

Original Article

Study of 5HT₃ and 5HT₄ Receptors Expression in HT29 Cell Line and Human Colon Adenocarcinoma Tissues

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Background: Serotonin (5HT) has been shown to be a mitogenic factor in several carcinomas. Its mitogenic effect is elicited through a wide range of 5HT receptor subtypes. In this study, the effects of 5HT, 5HT₃ (1-phenylbiguanide hydrochloride) and 5HT₄ (cisapride) agonists in promoting the growth of the HT29 cell line and the growth-inhibition effect of the 5HT₃ receptor antagonist (Y-25130 hydrochloride) and 5HT₄ receptor antagonist (RS 23597-190) were investigated. The expressions of 5HT₃ and 5HT₄ receptors in human colon cancer tissues and the HT29 cell line were studied.

Methods: The growth-promoting and growth-inhibition effects of 5-HT, 5HT₃ and 5HT₄ agonists and antagonists on the HT29 cell line were studied using MTT assay. Receptor expression has been demonstrated by western blotting.

Results: The results showed that 5HT, 5HT₃, and 5HT₄ agonists caused significant proliferation of HT29 cells. 5HT₃ and 5HT₄ receptor antagonists had an inhibitory effect on the growth of these cells. Western blot analysis gave bands from colon tissue extracts and the HT29 cell line.

Conclusion: The results indicate which 5HT₃ and 5HT₄ receptors are significantly expressed in both colon cancer tissue and the HT29 cell line. Expression for the 5HT₃ receptor is more potent. Furthermore, 5HT plays a mitogenic role in colon cancer cells and antagonists of 5HT₃, and 5HT₄ receptors can inhibit cancer cell growth.

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Introduction

Colorectal cancer is one of the major causes of cancer-related deaths in the world. Over the last decade, much progress has been made in the treatment of this malignancy and new chemicals have been introduced. The survival of people with metastatic colorectal cancer has improved with new therapeutics. Recently, selective serotonin reuptake inhibitors (SSRIs) have been suggested as preven-

tive agents for people at high risk of developing colorectal cancer,¹ and is based on reports indicating that SSRIs could decrease the risk of colorectal cancer in humans.² Laboratory studies have shown that fluoxetine, a novel SSRI drug, at doses of 10 – 100 μM may reduce the growth of cancer cells *in vitro*. This effect may be mediated by increasing serotonin levels in the peripheral nerve endings of the gastrointestinal tract.³ However; other reports suggest that SSRIs may induce mammary tumors in animals and breast cancer in humans.^{4,5}

Serotonin (5-hydroxytryptamine; 5HT) is a neurotransmitter that mediates a wide variety of physiological effects (both peripherally and centrally) by binding to multiple receptor subtypes. The major sites of 5HT synthesis and storage are located in the periphery, the gut enterochromaphin cells and blood platelets, respectively. The great

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diversity of 5HT functions is due to different 5HT receptors and the pharmacological complexity of these receptors, reflecting the second messenger system to which the receptors are coupled. The 5HT family, including 5HT₁ and 5HT₅ subtypes of receptors negatively interact with adenylate cyclase and the 5HT₂ subfamily of receptors is coupled to the activation of phospholipase C-β. Adenylate cyclase is activated by the 5HT₄, 5HT₆, and 5HT₇ subtypes of receptors.⁶

5HT₃ receptors are cation channels that tend to depolarize the membrane when activated.⁷ 5HT₃ receptors contain four transmembrane domains and exist as homo- or hetero-pentamers in the membrane. Two 5HT₃ receptor subunits have been identified: 5HT_{3A} and 5HT_{3B}.^{8,9} The 5HT_{3A} subunit can form a functional channel as a homo-pentamer, but the 5HT_{3B} subunit alone does not. In addition to two known 5HT₃ subunits, two splice variants of 5HT_{3A} have been identified by Brüss et al.¹⁰ which further increases the functional diversity of these receptors. It is possible that additional, undiscovered 5HT₃ receptor subunits exist.

