Histochemistry and Cell Biology (2019) 152:155–166
https://doi.org/10.1007/s00418-019-01791-7

ORIGINAL PAPER

TAAR1 levels and sub-cellular distribution are cell line but not breast cancer subtype specific

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Accepted: 7 May 2019 / Published online: 21 May 2019

Abstract

Trace amine-associated receptors are G protein-coupled receptors of which TAAR1 is the most well-studied. Recently, Vattai et al. (J Cancer Res Clin Oncol 143:1637–1647 https://doi.org/10.1007/s00432-017-2420-8, 2017) reported that expression of TAAR1 may be a marker of breast cancer (BC) survival, with a positive correlation also suggested between TAAR1 expression and HER2 positivity. Neither a role for TAAR1 in breast tissue, nor in cancer, had previously been suspected. We, therefore, sought to provide independent validation and to further examine these putative relationships. First, a bioinformatic analysis on 58 total samples including normal breast tissue, BC-related cell lines, and tumour samples representing different BC sub-types found no clear correlation between TAAR1 mRNA levels and any BC subtype, including HER2 + . We next confirmed the bioinformatics data correlated to protein expression using a well validated anti-human TAAR1 antibody. TAAR1 mRNA levels correlated with the relative intensity of immunofluorescence staining in six BC cell lines (MCF-7, T47D, MDA-MB-231, SKBR3, MDA-MB-468, BT-474), but not in the MCF-10A immortalized mammary gland line, which had high mRNA but low protein levels. As expected, TAAR1 protein was intracellular in all cell lines. Surprisingly MCF-7, SKBR3, and MDA-MB-468 showed pronounced nuclear localization. The relative protein expression in MCF-7, MDA-MB-231, and MCF-10A lines was further confirmed by semi-quantitative flow cytometry. Finally, we demonstrate that the commercially available anti-TAAR1 antibody has poor selectivity, which likely explains the lack of correlation with the previous study. Therefore, while we clearly demonstrate variable expression and sub-cellular localization of TAAR1 across BC cell lines, we find no evidence for association with BC subtype.

Keywords Trace amine-associated receptor 1 · Breast cancer · Confocal microscopy · Bioinformatics · Flow cytometry

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00418-019-01791-7) contains supplementary material, which is available to authorized users.

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Abbreviations

BC	Breast cancer
BL1	Basal-like 1
BL2	Basal-like 2
CNS	Central Nervous System
ER	Estrogen receptor
GEO	Gene expression Omnibus
GPCR	G protein-coupled receptor
HER2	Human epidermal growth factor receptor 2
IF	Immunofluorescence
PR	Progesterone receptor
RMA	Robust multi-array normalization
TAAR	Trace amine-associated receptor
TNBC	Triple negative breast cancer

Introduction

Trace amine-associated receptors (TAARs) are a family of G protein-coupled receptors (GPCRs) that are an emerging therapeutic target in particular for neuropsychiatric indications and disorders associated with disruptions in energy metabolism (Berry et al. 2017). Although the majority of TAARs remain orphan receptors, with no clearly established endogenous ligand, TAAR1 is activated by a number of endogenous compounds including the amino acid derivatives 2-phenylethylamine and *p*-tyramine, the dopamine metabolite 3-methoxytyramine, and the thyroid hormone metabolite 3-iodothyronamine (Gainetdinov et al. 2018). The available evidence suggests that TAAR1 is heterogeneously expressed in low levels throughout the body (Berry et al. 2017) and is located almost exclusively intracellularly on uncharacterized intracellular membranes (Revel et al. 2013; Szumska et al. 2015; Raab et al. 2016), possibly due to the absence of *N*-terminal glycosylation sites (Barak et al. 2008). On agonist binding, translocation to the plasma membrane can occur following heterodimerization with other receptors (Harmeier et al. 2015; Espinoza et al. 2015). Signal transduction occurs through the $G\alpha_s$ protein leading to an accumulation of intracellular cAMP (Borowsky et al. 2001; Bunzow et al. 2001). Independent of G protein signaling, TAAR1 can also signal via the β-arrestin 2 cascade (Espinoza et al. 2011, 2015; Harmeier et al. 2015).

Although research has primarily focused on the role of TAAR1 in the central nervous system (CNS), TAAR1 is also expressed in a number of peripheral tissues, including pancreatic β-cells, stomach, and intestines of humans, rats, and mice (Kidd et al. 2008; Ito et al. 2009; Regard et al. 2008; Chiellini et al. 2012; Revel et al. 2013; Adriaenssens et al. 2015), suggesting a role outside the CNS. TAAR1 is also expressed in human and mouse leukocytes where it may have an immunomodulatory role (D'Andrea et al. 2003; Nelson et al. 2007; Panas et al. 2012; Wasik et al. 2012; Babusyte et al. 2013). Although it was initially reported that TAAR1 was also expressed in the liver, kidneys, lungs, and testes (Borowsky et al. 2001; Chiellini et al. 2012), more recent studies using a highly selective and well-validated anti-human TAAR1 antibody, corroborated with LacZ/dsRED knock-in/TAAR1 knockout rodent lines, have not confirmed these findings (Revel et al. 2013; Raab et al. 2016; Berry et al. 2017).

