All Sciences Proceedings

1st International Conference on Pioneer and Innovative Studies

June 5-7, 2023 : Konya, Turkey

ICPIS

All Sciences Proceedings
http://as-proceeding.com/

https://as-proceeding.com/index.php/icpis

Investigation of Clock Gene Variations in *Nannospalax* Cytotypes

İlkay Civelek*, Teoman Kankılıç [2] and Dilara Fatma Akın [3]

[1]*Department of Biotechnology, Niğde Ömer Halisdemir University, Turkey*
[2]*Department of Biotechnology, Niğde Ömer Halisdemir University, Turkey*
[3] *Department of Basic Medical Sciences, Niğde Ömer Halisdemir University, Turkey*

(ilkaycivelek@ohu.edu.tr)

Abstract – Blind mole rats (*Nannospalax*) are subterranean mammals noted for their longevity and cancer resistance. It is known that these animals' vestigial eyes, particularly the Harder gland around the eyes, may generate considerable amounts of melatonin. Furthermore, the melatonin production mechanism in the circadian rhythm cycle of blind mole rats is regarded to be different from that of other living beings. The melatonin and Clock genes are hypothesized to be linked to the formation, development, and spread of cancer, but the researchers still cannot explain their extraordinary cancer resistance.

In this study, we hypothesized that the melatonin production mechanism in the circadian rhythm cycle of blind mole rats, which have been shown in the literature to be cancer-resistant, may differ from that of other living species due to the difference in their amino acid variations. Differences in the DNA of the Clock genes (Cry1 and Per1) involved in melatonin biosynthesis in blind mole rats were studied compared to other model species (Spalax galili, Mus musculus, Heterocephalus glaber, Rattus norvegicus, and Homo sapiens). As a result, while no variations were found in the *Cry*1 gene; only p.G7R variation was found in the *Per1* gene. The SNAP2 software had demonstrated that the human analogs of this variation harmful effects. It was suggested that more or full exon sites, more samples and genes should be studied to observe more variants in *Nannospalax* species. Thereby, the cancer resistance of blind mole rats may be explained better by these variations and the functions of protein domains where these variants are located in.

Keywords – Nannospalax, Clock genes, Cry1, Per1, Cancer.

I. INTRODUCTION

Nannospalax is a member of Spalacidae family, which is one of the most extreme cases of rodents adopting subterranean ecological niche [1], [2]. The blind mole rats, which have both behavioral and physiological adaptations to underground life, dig underground tunnels and rarely rise above ground. Their eyes are protected by a thick layer of skin and dark fur [3]. The physical features of their vestigial eye are mostly aberrant, however, there is still a distinct retinal layer remaining. Although photic information is unlikely to reach retina, it appears that the eye of the blind mole rats can perceive photoperiod. The blind mole rat's eye does not have an evoked visual potential; yet the retina may still be light sensitive and functionally implicated in the photoperiodic response [4]. It has been demonstrated that these animals' vestigial eyes, particularly Harder gland around the eye, can synthesize substantial amounts of melatonin and 5-methoxy tryptophol [5]. Melatonin synthesis is controlled by the circadian cycle of living organisms. Light is one of the primary physical elements that has a direct impact on the melatonin synthesis [6]. The biological clock and consequently the 24-hour circadian rhythm are highly connected in the production of the melatonin. Melatonin production peaks at night and then reduces precipitously when exposed to light throughout the day [7]. The regulation of the circadian rhythm of melatonin has been

emphasized by the persistence of the cycle in animals kept in the dark [8].

Some details, such as melatonin secretion and circadian rhythm in the blind mole rat species, are still unknown. The key information that researchers are interested in this regard are how this underground species, which has no vision, might adapt to variations in the daily rhythm of light. According to some researchers, this is because blind mole rats, which lack the ability to distinguish visual images, have a complex photo-sensing system and subcutaneous atrophic eyes that respond rhythmically to light [9], [10].