The 5HT₄ receptor was first characterized in mouse collicular neurons.¹¹ It is also found in the hippocampus and in peripheral tissues such as the guinea pig ileum, rat esophagus, and human atrium.¹² The 5HT₄ receptor has been suggested to contribute to tumor cell progression by secretion of hormones and other growth factors.¹³

5HT₃ receptor is expressed in the enteric nervous system^{14,15} and plays an important role in enteric physiology. Although there is some evidence regarding the mitogenic effects for 5HT in certain malignancies such as breast, prostate, and bladder cancer¹⁶⁻¹⁸; to date, however, there have been no studies specifically assessing the role of 5HT and expression of 5HT₃ and 5HT₄ receptors in colon cancer. There are some reports concerning the mitogenic role of serotonin in colon cancer. For example, in one study, the influence of serotonin in dimethylhydrazine-induced adenocarcinoma of the colon has been reported.^{19,20} However, few studies have been conducted that examine the possible role of 5HT₃ and 5HT₄ receptors' agonists and antagonists in tumor regulation. In the present study we have determined the effect of 5HT, 5HT₃, and 5HT₄ agonists and antagonists on the proliferation of the HT29 human colon adenocarcinoma cell line. We also studied the expression of 5HT receptor subtypes 3 and 4 in this cell line and in colon cancer tissue.

Materials and Methods

Tissues and cell line

Human colon cancer tissue samples were obtained during surgery from five patients (Imam Hospital, Tehran, Iran). Tissue samples were used for western blot analysis.

The HT29 cell line was kindly provided by the National Cell Bank of Iran (NCBI), Pasteur Institute of Iran. It must be noted that HT29 is a current human cell line with potent metastatic potential which has been widely used in colorectal carcinoma studies. With respect to this and according to other studies,²¹ we selected this cell line for our study. The cells were maintained in exponential growth phase in 25-cm² flasks in RPMI 1640 (Gibco, Germany) medium supplemented with 10% heat-inactivated fetal calf serum (Gibco, Germany), 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (all from Sigma, Germany) in a humidified atmosphere of 5% CO₂ at a constant temperature of 37°C. Cells were split when they reached a confluency of approximately 90 – 95%.

MTT proliferation assay

HT29 cells were washed with Phosphate buffer saline (PBS) and harvested with a 0.5% trypsin (Sigma, Germany) solution at 50 – 60% confluency. Cells were then added to wells at a density of 10⁴ cells/well in a 96-well plate to a final volume of 100 µL/well. After 24 hours of incubation at 37°C in a 5% CO₂ atmosphere, the culture medium was replaced with 200 µL fresh culture medium containing 5HT hydrochloride (Sigma, Germany), 1-phenylbiguanide hydrochloride (5HT₃ agonist), cisapride (5HT₄ agonist), Y-25130 hydrochloride (5HT₃ antagonist), or RS 23597-190 (5HT₄ antagonist; all from Tocris Laboratories, UK) at concentrations of: 3.125, 6.25, 12.5, 25, 50, and 100 µM. Cells cultured solely in media served as negative controls. After 48 hours of incubation at 37°C in a 5% CO₂ atmosphere, the culture medium was removed and 8 µL MTT reagent (diluted in PBS at a concentration of 4 mg/mL; Sigma, Germany) was added to 50 µL of fresh culture medium at a final concentration of 0.55 mg/mL. The optimum incubation period time was determined in a pilot study.

After incubating the plates again for a 4 hour at 37°C in 5% CO₂ atmosphere, the resulting formazan product was resolved in 50 µL dimethyl sulfoxide (Merck Germany) and measured

spectrophotometrically at 570 nm (with a reference wavelength of 690 nm). The mitogenic effect was determined using the following formula: (mean absorbance (570 – 690 nm) of treated wells/mean absorbance (570 – 690 nm) of control wells) $\times 100$.

Results were expressed as the mean of five assays (three replicates for each concentration of each assay).

Protein extraction

HT29 cells were harvested with a 0.5% trypsin solution at 90 – 95% confluency and resuspended in lysis buffer containing a complete protease inhibitor (Roche, Germany), 1% SDS, tris 50 mM, pH 7.4; 0.5% sodium deoxycholate (Sigma, Germany). The lysate was incubated for 30 minutes at 4°C. Subsequently, the suspension was centrifuged at 12000 g for 20 minutes at 4°C and supernatant was kept at -70°C.