Breast cancer (BC) is the most common cancer in women and is the second leading cause of death from cancer in women worldwide (Harbeck and Gnant 2016). BC can be classified on the basis of the expression of estrogen (ER) and progesterone receptors (PR), or the overexpression of human epidermal growth factor receptor 2 (HER2) in the tumour (Fulford et al. 2006; Desmedt et al. 2008). HER2 positivity has been well established to be associated with more aggressive tumour progression, increased metastatic potential, and poorer prognosis (Yarden and Sliwkowski 2001; Tan and Yu 2007; Gonzalez-Angulo et al. 2009; Ahmed et al. 2015). Triple negative BC (TNBC) lacks the expression of ER and PR as well as not overexpressing HER2 (Fulford et al. 2006; Desmedt et al. 2008). TNBC can be further sub-classified based on distinct gene expression patterns with at least 2 different classification schemes proposed: luminal-like, basal-like, or mesenchymal-like each with distinct prognoses (Sørlie et al. 2001; Sotiriou et al. 2003; Brenton et al. 2005); or basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory, mesenchymal, mesenchymal stem-like, and luminal androgen receptor (Lehmann et al. 2011; Hon et al. 2016). Luminal and basal-like phenotypes can be distinguished based on the expression of cytokeratins in the tumour cells. Among TNBC subtypes, BL1, BL2, and mesenchymal subtypes display higher levels of basal-like cytokeratins, and luminal androgen receptor subtype tumours express high levels of luminal cytokeratins (Lehmann et al. 2011).

The thyroid hormone metabolite, 3-iodothyronamine, can act as a high affinity agonist of TAAR1 (Scanlan et al. 2004) and previous studies have found a correlation between altered thyroid function and BC (Rasmusson et al. 1987; Turken et al. 2003; Kuijpens et al. 2005). Specifically, hyperthyroidism and hypothyroidism have been associated with increased and decreased risk of BC, respectively (Søgaard et al. 2016). At initial diagnosis, BC patients have been reported to show increased blood concentrations of thyroxine, triiodothyronine, and antibodies against thyroid stimulating hormone and thyroidal peroxidase (Ditsch et al. 2010). Furthermore, it has been well established that leukocytes play important roles in the development and progression of various cancers, and in particular BC (Cimino-Mathews et al. 2015; Stanton et al. 2016). The activation of TAAR1 by a thyroid hormone metabolite, and putative role of the receptor in immune system modulation, therefore, may be relevant to the development and progression of BC. Consequently, Vattai et al. (2017) recently analyzed TAAR1 expression in BC patient samples and reported a positive correlation between TAAR1 expression and the survival of BC patients, as well as a positive correlation between increased TAAR1 expression and HER2 overexpression. Thus, this group suggested that TAAR1 may be a novel and independent predictor of BC survival through unknown molecular mechanisms (Vattai et al. 2017). How TAAR1 can be positively correlated with both patient survival and HER2 overexpression requires clarification given the well-established negative relationship of HER2 overexpression to patient outcomes. We, therefore, sought to further examine these possible relationships while providing an independent verification of the

findings of Vattai and colleagues, by determining for the first time the TAAR1 protein and mRNA expression profiles in BC cell lines, a key first step to elucidating the potential molecular mechanisms of action for TAAR1 in BC.

Here, we report our results from an in-depth analysis of TAAR1 mRNA expression across 20 BC cell lines as well as from 6 normal and 31 individual BC patient samples available within the Gene Expression Omnibus (GEO) database. Within these samples, we found no correlation of TAAR1 mRNA expression levels and BC sub-type. We then validated the findings by determining the protein expression by immunofluoresence (IF) in 6 BC cell lines as well as the normal MCF-10A cell line. Additional validation with quantitative analysis by flow cytometry in MCF-7, MDA-MB-231, and MCF-10A cells was also performed. Of high importance, all protein analysis was performed with a validated and highly specific monoclonal antibody (Raab et al. 2016). Both the mRNA expression and lack of correlation of TAAR1 expression with BC subtype was confirmed at the protein level, with the exception of the normal MCF-10A cells where TAAR1 protein and mRNA expression did not correlate.

Materials and methods

Bioinformatics: gene expression analysis

Microarray-based whole transcriptome data was retrieved from the GEO database (Supplemental Tables 1 and 2). The data was robust multi-chip average (RMA) normalized using the Oligo package and the pd.hg.u133.plus.2 annotation file through R 3.4.1 via R Studio 1.0.153 (Gentleman et al. 2004; Carvalho and Irizarry 2010; R Core Team 2013; Huber et al. 2015). The probe IDs for the selected genes of interest (Supplemental Table 2) were identified using Affymetrix's online NetAffx tool (Liu 2003). The genes selected included the target gene: TAAR1, along with ESR1 (ER alpha), PGR (PR), and ERBB2 (HER2) to confirm BC subtype (Supplemental Table 2) (Lehmann et al. 2011; Chen et al. 2012). TNBC subtype genes were also included in an attempt to differentiate between the six TNBC subtypes that have been identified based on these genetic markers. Cytokeratin genes were also selected to differentiate between basal and luminal TNBC subtypes (Lehmann et al. 2011; Chen et al. 2012). The RMA normalized data retrieved via GEO was then filtered using probe IDs and individual gene expression data was averaged between replicates for the selected genes. Expressed genes were identified using the Limma (Ritchie et al. 2015) and Affycoretools (Macdonald 2008) packages. Gene expression profiles were analyzed through hierarchical clustering using Genesis 1.8.1 (Sturn et al. 2002) with Euclidian average-linkage clustering.

