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Original Article

Alterations in *APC*, *BECN1*, and *TP53* gene expression levels in colon cancer cells caused by monosodium glutamate

Alterações nos níveis de expressão gênica APC, BECN1 e TP53 em células de câncer de cólon causadas por glutamato monossódico

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Abstract

Colorectal cancer (CRC) is a disease with high incidence worldwide. As of 2018, it is the second leading cause of cancer deaths in the world. In Saudi Arabia, the incidence of this disease has been increasing in the younger population. Both genetic and lifestyle factors may have contributed to its increased incidence and pathogenesis. Monosodium glutamate (MSG) is a food flavor enhancer that can be found in many commercial foods, and it can sometimes be used as a substitute to table salt. MSG has been investigated for its possible genotoxicity, yielding controversial results. In the present study, the effect of MSG on cell viability and its effect on expression of *APC*, *BECN1*, and *TP53* genes in SW620 and SW480 colon cancer cell lines were studied. *TP53* is a tumor suppressor gene that functions in modifying DNA errors and/or inducing apoptosis of damaged cells, and both *APC* and *BECN1* genes are involved in CRC and are of importance in cellular growth and metastasis. Cancer cell viability was analyzed using MTT assay, and the results showed a significant increase in the number of viable cells after 24 h of treatment with MSG with different concentrations (0.5, 1.0, 10, 50, and 100mM). Moreover, gene expression results showed a significant increase in the expression levels of *APC* and *BECN1* under specified conditions in both cell lines; conversely, *TP53* showed a significant decrease in expression in SW620 cells. Thus, it can be concluded that MSG possibly confers a pro-proliferative effect on CRC cells.

Keywords: MSG, SW620 and SW480 cell lines, APC, BECN1, TP53.

Resumo

O câncer colorretal (CCR) é uma doença com alta incidência mundial. Desde 2018, é a segunda principal causa de mortes por câncer no mundo. Na Arábia Saudita, a incidência dessa doença vem aumentando na população mais jovem. Tanto fatores genéticos quanto de estilo de vida podem ter contribuído para o aumento da sua incidência e patogênese. O glutamato monossódico (MSG) é um intensificador de sabor de alimentos que pode ser encontrado em muitos alimentos comerciais e às vezes pode ser usado como um substituto do sal de cozinha. O MSG tem sido investigado por sua possível genotoxicidade, produzindo resultados controversos. Neste estudo, foram estudados o efeito do MSG na viabilidade celular e seu efeito na expressão dos genes APC, BECN1 e TP53 em linhas de células de câncer de cólon SW620 e SW480. TP53 é um gene supressor de tumor que atua modificando erros de DNA e/ou induzindo apoptose de células danificadas, estando os genes APC e BECN1 envolvidos no CRC e sendo importantes no crescimento celular e metástase. A viabilidade das células cancerosas foi analisada por meio do ensaio MTT, e os resultados mostraram um aumento significativo no número de células viáveis após 24 h de tratamento com MSG em diferentes concentrações (0,5; 1,0; 10; 50 e 100mM). Além disso, os resultados da expressão gênica mostraram um aumento significativo nos níveis de expressão de APC e BECN1 sob condições especificadas em ambas as linhagens celulares. Por outro lado, TP53 mostrou uma diminuição significativa na expressão em células SW620. Assim, pode-se concluir que, possivelmente, o MSG confere um efeito pró-proliferativo às células CRC.

Palavras-chave: MSG, linhas de células SW620 e SW480, APC, BECN1, TP53.