Previous studies showed that disruption of melatonin production or Clock gene expression could promote inflammation or cancer development which could be caused by a variety of disorders (including Alzheimer's disease and liver disease) [11]. Multiple studies have indicated that melatonin has oncostatic effects on many kinds of cancers [12]. The melatonin has been reported to lower the incidence of experimentally induced malignancies [13], [14], [15] and considerably inhibit the growth of some human breast tumors [16].

Any disruption in the circadian rhythm, which controls biological processes in all organisms, causes an abnormal function in the expression of genes responsible for this process (Clock), including rats, and has related to a variety of cancers, including some hormone-dependent and hormone-independent cancers. Tumor development refers to a number of complex processes. Understanding the diurnal patterns of the nucleus clock genes and their clock-controlled genes, on the other hand, is critical for determining the molecular process behind the cancer phenotype. Because the melatonin production and secretion occur in a rhythmic manner during the light-dark cycle, understanding this information can aid in the implementation of a more realistic strategy for both diagnosis and treatment, along with the progression of the disease [17].

On the other hand, spontaneous tumors were not discovered among thousands of individuals (Israeli mole rats) in cancer research conducted on mole rats over a long period of time (50 years). Animals are extremely resistant to chemically induced cancers [18].

In general, studies have shown a lot of evidence associating the circadian rhythm mechanism and circadian rhythm-related clock genes with cancer development and progression, but more research is required in this area. The results show that there is a relationship between melatonin and clock genes and the development of cancer. The entire mechanism is connected to the 24-hour circadian rhythm cycle.

In this study, variants of Clock genes (*Cry 1* and *Per1*), which are involved in the biosynthesis of melatonin and play a role in the regulation of many biological functions such as sleep, circadian rhythm, adrenal gland and thyroid gland functions, reproduction, aging, the antioxidant system, and immunity, were investigated on blind mole rats. Additionally, this study presented DNA-based information for the *Nannospalax* species (*Nannospalax xanthodon, Nannospalax ehrenbergi* and *Nannospalax leucodon*) living in Turkey. We hypothesize that the melatonin biosynthesis mechanism in the circadian rhythm cycle of blind mole rats, which are known to be cancer-resistant according to the literature, may be different from that of other living creatures. As a result, the differences in the DNA of the Clock genes (*Cry1* and *Per1*) involved in melatonin biosynthesis in blind mole rats were researched in comparison to other living model organisms (*Spalax galili*, *Mus musculus, Heterocephalus glaber, Rattus norvegicus*, and *Homo sapiens*).

II. Materials and Method

A. Sampling

Ten different cytotypes (two samples from each cytotype) belonging to 3 different blind mole rat species (2n = 36, 48, 52, 54, 56 for *N. xanthodon*; 2n = 48, 52, 54, 56 for *N. ehrenbergi*; 2n = 56 for *N. leucodon*) distributed in Turkey were used in the study. For each gene, a total of 20 samples were studied. The liver, kidney, and muscle tissues of mole rats obtained from our previous studies were used for extracting DNA.

B. DNA Isolation and Polymerase Chain Reaction

CTAB (Cetyltrimethylammonium bromide) DNA isolation method was used previously described by [19]. Following conditions were used for PCR cycle: a 5-minute initial denaturation at 95°C; 40 cycles of denaturation (4 minutes at 94°C), annealing (1 minute at 58°C), and extension

(1 minute at 72°C); and a 5-minute final extension at 72°C.

The primer pairs used in the PCR amplifications were: Cry1 (Exon 7) forward primer 5'-ATAGTTCCCCTCCCCTTTCT-3'; *Cry1*, reverse primer 3'-CCAGCCCCCAGAAACTTTTA-5' and *Per1* (Exon 2) forward primer 5'-GTGACTGAATTTGTCAGCCC-3'; *Per1* (Exon 2) reverse primer 3-'GAGGGTCAGGAGATCAACAG-5'. The PCR amplification was carried out in 25 µl reactions with 5 µl template DNA, 5U Taq DNA polymerase (Thermo Scientific™), 1X Taq Buffer, 10 mM dNTP, 25 mM $MgCl_2$ (Thermo Scientific™), and 10 pmol/l of each primer. The PCR products were separated on a 1% agarose gel in Tris-acetate-EDTA buffer (TAE) and stained with ethidium bromide.