Cells and treatments

Prior to use, all cell lines were determined to be mycoplasma-free using the MycoAlert™Plus Mycoplasma Detection Kit from Lonza (Basel, Switzerland) and their identity validated by short tandem repeat profiling of DNA using Promega's GenePrint® 10 System (B9510) at The Centre for Applied Genomics: Genetics Analysis Facility (Toronto, ON, Canada). All reagents were obtained from Life Technologies Co. (Burlington, ON, Canada) unless otherwise indicated. MCF-7, MDA-MB-231, SKBR3, BT-474, T47D, and MDA-MB-468 cells (ATCC, Virginia, USA) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin, with media changes every 2–3 days. The immortalized mammary gland cell line, MCF-10A (ATCC), was maintained in DMEM/F12 medium supplemented with 5% horse serum, 20 ng/mL epidermal growth factor, 10 µg/mL insulin, 0.5 µg/mL hydrocortisone, and 1% penicillin–streptomycin. All cells were passaged when they reached 70-80% confluency and used prior to passage number 30.

Immunofluorescence

Cells were plated (1×10^5 cells/well in 1 mL) in BD Falcon™ CultureSlides, (BD Biosciences, Mississauga, ON, Canada) and grown for 24 h. Cells were washed once with 800 µL phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and then fixed with 4% paraformaldehyde (Sigma-Aldrich, Oakville, ON, Canada) for 20 min at room temperature, followed by 3 washes with PBS. The fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min at 4°C. Cells were then washed once with PBS followed by 3 washes with 500 µL 100 mM glycine in PBS for 15 min each at room temperature with shaking at low speed on a multipurpose rotator (Thermo Fisher Scientific, Burlington, ON). Next, 400 µL of blocking buffer (10% donkey serum (Sigma-Aldrich) in immunofluorescence (IF) buffer (0.1% bovine serum albumin (BSA), 0.2% Triton X-100, 0.05% TWEEN® 20 in PBS, pH 7.4) was added to each well and incubated with shaking at room temperature for 1 h. For TAAR1 staining, 16.2 µg/mL of mouse anti-human TAAR1 antibody (Roche clone 6/6; Hoffmann-La Roche, Basel, Switzerland) was incubated for 14–16 h at 4 °C. This antibody was initially isolated based on binding to SF9 cells transfected with full length human TAAR1 (TAAR are not present in insects being vertebrate specific) with further validation by showing no immunoreactivity in primary human samples incubated with secondary antibody alone, or with primary antibody pre-incubated with purified human TAAR1 (Raab et al. 2016). Although the use of primary human samples by Raab and colleagues precluded further validation by receptor

knockdown, they did further demonstrate that immunochemical staining in the primary human samples showed the same distribution as demonstrated for TAAR1 in transgenic TAAR1 knock-out/LacZ knock-in mice.

Following primary antibody incubation, the wells were washed 3 times with 500 µL IF buffer for 15 min each at room temperature with gentle shaking. All subsequent steps were performed in the dark. Alexa Fluor® 594-conjugated donkey anti-mouse secondary antibody (Thermo Fisher Scientific, Burlington, ON) (300 µL of 1:250 dilution), or blocking buffer alone, was added along with Alexa Fluor® 647 phalloidin solution (1:20 in blocking buffer) to each well and incubated with low speed shaking for 1 h at room temperature. Following incubation each well was washed 4 times with 500 µL of IF buffer for 15 min with low speed shaking and then the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Burlington, ON) (0.030 µM in PBS) for 15 min at room temperature, followed by a 5 min wash with PBS. ProlongGold Antifade Reagent (Thermo Fisher Scientific) was added to each well and after 2–3 min coverslips were applied and the slides dried overnight at room temperature. Slide edges were sealed with nail polish and stored in the dark at 4 °C until imaged.

Confocal microscopy

Images were taken by confocal microscopy using a Nikon T_i-E A1 + microscope (Melville, New York, USA) with NIS-Elements Confocal Microscope Imaging software. Four random fields of view were imaged per well at a depth midway through the cells, as defined by depth measurements. Where specified, two z-stack composite images were obtained, with images taken every 0.5 µm from the bottom to the top of each cell, over two random fields of view, resulting in at least 2 composite images per well per experimental condition. DAPI staining was detected using a 405.0 nm laser, Alexa Fluor® 594-conjugated donkey anti-mouse secondary antibody and Alexa Fluor® 647 phalloidin staining were detected using a 561.0 nm laser. Bright field imaging was performed to confirm cell boundaries at 60× magnification.

Flow cytometry

Cells were collected by trypsinization and washed twice with 1 mL of FACS buffer (1% heat inactivated FBS in PBS, pH 7.4) and 6.5×10^5 cells/sample were fixed in 100 µl of IC Fixation Buffer (Cat. No. 00-8222-49, Invitrogen, Burlington, ON) for 35 min on ice. All incubations were done in the dark. Cells were washed twice with 1 mL of 1× permeabilization buffer (Cat. No 00-8333-56, Invitrogen,) and then re-suspended in 1 mL of an 81 µg/mL solution of anti-TAAR1 antibody or mouse IgG isotype control (SC-3878, Santa Cruz Inc.), prepared in 1× permeabilization buffer, for 35 min on ice. Sufficient volumes of antibody solution were prepared so that all 3 cell lines were incubated with the same antibody mastermix to minimize intra-assay variability. Cells were then washed twice with 1 mL 1× permeabilization buffer and re-suspended in a 100 µL solution of 5 µg/mL R-phycoerythrin-conjugated Affinipure $F(ab)_2$ fragment donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Baltimore Pike Pennsylvania, USA) in 1× permeabilization buffer on ice for 35 min. Following incubation cells were washed twice with 1 mL FACS buffer and resuspended in 500 µL FACS buffer for analysis.

