A Molecular Case-Control Study on the Association of Melatonin Hormone and rs#10830963 Single Nucleotide Polymorphism in its Receptor MTNR1B Gene with Breast Cancer

Nadia A Abd El Moneim*, Hisham El Masry**, Mina Mamdouh Sorial**, Taha I Hewala***♦, Amira Embaby**, Salah Sheweita**

*Department of Cancer Management and Research, Medical Research Institute, University of Alexandria, Alexandria, Egypt
**Department of Biotechnology, Institute of Graduate Studies and Research, University of Alexandria, Alexandria, Egypt
*** Radiation Science Department, Medical Research Institute, University of Alexandria, Alexandria, Egypt

Abstract

Background: The main function of the pineal hormone melatonin which is mediated via its two receptors, MTNR1A and MTNR1B, is to mediate dark signals in addition to anti-oxidation, immune system enhancement, protection from radiation, and anti-cancer functions. A common single nucleotide polymorphism in the MTNR1B gene is rs#10830963, which is well known as a risk factor for type 2 diabetes mellitus. This study intends to figure out the role of melatonin and its receptor MTNR1B gene rs#10830963 polymorphism in breast cancer incidence, diagnosis and prognosis.

Methods: This study included 43 females with breast cancer and 45 apparently normal healthy females. Restriction fragment length polymorphism-PCR was used for amplification and genotyping of the MTNR1B gene rs#10830963 polymorphism in whole blood. Serum melatonin levels were measured using a ready-for-use radioimmunoassay kit.

Results: For the MTNR1B gene rs#10830963 polymorphism, we observed a significantly higher GG genotype frequency among cases (72.1%) than controls (13.3%), with a diagnostic sensitivity of 83.78% and specificity of 76.47%. The cases had a frequency of 11.6% for the CC genotype and 16.3% for the CG genotype which was significantly lower compared to controls that had a 44.4% frequency of the CC genotype and 42.2% frequency of the CG genotype. The GG genotype had a significant association with larger tumor volume (*P*=0.048). Serum melatonin levels were significantly lower among breast cancer patients than controls. Using the ROC curve analysis, serum melatonin showed a significant AUC (72.6%, *P*<0.001) with diagnostic sensitivity of 91.1% and specificity of 58% at a cut-off level of 39.5 pg/ml. Females with serum melatonin levels ≤39.5pg/ml were at significantly increased risk for breast cancer incidence by about 15 times more than females with levels >39.5 pg/ml.

Conclusion: The risk for breast cancer incidence increased as the serum levels of melatonin decreased and in females homozygous for the G allele (GG genotype) of the MTNR1B gene rs#10830963 polymorphism. The GG genotype was found to be associated with increased breast tumor volume as a marker of a poor prognosis breast cancer.

Keywords: Breast cancer, MTNR1B gene rs#10830963SNP, Melatonin hormone, Incidence, Diagonsis, Prognosis

♦Corresponding Author:
Taha I. Hewala; PhD
Radiation Science Department, Medical Research Institute, University of Alexandria, Alexandria, Egypt
Email: tahahewala@hotmail.com

Introduction

Among women, breast cancer remains the most commonly diagnosed cancer. The strongest risk factors for breast cancer include age, positive family history, lifestyle, environmental and hormonal factors.[1] Additionally, melatonin hormone level and polymorphisms in its receptor genes MTNR1A and MTNR1B are recently investigated risk factors.[2,3]

The pineal hormone melatonin is involved in circadian regulation and facilitation of sleep. Secretion of melatonin, the hormone of darkness, is related to the duration of darkness. The main function of melatonin, which is mediated via its two receptors, MTNR1A and MTNR1B, is to control dark signals in addition to antioxidant, immune system enhancement, protection from radiation, and anti-cancer functions. Previous studies have suggested that melatonin may act on cancer growth through a variety of mechanisms, most notably by direct anti-proliferative effects on breast cancer cells and via interactions with the estrogen receptor (ER) pathway.[3,4]

Individuals, such as night shift workers, who are exposed to light at night on a regular basis experience circadian disruption that includes circadian phase shifts, nocturnal melatonin suppression, and sleep disturbances. These individuals are not only immune suppressed, but they are also at an increased risk of developing different types of cancer.[3]

Three genes are responsible for mediating the downstream effects of melatonin: melatonin receptors 1 and 2 (MTNR1A and MTNR1B) and the arylalkylamine N-acetyltransferase (AANAT) gene. It is hypothesized that genetic variations in these genes may lead to altered protein production or function.[4] The MTNR1B gene, spanning 13.16 kb, includes 2 exons, 1 intron, as well as 5 kb and 3.5 kb of its 5'- and 3'-flanking regions, respectively. rs#10830963 single nucleotide polymorphism (SNP) is located mid-way from exon 1 (+5.6 kb) and exon 2 (−5.9 kb) of the MTNR1B in a non-conserved sequence across species.[5]