1. Introduction

Colorectal cancer (CRC) is the second leading cause of cancer deaths around the world (Bray et al., 2018; Stewart and Wild, 2014). In Saudi Arabia, the incidence of CRC is increasing among the younger population according to studies around different regions of the country (Mansoor et al., 2002; Guraya and Eltinay, 2006; Amin et al., 2012). CRC has been defined as a malignant tumor made up of the epithelial cells of the colon and/or rectum (Hamilton and Aaltonen, 2000). It can be caused by multiple factors that are either genetic or environmental in nature. It has

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been found that 35% of the causes of CRC are partially or completely genetic (Burn et al., 2013). Environmental factors including food intake, smoking, obesity, sedentary lifestyle and environmental changes such as pesticides exposure and dietary habits are also associated with increased risk for CRC (Bishehsari et al., 2014).

It has also been suggested that environmental and lifestyle factors can stimulate genetic alterations that can lead to cancer. (Lindor et al., 2008; Lee et al., 2015) . In both familial and sporadic CRCs, genetic abnormalities have been associated with the development of the disease (Fearon, 2011). Some of the several genes involved in the development and progression of CRC are *Adenomatous Polyposis Coli* (*APC*), *Beclin1* (*BECN1*), and *Tumor Protein p53* (*TP53*) (Hamilton and Aaltonen, 2000; Wu et al., 2015; Zhan et al., 2017).

The gene TP53 is the most commonly mutated gene in all cancers and is a center of interest in cancer research (Soussi, 2000). *TP53* acts by preventing mutations during cell growth and maintaining cell stability; when DNA damage occurs, *TP53* functions in DNA repair and/or induction of apoptosis in damaged cells (Rowland and Peeper, 2006). However, most CRC cases start with a mutation in the *APC* gene (Zhan et al., 2017). *APC* is a tumor suppressor gene that is a part of the Wnt signaling pathway, which is involved in cellular proliferation, differentiation, and migration in embryonic development; the Wnt pathway is also involved in regeneration and cellular hemostasis processes in adulthood (Krausova and Korinek, 2014). *BECN1* is another gene that is involved in CRC. It has an important role in CRC growth and metastasis by enabling the reuse of waste materials as an energy source in order to maintain cellular homeostasis and survival in a process called autophagy (Koukourakis et al., 2010; Gil et al., 2017)

There is increasing evidence proving the correlation between the risk of CRC and diet and lifestyle (Winkels et al., 2014). Food can directly modify colonic health due to its direct contact with the colonic epithelium (O'keefe, 2016; Grosso et al., 2017) . A common food flavor enhancer that is used worldwide, monosodium glutamate (MSG), has been longer under investigation due to its possible toxic effects (Quines et al., 2014). It is one of the major flavoring enhancers used globally. It can be found in abundance in Chinese food, fast food, potato chips, as well as in many other commercial foods (Livingstone, 1981). It is a center of interest in research as it has yielded hugely contradictory results in relation to its safety and mutagenic effects; thus, it requires further investigation for more definitive conclusions, especially since many studies have suggested its genotoxic and mutagenic effects (Ataseven et al., 2016). Some researchers found evidence suggesting that the consumption of MSG poses risks to humans, especially to children (Plaitakis and Shashidharan, 2000). It has been demonstrated that Monosodium glutamate has a remarkable neurotoxicity impact on the short-term spatial memory of rats through oxidative stress-induced degenerative changes and apoptosis of brain tissue as well as consistent systolic hypotension (Clough et al., 1986; Hazzaa et al., 2020).

As a flavor enhancer, MSG is commonly added to foods due to its unique taste of umami (Plaitakis and Shashidharan, 2000). Umami taste receptors T1R1 and T1R3 are found to be expressed in tissues of the gastrointestinal tract, including the stomach, small intestine, and colon (Bezençon et al., 2007). A study reported by Hata and colleagues showed that treatment of mice with subcutaneous MSG caused hyperinsulinemia, diabetes, hypertriglyceridemia, obesity, hyperlipidemia, and insulin resistance; these abnormalities turned the mice more vulnerable to the development of colon cancer induced by azoxymethane (Hata et al., 2012). Additionally, Shoji and colleagues observed an increase in the gene expression of T1R1 and T1R3 via quantitative reverse transcription PCR (qRT-PCR) after stimulating the tongue with MSG (Shoji et al., 2015). According to Zhang *et al*., those genes were also found to be upregulated in both the stomach and jejunum of piglets upon oral consumption of MSG (Zhang et al., 2013).

