviolet (UV) radiations, which represents the most prominent physical carcinogen in natural environment of humans. Approximately 5–10% of melanoma cases are familial, suggesting that gene variants might be responsible for susceptibility of this cancer. Indeed, several genes responsible for inheritable melanoma predisposition have been identified; however, our knowledge on this field is still limited.[20] In particular, correlation between the circadian status and melanoma occurrence may be explored in further details. Skin cells indeed, possess an intrinsic and complex circadian clock organization.[21] Although altered expression of circadian clock genes has been observed in human skin and melanoma biopsies,[22] there has been no systematic exploration of variant circadian clock genes to date in melanoma, which is the most severe form of skin cancer with high mortality rate and a dramatic increase in incidence. One hypothesis linking circadian rhythm to melanoma is that DNA damage and repair are under direct influence of external stimuli, e.g. light, and is particularly sensitive to UV radiation.[14] We hypothesized that further understanding of clock gene involvement and perturbation of circadian regulation in melanoma might contribute new data regarding melanoma pathogenesis. We now report new genomic sequence data of the putative promoter and 5′ untranslated region of the *NPAS2* gene in the Croatian population providing evidence for a genetic variant that might influence risk of predisposition for melanoma.

Patients and methods

Patient recruitment

A total of 100 melanoma patients (70 males and 30 females) evaluated through the Department for Plastic and Reconstructive Surgery, Clinic for Surgery, University Hospital Centre, Rijeka, Croatia, were included in the study. Pathohistological determination of stages I to IV was the main inclusion criteria for the study and was performed according to the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology for Melanoma (Version 3.2015) (Supplementary material). The control group consisted of 100, age and sex matched, healthy blood donors, with no family history of melanoma. The study protocol was approved by the Ethics Committee of the Clinical Hospital Centre Rijeka and the written consent was obtained from all included patients, as well as control subjects.

Genetic analysis

All studied subjects underwent genetic testing for *NPAS2* promoter mutations. The region of interest is 1459 bp long and goes from position −85.912 to position −84, from the translational start site and contains the untranslated exon 1 of the gene *NPAS2*. Genomic DNA was extracted from 200 μl peripheral blood using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), on a QIAcube robotic workstation (Qiagen), according to the manufacturer's manual. DNA quality and concentration were measured using a BioDrop spectrophotometer. The *NPAS2* promoter region was divided into four fragments and amplified by polymerase chain reaction (PCR). The list of primers (direction $5' \rightarrow 3'$) used for amplification of *NPAS2* is presented in Table 1. Annealing temperatures were as follows: fragment 1, 67.2°C; fragment 2, 67.2°C; fragment 3, 65.0°C; fragment RORE 68.5°C. For all fragments, other PCR parameters were identical: denaturation, 95°C for 45 s; annealing time, 60 s; elongation, 72°C, 60 s; final extension, 72°C for 7 min. For all fragments, 30 cycles of amplification were performed. The reagents used to set up the PCR were the GoTaq colorless Master Mix (PROMEGA, Madison, WI, USA) and the AccuPrime GC-Rich DNA polymerase (INVITROGEN, Carlsbad, CA, USA). All PCR products were sequenced from both ends. Sequence analysis was performed on a 3500 DX Sequencer (Life Technologies) analyzer using the BigDye Terminator kit v3.1 (Applied Biosystems, Foster City, CA, USA); the

Table 1 Primers used for DNA sequencing

Fragment	Forward primer	Reverse primer
Rore	5′-GACCTTTCCCCTCTCCCG-3′	5′-CCTAGTGTAGACCCTCGCAG-3′
1	5′- AAAAGGAGAGGAGGGCAGC-3′	5′-TGACAGTCGCGTGCTCGT-3′
2	5′- GAGGACAGTGTGGAGGGGG-3′	5′-CTGCGTGCGGAAGAGTTTG-3′
3	5′- GTGGAGAGGGAGGAGGGT-3′	5′-AGGGTGGGTAGTACGTGCG-3′