The association of breast cancer development and serum melatonin hormone level has been extensively investigated over the years, usually confirming a definite inverse relationship.[6] On the other hand, a number of studies have reported an association between MTNR1B gene rs#10830963 SNP and the risk of hyperglycemic diseases.[5] This wave has been extended to cover many other diseases, but not cancer. Very recently, Deming et al.[4] considered to be pioneers in the search to find an association between breast cancer susceptibility and both MTNR1A rs#7665392 and MTNR1B rs#10765576 SNP have reported that this association may vary by menopausal status. To the best of our knowledge, to date no study has been published that associates the MTNR1B gene rs#10830963 SNP with breast cancer. This study aimed to determine the association of melatonin hormone levels and rs#10830963 SNP in its receptor MTNR1B gene with the risk for breast cancer incidence.

Subjects and Methods

Subjects

This study was conducted on 88 females admitted to the Department of Cancer Management & Research of the Medical Research Institute, Alexandria University, Egypt within the period from November 2011 to December 2012. Females were divided into two groups: Group I (breast cancer group) consisted of 43 females proven to have histologically and mammography confirmed breast cancer, recently detected with clinical stages II or III, with no surgical, chemotherapy or radiotherapy interventions. Patients had a mean age of 52.5±10.7 years. Group II (normal healthy control group) consisted of 45 normal healthy female volunteers with no breast pathology, matched for age (48.4±9.3 years), menstrual status and socioeconomic status with the patients of group I.

Methods

This research protocol was approved by the Ethical Committee of the Medical Research Institute, Alexandria University, Egypt. Each patient underwent full history recording, thorough clinical examination, routine laboratory investigations that included complete blood count (CBC), breast

mammography and ultrasonography of the abdomen and liver, radiological investigations that included chest X-ray, CT scan and bone scan when necessary, and fine needle aspiration cytology (FNAC) of the breast mass to establish the pathological diagnosis of breast cancer.

The clinicopathologic data were obtained from patients' pathology reports. The collected data included tumor size, pathological tumor grade, axillary lymph node involvement, Her-2 expression, vascular invasion and status of ER and progesterone receptor (PR). Each patient's clinical stage was determined by the oncologist according to the TNM classification system.[7] Tumor volume (cm^3) was calculated from tumor size (cm) using the following formula:[8]

$$V = \frac{\pi}{6}(length) \cdot (width) \cdot (height)$$

All 43 breast cancer patients underwent modified radical mastectomies,[9] then received adjuvant combination chemotherapy [5-fluorouracil, adriamycin and cyclophosphamide (FAC)][10] for six cycles. After six cycles of chemotherapy, breast cancer patients underwent clinical, laboratory analyses and radiological evaluations in order to evaluate the clinical response.

After obtaining an informed consent from each female participant, peripheral venous blood samples were taken from both patients and controls early in the morning and at the same time. Immediately, each sample was divided into two portions: one portion was collected in EDTA-coated tubes from which genomic DNA was extracted from whole blood and the other portion was collected in plain tubes from which serum was obtained for determination of melatonin levels.

Genomic DNA extraction from whole blood

This procedure was carried out according to Sambrook et al.[11] followed by 1% agarose gel electrophoresis.

Amplification of MTNR1B gene by polymerase chain reaction (PCR)

MTNR1B gene, localized on chromosome 11q21-q22, was amplified via polymerase chain reaction (PCR) using isolated genomic DNA from whole blood of patients and controls. This gene contained the rs#10830963 SNP. The following primers were used: forward (5'-ATG CTA AGA ATT CAC ACC AGC T-3') and reverse (5'-CAC AGT GCA GAC TGT TTT CTA ATC-3').[12] Primers were HPLC purified and obtained from AccuOligo® (Bioneer Co., Korea). PCR reaction was carried out in a final volume of 25 µl which included: 12.5 µl PCR GoTaq Green Promega Master Mix, 4 µl DNA template, 1 µl of a stock (10 pmol/µl) of each forward and reverse primer and 6.5 µl nuclease-free water. The reaction tubes were held in the PCR thermocycler (pEQ Lab Primus 25, Germany). PCR conditions were set as follows: an initial denaturation of 95°C for 5 min, followed by 35 cycles, each composed of 94°C for 1 min, 60°C for 1 min, and 72°C for 25 sec, and a final extension of 72°C for 10 min. After termination of the reaction, 5 µl of the PCR product was loaded onto a 2% agarose gel electrophoresis and visualized under UV-transilluminator. The expected PCR product was 125 base pair (bp).