C. Bioinformatic Analysis

The sequence results were cleaned in the Bioedit software. The similarities between the sequences evaluated in the BLAST database and the sequence in the database were investigated, and the differentiated bases were identified. First, the variations in the amino acid sequences of *Nannospalax galili* and *Homo sapiens, Mus musculus, Rattus norvegicus,* and *Heterocephalus glaber* were determined using the ENSEMBL database [20].

After the variations in *Cry1* and *Per1* genes of humans were researched, the pathogenic features of these variants were investigated using a software program such as SNAP2. SNAP2 software was used to quantify the effect of single amino acid sequence variations on protein function in the human *Cry1* and *Per1* genes. The SNAP2 software was used to estimate the functional effects of single amino acid sequence variations [21].

III. RESULTS

The Bioedit sequence alignment editor was used to analyze and align the DNA sequence data. The NCBI-Blast database was used to screen all obtained DNA sequence results. Fig. 1 represents that Sequence alignment of *Cry1* gene in the Bioedit software and Fig. 2 shows the alignment of the *Per1* gene sequence with the reference to the *Per1* gene in the GenBank Blast database.

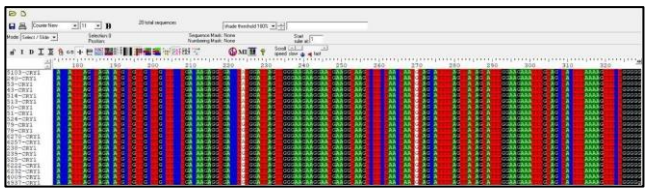

Fig. 1 Sequence alignment of *Cry1* gene in the Bioedit software

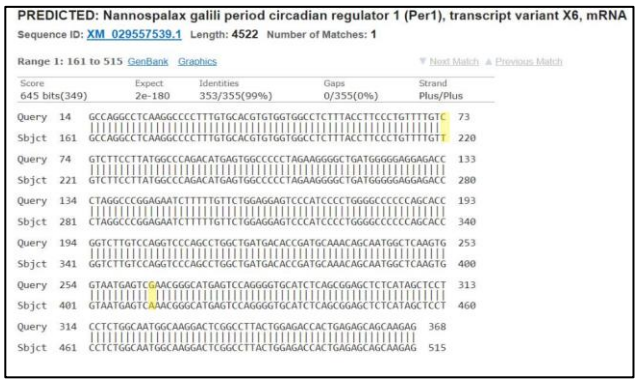

Fig. 2 The alignment of the *Per1* gene sequence with the reference *Per1* gene in the NCBI-GenBank Blast database

A. Evolutionary Analysis Results in Individual Genes Between Species

In Fig. 3 the domain regions and in Fig. 4 mutation domain regions [22] of each gene were given.

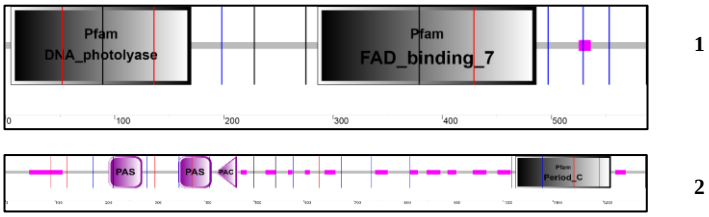

Fig. 3 1. *Cry1* domains, 2. *Per1* domains

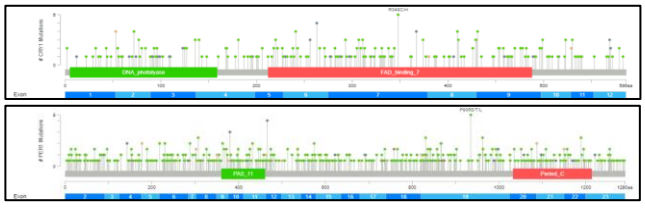

Fig. 4 Mutation domain regions of each gene

DNA sequence results of *Cry1* gene of *Nannospalax* species were examined and the base changes seen in the DNA sequence of the samples are single nucleotide changes. These changes did not cause an amino acid change in the codon; The

amino acids p.370L* (CTA>CTG) and p.312N* (AAC>AAT) were conserved in all species (Fig. 5).

