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ORIGINAL ARTICLE

Expression of MT_1 receptor in patients with gastric adenocarcinoma and its relationship with clinicopathological features

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Abstract

Gastric cancer accounts 8% of the total cancer cases leading to 10% of total cancer deaths worldwide. The indoleamine N-acetyl-5-methoxytryptamine, better known as melatonin, is the principal hormone produced by the pineal gland. Recently, it has been well documented some anti-cancer roles of melatonin in some malignancies as breast and colon cancer; as well as some its protective roles in the GI tract that have been known as free radical scavenger, antimitogenic and apoptotic properties. According to the anti-cancer effects of melatonin, wide distribution of this neurohormone in GI tract and some proposed physiologic and pharmacologic roles for this neurohormone and following our previous study which has shown expression of MT_2 receptor in gastric adenocarcinoma, this study initially scheduled to determine the expression of melatonin receptor MT_1 in tissue samples of adenocarcinoma cancer patients. A total of 10 gastric adenocarcinoma patients and 10 normal individuals were examined for MT_1 gene expression by real-time PCR. Additionally, for screening of different alleles of MT_1 in our samples, the SSCP-PCR procedure was developed. Our results have shown interestingly high expression for MT_1 receptor in cancer and marginal cancer groups comparing with normal group. Our findings also have shown that a remarkable association between MT_1 receptor mRNA levels and grade in individuals over age 50. PCR-SSCP analysis results showed a variation between individuals which may be effective on their gene expression patterns. According to our knowledge, for the first time this study evaluated the expression of MT_1 receptor gene in gastric adenocarcinoma tissues which consistent with our previous study but with some difference in comparisons between kind of tissue expression and difference in polymorphisms. Moreover, these results show the defending role of melatonin in the GI system.

INTRODUCTION

Gastric adenocarcinoma is the fourth most common cancer in the world and the second most frequent cause of cancer deaths, accounting for 10.4% of cancer deaths worldwide (Parkin *et al.* 2001). There are 900000 new cases and 700000 gastric cancer-related deaths worldwide per year (Parkin *et al.* 2005). This cancer is more frequently affected men than women and the disease usually occurs in those over the age of 55 (Pinto *et al.* 1994; Axon 2006). *H. pylori* infection is one of the main risk factors in 65–80% of gastric cancers. Nitrates and nitrites in cured meats could be converted by certain bacteria as *H. pylori* into compounds that have been found to cause gastric cancer. Smoking increases the risk of developing gastric cancer significantly. Also several studies have shown the increased risk of gastric cancer with alcohol consumption as well. The lipophilic indoleamine, N-acetyl-5-methoxytryptamine, known as Melatonin, is the principal hormone mainly synthesized and released during darkness by the pinealocytes in the pineal gland or epiphysis (Underwood *et al.* 1987). Melatonin's synthesis and also its release has been found in periphery as retina, the gastrointestinal tract, skin, bone marrow and lymphocytes, platelets, immune system cells and reproductive organs (Parkin *et al.* 2001; Stefulj *et al.* 2001; Pandi-Perumal *et al.* 2008). So far, the roles of melatonin have been well described by numerous researchers, including regulation of circadian and seasonal rhythms, regulation of estrogen and progesterone production (Fiske *et al.* 1984), controlling sleep-wake rhythm, renal function, blood pressure, bone metabolism, aging process, regulation of cardiovascular and the immune response, cancer cell growth and tumor suppression (Brzezinski 1997). Many biological effects of melatonin associated with its powerful free radical scavenger properties and its binding to membrane and nuclear receptors (Boutin *et al.* 2005; Reiter *et al.* 2007). Melatonin is also an antioxidant which can easily crosses through cell membranes and blood-brain barrier (Hardeland 2005, Reiter *et al.* 2010) and its metabolites reduce oxidative damage by hindering the formation of free radicals (Reiter *et al.* 2007). Melatonin has a preventive role against oxidative stress by scavenging free radicals, stimulating the synthesis of antioxidative enzymes and proteins and inhibiting activity of free radical generating enzymes. Two high affinity receptors of the G protein coupled receptor superfamily (GPCRs) for melatonin have been characterized: MT_1 (formerly known as Mel 1a or MTNR1A) and MT_2 (already known as Mel 1b or MTNR1B) (Reppert *et al.* 1994, Reppert *et al.* 1995). Both of melatonin receptors are linked to G proteins and with melatonin binding, formation of cyclic adenosine monophosphate (cAMP), protein kinase A (PKA) activity and cAMP response element-binding protein (CREB) into P-CREB or increasing in phosphoinositide (PI) hydrolysis and protein kinase C (PKC), were affected. The membrane of gastrointestinal tract is a major source of extra-pineal melatonin. Melatonin is probably produced in the serotonin-rich enterochromaffin cells of the gastrointestinal tract mucosa. Recent studies offer anti-neoplastic role for melatonin because of its antioxidant, cell preservation and oncostatic properties (Coughlin 2007). The aforementioned studies suggest that melatonin could act as an autocrine/paracrine hormone affecting the function of gastrointestinal tract epithelium, lymphatic tissues of the immune system and the smooth muscles of the digestive tract. Moreover, it has been suggested that increase of this hormone in gastrointestinal tract can prevent the development of cancer (Coughlin 2007; Lanoix *et al.* 2006) demonstrated a possible paracrine/autocrine function for melatonin in human placenta by evaluating the expression of the MT_1, MT_2 and RORa1 melatonin receptors in human term placental tissues and in choriocarcinoma cells with RT–PCR, western blotting and confocal microscopy methods (Lanoix 2006). Previous studies on Colon 38 adenocarcinoma cells confirmed this concept that both membrane and nuclear receptors (MT_1 and MT_2) are involved in the oncostatic action of melatonin (Karasek *et al.* 2002). It has suggested that melatonin or melatonin receptor agonists may have useful biologic properties in treatment of some forms of triple negative breast cancers (Oprea-Ilies *et al.* 2013). Additionally, our previous study confirmed the high expression of MT_2 receptor in gastric adenocarcinoma (Nasrabadi *et al.* 2014). According to the anti-cancer role of melatonin and some of its protective role in gastrointestinal tract and as there has not been any precise study about MT_1 receptor expression in gastric adenocarcinoma, this study programmed to determine the expression of MT_1 melatonin receptor in tissue samples of gastric adenocarcinoma patients.