Frozen samples of colon cancer tissue and rat brain as positive controls were homogenized in lysis buffer and the proteins were extracted as described above. Protein concentrations of supernatants were determined by the Bradford assay, with BSA as a standard.

Western blot analysis

Equal amounts of protein samples were heated at 100°C for 5 minutes in 6 \times SDS gel-loading buffer consisting of 375 mM Tris-HCl (pH 6.8), 12% SDS, 60% glycerol, 30% 2-mercaptoethanol, and 0.6% bromophenol blue. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 5% stacking and 10% separating gels. Proteins were then transferred to an Immobilon-P PVDF membrane (BioRad, Germany). Nonspecific binding sites were blocked by 1% bovine serum albumin in PBS (BSA-PBS). The membrane was subsequently incubated for 1 hour with goat anti-5HT₃ or goat anti-5HT₄ antibody (Santa Cruz, USA). After incubation with antibody, the membrane was washed three times, for 15 minutes each, with 50 mL of PBS containing 0.05% Tween20 (PBS-T). The membrane was then incubated for 1 hour with peroxidase conjugated donkey anti-goat IgG (Santa Cruz, USA) and was washed as mentioned above. The membrane was developed with diaminobenzidine (DAB; Sigma, Germany). Normal marginal tissues from the same patients were used as negative controls.

Statistical analysis

The results were expressed as the mean \pm SD.

Statistical differences were evaluated by one-way ANOVA. $P \leq 0.05$ was considered significant.

Results

MTT assay

The MTT assay was used to determine the effects of 5HT, 5HT₃, and 5HT₄ receptor agonists and antagonists on human colon cancer cell line proliferation. Cells were treated with increasing concentrations of drugs and the numbers of cells after incubation were determined spectrophotometrically using the MTT reagent.

As shown in Figure 1, 5HT, 5HT₃ agonist (1-phenylbiguanide hydrochloride), and the 5HT₄ agonist (cisaperide) caused a dose dependent proliferation of HT29 cells after 48 hours incubation. The maximum proliferation was at a

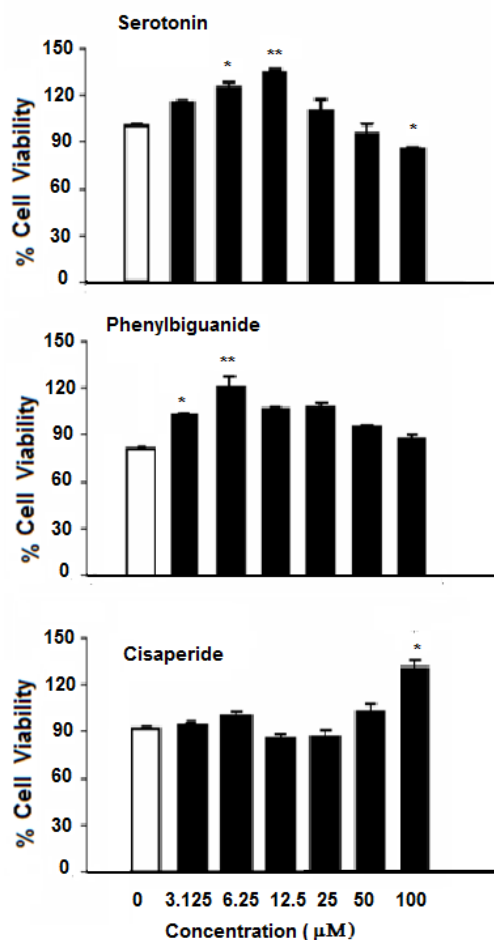


Figure 1. Effects of 5HT, 5HT₃, and 5HT₄ agonists on human colon cell proliferation. Cells were treated with increasing concentrations of drugs and cell proliferation was determined by MTT proliferation assay. Results represent the mean \pm SD values. (* $P \leq 0.05$, ** $P \leq 0.01$)

5HT concentration of 12.5 μM ($P \leq 0.01$). 5HT₃ agonists significantly stimulated the growth of cells at concentrations of 3.125 μM ($P \leq 0.05$) and 6.25 μM ($P \leq 0.01$). Stimulation of cell growth with 5HT₄ receptor agonist (cisapride) needed a higher concentration of related agonist. A significant growth stimulatory effect was observed at 100 μM ($P \leq 0.05$).