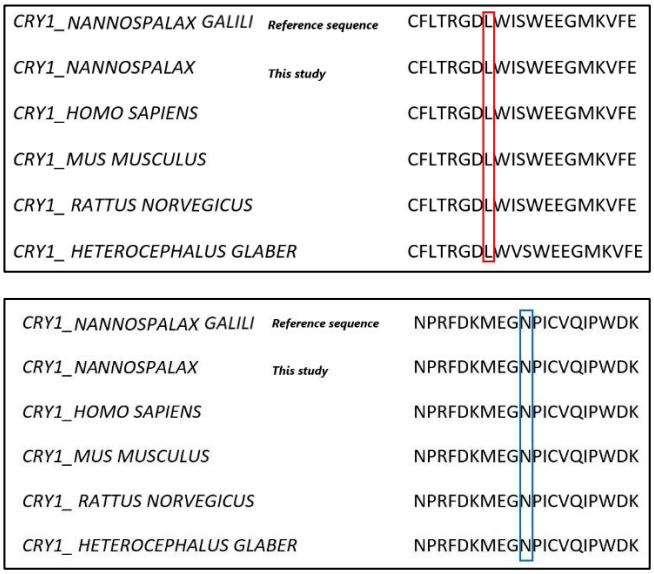

Fig. 5 Peptide sequence alignment of the Cry1 protein in different species; *Nannospalax* variants A) p.370L*, B) p.312N*

Amino acid changes in the codons and their locations were shown in Table 1. For *Per1* gene, codon 6 (GA**A**>GA**G**), codon 25 (GT**C**>GT**T**), codon 27 (TC**C**>TC**T**), codon 57 (TC**A**>TC**G**), codon 58 (AA**C**>AA**T**), codon 63 (AG**G**>AG**A**), codon 79 (AA**G**>AA**A**), codon 81 (TC**G**>TC**A**), codon 85 (GA**G**>GA**A**). Although there were single nucleotide changes, amino acids were preserved in all species. These were shown as p.6E*, p.25V*, p.27S*, p.57S*, p.58N*, p.79K*, p.81S*, p.85E*, p.88E* respectively (Fig. 6, Fig 7 and Fig. 8). However, in codon 7 (**G**GG>**A**GG) p.G7R variant was observed (Fig. 6-8).

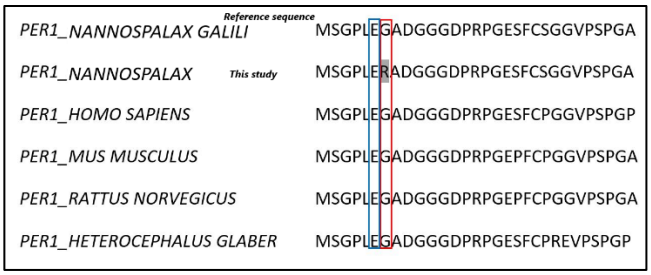

Fig. 6 Peptide sequence alignment of the Per1 protein in different species; *Nannospalax* variants; p.6E*, p.G7R

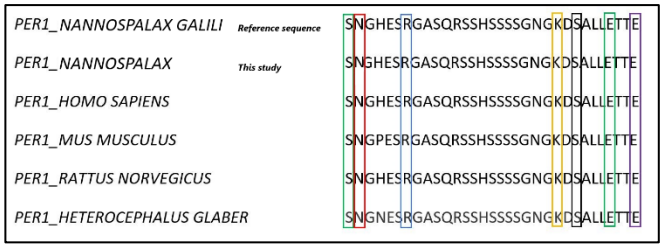

Fig. 7 Peptide sequence alignment of the Per1 protein in different species; *Nannospalax* variants; p.57S*, p.58N*, p.63R*, p.79K*, p.81S*, p.85E*, p.88E*

