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Differential expression of bitter taste receptors in non-cancerous breast epithelial and breast cancer cells

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ABSTRACT

The human bitter taste receptors (T2Rs) are chemosensory receptors that belong to the G protein-coupled receptor superfamily. T2Rs are present on the surface of oral and many extra-oral cells. In humans 25 T2Rs are present, and these are activated by hundreds of chemical molecules of diverse structure. Previous studies have shown that many bitter compounds including chloroquine, quinidine, bitter melon extract and cucurbitacins B and E inhibit tumor growth and induce apoptosis in cancer cells. However, the existence of T2Rs in cancer cell is not yet elucidated. In this report using quantitative (q)-PCR and flow cytometry, we characterized the expression of T2R1, T2R4, T2R10, T2R38 and T2R49 in the highly metastatic breast cancer cell line MDA-MB-231, poorly metastatic cell line MCF-7, and non-cancerous mammary epithelial cell line MCF-10A. Among the 5 T2Rs analyzed by qPCR and flow cytometry, T2R4 is expressed at 40–70% in mammary epithelial cells in comparison to commonly used breast cancer marker proteins, estrogen receptor and E-cadherin. Interestingly, the expression of T2R4 was downregulated in breast cancer cells. An increase in intracellular calcium mobilization was observed after the application of bitter agonists, quinine, dextromethorphan, and phenylthiocarbamide that are specific for some of the 5 T2Rs. This suggests that the endogenous T2Rs expressed in these cells are functional. Taken together, our novel findings suggest that T2Rs are differentially expressed in mammary epithelial cells, with some T2Rs downregulated in breast cancer cells.

1. Introduction

Membrane receptors are excellent drug targets because of their important physiological role in mediating communication between the cell and its environment. The largest group of these membrane receptors belong to the family of G protein-coupled receptors (GPCRs) [1]. GPCRs contain seven transmembrane (TM) helices and signal predominantly through heterotrimeric G-proteins in response to diverse extracellular stimuli including neurotransmitters, light, taste and smell [1]. The human bitter taste receptors (T2Rs) are a group of 25 chemosensory receptors that belong to the GPCR superfamily, and mediate signal transduction in response to stimulation by a wide variety of bitter agonists [2]. Recent studies on T2Rs showed that these receptors are expressed in many extraoral tissues, including the respiratory system, brain, reproductive tissues, and airways [3]. They mediate protective reflexes by performing different physiological roles in extraoral tissues including bronchodilation [4], and the susceptibility of humans to infections of the upper respiratory tract [5]. However, the expression and possible chemosensory function(s) of T2Rs in cancer cells was not elucidated thus far.

Breast cancer is the most common malignancy in females [6], with women having a lifetime risk of over 10% for its occurrence [7]. Many GPCRs such as chemokine receptors CXCR4 and CCR7 [8,9], protease-activated receptors (PARs) and lysophosphatidic acid receptors (LPAs) are upregulated in breast tumor cells and tissues [10,11]. Thus, GPCRs and their ligands are important therapeutic targets in treatment of breast cancer. Previous studies showed that bitter agonists, quinidine and chloroquine, triggered apoptosis in MCF-7 breast cancer cells through the p53 dependent pathway [12]. In addition, the bitter melon (*Momordica charantia*) extract was shown to inhibit breast cancer cell proliferation by modulating cell cycle regulatory genes and promotes apoptosis in breast cancer cells [13]. Recently, the molecular receptor targets (T2Rs) for most of these bitter compounds were characterized, *in vitro* [14]. However, the presence of T2Rs in cancer cells, including breast cancer has not been elucidated. Our hypothesis was that T2Rs are expressed in breast cancer and play a chemosensory role in migration and/or invasion of breast cancer. As a first

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step to validate our hypothesis, we studied the expression of 5 of the 25 human T2Rs in the highly metastatic breast cancer cell line MDA-MB-231, poorly metastatic cell line MCF-7, and non-cancerous mammary epithelial cell line MCF-10A (considered as normal epithelial cell).