MATERIALS AND METHODS

Sample collection and processing

Ten patients with gastric cancer and ten normal control individuals were considered for this study from 2011 to end of 2012 and the samples obtained by means of endoscopy at Tooba expert clinic of the Mazandaran University of Medical Sciences, Sari, Iran. Written informed consent was obtained from all participants and approved by the ethical committee of Mazandaran University of Medical Sciences (Ethics Committee Code: 909).

Two tissue samples from tumoral section and non-tumoral (marginal) section of stomach tissue of the one patient were taken from each patient during endoscopy. As negative control, normal samples were taken from peoples whose stomach tissues were reported non-tumoral after endoscopy. Hence the expression of MT_1 compared relatively between three groups of samples tumoral, non-tumoral (marginal) and negative control.

Tab. 1. Sequences of the PCR primer sets

Gene	GeneBank accession no.	Forward Primer	Reverse Primer	Size (bp)
HGPRT	NM_000194	CTAATTATGGACAGGACTGAACG	TTGACTGGTCATTACAATAGC TC	211
MT_1	NM_005958.3	CTCAGGAACGCAGGAAAC	ATGTTGAATATGGAGCCGATG	182

Total RNA isolation and cDNA synthesis

The biopsy specimens were immediately stored in RNA later solution *(Cat. no: 148012363)* and transferred at -20°C until isolation of RNA process. Total RNA isolation was performed using the method of *RNeasy® Plus Mini Handbook (Qiagen, 2010) (Cat No. /ID: 74904)*. For this purpose, approximately 30 mg RNA later stabilized tissue was disrupted by using a mortar and pestle followed by homogenization using needle and syringe and then the weighed (RNAlater stabilized) tissue immediately placed in liquid nitrogen and grinded thoroughly with a mortar and pestle Further processing was performed following the manufacturer's protocol of *RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) (Cat No./ID: 74904)*. Also a DNase treatment has been included in procedure for elimination of any genomic DNA (Qiagen, Hilden, Germany)*(Cat No./ID: 74192)*. Extracted RNAs were eluted in 40 µl RNase-free water and stored at -80°C. The quality of extracted total RNA was verified by agarose gel electrophoresis and the concentration of each RNA sample was measured at A260 using the Pico Drop 2000 (Thermo Fischer Scientific Inc). For reverse transcription of total RNA to complementary DNA (cDNA), the cDNA synthesis kit Thermo Scientific Germany,) *(Cat.no: #K1622)* has been used. Evaluation of concentration and purity of cDNA was performed using Pico Drop 2000 (Thermo Fischer Scientific Inc) (Behjati *et al.* 2005; Mowla *et al.* 2005).