Taking all of the above into account, together with the available data from preclinical studies and clinical trials regarding the toxic effects of MSG chronic dietary human exposure (Zanfirescu et al., 2019), it was interesting to evaluate the relevance of MSG as colorectal carcinogenic substance at the genetic level.

In the current study, the effects of MSG treatment on cell viability and the expression levels of *APC*, *BECN1*, and *TP53* were tested in CRC cell lines SW620 and SW480.

2. Materials and Methods

2.1. Cell culture

Human colorectal adenocarcinoma cells SW620 and SW480 originally obtained from the American Type Culture Collection (ATCC, USA) were kindly provided by the core facility of the Central Laboratory at King Saud University, Riyadh, Saudi Arabia. Cells were cultured in Dulbecco's Modified Eagle Media (DMEM; LOT # 70831-1, Millipore, Merck, USA) optimized by supplementing with 10% fetal bovine serum (FBS; LOT # 1579062, Gibco, Life technologies, USA), 1% L-glutamine (GIBCO, LOT # 803633, Invitrogen, UK), and 1% penicillin-streptomycin (Gibco, LOT # 1824315, Life technologies, USA) in a 5% CO_2 incubator at 37°C. Cells were allowed to grow until 70–90% confluence. Cell lines health monitoring was conducted before study started, by using Trypan Blue to measure cell viability and also, through counting the cells by means of a cell counter.

2.2. MTT assay

The cell lines SW620 and SW480 were seeded in 96-well tissue culture plates at a density of 2×10^5 cells per well in 100 µL optimized DMEM media; then, cells were allowed to settle for 24 h prior to the treatment. Treatment with different concentrations of MSG took place the next day by adding 100 µL of 0 mM (control), 0.5 mM, 1.0 mM, 10 mM, 50 mM, or 100 mM MSG dissolved in optimized DMEM media; each treatment concentration was performed in four replicates. Cells were incubated for 24 h and 48 h as recommended in the literature (Schønberg et al., 2006; Badrzadeh et al., 2014), and then the cells were treated with 100 µL of 5 mg/mL 0.22-µm filter-sterilized MTT

(SIGMA, LOT # MKBN7264V, Sigma-Aldrich, USA). The plates were kept in the dark for 2 h. The optical density of the samples was measured at an absorbance of 540 nm using the SpectraMax Plus 384 Microplate Reader (Molecular Devices).

2.3. MSG Treatment

The treatment with MSG was performed after the cells were settled in five 10-mm cell culture dishes at a concentration of 1.3×10^6 cells/mL. Cells were treated with 0 mM (control), 10 mM, or 100 mM MSG for periods of 24 h and 48 h. The treatment concentrations were chosen based on the MTT assay results; these two concentrations (10mM and 100mM) allowed us to have both high and moderate concentrations of MSG treatment.

2.4. RNA Extraction

Cells were collected in a 15-mL centrifuge tube, centrifuged at 2000×*g* for 5 min at 4°C, and the supernatant was discarded. RNA extraction was performed using PureLink RNA Mini kit (Ambion, Cat # 12183020, Thermo Fisher Scientific, USA) following the manufacturer's protocol.

2.5. RNA Quality and Quantity Determination

NanoDrop Spectrophotometer was used to measure the quality and quantity of the RNA. For further confirmation of RNA integrity, samples were run in a 2% agarose gel prepared by dissolving 1 g of agarose powder (Promega, LOT # 290218, Spain) in 50 mL of 1× TBE Buffer (Gibco, Cat. # 15581-044, Life Technologies, USA); the mixture was microwaved until fully dissolved and clear, and then added with 1.5 µL of ethidium bromide (Promega, Cat # H6041, Spain). The gel was left to solidify in the chamber, and then the samples were loaded by mixing 3 µL of the sample with 4 µL of the orange tracking dye. The results were then read using the BIO-RAD Gel Documentation System.