Genotyping of the MTNR1B gene rs#10830963 polymorphism using the restriction fragment length polymorphism (RFLP) technique

MTNR1B gene rs#10830963 polymorphism genotyping was performed by restriction fragment length polymorphism (RFLP) analysis. The resultant 125 bp PCR product was digested with the restriction enzyme FastDigest® Proteus vulgaris Type II (PvuII; Fermentas Chemical Co.). The digestion reaction volume (20 µl) consisted of 10µl of resultant PCR product, 1 µl PvuII enzyme, 2 µl enzyme buffer (10×) and 7 µl nuclease-free water. The reaction was allowed to incubate at 37°C for 10 min. Subsequently, products of restriction digestion were loaded onto a 4% agarose gel electrophoresis along with the 50 bp ladder and analyzed.

Determination of serum melatonin levels using a ready-for-use radioimmunoassay (RIA) kit

Serum melatonin levels were determined using a ready-for-use radioimmunoassay (RIA) kit (DIAsource ImmunoAssays S.A., Belgium)

Table 1. Distribution of polymorphic variants of the MTNR1B gene rs#10830963 polymorphism among breast cancer patients and controls.

Genotype	Controls (n=45)		Cases (n=43)		P-value
	No.	%	No.	%	
CC	20	44.4	5	11.6	<0.001*
CG	19	42.2	7	16.3	<0.001*
GG	6	13.3	31	72.1	<0.001*

*: P<0.05 was statistically significant.; No.: Sample size

Table 2. The diagnostic sensitivity and specificity of the GG genotype of MTNR1B gene rs#10830963 polymorphism among breast cancer patients.

Genotype	Controls (n=45) No. (%)	Cases (n=43) No. (%)
CC+CG (-ve)	39 (86.7)	12 (27.9)
GG (+ve)	6 (13.3)	31(72.1)
Sensitivity	83.78%	
Specificity	76.47%	

according to the manufacturer's protocol. Briefly, 20 µl of each standard and quality control were pipetted into their respective tubes followed by the addition of 200 µl of equalizing reagent into each tube. Next, 200 µl of each sample were added to their respective tubes. 50 µl of enzyme were added to all tubes, followed by mixing. All tubes were incubated for 1 h at room temperature. 100 µl of assay buffer were pipetted into all tubes with mixing. A total of 50 µl of melatonin antiserum were added to all tubes, mixed thoroughly, and incubated at room temperature for 1 h. Next, 50 µl of ^{125}I-labeled melatonin were added to each tube and mixed thoroughly. All tubes were covered and allowed to incubate overnight at room temperature.

The following day, 1000 µl of cold precipitating reagent were pipetted into all tubes and mixed on a vortex, followed by incubation for 20 min at 2-8°C, then centrifuged for 20 min at 6000 rpm in a cooling centrifuge. The supernatant was decanted with extreme care prior to blotting the tubes for dryness by leaving them upside down for 2 min. Finally all tubes were counted for 1 min in a gamma counter (Perkin-Elmer, Finland).

Statistical analysis

Data were analyzed using the Predictive Analytics Software (PASW Statistics 18) for Windows (SPSS Inc., Chicago, IL, USA). The distributions of quantitative variables were tested for normality using the Kolmogorov-Smirnov test. If the data was distributed normally it was described as mean±standard deviation, and the parametric tests were applied (independent t-test). In cases where the data were abnormally distributed, it was described by the median, minimum and maximum, and the non-parametric test was used (Mann-Whitney test).

The frequencies of distribution of MTNR1B gene rs#10830963 polymorphism genotypes for patients and controls were compared using the chi-square test. When more than 20% of the cells had an expected count less than 5, correction for the chi-square was conducted using Fisher's exact test. Odds ratio (OR) and 95% Confidence interval (CI) were also calculated to quantify the association between breast cancer risk and MTNR1B gene rs#10830963 polymorphism. Significance test results were quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.
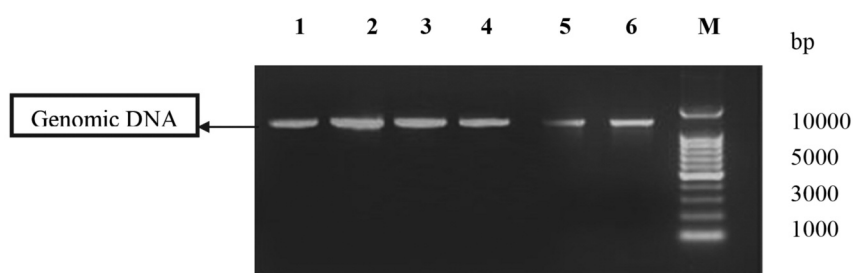
Figure 1. 1% agarose gel electrophoresis for DNA isolated from whole blood of some study subjects. Lanes 1-6: genomic DNA, Lane M: 1 Kbp DNA ladder.
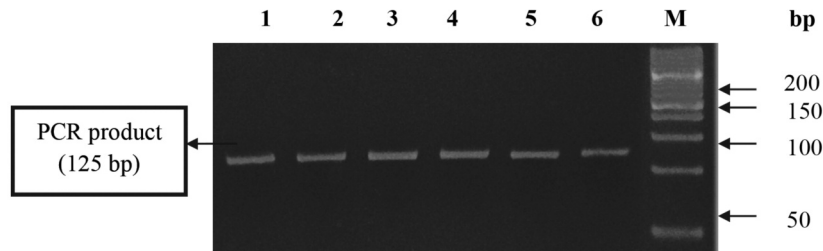
Figure 2. 2% agarose gel electrophoresis for PCR products of the MTNR1B gene amplified from some of the study subjects. Lanes 1-6: PCR products (125 bp), Lane M: 50 bp DNA ladder.

