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Alterations in Oral Cancer Gene Expression in Response to Melatonin

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Melatonin (MLT) exerts oncostatic effects on numerous tumour types presumably by inhibiting cell proliferation and promoting apoptosis. The primary aim of this study was to investigate melatonin-induced changes in gene expression patterns in two different oral squamous carcinoma cell lines (OSCC).
Methodology: This was a prospective non-randomized experimental study design that was conducted at Department of Biomedical Sciences at the University of Nevada, Las Vegas – School of Dental Medicine between May 2016 and March 2017. The SCC25 and CAL27 cell lines were cultured with and without MLT (10 ug/mL) for 72 hours, and total cellular RNA was isolated and converted to cDNA. The expression of 92 genes associated with the molecular mechanisms of cancer and four endogenous control genes were examined by qRT-PCR. The fold change with respect to control levels were calculated using the comparative method or delta-delta ddCt algorithm.

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Results: Gene expression was compared between the untreated and treated cells, SCC25 and CAL27. Although 40% of the genes (n=37/92) in SCC25 cells demonstrated different expression levels, only six were outside the relative-fold change values observed with all other genes (PTEN, MAPK3K5, BCL2, TAGA2B, MAX, and NFKB-IA). In CAL27 cell over 70% (n=65/92) genes exhibited different expression levels, with only two outside the relative fold-change values observed with all other genes (MAPK3K5, FZD1).
Conclusion: MLT administration to oral cancer cells may induce substantial changes in the expression levels of genes associated with the molecular mechanisms of cancer. However, relatively few of these changes were outside the range of observed values. Therefore, continued analysis and verification of these results in other oral cancers may reveal common MLT-induced changed and provide insights into the potential mechanisms of MLT-induced oncostatic effects in oral cancers.

Keywords: Oral squamous cell carcinoma; cancer gene expression; melatonin.

1. INTRODUCTION

Melatonin (MLT) is known to exert tumour suppressive and oncostatic effects on numerous tumour types, including breast, lung, colorectal, prostate, liver and some head and neck cancers [1-3]. It is thought these effects include inhibition of cellular growth and angiogenesis, as well as promoting apoptosis [4-6]. Despite the growing body of evidence regarding these anti-tumour effects, relatively few of these studies have focused specifically on oral squamous cell carcinomas [2,7,8].

Preliminary studies from this group demonstrated that MLT administration is sufficient to inhibit proliferation and decrease the viability of oral squamous cell carcinomas *in vitro* [9]. Additional research has revealed evidence for biphasic effects of MLT at both physiologic and supraphysiologic concentrations, which may explain these effects observed among patients under normal conditions, as well as under MLT supplementation [10]. These studies may help to explain the role of sleep-wake cycles and MLT balance in cancer progression, as well as the stronger and dose-dependent evidence of MLT effects under supraphysiologic supplementation [11-12].

However, few studies to date have yet examined the effects of MLT on gene expression and mRNA transcriptional regulation in oral cancers [13]. Based upon this information, the primary aim of this study was to investigate MLT-induced changes in gene expression patterns in two different oral squamous carcinoma cell lines (OSCC) to more fully understand the mechanisms that may underlie these phenomena.

2. MATERIALS AND METHODS

2.1 Cell Culture

Experimental oral cancer tissue was obtained from the American Tissue Culture Collection or ATCC (Manassas, VA). SCC25 (CRL-1628) and CAL27 (CRL-2095) cells were grown in Dulbecco's Modified Eagles Medium or DMEM from Hyclone (Logan, UT) that contained 4.0 mM L-glutamine, 4.5 g/L glucose and 110 mg/L sodium pyruvate. Cell culture media was then supplemented with Penicillin-Streptomycin (100 U/mL, 100 ug/mL, respectively) and 10% fetal calf serum (FCS). Cell cultures were maintained in tissue culture-treated flasks and grown at 37°C and 5% CO_2 in humidified incubators.