Polymerase chain reaction in real time (Real-Time PCR)

Quantitative real-time RT-PCR was performed using specific primers for MT_1 and Housekeeping HGPRT genes (chosen as a reference gene) (Table 2) with the *Quanti Fast SYBR Green PCR Master Mix (Qiagen, Hilden, Germany (Cat No./ID: 204141)* (Karasek *et al.* 2002), and run on the Rotor-gene 6000 instrument *(Serial number: 9001550*, Germany). Primers were designed with Beacon Designer 7 software based on the full human MT_1 receptor cDNA sequences (Accession No. NM_005959) and HGPRT as reference gene (Accession No. NM_000194) (Table 1).

Quantitative real-time PCR reactions were performed in a 15 µl volume containing 7.5 µl of Quanti-Tect SYBR Green PCR master mix 1X (Qiagen, Hilden, Germany, (Cat No./ID: 204141) 1 µl of each forward and reverse primers (10 pM) and 1 µl of first strand cDNA according to manufacturer's procedure (Karasek *et al.* 2002). After an initial 5-minute heating at 95°C as activation step, 40 cycles of denaturation at 95°C, 10 S; annealing and extension at 60°C, 30 S have been carried out. In each PCR run, preparation of standard curve was carried out by serial dilution of cDNA from each sample (one pooled sample). The mRNA level of MT_1 gene was assayed by comparison of the test with standard curve of the specific target and reference gene in each PCR run. Also Melting curve has been designed to show confidence of production.

Evaluation of DNA variation among samples by SSCPA

PCR products were resolved by SSCPA (Single-Strand Conformational Polymorphism Analysis). For SSCPA a 4 µl aliquot of each PCR product was mixed with 7 µl of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, and 0.025% xylene cyanol). After denaturation at 95°C for 5 min, samples were rapidly cooled on ice for 10 min to prevent reannealing of the single-stranded product and then loaded on 14% acrylamide: Bis-acrylamide (29:1) gels. Electrophoresis was performed using Vertical Slab Unit, VSS-100 (Akhtarian), at 4-5°C with 250 V for 14 h in 1% TBE buffer. A constant temperature was essential for band sharpness and reproducibility of strand separation. Then the electrophoresis unit was coupled to a refrigerator at 4°C. DNA fragments were visualized by the silver staining.

Statistical Analysis for Genotyping Results

The genotype frequency of MT_1 gene were tested using the Popgene version 1.32 software for Hardy-Weinberg equilibrium (HWE). P-value > 0.05 was considered deviate from the equilibrium. Logistic regression model was carried out to analyze the distribution of MT_1 polymorphism between case and control groups and the clinicopathological characteristics of gastric cancer. All statistical tests were considered significant with a level of $p \leq 0.05$. Odds ratios (OR) adjusted for age were calculated. All statistical analyses in our study were carried out with SAS 9.1.

Statistical analysis for Real Time Results

All measurements were done at least duplicates and Data were analyzed using GLM procedures of SAS software version 9.1. Differences between means were tested using Tukey test. Differences were considered to be significant at $P<\neg 0.05$. Additionally, to examine the relationship between gene expression with progress and progression of tumor, we considered some clinicopathological figures as age, tumor location, Laurens score and tumor grade and the differences between

MT_1 expression with theses parameters were assayed by Tukey test and $\chi 2$ (Table 2).

Tab. 2. A brief description of patients with gastric cancer

Characteristics	Value
Mean age, years	64.4+12.3
Age range, years	36–83
Sex	
Male	4(40%)
Female	6(60%)
Tumour type	
Adenocarcinoma	10(100%)
SCC	0(0)
Tumor Location	
Cardia	7(70%)
Non-Cardia	3(30%)
Laurens Score	
Intestinal type	3(30%)
Diffused type	7(70%)
Mixed type	0(0%)
Tumor Grade	
Testosterone	1(10%)
Growth hormone	7(70%)
Vasopressin	2(20%)

RESULTS

Amplification stage

The standard curve which plotted based on the logarithm of the concentration of cDNA and threshold cycle (Ct) was linear (Figure 2A). Also the curves of amplification of MT_1 and HGPRT gene using Real-time PCR showed that the amplification has performed suitably with appropriate performance (Figure 2B). Results of melting curve for the designed primers showed that each pair of primers acted as especial shape and had no secondary structures and non-especial patches (Figure 3). With running Real-time PCR product on agarose gel, good qualitative results has been obtained and these results showed high qualified amplification of the desired fragments (Figure 4).