Results

Genomic DNA extraction from whole blood

Genomic DNA isolated from all subjects enrolled in this study was analyzed using 1% agarose gel electrophoresis as shown in Figure 1. The isolated DNA was found to be intact and suitable for genotyping of the MTNR1B gene polymorphism by RFLP-PCR.

Amplification of the MTNR1B gene by polymerase chain reaction (PCR)

A partial fragment of the melatonin receptor gene that contained SNP rs#10830963 was amplified via PCR. A PCR product of 125 bp was detected by running aliquots of the PCR product on 2% agarose gel electrophoresis (Figure 2).

Genotyping of MTNR1B gene rs#10830963 polymorphism using the restriction fragment length polymorphism (RFLP) technique

The obtained PCR product was digested by PuvII (fast digest) in order to genotype the MTNR1B gene polymorphism. Three possible genotypes could be detected from the resultant RFLP-PCR patterns. Homozygous genotype (CC alleles) appeared as a single uncut band (125 bp), homozygous genotype (GG alleles) appeared as two bands (105 bp and 20 bp) and the heterozygous genotype (CG alleles) appeared as three bands (125 bp, 105 bp and 20 bp). However, the 20 bp band was not visualized on the 4% agarose gels due to limited resolution of agarose for very small fragments beyond 50 bp (Figure 3).

Distribution of the polymorphic variants of the MTNR1B gene rs#10830963 polymorphism among patients and controls was shown in Table 1. There was a significantly higher frequency of the GG genotype among cases (72.1%) compared to controls (13.3%; $P<0.001$) whereas there was a significantly lower frequency of the CC (11.6%) and CG (16.3%) genotypes among cases compared to controls. Controls had a 44.4% frequency of the CC genotype and 42.2% frequency of the CG genotype ($P<0.001$). Compared with combined CC and CG genotypes (individuals with either CC or CG genotypes), the diagnostic sensitivity and specificity of the GG genotype was 83.78% and 76.47%, respectively (Table 2).

As shown in Table 3 the presence of the GG genotype significantly increased the risk for breast cancer by approximately 21 times more than the CC genotype (OR=20.67; 95% CI=4.77-99.33). On the other hand, there was no significant association of the CG genotype with the risk for breast cancer incidence (OR=1.47; 95% CI=0.34-6.61).

In terms of an association between the different genotypes of the MTNR1B gene rs#10830963 polymorphism and breast cancer clinicopathological data, the mean±SE values for tumor volume were significantly larger (26.79±0.45 cm³) among patients who had the GG genotype compared to those with either the CC or CG genotypes
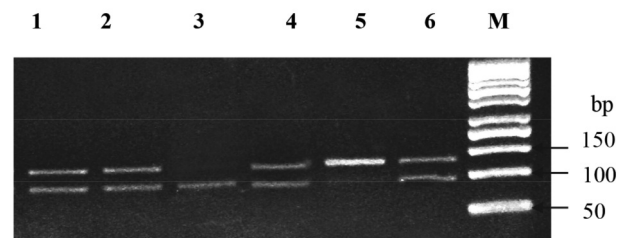

Figure 3. 4% agarose gel electrophoresis for restriction fragment length polymorphism (RFLP) of the amplified MTNR1B gene of some study subjects. Lanes 1,2,4,6: CG genotype (uncut allele C 125 bp, cut allele G 105 bp), Lane 3: GG genotype (cut allele G 105 bp), Lane 5: CC genotype (uncut allele C 125 bp), Lane M: 50 bp DNA ladder.

Table 3. Association of polymorphic variants of the MTNR1B gene rs#10830963 polymorphism with risk for breast cancer incidence.

Genotype	Cases (n=43)		Controls (n=45)		OR (95% CI)
	No.	%	No.	%	
CC ®	5	11.6	20	44.4	--
CG	7	16.3	19	42.2	1.47 (0.34-6.61)
GG	31	72.1	6	13.3	20.67 (4.77-99.33)*

*: Significant at *P*<0.05; ®: Reference genotype; OR: Odds ratio

(6.64±0.48 cm³; *P*=0.048; Figure 4). The other clinicopathological data showed non-significant associations with the different genotypes of the MTNR1B gene rs#10830963 polymorphism.