The commercially available rabbit anti-human TAAR1 polyclonal antibody (ab150646; Abcam, Toronto, ON) was used at a 10 µg/ml concentration, with a rabbit IgG isotype control (SC-3888; Santa Cruz) included, and visualization by R-phycoerythrin conjugated goat anti-rabbit IgG secondary antibody (P2771MP; Invitrogen) at a concentration of 10 µg/ml. Samples were pre-incubated (room temperature x 15 min) with a human Fc block solution (Human Seroblock BUF070, Bio-Rad Laboratories, Mississauga, ON) at a 1:20 dilution as per the manufacturers recommendations to decrease non-specific binding. All other incubation times and conditions were as previously described with the exception that Seroblock was maintained in all solutions throughout the staining procedure. Parallel samples of the same cell populations were simultaneously analyzed with the Roche anti-TAAR1 antibody (81 µg/mL) replacing the Abcam anti-TAAR1 antibody.

In all instances data was collected with a BD FACS Aria flow cytometer and analyzed using FlowJo software v10.0.5 (Ashland OR, USA). Cells were selected for analysis based on FSC and SSC gating for every sample. Data were compared using GraphPad Prism 6 software (San Diego, CA, USA) by repeated measures two-way ANOVA with Holm–Sidak multiple comparisons post hoc test where significant main effects were observed.

TAAR1 knockdown

TAAR1 shRNA and scrambled sequence shRNA control plasmids were obtained from Santa Cruz Biotechnology (Catalogue numbers sc-61646-SH and sc-108060) and concentrated to 1 µg/mL following ethanol precipitation. Plasmids were added to MCF-7 cell suspensions (5×10^6/mL) to give a final plasmid DNA concentration of 0.1 µg/µL and cells transfected by electroporation using a Neon Transfection system using the following parameters: pulse voltage = 1250 V; pulse width = 20 ms; pulses = 2. Following electroporation cells were immediately transferred to 1 mL of pre-warmed RPMI medium containing 10% FBS, and allowed to recover at 37 °C, 5% CO_2 × 24 h. Following recovery, transfected cells were selected for puromycin

resistance by resuspension in RPMI growth medium supplemented with 3.0 µg/mL puromycin and incubation under standard cell culture conditions for 80 h. Transfected polyclonal cell populations were then expanded to sufficient numbers to allow for analysis of TAAR1 expression by flow cytometry using the Roche anti-TAAR1 antibody as described above.

Results

Bioinformatics analysis reveals that TAAR1 mRNA transcript levels does not correlate with BC subtype

To determine if TAAR1 mRNA transcript levels correlate with BC subtype, we performed an unsupervised hierarchical analysis of transcriptomic data for 20 different BC cell lines, the immortalized non-tumorigenic breast-derived epithelial cell line MCF-10A, along with 6 normal breast, 7 HER2 positive, 5 Luminal A, 9 Luminal B, and 10 TNBC patient samples obtained from the GEO data repository (total 58 samples; Supplementary Table 1). Transcript levels of HER2 (ERBB2), ER, PR, as well as various TNBC subtype-specific genes and cytokeratin genes (Supplementary Table 2) clustered the different BC subtypes and normal cells as expected (Fig. 1a). Although there were clear differences in the TAAR1 transcript levels between the various samples, there was no obvious correlation between BC subtype and TAAR1 transcript levels (Fig. 1a, b). Interestingly the immortalized non-tumorigenic breast-derived cell line MCF-10A and normal breast tissue samples tended to have relatively higher TAAR1 mRNA levels compared to the majority of BC samples which generally had much lower TAAR1 transcript levels (Fig. 1a, b).

TAAR1 protein expression by IF shows nuclear localization with relative protein abundance similar to mRNA in BC but not normal cells

The results of the bioinformatics analysis suggested that TAAR1 expression varies between individual cell lines and tissue samples, but independently of BC subtype. Based on