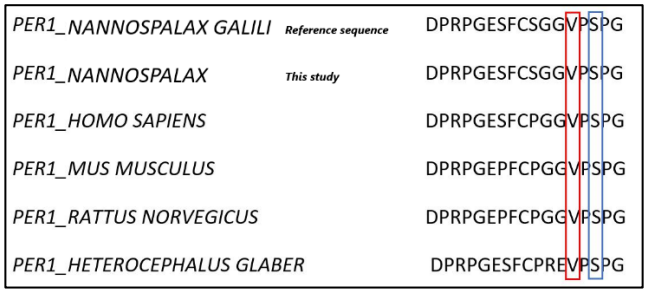

Fig. 8 Peptide sequence alignment of the Per1 protein in different species; *Nannospalax* variants; p.25V*, p.27S*

In Table 1, amino acid changes in codons and their locatins are given both for Reference genome and *Nannospalax* species. Aa represents amino acid names and abbreviations of aminoacid names are as follows; (L: Leucine, R: Arginine, A: Alanine, P: Proline, E: Glutamine, V: Valine, S: Serine, N: Asparagine, K: Lysine). In some codons, even base pair changes, amino acid does not change such as gene *Cry1* CT**A**>CT**G** change, but in some other examples the change is resulted with an aminoacid change in the codon such as *Per1* gene, **G**GG>**A**GG).

Table 1. Amino acid changes in the codons and their locations

Gene	Reference Genome		This Study		Aa Location
	Codon	Aa	Codon	Aa	
Cry1	CTA	L	CTG	L	p.370L*
Cry1	CGC	R	CGT	R	p.181R*
Cry1	GCC	A	GCT	A	p.184A*
Cry1	CCC	P	CCT	P	p.192P*
Per1	GAA	E	GAG	E	p.6E*
Per1	GGG	G	AGG	R	p.G7R
Per1	GTC	V	GTT	V	p.25V*
Per1	TCC	S	TCT	S	p.27S*
Per1	TCA	S	TCG	S	p.57S*
Per1	AAC	N	AAT	N	p.58N*
Per1	AGG	R	AGA	R	p.63R*
Per1	AAG	K	AAA	K	p.79K*
Per1	TCG	S	TCA	S	p.81S*
Per1	GAG	E	GAA	E	p.85E*

B. In-Silico Analysis of Oncogenic Pathogenic Effects in Detected Variants

In Cry1 protein, since no alterations were detected in any species, no pathogenic effects were analyzed for humans in the SNAP2 software. On the other hand, the SNAP2 software was utilized to evaluate if human variations of Per1 proteins are pathogenic. According to the SNAP2 *in-silico* results, p.G7R, variant in human Per1 protein were found in 65 score, and the estimated pathogenic effect values were positive (Fig. 9).

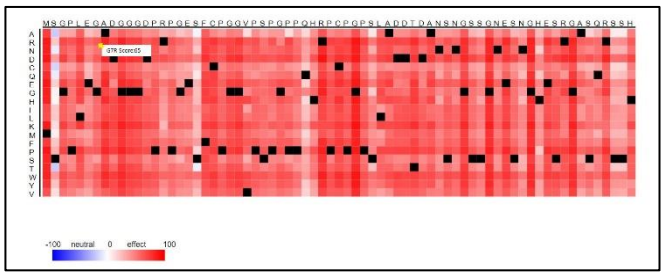

Fig. 9 In-silico analysis of the oncogenic pathogenic effects of p.G7R variants on humans of the Per1 protein detected by the SNAP2 Program

IV. DISCUSSION

The evolution of the eye is physiologically influenced by light signals in the eye, which are the primary cause of the circadian rhythm. In this scenario, the question of potential function loss comes to mind. The functionality of blind mole rats' eyes, which have been shielded from light for millions of years, is demonstrated by their protection of the eye function [23]. The *Spalax* circadian clock functions similarly to other rodents via a group of clock genes [24]. Here, the genes in blind mole rats were compared with human and other model organisms to determine whether any variation in the Clock gene confers cancer resistance in this evolutionary history of blind mole rats.