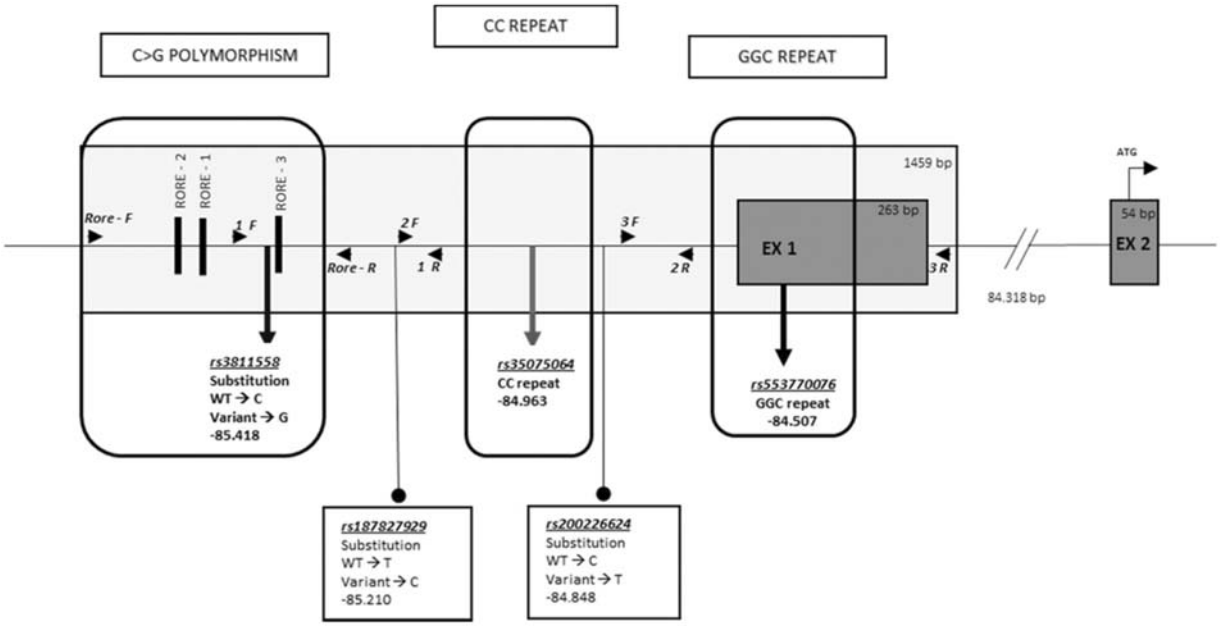

Figure 1 The sequenced region of the *NPAS2* gene depicted schematically. Arrowheads indicate positions of primers used for amplification. Numbers indicate base pairs upstream from the ATG and results are presented in boxes: a C > G variation at position −85.418; a CC repeat at position −84.963; a GGC repeat at −84.507 inside the untranslated exon 1 and microsatellite comprising a GGC repeat. RORE: retinoid related orphan receptor response elements; EX: exon. Black dots indicate variations found in only one subject

software Sequencing Analysis v5.4 (Life Technologies) was used for mutation detection. A portion of the region of interest, specifically the one included between positions −84.692 and −84.288, which contains the untranslated exon 1, was also evaluated by sizing analysis. The primers used were those used to amplify fragment 3; the forward primer is marked with 6FAM. Sizing analysis was performed on a 3500 DX Sequencer (Life Technologies) using the 600 LIZ Size Standard v2.0. The GeneMapper software v4.1 (Life Technologies) was used to determine the length of fragments. Differences between genotype and allelic frequencies were evaluated by the online QuickCalcs software (http://www.graphpad.com/quickcalcs/) using a Chi-square, two-tailed comparison between expected and observed frequencies.

Results

Ninety-three patients and ninety-six control subjects were included in data presentation of variations in the putative promoter and 5′ untranslated region of the *NPAS2* gene. Discrepancy from the total number of recruited subjects is due to low DNA quality and low genotyping efficiency for seven patients and four controls. The analyzed region, along with identified sequence changes, is depicted schematically (Figure 1), where numbers indicate base pairs upstream from the ATG of *NPAS2* gene. This region might be in our opinion relevant for the activity of the gene, and herein presented data provide evidence for variant gene organization in patients diagnosed with melanoma. Specifically, frequent variations include three polymorphic sites: a C > G variant at position −85.418 (rs3811558); a CC repeat at position −84.963 (rs35075064); and a GGC repeat at −84.507 (rs553770076) within the untranslated exon 1. The CC repeat polymorphism consists of two alleles having either three or four repeats. Of particular interest, we identified a novel microsatellite comprising a GGC repeat with variant alleles ranging from 7 to 13 repeats. Two other variants were detected in only one subject: a T > C substitution at position −85.210 (rs187827929) and a C > T substitution at position −84.848 (rs200226624).