Variations at MT_1 locus

For genotyping and screening of different alleles of MT_1 in our samples, the SSCP-PCR procedure was developed. Then, Real time PCR products obtained were analysed by electrophoresis on Acryl amide gel. Amplicons comparable to human amplicon length (182bp) were obtained from human cDNA using the PCR primers. These amplicons exhibited polymorphism upon SSCP analysis, and two unique banding patterns could be identified under the SSCP conditions described (Figure 1). Either one or two of these patterns was observed for each individual, reflecting the presence of homozygous or heterozygous genotypes at the human MT_1 locus.

The most common allele present in was allele A with a frequency of 56.66%, and the second allele was B

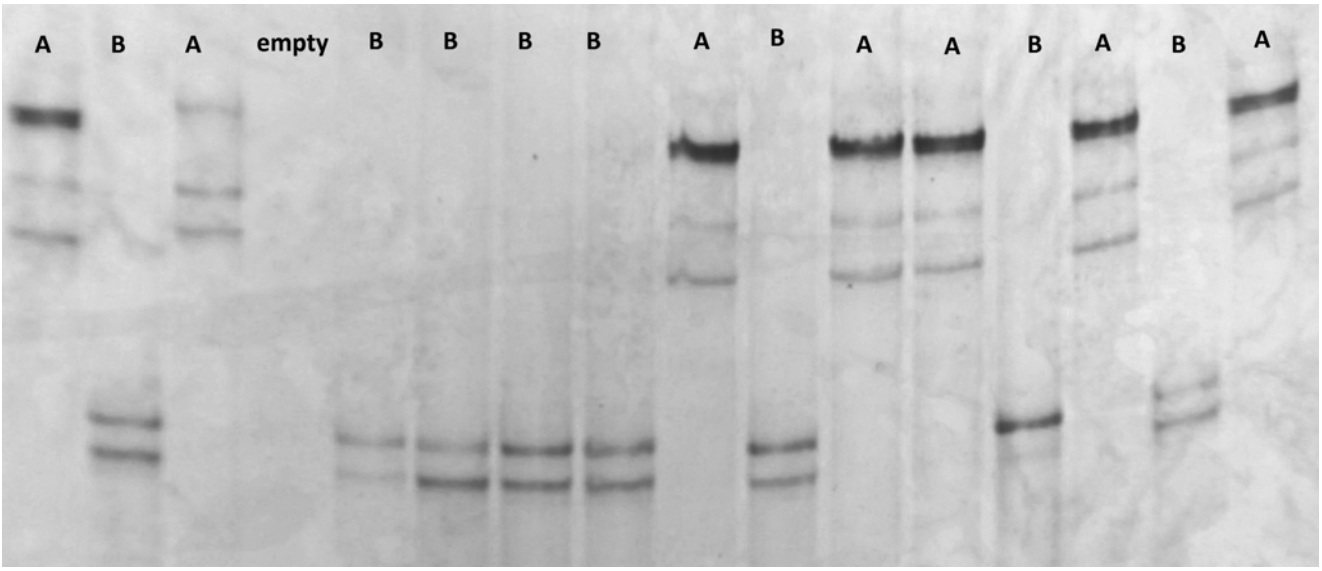

Fig. 1. PCR-single-strand conformational polymorphism of the MT_1 gene. Two unique SSCP patterns were detected using the electrophoresis conditions

allele 43.33%. These results show that there is a variation between individuals which may be affect their expression gene patterns. Although the SSCPA showed the variation between individuals, the results must be confirmed by direct sequencing technique and more samples size.

The MT_1 genotype distribution

The gastric cancer patients and healthy controls were all Mazandaran mainland women and men and we found a statistically significant difference between the two groups in the matching characteristic. Chi-square test was used to determine whether the subjects met the Hardy-Weinberg equilibrium. We confirmed that both case and control groups were not compatible with the HWE, the $\chi 2$ value for in case and control groups was 0.00001 and 0.0008, respectively. As shown in Table 3, in the logistic regression model, MT_1 band patterns have shown none of significant association with a decreased risk for gastric cancer.