2.2 Melatonin

Melatonin (N-acetyl-5-methoxy-tryptamine) was obtained from Thermo Fisher Scientific (Fair Lawn, NJ). RNA isolation was performed with the addition of MLT to the cell culture medium at 10 ug/mL for 72 hours to approximate serum and salivary concentrations of MLT administered as an over-the-counter supplement [14,15]. Non-treated cells were used as the negative control. These experiments were repeated in triplicate (n=3) for both cell lines and each condition (negative control, experimental).

2.3 RNA Isolation

Total RNA was isolated from the oral cancer cell lines at approximately 1.0 x 10^6 cells/mL using the ABgene Total RNA Isolation Kit from Epson (Surrey, UK) and the recommended protocol for tissue culture isolation. Purity and quantity of RNA was obtained from spectrophotometric absorbance readings at A260 and A280. In brief,

RNA purity was calculated from the ratio of A260:A280, which ranged between 1.65 and 2.00.

2.4 cDNA Synthesis and qPCR Screening

RNA was then processed using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Foster City, CA) using the protocol recommended by the manufacturer. In brief, quantitative conversion of 2 µg of total RNA was performed to yield single-stranded cDNA for use in applications, such as qPCR screening. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) was performed using the TaqMan Array Human Molecular Mechanisms of Cancer 96-well plate with 92 assays for genes associated with the known molecular mechanisms of oncogenesis, as well as four assays to candidate endogenous control genes.

2.5 Statistical Analysis

Quantification of average mRNA expression of genes (determined by qRT-PCR) was calculated using the numerical average of the three replicates from each cell line (CAL27, SCC25) under each condition (non-treated control, experimental). Two-tailed t-tests were used to determine if the average mRNA expression under MLT administration was different than the average mRNA expression under non-treated (control) conditions, with the significance level set at alpha=0.05.

Although absolute changes in mRNA expression between experimental and control cells provides valuable information, normalisation of these data using the four (n=4) internal control genes not associated with molecular carcinogenesis was also performed to assess the magnitude of relative expression changes. This was accomplished using the average mRNA expression change of the internal control genes between control (non-treated) and MLT (experimental) cells as the standard to which all other changes in mRNA expression would be compared. Comparison of relative-fold expression outside of the normal range of the internal control genes (determined as less than one-fold or greater than two-fold relative change in comparison to the internal control average) was used to determine which genes exhibited the most change in overall mRNA expression.

3. RESULTS

RNA was successfully isolated from both oral cancer cell lines (SCC25, CAL27) under experimental (melatonin) and control (no melatonin) conditions (Table 1). These data demonstrated that the quantity of RNA isolated from the SCC25 negative control cells (411.3 ng/µL) was not significantly different than the RNA isolated from SCC25 cells incubated with melatonin (391.2 ng/uL). In addition, the quantity of RNA isolated from the CAL27 negative control cells (383.2 ng/uL) was also not significantly higher than from CAL27 cells under melatonin administration (376.1 ng/uL), p=0.1555 ($\chi 2$=2.018, d.f.=1). Finally, quality of RNA purity was found to be within the expected range of 1.65 – 2.00 for both cell lines and all conditions (range 1.79 – 1.92).

Table 1. Quantification of RNA isolation

Sample	RNA concentration (ng/uL)	RNA purity (A260:A280)
SCC25 – melatonin	391.2	1.88
SCC25 – control	411.3	1.92
CAL27 – melatonin	403.1	1.79
CAL27 – control	383.2	1.82

Having determined the quality and quantity of RNA, qRT-PCR was used to screen for any changes in gene expression following MLT administration, compared with non-treated controls (Fig. 1). These results demonstrated that approximately 40% of these mRNAs (n=37/92) exhibited differential expression in these two cell lines under MLT administration (with the 37 differing gene expressions in CAL27 and SCC25 shown). The relative fold difference in expression compared average mRNA expression among non-treated cells compared with average mRNA expression of treated cells, which revealed 37 genes that were evenly distributed between higher expression among the SCC25 cells (n=19/37) and slightly lower expression (n=18/37) – with no quantitative differences in expression among the remaining 60% of genes under MLT administration (n=55).