Frequencies of identified polymorphisms were assessed in melanoma versus control subjects (Table 2). The only significant difference was observed in the repeat number of the

Table 2 Genotype and allele frequencies of the identified polymorphisms in melanoma and control subjects

Genotype frequency				Allele frequency[a]			
Genotype	**Controls (%)**	**Patients (%)**	***P* value**	**Allele**	**Controls**	**Patients**	***P* value**
C > G −85418							
C/C	60 (62.5)	57 (61.3)	>0.05	**C**	0.79	0.78	>0.05
C/G	29 (30.2)	33 (35.5)	>0.05	**G**	0.21	0.22	>0.05
G/G	7 (7.3)	3 (3.2)	>0.05				
CC repeat −84963							
4/4	49 (51.0)	47 (50.5)	>0.05	**4**	0.73	0.71	>0.05
4/3	42 (43.7)	39 (41.9)	>0.05	**3**	0.27	0.28	>0.05
3/3	5 (5.3)	7 (7.6)	>0.05				
GGC repeat −84507							
7/7	25 (26.0)	29 (31.1)	>0.05	**7**	0.53	0.50	>0.05
7/9	49 (51.1)	36 (38.7)	>0.05	**9**	0.42	0.46	>0.05
7/12	1 (1.0)	0 (0)	>0.05	**12**	0.04	0.03	>0.05
9/9	13 (13.5)	21 (22.6)	**0.0206***	**13**	0.01	0.01	>0.05
9/12	6 (6.3)	5 (5.4)	>0.05				
9/13	2 (2.1)	2 (2.2)	>0.05				

Note: The only significant difference was observed in the GGC microsatellite for the genotype 9/9 more prevalent in melanoma than in control subjects (22.6% and 13.5%, respectively, *P*: 0.0206).
[a]For each polymorphism allele frequency is expressed as a fraction of total.
*$P \leq 0.05$.

GGC microsatellite. In this case, the 9/9 genotype (homozygous for the nine repeat variant) was more prevalent in melanoma than in control subjects (22.6% and 13.5%, respectively, *P*: 0.0206). The 7/9 genotype (heterozygous for the seven and nine repeat variants) was underrepresented in the melanoma subjects, but the difference was not statistically significant.

Discussion

There is a growing body of evidence showing that circadian rhythms are essential for physiological homeostasis in mammals, and that disruption of their function has been associated with various pathological conditions. It is also generally accepted that key circadian clock genes can exert a tumor suppressor role by regulating proliferation of malignant cells. Thus, it is not surprising that deregulation of the circadian clock may lead to deregulation of the cell cycle control, which is one of the major hallmarks of cancer cells.[2,23] There is little known to date about molecular clock functions in melanoma, which is the most malignant form of skin cancers. A deeper and more complete understanding of circadian rhythm deregulation in melanoma patients may provide novel insights into disease predisposition and pathogenesis. The rationale of the study undertaken in this report was to expand our understanding of one of the main circadian regulators in humans, the *NPAS2* gene, as it relates to risk of melanoma. The *NPAS2* gene, which is expressed both in the brain and periphery,[13] is known to be involved in DNA damage response mechanisms and is recognized a potential risk biomarker in human malignancies.[10,16,18] Since published data strongly support the involvement of the *NPAS2* gene in tumorigenesis, we undertook a study to investigate the potential association of melanoma with the genetic status of the *NPAS2* gene promoter. Indeed, a variant *NPAS2* promoter sequence, e.g. polymorphism or mutation, may result in alterations in its transcription. For example, the *NPAS2* promoter contains two functional ROREs that bind the nuclear receptors ROR and REV-ERBs and that are separated from each other by 160 bp.[13] The REV-ERBs are circadian regulators but are also major regulators of metabolic processes, including glucose metabolism.[24,25] Moreover, RORE1 is essential for nuclear receptor-mediated cell-autonomous circadian transcription of *NPAS2*.[26] In this study, however, we did not detect any alterations in ROREs in the region upstream of the *NPAS2* transcription start site. Nevertheless, we found a C > G variant (rs3811558) and a CC repeat (rs35075064) in the *NPAS2* gene promoter region (Table 2). Consistent with published data which show no correlation between these polymorphisms and predisposition to human malignancies, these polymorphisms showed no associations with melanoma risk. We did, however, identify a polymorphic microsatellite GGC repeat in the untranslated (first) exon of *NPAS2* with a 9/9 genotype that was more frequent in melanoma subjects than in controls (Table 2). To our knowledge, this is the first report of an *NPAS2* genotype to show association with melanoma risk. In particular, microsatellite repeats are found to be abundant in human promoters and are associated with regulatory elements. This might have implications on disease pathogenesis since effects on *NPAS2*-regulated cell processes are central to cell fate and cell homeostasis. Promoter microsatellites, moreover, tend to be G/C rich, which is consistent with data for a number of promoter microsatellites discovered within or near 5′ UTRs, CpG islands, and G4 structures.[27] Importantly, it has been