2.6. Reverse transcription

Reverse transcription was carried out in the Applied Biosystems Veriti 96 Well Thermal Cycler using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, USA) following the manufacturer's protocol.

2.7. Gene expression

Gene expression was measured by qRT-PCR using the QuantiTect SYBR Green PCR Kit (Qiagen, Cat # 204143, USA) and the Applied Biosystems ViiA7 (Life Technologies) following the manufacturer's protocol and the expression levels were then calculated using the formula $2^{-\Delta\Delta C_T}$ (Winer et al., 1999). *GAPDH* was used as the housekeeping gene in this experiment. Primers sequences were presented in the Table 1.

2.8. Statistical analysis

Two statistical methods were used in this experiment using the SPSS statistics software version 25.0 (IMB Corporation, New York, U.S.). For the MTT Assay, one sample t-test was applied with 100% being the test value for each timeframe at p=0.05 in reference to the control sample. For gene expression results, both one-way ANOVA and one sample t-test were applied for each gene separately at p=0.05, the statistics were to compare different doses and different timeframes in reference to the control.

3. Results

3.1. MTT assay

This test was performed to evaluate the percentage of viable cancer cells after being treated with different concentrations of MSG for either 24 h or 48 h in reference to the control sample (100%).

MTT assay showed a highly significant increase in the number of viable cancer cells after treatment with various concentrations of MSG for 24 h for both cell lines SW620 and SW480 ($p \leq 0.05$); there was also an increase after treatment with the same concentrations for 48 h, but it was not statistically significant for SW620 cells ($p > 0.05$) (Figure 1). The concentrations 10 mM and 100 mM were chosen and used for subsequent experiments as these concentrations yielded good results for both 24 h and 48 h treatment durations; in addition, these two concentrations

Table 1. Primers for real-time RT-PCR.

Gene Symbol		Sequences
GAPDH	Forward:	AATGGGCAGCCGTTAGGAAA
	Reverse:	GCCCAATACGACCAAATCAGAG
APC	Forward:	GACTCGGAAATGGGGTCCAA
	Reverse:	TCTTCAGTGCCTCAACTTGCT
BECN1	Forward:	GTAAAACGACGGCCAGTGCTGTTTGGAGATCTTAGAGCAA
	Reverse:	CAGGAAACAGCTATGACCAGCTCATCATCCAGCTCCAG
TP53	Forward:	GTAAAACGACGGCCAGTGTCTGGGCTTCTTGCATTCT
	Reverse:	CAGGAAACAGCTATGACCCCAAATACTCCACACGCAAA

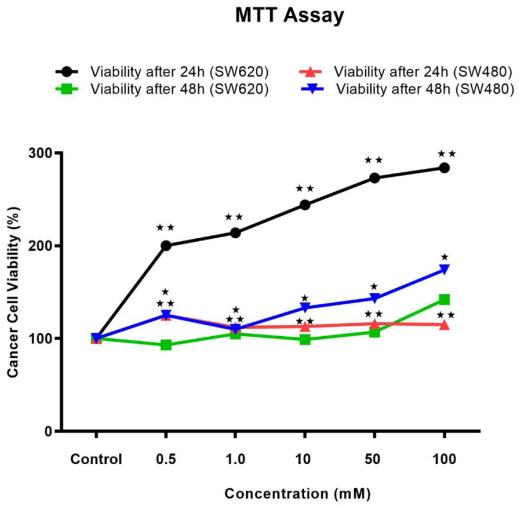

Figure 1. Cancer cell viability in percentage after treatment with MSG for 24 h or 48 h; *$p \leq 0.05$ vs control (0 mM) using one-sample t-test.

allowed us to have both high and moderate concentrations as treatments.