Although the absolute change in gene expression provides valuable information – in order to standardise the analysis of these observations, these data were replotted using the relative fold change in gene expression in comparison to the four endogenous control genes under these same conditions (Fig. 2).

These data revealed that melatonin administration resulted in gene expression changes of 57 genes (62%) in SCC25 cells, with most exhibiting a non-significant increase. SCC25 cells exhibited significant changes in a smaller subset of genes, with up-regulation of BCL2, ITGA2B, MAX, NFKB-IA greater than two-fold higher than the internal control genes. Two notable exceptions to this trend were the down-regulation of MAPK and PTEN in SCC25 cells under melatonin administration.

In CAL27 cells, melatonin administration resulted in non-significant changes in gene expression of 65 genes (70%), with all of the genes except one demonstrating higher expression. The gene with the most significant up-regulation was FZD1, which was much more highly expressed following

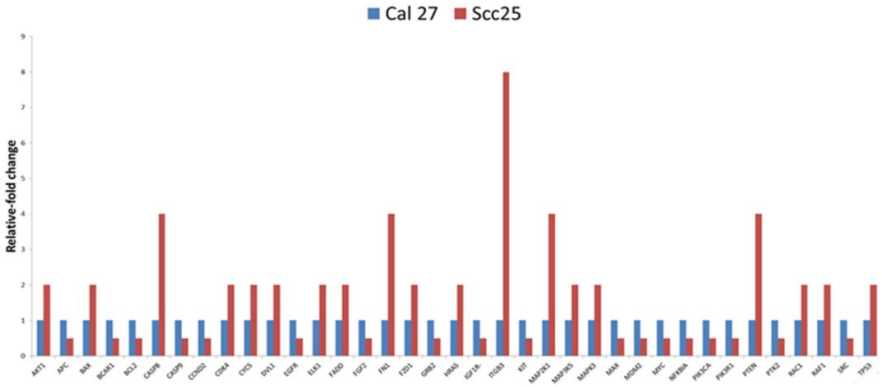

Fig. 1. Differential expression of 37 genes between CAL27 and SCC25 associated with the molecular mechanisms of cancer. Quantification of gene expression among SCC25 and CAL27 oral cancer cell lines demonstrated a majority or 60% (n=55/92) of the selected mRNAs were approximately the same between these two cell lines. A smaller subset of genes (40%, n=37/92) were found to exhibit differential expression, which was nearly equally distributed between higher expression (n=19/37) and lower expression (n=18/37) in SCC25 cells

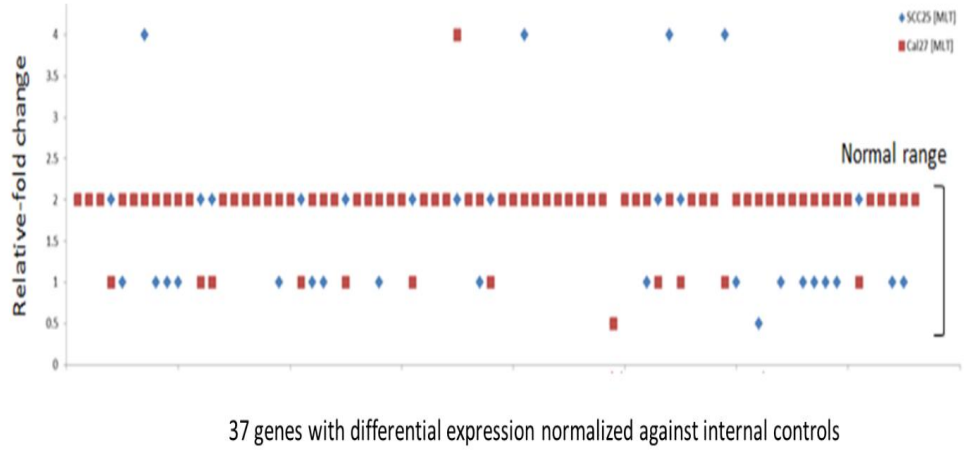

Fig. 2. Differential expression of 37 genes under melatonin administration normalised using four endogenous control genes. The administration of melatonin (10 ug/mL) was sufficient to induce non-significant changes to gene expression in SCC25 (62%, n=57/92) and CAL27 (70%, n=65/92). Significant changes, which were outside the normal range of the internal control genes, were observed only n a minor subset of genes. These results demonstrated an up-regulation of BCL2, ITGA2B, MAX, NFKB-IA and down-regulation of MAPK and PTEN in SCC25 cells. In contrast, CAL27 cells up-regulated FZD1 and down-regulated MAPK3K5