5HT₃ receptor antagonist (Y-25130 hydrochloride) and 5HT₄ antagonist (RS 23597-190) had dose-dependent inhibitory effects on cell growth (Figure 2). The 5HT₃ antagonist significantly inhibited proliferation of HT29 cells at concentrations of 50 and 100 μM ($P \leq 0.01$). The 5HT₄ receptor antagonist inhibited proliferation of the colon cell line at only the 100 μM concentration ($P \leq 0.01$).

Western blot analysis

Western blot analysis of HT29 cells and human colorectal tumor tissues has confirmed expression of 5HT₃ and 5HT₄ receptor proteins. At least three different western blots were performed with total proteins extracted from each sample. Polyclonal anti-5HT₃ IgG recognized a single band of approximately 48 kDa, and anti-5HT₄ IgG showed a band of approximately 44 kDa in the HT29 cell line and cancer tissues. Proteins isolated from the rat

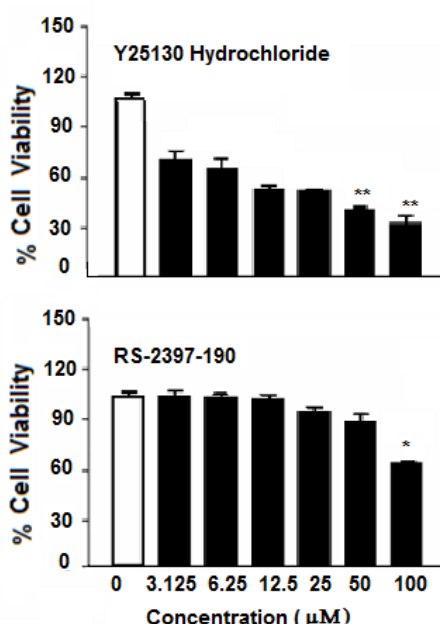


Figure 2. Antagonist to receptor 5HT₃ (Y-25130 hydrochloride) significantly inhibited proliferation of HT29 cells at concentrations of 50 and 100 μM ($P \leq 0.01$). 5HT₄ antagonist (RS 23597-190) inhibited proliferation of colon cell line at 100 μM ($P \leq 0.01$)

brain (positive control) revealed similar bands to those of proteins isolated from the HT29 cell line and human colorectal tumor tissues (Figure 3).

Discussion

In the present study, for the first time, we have demonstrated that 5HT₃ and 5HT₄ serotonergic receptors are expressed in a human colon cancer cell line (HT29) and in the tumor tissues from patients with colon carcinoma.

Moreover, MTT proliferative assays which measure cell viability through the reductive activity of mitochondria revealed that 5HT, 5HT₃, and 5HT₄ agonists stimulate HT29 cell proliferation. It has been shown that 5HT acts as a growth factor for several types of non-tumor and tumor cells including the choriocarcinoma, breast, prostate, and bladder cancer cell lines.^{16-18,22} Given the wide distribution of 5HT₃ and 5HT₄ receptors in the alimentary tract, we were interested to determine if the mitogenic effect of 5HT was mediated by these receptors. The