Association between MT_1 polymorphism and known clinicopathological variables

Table 4 listed the association of A and B band patterns with clinicopathological characteristics, including age at diagnosis, pathological diagnosis, Laurens Score, Tumor Location. Furthermore, the association of different patterns with the age at diagnosis was significant ($p<0.036$). However, we found no association of the polymorphism with sex, Laurens Score, Tumor Location.

As the clinical study, the sample size in the present study is too small to claim that there was a polymorphism distribution. Although such a small number of patients could not represent the population distribution, the DNA variations may have associated with gastric cancer and more research is needed. However, these results could be considered as a fundamental information to design a project on evaluating the effect of gene variation of this marker site on risk for gastric cancer in following research works.

Tab. 3. The MT_1 genotype distribution in patients and controls

Genotype	No. of subjects (%)		Non-adjusted[b]		Adjusted[c]	
	Case[a]	Control[a]	p value	OR	p value	OR
A pattern	11 (55)	6 (60)		1.00		1.00
B pattern	9 (45)	4 (40)	0.7946	1.22	0.8643	1.15
MT_1	20	10				0

[a], The χ^2 for HWE of case and control group is 0.00001** and 0.0008** respectively.
[b], Logistic regression model, non-adjusted.
[c], Logistic regression model, adjusted for diagnostic age.
All statistical tests were two-sided with a significance level of $p \leq 0.05$.

Tab. 4. Relationship between MT_1 polymorphism patterns and known clinicopathological variables

Clinicopathological Variables	All	Pattern (%)		Adjusted[a]	
		A	B	p value	OR
Age					
≤ 50	11(36.7%)	6(54%)	5(46%)		1.00
> 50	19(63.3%)	11(57%)	8(43%)	**0.0366***	5.969
Score					
Diffused Type	14(70%)	7(50%)	7(50%)		1.00
Intestinal Type	6 (30%)	4(66%)	2(34%)	0.6075	0.583
Location					
Non Cardia	6 (30%)	4(66%)	2(34%)		1.00
Cardia	14(70%)	8(57%)	6(43%)	0.6075	1.714
Sex					
Female	12(40%)	9(75%)	3(25%)		1.00
Male	18(60%)	8(44%)	10(56%)	0.1309	3.573

[a] Logistic regression model adjusted for diagnostic age.
All statistical tests were two-sided with a significance level of $p \leq 0.05$.

MT_1 expression results

Expression results showed that MT_1 gene was expressed in each of three types of tissues (control, tumoral and nontumoral (marginal)). The MT_1 mRNA levels in tumoral and marginal groups significantly increased compared with control group. Although the expression of MT_1 gene in tumoral group was increased compared with marginal group, the difference was not significant statistically (Figure 4). Additionally, the relationship between MT_1 gene expression and clinicopathological features of patients including tumor type, tumor location, Laurens (scores), tumor grade was evaluated and these associations were not significant (Table 5).