As shown in Figure 5, patients had significantly lower mean±SE levels of serum melatonin (39.2±3.2 pg/ml) than controls (58.9±5.9 pg/ml; *P*=0.021). According to ROC curve analysis (Figure 6), serum melatonin showed a significant area under the curve (72.6%, *P*<0.001) with a diagnostic sensitivity of 91.1% and specificity of 58% at a cut-off level of 39.5 pg/ml.

As shown in Table 4, females with serum melatonin levels ≤39.5 pg/ml had significantly increased risk for breast cancer incidence. This risk was approximately 15 times more than females who had levels >39.5 pg/ml (OR=14.24; 95% CI=4.32-46.90).

The mean±SE levels of serum melatonin were non-significantly lower among patients who carried the GG genotype of the MTNR1B gene rs#10830963 polymorphism (46.48±5.28 pg/ml) compared to those with either CC+CG genotypes (50.25±16.82 pg/ml; *P*=0.255).

Discussion

Numerous studies on humans have focused on evaluating changes in melatonin levels during tumor growth in order to define the possible involvement of the pineal gland in breast carcinogenesis, as well as to search for the value of serum melatonin hormone level changes as a diagnostic and prognostic marker. Cohen M.[13] was the first to suggest that a decrease in pineal activity would contribute to the etiology of breast cancer by causing a state of relative hyper-estrogenism.

The results of the present study showed significantly lower serum melatonin levels in cases compared to controls (*P*=0.021). Our results confirmed the results of numerous previous studies. Bartsch et al.[6] published the first study which demonstrated that urinary melatonin levels diminished in patients with breast cancer. The melatonin excretion patterns of the cancer patients were not synchronized compared to synchronized patterns of the controls. Bartsch et al.[6] suggested that pineal melatonin secretion might be modified in quantity as well as rhythmicity in breast cancer patients. In a small study of 10 women with stages I or II breast cancer, Tamarkin et al.[14] showed that women with breast cancer had lower levels of peak plasma melatonin concentration than control subjects.

The results of the current study showed that females with lower serum melatonin levels of ≤39.5 pg/ml were at a significantly increased risk for breast cancer incidence. This risk was approximately 15 times more than females with levels >39.5 pg/ml (OR=14.24; 95% CI: 4.32-46.90). Our results supported numerous studies in humans that measured circulating melatonin levels in women with breast cancer which sought to determine the
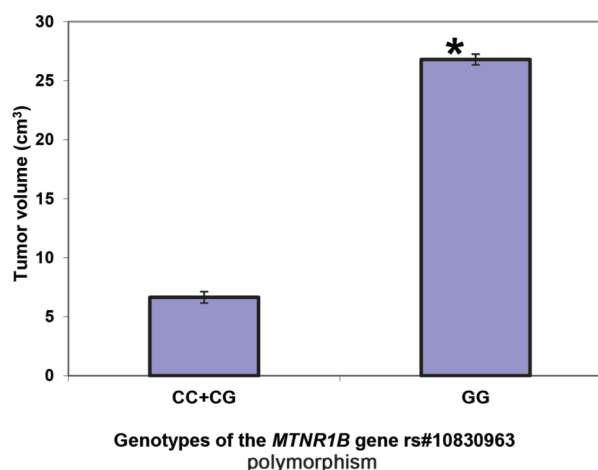

Figure 4. Association of polymorphic variants of the MTNR1B gene rs#10830963 polymorphism with tumor volume (cm³) of breast cancer patients. *: Significance was compared with CC+CG genotypes.

Table 4. Association of serum melatonin levels with risk for breast cancer incidence.

| Melatonin cut-off (pg/ml)	Controls (n=45)		Cases (n=43)		OR (95% CI)
	No.	%	No.	%	
>39.5 ®	41	91.1	18	41.9	14.24 (4.32-46.90)
≤39.5	4	8.9	25	58.1	

®: Reference genotype; OR: Odds ratio; No.: Sample size

relationship between pineal function and the incidence and evolution of this type of tumor.[15,16] All reported that lower melatonin hormone levels were a valuable risk factor for breast cancer occurrence. Wu et al.[17] hypothesized that an inverse association between sleep duration and breast cancer risk was possibly due to greater overall melatonin production in longer sleepers. In contrast, Pinheiro et al.[18] reported no such association, possibly because the selected residential nurses that were studied had rotating-shift work and variations in the timing of their sleep. Hence data collected from their study might be inappropriate.