In this study, using quantitative (q)-PCR and flow cytometry analysis we show the differential expression of T2R1, T2R4, T2R10, T2R38 and T2R49 in normal and breast cancer cells. T2R4 was expressed at significant levels of 40–70% of the breast cancer markers used in this study. Functional studies on these cells indicated an increase in intracellular calcium mobilization after the application of natural and synthetic bitter agonists, suggesting that the endogenous T2Rs in these cells are functional. Our novel findings show that T2Rs are expressed at varying levels in normal and breast cancer cells, and suggest downregulation of T2Rs in breast cancer.

2. Materials and methods

2.1. Materials

Cell culture media, Fluo-4 NW calcium assay kit and culture supplements were purchased from Invitrogen (Carlsbad, CA, USA). Dextromethorphan hydrobromide (DXM), quinine hydrochloride, Phenylthiocrabamide (PTC) were purchased from Sigma (Toronto, ON, Canada). The polyclonal antibodies against human T2R1, T2R4, T2R10, T2R38, T2R49 and estrogen receptor (ER) were purchased from Abcam (Toronto, ON, Canada) and Santa Cruz (Dallas, TX, USA), respectively. RNA isolation kit and cDNA synthesis kit were purchased from Qiagen (Toronto, ON, Canada) and Invitrogen. The synthetic oligonucleotide primer sequences for human TAS2R1, TAS2R4, TAS2R10, TAS2R38, TAS2R49, ER and GAPDH were purchased from Invitrogen. The HUGO gene nomenclature of TAS2R is used wherever the gene is mentioned.

2.2. Cell culture

Human mammary epithelial cell lines MCF-10A, MCF-7 and MDA-MB-231 were kind gifts from Drs. Etienne Leygue and James Davie, Cancer Care Manitoba, MB, Canada. MCF-7 and MDA-MB-231 breast cancer cells were maintained in DMEM with 10% FCS, whereas MCF-10A were maintained in DMEM supplemented with 5% horse serum, hydrocortisone (0.5 μg/ml), insulin (10 μg/ml), epidermal growth factor (20 ng/ml), and penicillin-streptomycin (100 μg/ml each). MDA-MB-231 cells are estrogen receptor (ER), progesterone receptor (PR), and E-cadherin negative, whereas MCF-7 cells are ER, PR and E-cadherin positive.

2.3. RNA isolation

Total RNA from MCF-7, MCF-10A and MD-MBA-231 cells was isolated using RNeasy mini kit (Qiagen) according to manufacturer's protocol. Isolated RNA was treated with DNase I and then used for cDNA synthesis with SSIII RT (superscript III reverse transcriptase), dNTPs, Oligo dT primer and first strand buffer (Invitrogen).

2.4. Quantitative (q)-PCR

For analysis by real-time PCR, reverse transcription was carried out as described above. Reaction mixtures, with a final volume of 10 μl consisted of 0.5 μl reverse transcribed cDNA, 0.4 μM primers, 1X SYBR Green containing dNTPs mix and Taq polymerase. The reaction consisted of the following steps; an initial denaturation step of 15 min at 95 °C then 50 cycles of 94 °C for 30 s, annealing at 58 °C for 30 s for TAS2R1, TAS2R10 and TAS2R4, and 60 °C for 30 s for TAS2R38, TAS2R49, and ER, and extension at 72 °C for 30 s and a final extension at 72 °C for 2 min. This was followed by melt curve analysis from 73 to 95 °C at every 1 °C increase in temperature for about 1 s for 23 cycles. Melt curve analysis confirmed the presence of a single PCR product in each reaction. A real time PCR machine, illumina, from Montreal Biotechnology Inc. was used for this experiment.