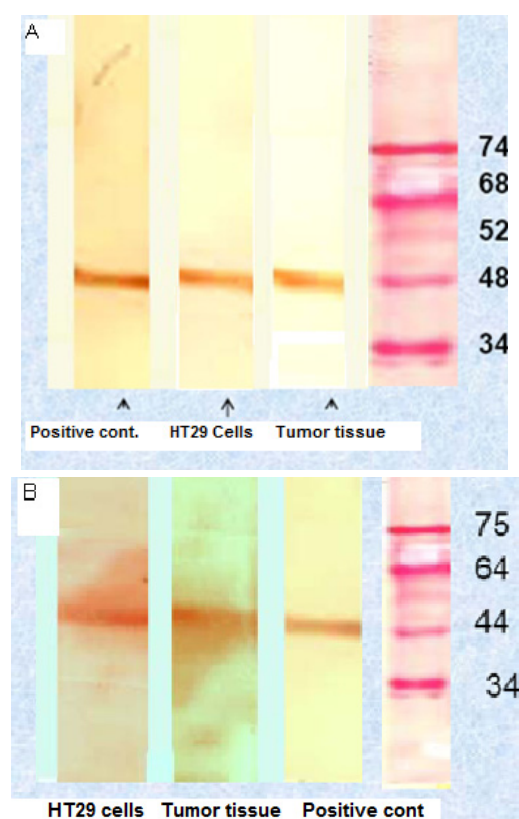


Figure 3. Western blot analysis showing expression of 5HT₃ and 5HT₄ receptors in HT29 cell line and human colon cancer tissue. Polyclonal anti-5HT₃ IgG recognized a single band of approximately 48 kDa (A), and anti-5HT₄ IgG showed a band of approximately 44 kDa in HT29 cell line and cancer tissues.

proliferation of the HT29 cell line in response to 5HT₃ and 5HT₄ agonists suggested that the mitogenic effect of 5HT on this cell line was mediated in part by 5HT₃ and 5HT₄ receptors. Siddiqui et al. have shown that 5HT₁ but not 5HT₂ and 5HT₃ exert cell growth in bladder cancer cells.¹⁶ This effect is mediated by 5HT₂ in a breast cancer cell line.¹⁷ Although 5HT₁ and 5HT₂ receptors are known as mitogenic receptors, the mitogenic activity of 5HT₃ receptors is a novel finding. The mitogenic effect of 5-HT via a 5HT_{2A} receptor has been described as being mediated by different signal transduction pathways, including the Jak/STAT²³ and Erk/MAPK activation.^{24,25} The signal transduction pathway of the mitogenic effect of 5HT₃ receptor remains to be revealed.

Likewise, the 5HT₄ receptor agonist results in HT29 cell line proliferation. Receptor subtype 4 has been shown to stimulate hormone secretion. The agonist to 5HT₄ stimulates cortisol secretion in patients with adrenal tumors and IGF-1 secretion in mice fibroblasts.²⁶ It has been suggested that 5HT₄ may contribute to tumor cell progression by stimulating hormonal secretion and other growth factors, probably via activation of adenylate cyclase.²⁷

Moreover, 5HT₃ and 5HT₄ antagonists inhibited HT29 cell growth. In line with our findings, the anti-proliferative effect of RS 23597-190 (5HT₄ antagonist) has also been reported in the DU145 prostate cancer cell line.²⁸ However, the 5HT₃ and 5HT₄ antagonists had no significant inhibitory effects on cell growth of the bladder cancer cell line 16 and PC3 prostate cancer cells.^{16,29}

To further confirm the role of 5HT₃ and 5HT₄ receptors in regulation of the colon cell line growth, western blot analysis was carried out. In the present study western blot analysis identified 5HT₃ and 5HT₄ receptor proteins in the HT29 colon cell line and colon cancer tissues. A similar profile with HT29 cells, colon tumor tissues, and rat brain demonstrated major protein bands at 48 and 44 kDa for 5HT₃ and 5HT₄ receptors, respectively. Also this immunoblot expression for the 5HT₃ receptor was completely significant when compared with the negative control (normal human marginal tissue) and as strong as the positive control (brain tissue). However, for the 5HT₄ receptor, this expression was mild. According to other protein expression studies, it has been previously demonstrated that the 5HT₂ receptor expressed profoundly in breast cancer.¹⁷ Other western blot and immunohistochemistry studies have shown that 5HT_{1A} and 5HT_{1B} receptors

expressed in bladder and prostate cancer.^{16,18} In those studies, immunohistochemistry assays showed expression of 5HT receptors in the cell membrane and cytoplasm of colon epithelial cells which are the site of localization of the G-protein coupled receptors (GPCR) as 5HT receptors. Therefore, our study as long as previous researches, describes the expression of 5HT receptors in cancer. But further studies are needed to clarify the pattern of these receptor expressions.

In summary, our results demonstrate for the first time that the 5HT₃ and 5HT₄ receptors are expressed in both human colon cancer tissue and cell line HT29. This expression for the 5HT₃ receptor is much stronger and more important than 5HT₄. Our data also show that 5HT, in part via the 5HT₃ and 5HT₄ receptors, has a mitogenic effect on HT29 cell proliferation.

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