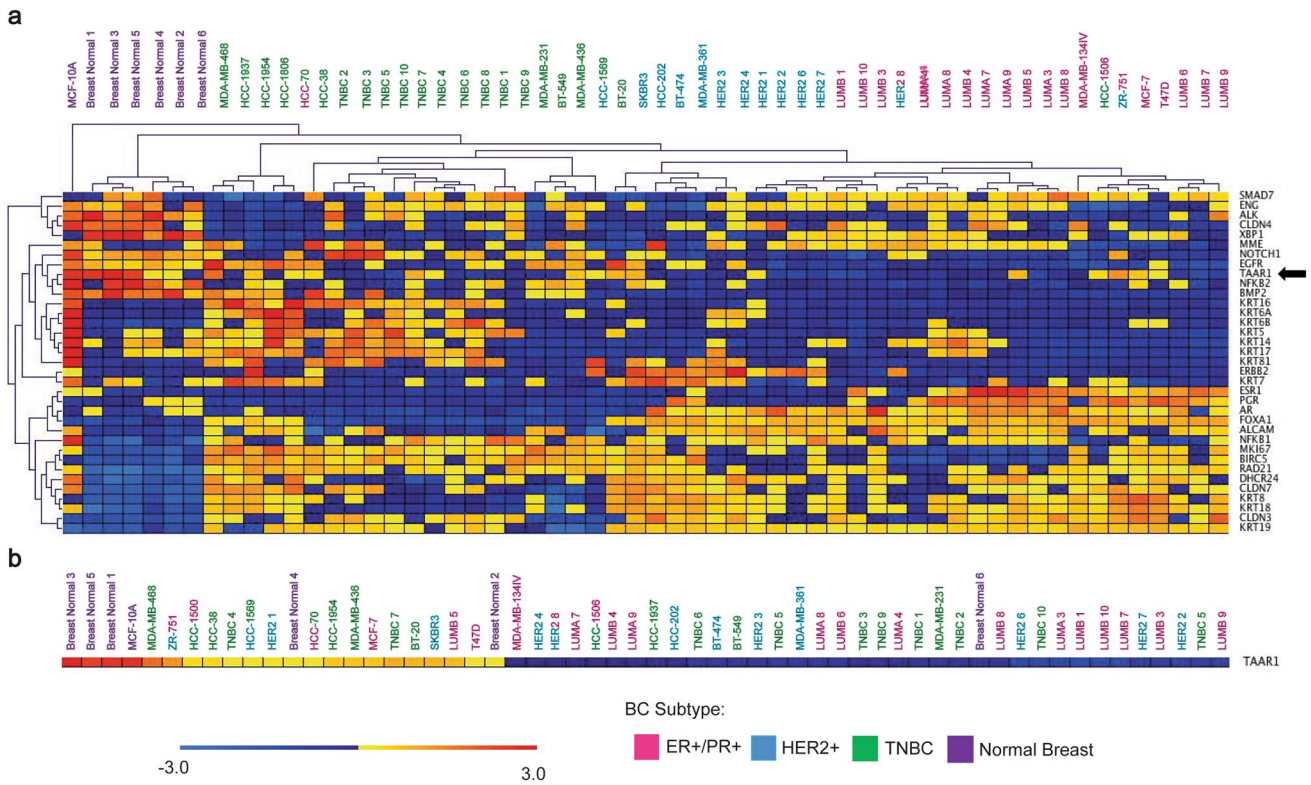

Fig. 1 Hierarchical clustering of the TAAR1 mRNA expression reveals that it does not cluster by BC subtype. **a** A total of 58 samples from 21 cell lines, 6 normal breast tissues, 7 HER2+, 5 Luminal A, 9 Luminal B, and 10 TNBC tissue samples were analyzed based on the expression of 35 genes representing the different molecular subtypes. The unsupervised hierarchical cluster analysis is shown with the sample labels colour-coded as per the legend shown. When samples were identified by histology as one sub-type but by our clustering as a different sub-type both colours are used. The location of TAAR1 is indicated by the arrow. **b** Samples were ordered based on expression of the TAAR1 gene and sample labels are colour-coded as indicated in **a**. Red denotes higher expression and blue indicates lower expression as per the colour bar

these findings, seven cell lines were selected for TAAR1 protein analysis representing different histological subtypes, and based on differential TAAR1 mRNA levels.

Using a specific mouse anti-human TAAR1 monoclonal antibody (Raab et al. 2016), IF staining revealed relative TAAR1 protein expression levels as predicted by mRNA transcript levels in all cell lines with the notable exception of MCF-10A cells (Fig. 2; Table 1). Comparatively, the MDA-MB-468 cell line appeared to have the most TAAR1 staining of the BC cell lines, followed by the MCF-7 cells. SKBR3 and T47D had intermediate staining while MDA-MB-231, BT-474 and MCF-10A cell lines showed TAAR1 staining indistinguishable from that of the secondary only controls (Fig. 2). As reported elsewhere in other cell types (Barak et al. 2008; Leo and Espinoza 2016), TAAR1 staining was almost exclusively localized intracellularly. Interestingly, z-stack analysis revealed that TAAR1 protein was nuclear, as it fully overlapped with DAPI staining, in the MCF-7, MDA-MB-468, and SKBR3 cell lines, but not in the T47D cells (Fig. 3).

FACS-based quantitation confirms significant differences in TAAR1 protein levels between cell lines

Based on the IF data, three cell lines (MCF-7, MDA-MB231, and MCF-10A) were selected for quantitation of TAAR1 protein levels by intracellular flow cytometry. There