2.5. Flow cytometry

Analysis of expression of T2Rs in different cell lines, were carried out using BD FACS canto flow cytometry. Normal mammary epithelial cells MCF-10A and breast cancer cells MCF-7 and MDA-MB-231 were grown in 6-well tissue culture dishes and 1×10^6 viable cells were collected for the experiment. The cells were washed 2–3 times with FACS buffer (PBS along with 0.5% BSA) and incubated with primary antibodies 1:300 dilution against T2R1, T2R4, T2R10, T2R38 and T2R49, and with E-cadherin, a cell surface breast cancer marker as positive control, and isotype control and cells with either primary or secondary antibody as negative control, for 1 h on ice. After 1 h cells were washed thrice with FACS buffer by centrifugation. Next, cells were incubated with 1:1000 dilution of Alexa 488 goat anti-rabbit for T2R1, T2R4, T2R10 and E-cadherin, whereas rabbit anti-goat for T2R38 and T2R49 for 1 h on ice. The cells were washed thrice with FACS buffer and then resuspended in 300 μl PBS. The fluorescence signals of 1×10^6 cells/tube were measured using single colour analysis by BD FACS Canto II analyzer. The results were analyzed using FACS Diva software. Cell surface receptor expression for T2Rs were expressed in Mean fluorescence unit (MFI) and expressed as percentage of E-cadherin taken as 100%.

2.6. Functional assays

Calcium mobilization experiments were carried out as described earlier [15]. Briefly for functional analysis, MCF-7, MCF-10A and MDA-MB-231 cells (1×10^5) were seeded into 96-well tissue culture treated BD-optilux plates. Receptor activation was determined by measuring changes in intracellular calcium after application of bitter ligands, DXM, quinine and PTC, and the signals read using Flex station 3 fluorescence plate reader (Molecular Devices, CA, USA) at 525 nm, following excitation at 494 nm. Data were collected from 2 to 3 experiments done in triplicate. EC_{50} values were plotted using PRISM software version 4.03 (Graph Pad Software Inc. San Diego, CA) after subtracting the responses of cells treated with assay buffer.

3. Results and discussion

Antibodies required to detect expression of T2R protein by flow cytometry, are not currently available for all the 25 human T2Rs, this led us to focus on only a few T2Rs. Another reason for selecting these T2Rs is the agonist specificity. For example, T2R1 and T2R10 are activated by only dextromethorphan (DXM), and T2R38 is activated only by phenylthiocarbamide (PTC). Quinine is one of the most intense bitter tasting compound [15], and though it stimulates 10 T2Rs [14], it activates T2R4 with the highest efficacy [16]. We have used two breast cancer markers, ER and E-cadherin [17]. Since, ER is a standard breast cancer marker, breast cancer cell lines are classified as ER positive or negative based on its presence. ER was used as a marker in our qPCR studies. However, ER is not expressed on the surface of cells, and since T2Rs like other GPCRs are cell surface proteins, we used E-cadherin which is expressed

on the surface of breast cancer cells, as a marker for our flow cytometry experiments.

3.1. Analysis of TAS2R gene expression by qPCR

It is well known that metastatic cells tend to show altered gene expression levels, and subsequent changes in protein expression patterns and signalling, contributing towards cell survival and growth. [18–20]. Such changes might be responsible for cancer cell survival or might contribute towards evasion of growth inhibition by normal tissue environment. To determine the expression of T2Rs in normal and breast cancer cells, five well studied T2Rs (T2R1, T2R4, T2R10, T2R49 and T2R38) and the breast cancer marker ER, as a reference gene, were analyzed by qPCR. The RNA was isolated from normal as well as breast cancer cells, the cDNA synthesized, and RT-PCR performed as described in methods. Results are shown in Fig. 1. MCF-7 cells are known to be ER positive cells, whereas MDA-MB-231 cells are considered as ER negative cells. Therefore, the qPCR data was normalized to the expression of ER in MCF-7 cells which was considered to be 100%. The amplicons for all 5 TAS2Rs was detected in the breast cancer cells and in non-cancerous mammary epithelial cells. The mRNA expression level of TAS2R4 in MCF-10A cells was 50 ± 6% followed by MDA-MB-231 and MCF-7 cells which were 20 ± 3% and 15 ± 2%, respectively. However, the expressions of other 4 TAS2Rs analyzed were at low levels in both breast cancer as well as in normal mammary epithelial cells (Fig. 1). We speculate that cancer cells in order to evade possible growth suppressive effects from a foreign microenvironment and metastasize might cause down regulation of some key genes, and TAS2R4 down regulation might be one of the mechanisms.