The current study revealed non-significant correlations between serum melatonin levels and clinicopathological data of breast cancer patients. In contrast to the present study, Bartsch et al.[19] found that the reduction in peak amplitude of melatonin to be directly correlated with tumor size. Increased tumor size staged as T1, T2, and T3 had reductions in peak melatonin amplitudes of 27% (T1), 53% (T2), and 73% (T3).[19] This contradiction could be attributed to the times at which blood samples were obtained and individual variations among the selected populations.

In addition to decreased melatonin hormone levels, several studies identified various disease-predisposing SNPs in the melatonin receptor MTNR1A and MTNR1B genes. The most common disease associated with these SNPs was type 2 diabetes mellitus (T2DM) and increased fasting blood glucose levels.[20] The importance of T2DM was a possible link that existed between this disease and breast cancer incidence.[21] Although the results of the current study have revealed no significant association between the GG genotype of the MTNR1B gene rs#10830963 polymorphism and the incidence of T2DM, numerous studies have shown a significant association between GG genotype of the MTNR1B gene rs#10830963 polymorphism and the incidence of T2DM.[22-24] This contradiction could be explained by the finding that only 12/43 (27.9%) of our cases had T2DM; this sample size could be considered too small to confirm any
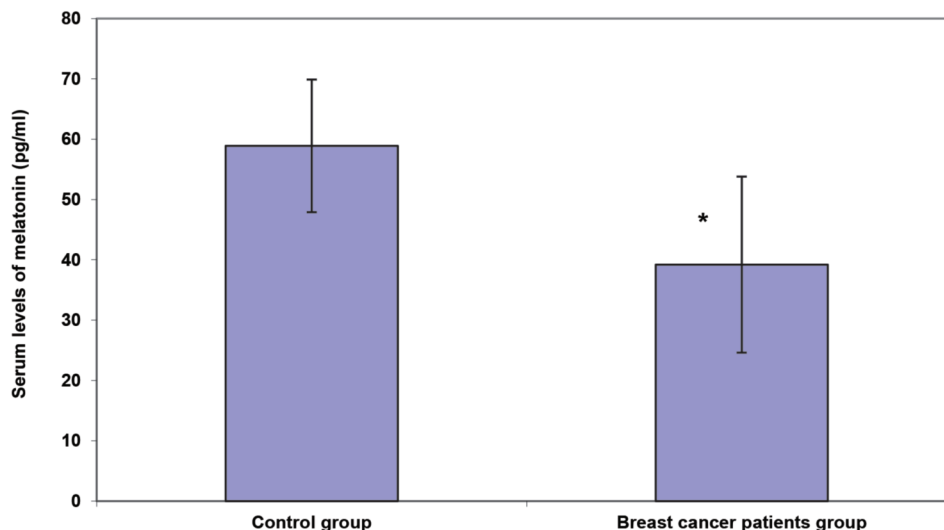
Figure 5. Serum melatonin levels among breast cancer patients and controls.
*: Significance was compared with the control group.

association.

The results of the current study showed a significantly higher frequency of having the GG genotype of the MTNR1B gene rs#10830963 polymorphism in cases compared to controls (P<0.001). However, the frequency of having the CC or CG genotype was significantly lower among the cases than controls (P<0.001 for both). The results of the current study revealed that in comparison with the CC genotype of the MTNR1B gene rs#10830963 polymorphism, an increased risk of breast cancer incidence was associated with the GG genotype (OR=20.67; 95% CI: 4.77-99.33). However we have shown no significant association of the CG genotype with breast cancer incidence (OR=1.47; 95% CI: 0.34-6.61). This means that the homogeneity for the G allele (i.e., the GG genotype) of the MTNR1B gene rs#10830963 polymorphism is a risk factor for breast cancer incidence; the presence of the C allele (either homogeneous CC or heterogeneous CG) is essential for protection from breast cancer.

The results of the present study have shown a significant association between the GG genotype of the MTNR1B gene rs #10830963 polymorphism and larger breast tumor volume (P=0.048). As breast tumor volume is a marker of poor prognosis in breast cancer, the GG genotype can be used to give an indication about the clinical outcome of breast cancer patients. However, the other breast cancer clinicopathological data were not significantly associated with the GG genotype of the MTNR1B gene.

In the current study, the diagnostic sensitivity of serum melatonin was 91.1% and its specificity was 58%. For the GG genotype of the MTNR1B gene rs#10830963 polymorphism, the diagnostic sensitivity was 83.78% and specificity was 76.47%. The GG genotype of the MTNR1B gene rs#10830963 polymorphism is associated with breast tumor volume, one of the markers of poor breast cancer prognosis. Serum melatonin has no association with breast cancer prognosis.