The lack of variation in the *Cry1* gene of the three *Nannospalax* species may be a coincidence, or it may be caused by the absence of variation in the sequenced exon in the study.

On the other hand, in the *Per1* gene, only one variation (p.G7R) was observed compared to the reference genome *Nannospalax galili*. On the other hand, one variation (p.G7R) was determined compared to the *Homo sapiens*. These changes were not situated on any domain. But it showed a harmful amino acid change in humans, which suggested that the variation was either effective or pathogenic.

V. CONCLUSION

In this DNA sequence-based study of two Clock genes *Cry1* and *Per1* involved in melatonin biosynthesis, although the relationship between eye evolution and cancer resistance in blind mole rats could not be clarified, one variation was found in *Per1* gene in *Nannospalax* species compared to the *Nannospalax galili* and *Homo sapiens*.

In conclusion, the differences seen in these organisms offer no explanation for why blind mole rats are more cancer-resistant than the other species. For more information it is recommended to increase the number of samples and expand the study with more genes and whole exon regions in future studies.

ACKNOWLEDGMENT

This study was supported by TUBITAK 1002 Hızlı Destek-B Project (No: 123Z007) of "Investigation of the Gene Variants of Clock Genes Involved in Melatonin Biosynthesis in *Nannospalax* (Mammalia: Rodentia) Cytotypes".