3.2. Analysis of expression of T2Rs by flow cytometry

Next, to determine the expression of T2Rs on the cell surface of non-cancerous and breast cancer cells, flow cytometry analysis was performed. The expression of different T2Rs in terms of MFI were normalised to E cadherin, a cell surface protein marker, whose expression is known to increase during breast cancer development [17]. Flow cytometry studies revealed that cell surface expression of T2R4 in MCF-10A cells was significantly higher $^{***}P < 0.001$ as compared to MDA-MB-231 and MCF-7 cells (Fig. 2). However, no statistically significant difference in expression was observed for the other T2Rs analyzed in the study. This result was consistent with our qPCR data, which showed higher expression of T2R4 in normal mammary epithelial cells compared to breast cancer cells. Previous studies using GPR56 have shown that down regulation of this receptor was important for tumour growth suppression both *in vitro* and *in vivo* [20]. Our studies with only 5 of the 25 T2Rs show down regulation in the invasive breast cancer cell lines compared to normal non-invasive cells. The possible mechanism of signalling, and how T2Rs affect tumor microenvironment, remains to be elucidated. However, we speculate T2Rs might act in a synergistic manner to modulate tumor microenvironment.

3.3. Functional assay using bitter agonists DXM, Quinine and PTC

T2R4 was expressed at significant levels in both normal and breast cancer cell lines, while other T2Rs are expressed at lower levels (Figs. 1 and 2). Among the five T2Rs analyzed, expression of T2R49 is the lowest; hence it was not targeted in functional assays. To analyze whether the endogenous T2Rs are functional, we pursued calcium mobilization assays using three bitter compounds, DXM, Quinine and PTC. The synthetic bitter compound DXM is known to activate two T2Rs, T2R1 and T2R10; while quinine activates 10 T2Rs but is known to predominantly act on T2R4, and PTC activates only T2R38. In our assays, the addition of DXM, quinine and PTC (micromolar doses) induced a dose dependant increase in intracellular calcium mobilization in both cancerous and non-cancerous cells (Fig. 3). Interestingly, the calcium mobilization for an intrinsic concentration (highest concentration) of the agonist or the E_{max} obtained from the dose response curves is highest for quinine, followed by DXM, and lowest for PTC. E_{max} values of 277 ± 37, 178 ± 16 and 88 ± 15 relative fluorescence units (RFUs) were observed for MCF-10A cells, MDA-MB-231 and MCF-7 cells stimulated with 2 mM DXM (Fig. 3A). E_{max} values of 306 ± 15, 186 ± 19 and 190 ± 24 RFUs were observed for