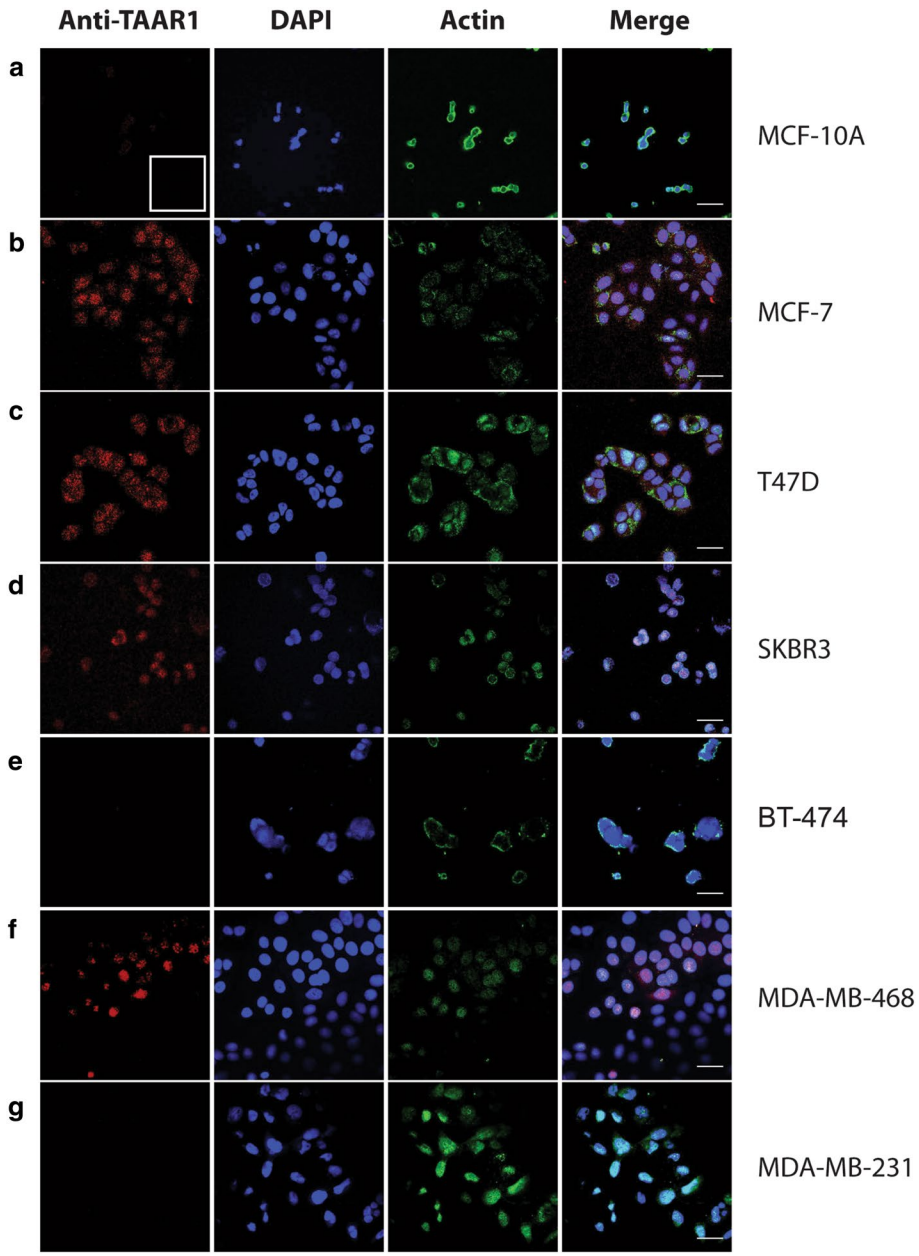

Fig. 2 TAAR1 immunofluorescence staining varies across six breast cancer and one normal cell lines and shows an intracellular localization. TAAR1 protein was visualized with a validated mouse anti-human TAAR1 antibody (Roche clone 6/6) followed by Alexa-Fluor 594-conjugated donkey anti-mouse secondary antibody (red). Nuclei were visualized with DAPI staining (blue) and actin with Alexa Fluor 647 phalloidin (green) and images captured by confocal microscopy. Merged images were generated using Image J software. Inset images in the TAAR1 panels represent negative control staining where primary antibody was omitted. Images are representative examples of 4 fields of view per culture, and 3–4 independent cultures at different passage numbers. Scale bar = 100 µm

Table 1 TAAR1 mRNA and protein levels correlate in BC cell lines but are not correlated to breast cancer subtype

Cell line	Subtype	ER	PR	HER2	Source	Tumor type	TAAR1 mRNA expression[a]	TAAR1 protein expression via IF staining[a]	TAAR1 protein expression via flow cytometry	% Metastatic potential of MDA-MB-231 cells[b]
MCF-10A	N/A	N/A	N/A	N/A	N/A	Non-tumourigenic epithelial	++++	+/−	+	N/A
MCF-7	Luminal A Epithelial	+	+	–	Pleural effusion	Metastatic adenocarcinoma	+++	+++	+++	20–40%
T47D	Luminal A Epithelial	+	+	–	Pleural effusion	Invasive ductal carcinoma	++	++	ND	0–20%
SKBR3	HER2+ Epithelial	–	–	+	Pleural effusion	Adenocarcinoma	++	++	ND	0–20%
BT-474	Luminal B Epithelial	+	+	+	Primary tumour	Invasive ductal carcinoma	+	+/−	ND	ND
MDA-MB-468	Basal A Epithelial	–	–	–	Pleural effusion	Metastatic adenocarcinoma	++++	++++	ND	0–20%
MDA-MB-231	Basal B Mesenchymal	–	–	–	Pleural effusion	Metastatic adenocarcinoma	+	+/−	++	100%

ND not determined

[a]Relative expression of TAAR1 mRNA or protein in the various BC cell lines, ranging from lowest (+/−) to highest (++++)

[b]From Thompson et al. (1992)

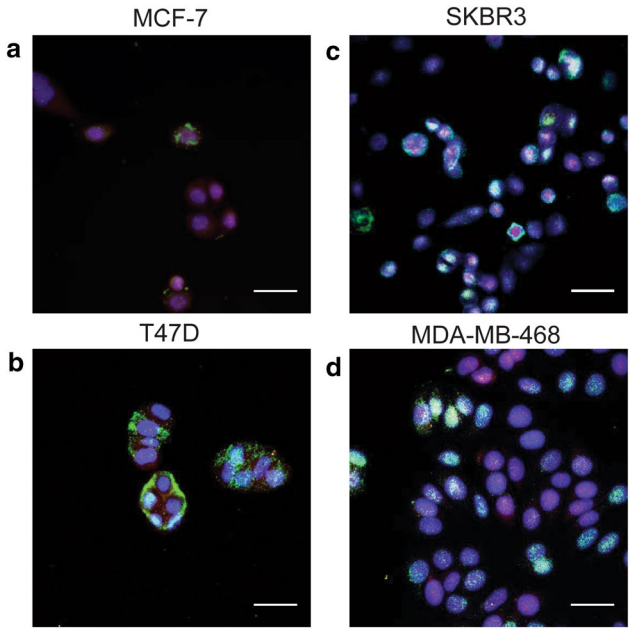

Fig. 3 TAAR1 protein is localized to the nucleus in some cell lines. Z-stack composite images were compiled in 0.5 µm increments using Image J software for the same cultures as shown in Fig. 2. Images are representative examples selected from two z-stack composites obtained per culture and 3–4 independent cultures at different passage numbers. Red staining = TAAR1, blue = nucleus (DAPI), green = actin, purple = TAAR1 + DAPI co-localization. Scale bar = 100 µm