3.2. Gene expression

The effects of MSG on the expression of *APC*, *BECN1*, and *TP53* were measured in comparison to that from a control sample (0 mM MSG) in cancer cell lines SW620 and SW480.

Gene expression analysis revealed a significant increase in the expression of *APC* in all treatment conditions in both cell lines compared to that in the control ($p \leq 0.05$) (Figure 2). In *BECN1* a statistically significant increase in expression ($p \leq 0.05$) was observed at 48 h of treatment with MSG in both cell lines, while the other treatment conditions the increase in expression was not statistically significant ($p > 0.05$) (Figure 3). *TP53* gene showed a significant decrease in its expression in SW620 cells under most of the treatment conditions employed in the experiment, while its expression in SW480 cells had non-significantly increased (Figure 4).

4. Discussion

In the present study, MSG, a common food flavor enhancer, was investigated for its effect on the proliferation of CRC cell lines and on the relative gene expression levels of *TP53*, *BECN1*, and *APC* via MTT assay and qRT-PCR, respectively, as none of these tests have been done before for MSG in CRC. However, many studies have identified toxic effects of MSG both at the cellular and molecular level (Olney, 1969; Rivera-Carvantes et al., 2017).

The MTT results suggest a proliferative effect of MSG on CRC cells. According to Sudha and colleagues, 10 mM MSG caused a decrease in viability of a culture of normal retina (Sudha et al., 2016); a decrease in viability was also detected by Shah *et al*. when they treated IMR-

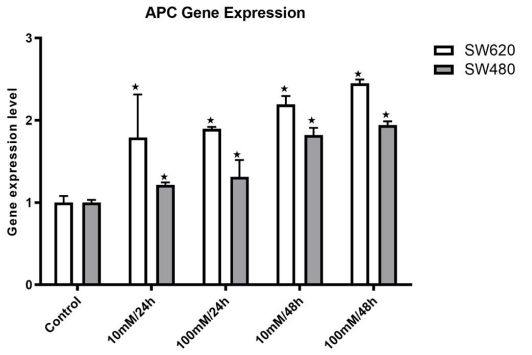

Figure 2. Expression levels of *APC* (mRNA *APC*/mRNA *GAPDH*) in SW620 and SW480 cells treated with either 10 or 100 mM MSG for either 24 or 48 h, data are expressed as mean ±SD (n = 2 samples/group); *$p \leq 0.05$ vs control value tested both by one-way ANOVA and one-sample t-test.

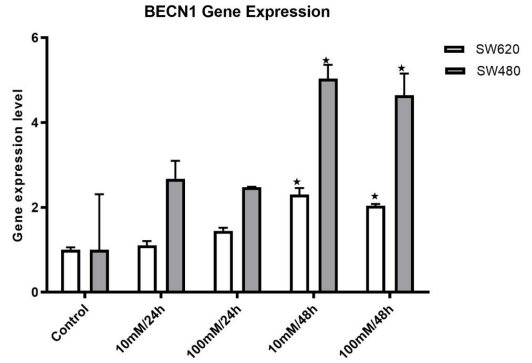

Figure 3. Expression levels of *BECN1* (mRNA *BECN1*/mRNA *GAPDH*) in SW620 and SW480 cells treated with either 10 or 100 mM MSG for either 24 or 48 h, data are expressed as mean ±SD (n = 2 samples/group); *$p \leq 0.05$ vs control value tested both by one-way ANOVA and one-sample t-test.

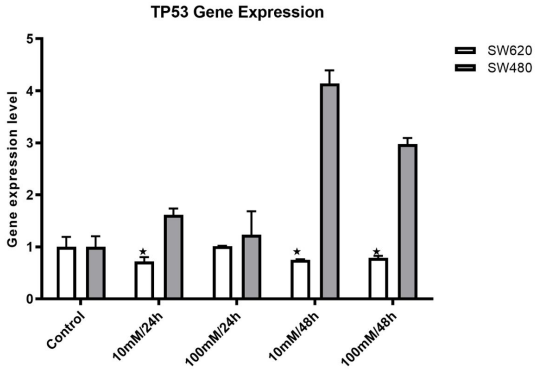

Figure 4. Expression levels of *TP53* (mRNA *TP53*/mRNA *GAPDH*) in SW620 and SW480 cells treated with either 10 or 100 mM MSG for either 24 or 48 h, data are expressed as mean ±SD (n = 2 samples/group); *$p \leq 0.05$ vs control value tested both by one-way ANOVA and one-sample t-test.