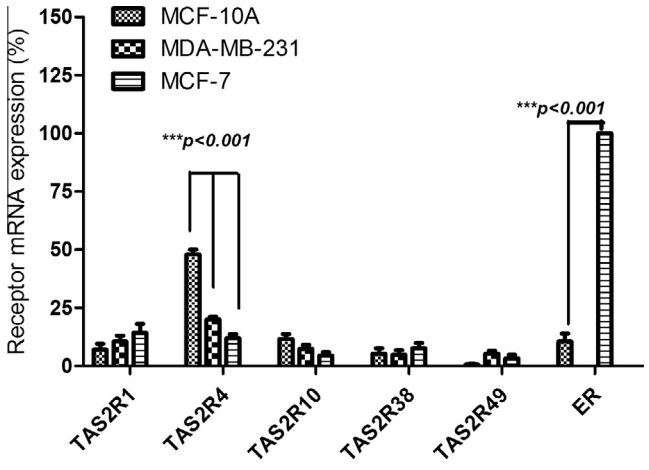

Fig. 1. Quantitative (q) PCR expression analysis of TAS2R1, TAS2R4, TAS2R10, TAS2R38 and TAS2R49 transcripts in non-cancerous breast epithelial cell line MCF-10A, and breast cancer cell lines MDA-MB-231 and MCF-7. Relative expression is normalized to that of estrogen receptor (ER) in MCF-7, which is considered as 100%. The MDA-MB-231 cell line is ER negative. Data presented are from 2 to 4 independent experiments done in triplicate. GAPDH was used as an internal control. Values are plotted as mean ± SEM. Relative expressions were computed using $2^{-\Delta CT}$ methods. Melt-curve analysis confirmed the presence of a single PCR product in each reaction. Statistically significant values are shown by asterisk.

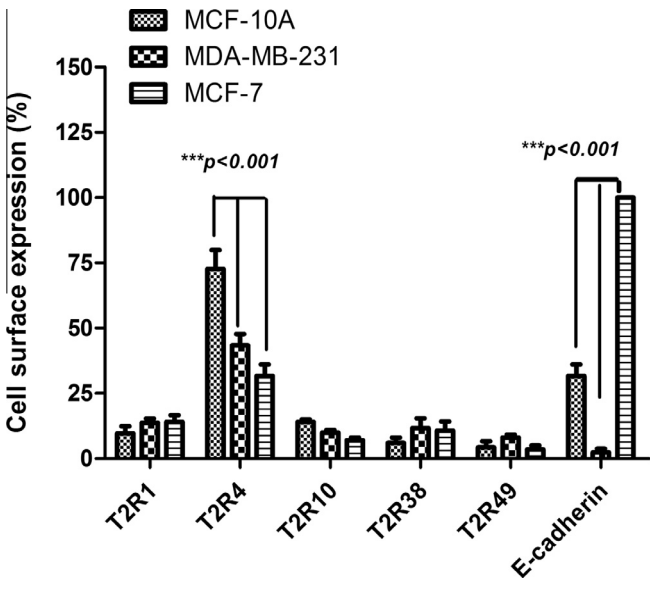

Fig. 2. Flow cytometry analysis of T2R1, T2R4, T2R10, T2R38, T2R49 and E-cadherin in non-cancerous breast epithelial cell line MCF-10A, and breast cancer cell lines MDA-MB-231 and MCF-7. The cell surface expression of T2Rs was normalized to the Mean Fluorescence Intensity (MFI) obtained from E-cadherin in MCF-7 cells which is considered as 100%. The results were analyzed using two way ANOVA. Only T2R4 showed statistically significant values.