Current studies have investigated the possible use of melatonin to control human cancers. Sharman et al.[25] used dietary melatonin *in vivo* to reduce tumor number and size in aged male mice. This confirmed the idea that melatonin hormone might be useful as a strategic plan for controlling and treating breast cancer. Seely et al.[26] have recommended that melatonin may benefit cancer patients who are also receiving chemotherapy, radiotherapy, supportive therapy, or palliative therapy by improving survival and ameliorating the side effects of chemotherapy. Wang et al.[27] have stated that melatonin, as an adjuvant therapy for cancer, led to substantial improvements in tumor remission and alleviation of radiochemotherapy-related side effects.

Finally, it can be concluded that the risk for breast cancer increases in females who are homozygous for the G allele of the MTNR1B gene rs#10830963 polymorphism (i.e., the GG genotype).
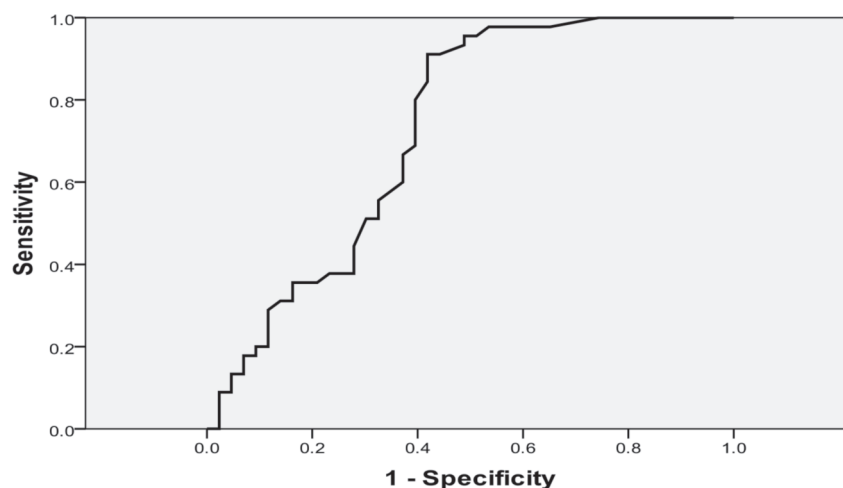

Figure 6. ROC curve for serum melatonin in breast cancer patients before surgery.

Additional risk follows those with deficiencies in blood serum melatonin levels. This can be explained as the abnormal variant of rs#10830963 in the MTNR1B gene may lead to functional and activity changes in melatonin receptor, as well as a deficiency in melatonin that possibly contributes to breast cancer. The GG genotype of the MTNR1B gene rs#10830963 polymorphism is associated with increased breast tumor volume which is one of the markers of poor prognosis for breast cancer, while serum melatonin has no association with breast cancer prognosis.

To the best of our knowledge this is the first study that has investigated the association of melatonin hormone levels and the rs#10830963 polymorphism in its receptor MTNR1B gene with the risk for breast cancer incidence by determining their diagnostic and prognostic values in breast cancer females. The results of the current study need to be verified by further studies with larger study populations.

Conflict of Interest

No conflict of interest is declared.