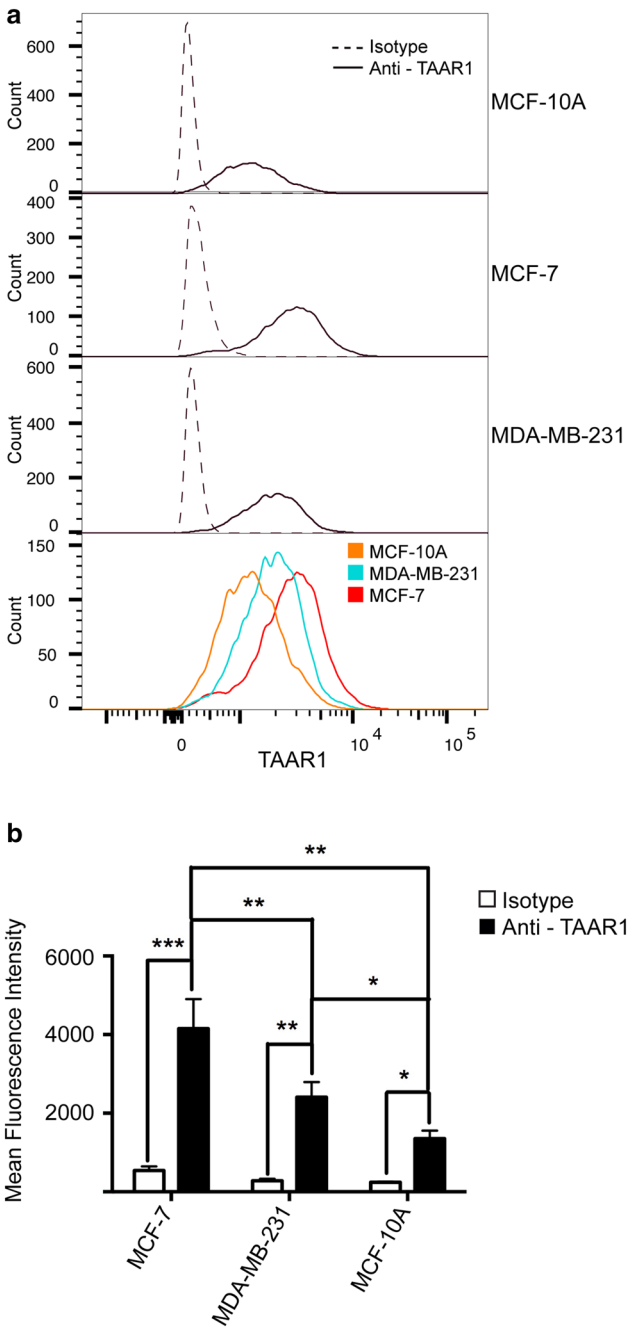

Fig. 4 TAAR1 expression determined by flow cytometry correlates with intensity of IF staining in a subset of cell lines. **a** Cells were stained for intracellular TAAR1. The isotype control contained mouse IgG in place of primary antibody to correct for non-specific staining, and was performed on paired samples to those treated with the anti-TAAR1 primary antibody. A mastermix of antibody solutions was prepared for each independent experiment, with each cell line treated simultaneously using the same mastermix. Images are representative samples of 3 independent experiments conducted at different cell passage numbers. **b** Mean fluorescence intensity for the isotype control and TAAR1 stained cells was determined. Data represent mean ± SEM of 3 independent experiments conducted with different passage numbers of each cell line. Data were compared by two-way ANOVA with Holm–Sidak's multiple comparison test for individual comparisons where significant main effects were observed. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

was no significant difference in isotype control staining levels between the three cell lines (Fig. 4, open bars), while in all three TAAR1 staining was significantly greater than the isotype controls (Fig. 4, open vs. closed bars), indicating the detection of TAAR1 protein. Furthermore, consistent with the qualitative IF staining, MCF-7 cells showed significantly greater staining than MDA-MB-231 cells, which in turn showed significantly greater staining than MCF-10A cells (Fig. 4, closed bars).

To investigate the apparent differences between our results and those previously reported by Vattai et al. (2017), we tested a commercially available anti-human TAAR1 antibody on MCF-7 and HEK293 cells. As the catalogue or clone number was not provided by Vattai et al. (2017) and both antibodies available from Abcam are rabbit polyclonal IgG, we selected the antibody reported to react with the extracellular domain of TAAR1, consistent with the area to which the Roche mouse monoclonal antibody was raised against (Raab et al. 2016). While the Roche mouse monoclonal antibody again showed clear specific labelling (data not shown), the commercial rabbit polyclonal antibody showed no difference in staining compared to rabbit isotype control in parallel cell samples, even when the Fc receptor was blocked to reduce non-specific binding (Supplemental Figure 1a). This lack of specific binding by the rabbit polyclonal

antibody was further confirmed in a second, non-breast, non-carcinogenic, human cell line, HEK293 cells (Supplemental Figure 1b). To further validate the Roche antibody, TAAR1 expression in the MCF-7 cell line was knocked down using shRNA. In these cells, an obvious loss of immunoreactivity toward the Roche antibody was observed (Supplemental Figure 2) in response to TAAR1-directed shRNA treatment, seen as a 2.75-fold increase in the number of cells devoid of TAAR1 immunoreactivity (scrambled shRNA 8.2%; TAAR1 shRNA 22.7%) and a 33% decrease in mean fluorescence intensity (Supplemental Figure 2).