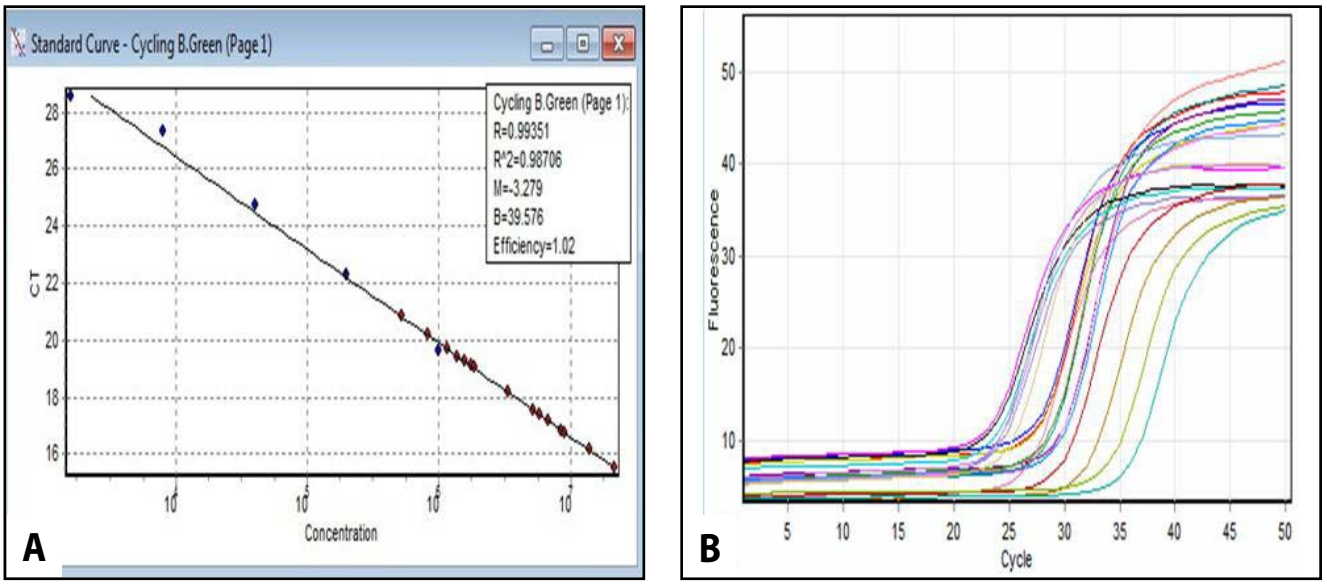

Fig. 2. A) standard curve B) Amplification curves of cDNA tissue samples of gastric cancer of MT_1 and HGPRT genes.

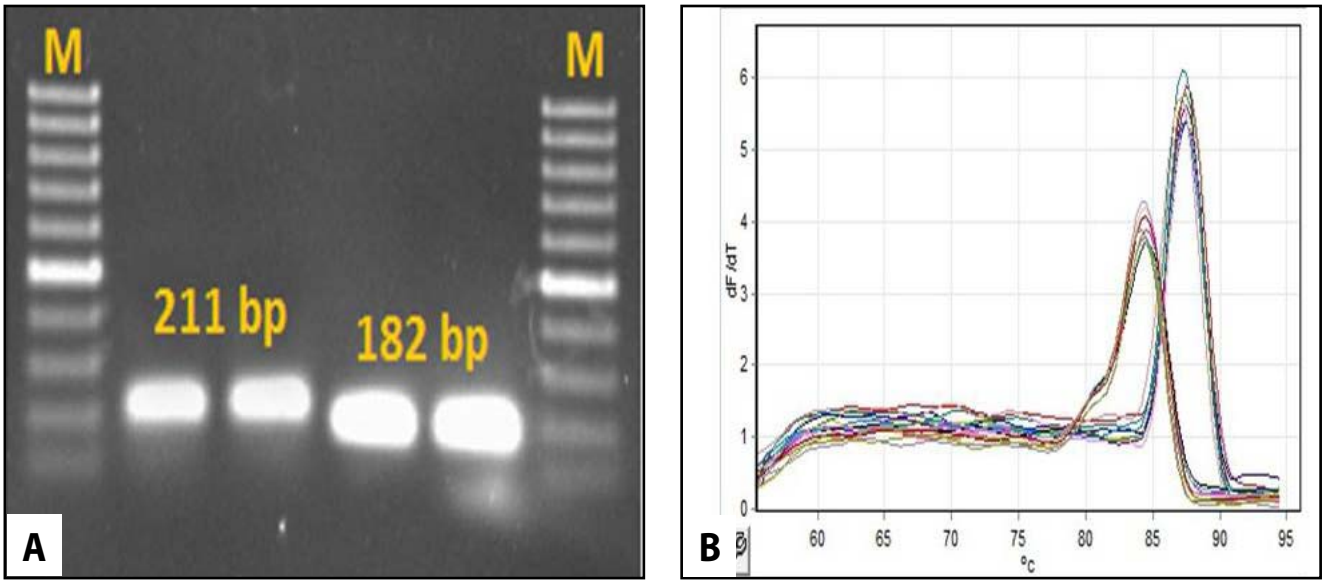

Fig. 3. A) Products of Real-time PCR on 2%Agarose gel. B) Melting curve analysis shows specific amplification of MT_1 and HGPRT by real-time RT-PCR.