References

1. Burstein, HJ; Harris, JR; Morrow, M. Malignant Tumors of the Breast. In: DeVita, VT; Lawrence, TS; Rosenberg, SA, editors. Cancer: Principles & Practice of Oncology. 8th ed. Lippincott: Williams & Wilkins; 2008:p.1606-54.
2. Bartsch C, Bartsch H, Bellmann O, Lippert TH. Depression of serum melatonin in patients with primary breast cancer is not due to an increased peripheral metabolism. *Cancer.* 1991; 67(6):1681-4.
3. Blask DE. Melatonin, sleep disturbance and cancer risk. *Sleep Med Rev.* 2009; 13(4):257-64.
4. Deming SL, Lu W, Beeghly-Fadiel A, Zheng Y, Cai Q, Long J, et al. Melatonin pathway genes and breast cancer risk among Chinese women. *Breast Cancer Res Treat.* 2012; 132(2):693-9.
5. Li C, Shi Y, You L, Wang L, Chen ZJ. Association of rs10830963 and rs10830962 SNPs in the melatonin receptor (MTNR1B) gene among Han Chinese women with polycystic ovary syndrome. *Mol Hum Reprod.* 2011; 17(3): 193-8.
6. Bartsch C, Bartsch H, Jain AK, Laumas KR, Wetterberg L. Urinary melatonin levels in human breast cancer patients. *J Neural Transm.* 1981;52(4):281-94.
7. Haskell, CM; Lowitz, BB; Casciato, AD. Breast cancer. In: Casciato, AD; Lowitz, BB, editors. Manual of clinical oncology 2nd ed. Boston: Little and Brown (Pub.);1985:p.150-65.
8. Wapnir IL, Barnard N, Wartenberg D, Greco RS. The inverse relationship between microvessel counts and tumor volume in breast cancer. *Breast J.* 2001; 7(3): 184-8.
9. Rintoul, RF. Operations on the breast. In: Farquhaerison, S, editor. Text book of operative surgery. 7th ed. New York:Churchill Livingstone (pub.);1986:p.270-81.
10. Abeloff, MD; Lichter, AS; Niederhuber, JE; Pierce, LJ; Aziz, DC. Breast. In: Abeloff, MD; Armitage, JO; Licher, AS; Niederhuber, JE, editors. Clinical oncology. New York: Churchil Livingstone Inc. (Pub.);1995:p.1617-714.
11. Sambrook, J; Frisch, EF; Maniatis, T. Molecular cloning: A Laboratory manual. 2nd ed. New York: Cold Spring Harbor laboratory Press, 1989.
12. Vlassi M, Gazouli M, Paltoglou G, Christopoulos P, Florentin L, Kassi G, et al. The rs#10830963 variant of melatonin receptor MTNR1B is associated with increased risk for gestational diabetes mellitus in a Greek population. *Hormones (Athens)* 2012; 11(1):70-6.
13. Cohen M, Lippman M, Chabner B. Role of pineal gland in etiology and treatment of breast cancer. *Lancet.* 1978; 2(8094):814-6.
14. Tamarkin L, Danforth D, Lichter A, Demoss E, Cohen M, Chabner B, et al. Decreased nocturnal plasma melatonin peak in patients with estrogen receptor positive breast cancer. *Science.* 1982; 216(4549):1003-5.
15. Danforth DN, Tamarkin L, Mulvihill JJ, Bagley CS, Lippman ME. Plasma melatonin and the hormone-dependency of human breast cancer. *J Clin Oncol.* 1985; 3(7):941-8.
16. Falkson G, Falkson HC, Steyn ME, Rapoport BL, Meyer BJ. Plasma melatonin in patients with breast cancer. *Oncology.* 1990; 47(5):401-5.
17. Wu AH, Wang R, Koh WP, Stanczyk FZ, Lee HP, Yu MC. Sleep duration, melatonin and breast cancer among Chinese women in Singapore. *Carcinogenesis.* 2008; 29(6): 1244-8.
18. Pinheiro SP, Schernhammer ES, Tworoger SS, Michels KB. A prospective study on habitual duration of sleep and incidence of breast cancer in a large cohort of women. *Cancer Res.* 2006; 66(10):5521-5.
19. Bartsch C, Bartsch H, Fuchs U, Lippert TH, Bellmann O, Gupta D. Stage-dependent depression of melatonin in patients with primary breast cancer. Correlation with prolactin, thyroid stimulating hormone, and steroid receptors. *Cancer.* 1989;64(2):426-33.
20. Ji LD, Xu J, Wu DD, Xie SD, Tang NL, Zhang YP. Association of disease predisposition polymorphisms of the melatonin receptors and sunshine duration in the global human populations. *J Pineal Res.* 2010; 48(2):133-41.
21. Giovannucci E, Harlan DM, Archer MC, Bergenstal RM, Gapstur SM, Habel LA, et al. Diabetes and cancer:

a consensus report. *Diabetes Care.* 2010; 33(7):1674-85.

22. Rönn T, Wen J, Yang Z, Lu B, Du Y, Groop L, et al. A common variant in MTNR1B, encoding melatonin receptor 1B, is associated with type 2 diabetes and fasting plasma glucose in Han Chinese individuals. *Diabetologia.* 2009; 52(5):830-3.

23. Langenberg C, Pascoe L, Mari A, Tura A, Laakso M, Frayling TM, et al. Common genetic variation in the melatonin receptor 1B gene (MTNR1B) is associated with decreased early-phase insulin response. *Diabetologia.* 2009; 52(8):1537- 42.

24. Liu C, Wu Y, Li H, Qi Q, Langenberg C, Loos RJ, et al. MTNR1B rs#10830963 is associated with fasting plasma glucose, HbA1C and impaired beta-cell function in Chinese Hans from Shanghai. *BMC Med Genet.* 2010; 11:59.

25. Sharman EH, Sharman KG, Bondy SC. Extended exposure to dietary melatonin reduces tumor number and size in aged male mice. *Exp Gerontol.* 2011; 46(1):18-22.

26. Seely D, Wu P, Fritz H, Kennedy DA, Tsui T, Seely AJ, et al. Melatonin as adjuvant cancer care with and without chemotherapy: a systematic review and meta-analysis of randomized trials. *Integr Cancer Ther.* 2011; 11(4):293-303.

27. Wang YM, Jin BZ, Ai F, Duan CH, Lu YZ, Dong TF, et al. The efficacy and safety of melatonin in concurrent chemotherapy or radiotherapy for solid tumors: a meta-analysis of randomized controlled trials. *Cancer Chemother Pharmacol.* 2012;69(5):1213-20.