Table 2. Melatonin-induced gene expression in CAL27 and SCC25 cells

		CAL27	SCC25
Down-regulated	MAPK3K5	**Down-regulated**	**Down-regulated**
	PTEN	No change	**Down-regulated**
Up-regulated	BCL-2	No change	**Up-regulated**
	FZD1	**Up-regulated**	No change
	ITGA2B	No change	**Up-regulated**
	MAX	No change	**Up-regulated**
	NFKB-IA	No change	**Up-regulated**

melatonin administration compared with the internal control standards. The one gene which was highly down-regulated in CAL27 cells was MAPK3K5, which was strongly and significantly inhibited by melatonin administration. The data from these assays that demonstrated the genes with the most significant change from the baseline negative controls has been summarised in Table 2.

4. DISCUSSION

Few studies have evaluated the effects of MLT on gene expression and mRNA transcriptional regulation in oral cancers, therefore the primary aim of this study was to investigate MLT-induced changes in gene expression patterns in two different oral cancer cell lines to more fully understand the potential mechanisms that may underlie the oncostatic effects of MLT. The results of this study demonstrated that expression of a small subset of genes associated with the known molecular mechanisms of cancer development are significantly altered by MLT administration.

More specifically, only MAPK3K5 expression was strongly and significantly down-regulated by MLT administration in both CAL27 and SCC25 cells. This finding supports other research, which has demonstrated that MLT also down-regulated MAPK in gastric, colon and breast cancer cells and may be a common mechanism of MLT-induced oncostatic effects [16-18]. The only other gene strongly and significantly down-regulated was PTEN in the CAL27 cell line, which also appears to confirm other research that demonstrates PTEN is not normally affected by MLT administration and has only been observed in one study of gliomas [19].

Conversely, the genes that were strongly and significantly up-regulated by MLT administration appear to be associated with mechanisms associated with cellular protection. For example, the increased expression of apoptosis-associated Bcl-2 has also been observed in the liver, pancreas, and lung cancers under MLT administration [20-22]. In addition, the up-regulation of the pro-inflammatory and immunomodulatory NFKB has also been observed in pancreatic cells under MLT administration [22].

These data may, therefore, suggest that some common mechanisms for MLT-induced cancer inhibition may exist between differing tissues and cell types [23,24]. However, based upon the paucity of evidence to date regarding these mechanisms and the lack of uniform cellular responses between these oral cancer cell lines – it is clear that more research will be needed to uncover the common and disparate mechanisms associated with MLT-induced oral cancer inhibition.

In order to more fully understand these results, a more thorough analysis and discussion of these oral cancer cell lines is warranted. For example, it has been noted that the SCC25 cell line harbours a deletion in the promoter region of cyclin-dependent kinase (cdk1), which may alter the responsiveness of these cells to transcriptional activation and repression following MLT administration [25]. In addition, other studies have found that the CAL27 cell line harbours a non-sense mutation in the SMAD4 gene, causing premature termination of a transcription factor that is an important regulator of transforming growth factor (TGF-β) [26]. However, these represent only two small genetic differences between these two oral cancer cell lines – many more common phenotypes may be found that underlie cellular responsiveness and behaviours in response to chemotactic stimuli that suggest more research may be needed to elucidate these phenomenon [27-29].

5. CONCLUSIONS

MLT administration to oral cancer cells may induce substantial changes in the expression

levels of genes associated with the molecular mechanisms of cancer. However, relatively few of these changes were outside the range of observed values. Therefore, continued analysis and verification of these results in other oral cancers may reveal common MLT-induced changed and provide insights into the potential mechanisms of MLT-induced oncostatic effects in oral cancers.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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