REFERENCES

[1] I. R. Savic and E. Nevo, “The Spalacidae: evolutionary history, speciation and population biology”, *Prog. Clin. Biol. Res.*, vol. 335, pp. 129-153, 1990.
[2] V.A. Topachevskii, “Fauna of the USSR: mammals mole-rats. Spalacidae,” Akad. Nauk USSR New Ser, vol. 99, pp. 308, 1969.
[3] R. Rado, H. Gev, B. D. Goldman, and J. Terkel, “Light and circadian activity in the blind mole rat,” J. Photochem. Photobiol. A, pp. 581-589, 1991.
[4] S. Sanyal, H. G. Jansen, W. J. De Grip, E. Nevo, and W. W. De Jong, “The eye of the blind mole rat, Spalax ehrenbergi. Rudiment with hidden function?,” IOVS vol. 31, 1398-1404, 1990.
[5] P. Pevet, G. Heth, A. Hiam, and E. Nevo, “Photoperiod perception in the blind mole rat (Spalax ehrenbergi, Nehring): involvement of the Harderian gland, atrophied eyes, and melatonin,” J. Exp. Zool., vol. 232, pp. 41-50, 1984.
[6] J. Arendt, “Melatonin: characteristics, concerns, and prospects,” J. Biol. Rhythms, vol. 20, pp. 291-303, 2005.
[7] S. Liu, C. O. Madu, and Y. Lu, “The role of melatonin in cancer development,” *Oncomedicine*, vol. 3, pp. 37-47, 2018.
[8] B. D. Goldman, “The circadian timing system and reproduction in mammals,” *Steroids*, vol. 64, pp. 679-685, 1999.
[9] Z. K. David-Gray, H. M. Cooper, J. W. Janssen, E. Nevo, and R. G. Foster (1999). Spectral tuning of a circadian photopigment in a subterranean ‘blind’mammal (*Spalax ehrenbergi*),” *FEBS Lett.*, vol. 461, pp. 343-347, 1999.
[10] J. Hannibal, P. Hindersson, E. Nevo and J. Fahrenkrug, “The circadian photopigment melanopsin is expressed in the blind subterranean mole rat, *Spalax*,” *Neuroreport*, vol. 13, pp. 1411-1414, 2002.
[11] K. Sato, F. Meng, H. Francis, N. Wu, L. Chen, L. Kennedy, Z. Tianhao, A. Franchitto, P. Onori, E. Gaudio, S. Glaser, and G. Alpini “Melatonin and circadian rhythms in liver diseases: Functional roles and potential therapies,” *J. Pineal Res.*, vol. 68, pp. e12639, 2020.
[12] S. M. Hill, D. E. Blask, S. Xiang, L. Yuan, L. Mao, R. T. Dauchy, F. Tripp, and T. Duplesis, “Melatonin and associated signaling pathways that control normal breast epithelium and breast cancer,” *J Mammary Gland Biol Neoplasia*, vol. 16, pp. 235-245, 2011.
[13] L. Tamarkin, D. Danforth, A. Lichter, E. DeMoss, M. Cohen, B. Chabner, and M. Lippman, “Decreased nocturnal plasma melatonin peak in patients with estrogen receptor positive breast cancer,” *Science*, vol. 216, 1003-1005, 1982.
[14] D. E. Blask, D. B. Pelletier, S. M. Hill, A. Lemus-Wilson, D. S. Grosso, S. T. Wilson, and M. E. Wise “Pineal melatonin inhibition of tumor promotion in the N-nitroso-N-methylurea model of mammary carcinogenesis: potential involvement of antiestrogenic mechanisms *in vivo*,” *J. Cancer Res. Clin. Oncol.*, vol. 117, pp. 526-532, 1991.
[15] S. R. Teplitzky, T. L. Kiefer, Q. Cheng, P. D. Dwivedi, K. Moroz, L. Myers, M. B. Anderson, A. Collins, J. Dai, J. Yuan, L. L. Spriggs, D. E. Blask, and S. M. Hill, “Chemoprevention of NMU-induced rat mammary carcinoma with the combination of melatonin and 9-cis-retinoic acid,” *Cancer Lett.*, vol. 168, pp. 155-163, 2001.
[16] S. M. Hill, L. L. Spriggs, M. A. Simon, H. Muraoka, and D. E. Blask, “The growth inhibitory action of melatonin on human breast cancer cells is linked to the estrogen response system,” *Cancer Lett.*, vol. 6464, pp. 249-256, 1992.
[17] L. G. de Almeida Chuffa, F. R. F. Seiva, M. S. Cucielo, H. S. Silveira, R. J. Reiter, and L. A. Lupi, “Clock genes and the role of melatonin in cancer cells: an overview,” *Melatonin Research*, vol. 2, pp. 133-157, 2019.
[18] I. Manov, M. Hirsh, T. C. Lancu, A. Malik, N. Sotnichenko, M. Band, and A. Avivi, I. Shams, "Pronounced Cancer Resistance in a subterranean rodent, the blind mole-rat, *Spalax*: *in vivo* and *in vitro* evidence," *BMC Biol.*, vol. 11, pp. 1-18, 2013.
[19] J. J. Doyle, and J.L. Doyle, “Isolation of plant DNA from fresh tissue,” *Focus*, vol. 12, pp.13-15, 1990.
[20] (2023) The Ensembl website. [Online]. Available: https://www.ensembl.org
[21] M. Hecht, Y. Bromberg, and B. Rost, “Better prediction of functional effects for sequence variants,” *BMC Genom.*, vol. 16, pp. 1-12, 2015.
[22] (2023) The cBioPortal website. [Online]. Available: https://www.cbioportal.org/
[23] A. Avivi, U. Albrecht, H. Oster, A. Joel, A. Beiles, and E. Nevo, “Biological clock in total darkness: the Clock/MOP3 circadian system of the blind subterranean mole rat,” *PNAS*, vol. 98, pp. 13751-13756, 2001.
[24] U. Albrech, “Invited review: regulation of mammalian circadian clock genes,” *J. Appl. Physiol.*, vol. 92, pp. 1348-1355, 2002.