32 neuroblastoma cells with various concentrations of MSG (1–10 mM)(Shah et al., 2018) .

APC gene expression was significantly increased in this study, and as mentioned previously, is mutated in the SW620 cell line, and mutations in this gene are associated with the loss of tumor suppression and the gain of functions that enhance proliferation, migration, and resistance to apoptosis (Zhang et al., 2013). It has also been seen by Huang and Guo that cells expressing mutant APC^{Q1338*}, as observed in SW620 and SW480, were resistant to valproic acid-induced apoptosis, while cells with wild-type *APC* were not resistant (Huang and Guo, 2006). This may explain the increase in viable CRC cells measured through MTT assay. On the other hand, the non-significant increase of SW620 CRC might be attributed to the dose and time dependent effect of MSG on mitotic colonal expansion phase (MCE) phase leading to complete inhibition of mitotic activity after 48 hours (Türküner and Özcan, 2020) .Beclin 1 has a central role in autophagy in mammalian cells and is considered as a key regulator of autophagosome formation. In this experiment, *BECN1* showed an increase in expression and according to a study conducted in China by Wu and colleagues they observed an increase in both gene and protein expression of *BECN1* in CRC tissues compared to that in normal tissues. Researchers suggested that the correlation between *BECN1* and cancer can be attributed to the fact that cancer cells are highly proliferative and are subject to loss of nutrients and metabolic stress; by increasing the expression of *BECN1*, cancer cells may undergo autophagy, which promotes vascularization, and induce cell survival and resistance to treatments(Wu et al., 2015) .

For *TP53*, the decrease in expression in SW620 and increase in expression in SW480 can be explained by the different mechanisms being employed by the sister cell lines SW620 and SW480 as to Schonberg and colleagues, clarifying that cells that are closely related can still exhibit different responses (Schønberg et al., 2006). However, these cell lines are known to be double mutants of *TP53*. Rochette *et al*. studied the *TP53* gene and its protein product in SW480 cells, and their results suggested that *TP53* with mutations R273H and P309S is still capable of activating genes and proteins associated with DNA repair (Schwitalla et al., 2013) . Since, in this experiment, MSG down-regulated $TP53^{R273H/P309S}$ in SW620 cells, which still have wild-type *TP53* properties, its down-regulation can lead to a loss of tumor suppression and promote colorectal tumorigenesis (Schwitalla et al., 2013); on the other hand, the up-regulation of the gene in SW480 was not statistically significant ($p > 0.05$).

In the present study, the recorded increase of CRC viability in MSG- treated cells can be attributed to intestinal epithelial dysfunction as toxic effect of MSG recently recognised by Türküner and Özcan (2020). Induced epithelial dysfunction, in turn can be related to the remarkable increase of gut bacterial translocation, increase of hepatic lipid droplets, and decrease of rough endoplasmic reticulum activity, collectively induce proliferation of CRC (Türküner and Özcan, 2020).

In conclusion, different treatment concentrations of MSG have a direct effect on CRC cells. The increase in viability of cancer cells with the increase in expression of both APC^{Q1338*} and *BECN1*, accompanied by the decrease in expression of $TP53^{R273H/P309S}$ led to the understanding that MSG potentially has a pro-proliferative effect on CRC cell lines, SW620 and SW480, both at the genetic and cellular level. Consumption of MSG as a flavor enhancer by CRC patients is unrecommended until further investigations are done.

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