suggested that many of the promoter-associated microsatellites have the potential to affect human phenotypes by generating mutations within regulatory elements, which can ultimately lead to disease as promoter function is affected. The G/C rich motifs containing CpG dinucleotides are potential sites for epigenetic modification and changes in repeat number of CpG-containing microsatellites might alter the number of potential methylation sites.[27] In fact, it was shown that several circadian clock genes can be transcriptionally silenced by promoter hypermethylation in different malignancies and that a clear correlation exists between methylation frequency and the clinical phases of diseases.[28–30] The *NPAS2* gene promoter specifically is hypomethylated in Parkinson's disease patients, leading to the suggestion that altered promoter methylation may contribute to abnormal expression of clock genes in this disease.[31] We suggest a possible correlation between promoter regulation and *NPAS2* deregulation in melanoma, although this postulate requires further investigation. In particular, nine GGC genotype was more prevalent in melanoma patients in comparison with controls then the other combinations which might correlate with decreased gene expression in 9/9 genotype patients. For example, it was suggested that CCG/CGG motif can form secondary structures and changes in the length of these microsatellites may modulate gene expression and even induce disease when extended. [27] Moreover, the number of potential methylation sites might be altered by variations in the repeat number for CpG-containing microsatellites.[27] It may be therefore speculated that patients with nine GGC genotype may have lower expression of *NPAS2* gene due to higher promoter methylation, though this assumption need further investigations to be proved. Consistent with this idea is the observation that knockdown of *NPAS2* suppresses expression of several cell cycle-related genes and genes involved with DNA repair, impairs checkpoint activation and DNA repair.[10] Furthermore, silencing of *NPAS2* promotes cell growth and invasion in DLD-1 cells and correlates with poor prognosis of colorectal cancer. In aggregate, these data illustrate the potential of *NPAS2* gene as a promising target or feasible prognostic indicator for malignant disease.[32]

In conclusion, we show that homozygous genotype of the nine GGC repeat within the *NPAS2* promoter region is more prevalent in melanoma patients than in control subjects and suggest that the circadian gene *NPAS2* may increase an individual's susceptibility to melanoma. Future directions of this research might include a larger cohort of melanoma patients or even analysis of particular clinical subgroups, *i.e.* metastatic patients. Furthermore, *NPAS2* allele type correlation with expression of transcriptional targets, mRNA or protein expression might be performed as well. At last, from a precision medicine perspective, this genotype might be used as one of several biomarkers indicative of increased melanoma predisposition.

Authors' contributions: All authors participated in the design, data interpretation and final approval of the paper; AF, CP, CM, MDL and GP conducted sequencing experiments, analyzed obtained data and prepared figures; EMC, SDP and SKP designed the study, performed sample preparation, wrote the manuscript and discussed all data, SKP and GD performed critical reading and revised the manuscript; DJ collected samples and patients anamnesis, SKP and GD secured funding for research.

ACKNOWLEDGMENTS

We greatly appreciate access to equipment in possession of University of Rijeka within the project "Research Infrastructure for Campus-based Laboratories at University of Rijeka," co-financed by European Regional Development Fund (ERDF). This research was funded through University of Rijeka research grant 13.11.1.1.11.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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(Received May 17, 2017, Accepted July 11, 2017)