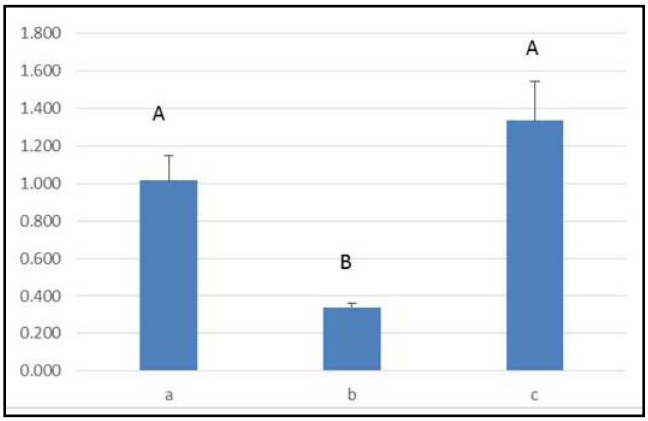

Fig. 4. Relative expression of MT_1 in tissue samples of non-tumoral (marginal) (a), normal tissues (b) and tumoral(c) ; A: significant compared with B; P<0.05

Tab. 5. The relationship between MT_1 gene expression and clinicopathological features of patients.

Type of tumor	Sex	Age	Tumor location	Laurens score	Tumor Grade
Tumoral	0.1713*	0.6670*	0.6251*	0.3305*	0.7679*
Non-Tumoral	0.7854*	0.3751*	0.1758*	0.6825*	0.7347*

* No significant P >0.05

DISCUSSION

The aim of this study was to investigate the expression of MT_1 gene in individuals with gastric adenocarcinoma and to compare this expression between patient and normal individuals to show whether any relationship was between gene expression and tumor stage and clinic pathological figures. Previous studies confirmed this concept that both membrane and nuclear receptors (MT_1 and MT_2) are involved in the oncostatic action of melatonin and it has been suggested that melatonin or melatonin receptor agonists may have useful biologic properties in treatment of cancers. Based on our previous study, MT_2 receptor expression has been increased in human gastric adenocarcinoma (Nasrabadi *et al.* 2014). By considering other studies, it seems that MT_2 receptor can stimulate the secretion of bicarbonate through stimulating of calcium release in mucosa of enterochromaffin cells. Melatonin has also been found to increase pancreatic secretion of amylase and cholecystokinin via activation of MT_2 receptors (Quastel *et al.* 1965).

In the present study, there is an increasing of MT_1 receptor expression in tumoral and non-tumoral (marginal) tissues of gastric adenocarcinoma patients compared to normal individuals while a remarkable difference has not observed between tumoral and marginal (non-tumoral) tissues of the same patient. To explain high expression of MT_1 receptor in patients, we can suggest up-regulation of surface melatonin receptors in gastric epithelial cells of tumoral and marginal region of cancer patients is a defending mechanism to response better to the diminished concentrations of melatonin in peripheral tissues. These findings were in agreement with previous results in cervical, breast and colon cancers (Barni *et al.* 1988; Cos *et al.* 1998). Also, in aforementioned studies, the melatonin plasma levels of patients were lower than that of healthy individuals, hence it was expected for up-regulation of melatonin receptors as a compensatory mechanism (Pandi-Perumal *et al.* 2006; Mao L et al 2012). Previously demonstrated the overexpression of the MT_1 receptor in transfected MCF-7 human breast cancer cells through inhibition of aromatase mRNA expression which was in relation with inhibition of human MCF-7 cell proliferation-derived mammary tumor formation in male mice .

On the other hand, the amplicons of gel-electrophoresis analysis in the present study exhibited two unique banding patterns resulting in variations by SSCP analysis, which were in parallel with other polymorphism studies in gastric cancer (Stefulj *et al.* 2001, Karasek *et al.* 2002, Lanoix *et al.*2006). Although these genotyping need to more sample size and even finding the actual SNP by direct sequencing technique, this information could confirm the existing of polymorphism in MT_1 receptor among individuals which may affect its gene expression. However, more research is needed to confirm the role of these obtained banding patterns on gastric cancer. According to our knowledge, for the first time our results showed role of MT_1 receptor in gastric adenocarcinoma and demonstrated that melatonin can have a preventive role in gastric adenocarcinoma through MT_1 receptor, especially for patients over age 50. Therefore, it can be suggesting melatonin usage in elderly to reduce the risk of gastric malignancy. It is noticed that some complementary studies especially around protein expression of melatonin receptors has been remained to do and to reveal better the mechanism of melatonin.

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