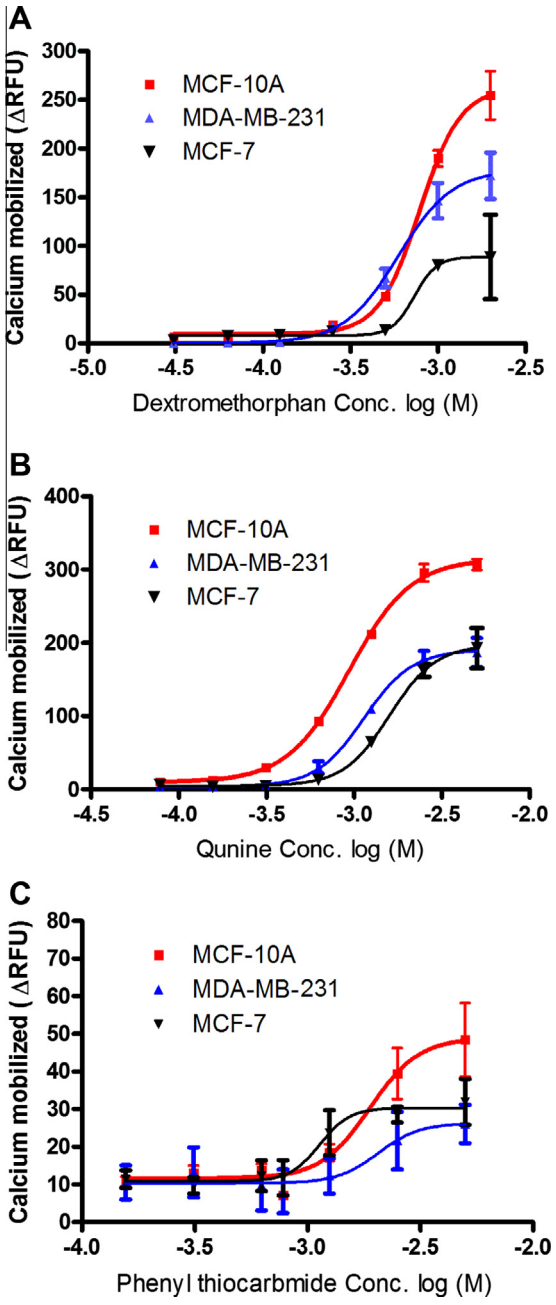

Fig. 3. Calcium mobilization induced by T2R agonists in non-cancerous breast epithelial cell line MCF-10A (red), and in breast cancer cell lines MDA-MB-231 (blue), and MCF-7 (black). Concentration-dependent changes in intracellular calcium [Ca^{2+}] induced by bitter ligands (A) Dextromethorphan (DXM), (B) Quinine, and (C) Phenylthiocarbamide (PTC). DXM is known to activate two T2Rs, T2R1 and T2R10; while quinine activates 10T2Rs but predominantly acts on T2R4, and PTC activates only T2R38. Data were collected from 2 to 3 independent experiments carried out in triplicate. Dose response curves were generated using Graph Pad Prism software, after subtracting the responses of cells stimulated with assay buffer. The calcium mobilization in the non-cancerous breast MCF-10A cells are higher compared to the breast cancer cell lines for all the three agonists tested (A–C), suggesting downregulation of T2Rs in breast cancer cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MCF-10A cells, MDA-MB-231 cells and MCF-7 cells stimulated with 5 mM quinine (Fig. 3B). Whereas E_{max} of 48 ± 21, 26 ± 4 and 31 ± 4 RFUs were observed for MCF-10A cells, MDA-MB-231 and MCF-7 cells stimulated with 5 mM PTC (Fig. 3C). However, no statistically signifiant changes in EC_{50} values were observed for a given compound within these cell lines. The functional data based on E_{max} values, is consistent with expression of the respective T2Rs in these cell lines.

Many phytochemicals which taste bitter, including phenols, flavonoids, isoflavones, and glucosinolates, have been shown to have antioxidant and anti-carcinogenic effects and a wide spectrum of tumor-blocking properties [21,22]. It is generally accepted that diets high in vegetables and fruits confer a degree of protection against cancer, including breast cancer [21,23]. For example, bitter vegetables block or decrease the matrix metalloproteinase (MMP) activity in MDA-MB-231 cells thus inhibiting tumor cell invasiveness *in vitro* [24]. These studies show many bitter compounds to negatively regulate breast cancer growth by inhibiting cell proliferation and inducing apoptosis in breast cancer. However, the role of T2Rs in inhibiting or promoting breast cancer growth and resistance to metastasis remains to be elucidated.

In this study, we characterized the mRNA and protein levels of 5 T2Rs in normal and breast cancer cell lines. We characterized the functionality of T2R1, T2R10, T2R4 and T2R38 endogenously expressed in these cell lines, by using T2R specific agonists and pursuing calcium mobilization assays. We observed that our expression data (mRNA and protein levels) is in agreement with the functional data, in the cell lines tested. Interestingly, we found these T2Rs are downregulated in breast cancer cells. Previous studies have shown that downregulation of expression of type III TGFβ receptor and subsequent immunosuppression resulted in the modulation of breast cancer microenvironment [25]. We speculate that downregulation of T2Rs might synergistically modulate tumour microenvironment. In addition, factors that promote this downregulation, including the role of microRNAs need to be investigated. Further studies are needed to elucidate the chemosensory roles of the T2Rs in migration and/or invasion of breast cancer.

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