Discussion

Here, we sought to systematically examine TAAR1 expression profiles across different BC subtypes, including ER/PR+, HER2+, and TNBC. Using a bioinformatics based approach we did not observe any evidence for a correlation between TAAR1 mRNA transcript levels and BC subtypes, in either cell lines or primary BC samples, although differential TAAR1 expression between individual samples was observed. A similar bioinformatics approach also recently identified differential TAAR1 mRNA transcript levels in breast cancer samples (Fleischer et al. 2018), although no examination of breast cancer subtypes was performed.

Through IF staining and flow cytometry, we found, in all but the MCF-10A cell line, that TAAR1 protein expression follows the same trends predicted in the bioinformatics screening (Table 1). Notably, with IF, TAAR1 staining in the MCF-10A and MDA-MB-231 cells was indistinguishable from background, flow cytometry confirmed that TAAR1 protein was present, albeit at low levels. This likely represents different levels of sensitivity between these two techniques. To our knowledge, this is the first time that TAAR1 protein expression by IF and flow cytometry has been demonstrated in human BC cell lines using a rigorously validated anti-TAAR1 antibody. Currently, there has been only one other study that has examined the expression of TAAR1 protein in BC where immunohistochemical analysis of TAAR1 expression suggested it as an independent predictor of BC survival while also being positively correlated with HER2 positivity in primary BC tissue (Vattai et al. 2017). This was surprising given the well documented negative correlation of HER2 positivity with patient outcomes (Yarden and Sliwkowski 2001; Tan and Yu 2007; Gonzalez-Angulo et al. 2009; Ahmed et al. 2015). While patient samples from 181 HER2− and 23 HER2+ non-metastatic BC tumours were analyzed, these varied in tumour size, tumour grade, ER/PR and HER4 receptor status, and tumour location; thus, creating a situation where patient and tumour heterogeneity may have confounded the analysis. This heterogeneity extended to TAAR1 status, with only 55.9% of all patient tumour samples positive for TAAR1 (Vattai et al. 2017), consistent with the inter-sample TAAR1 variability we have also observed (Fig. 1).

The suitability and selectivity of commercially available TAAR1 antibodies has recently been questioned (Berry et al., 2017) and may underlie our lack of replication of the findings of Vattai and colleagues (Vattai et al. 2017). Indeed, we found that in two unrelated human cell lines, the intensity of staining by one of the commercially available polyclonal rabbit anti-human TAAR1 antibodies is indistinguishable from the rabbit isotype control antibody (Supplemental Figure 1). In contrast, the previously thoroughly validated Roche antibody consistently provides labelling that is distinct from its isotype control (Fig. 4). We also provide further validation of the Roche antibody, demonstrating a reduction in immunoreactivity following shRNA-mediated knockdown of TAAR1 (Supplemental Figure 2). Although complete loss of TAAR1 immunoreactivity was not observed, a lack of correlation between TAAR1 mRNA and protein in normal tissues is commonly observed (Human Protein Atlas—https://www.proteinatlas.org/ENSG00000146399-TAAR1/tissue). This may represent a prolonged half-life of the mature TAAR1 protein or the use of poorly selective commercial antibodies. Together with previous validation studies, including antibody pre-incubation with immunogen, and omission of the primary antibody (Raab et al. 2016), it is unlikely that the immunoreactivity observed with the Roche antibody represents non-TAAR1 binding. As such, it is likely that our lack of replication of the previous results is due to non-specific reactivity of the commercial antibody.

Interestingly, we observed an apparent higher TAAR1 expression in normal breast tissue at the mRNA level, whereas the majority of BC samples exhibited markedly lower TAAR1 transcript levels (Fig. 1). This may be consistent with the previous suggestion that TAAR1 expression is a positive predictor of survival (Vattai et al. 2017). At this time, there is no clear explanation as to why TAAR1 transcript levels may be greater in normal breast tissue, or the physiological role of TAAR1 in the breast. Unlike in the BC cell lines, however, high TAAR1 mRNA levels were not indicative of high TAAR1 protein expression, at least in the immortalized breast tissue cell line, MCF-10A (Figs. 2, 3, 4). This may be due to translational regulation in normal cells that is lacking in cancer cells, however, the mechanism and biological consequence of this observation remains to be investigated.

Previous studies have reported that TAAR1 is preferentially located intracellularly (Revel et al. 2013; Raab et al. 2016). Translocation to the plasma membrane can occur following agonist-induced heterodimerization with other GPCRs (Espinoza et al. 2015; Harmeier et al. 2015). Here we provide the first demonstration that intracellular TAAR1

is localized to the nucleus in some breast cancer cell lines. In fact, z-stack image analysis showed TAAR1 staining was almost exclusively intracellular in all cell lines showing detectable TAAR1 protein; however, in only some cell lines (MCF-7, MDA-MB-468, and SKBR3) does it appear to be present in the nucleus (Fig. 3), while in the remaining cell lines there was no evidence of TAAR1 staining overlapping with the nuclear DAPI staining. One previous study, using a commercially available anti-TAAR1 antibody, provided evidence of nuclear TAAR1 protein in human astrocytes (Cisneros and Ghorpade 2014), however, the consequence of this localization in these cells is also unknown. Since the anti-TAAR1 antibody used in our study is not suitable for western blot protocols (Berry et al., 2017), it likely only recognizes the native conformation of TAAR1, suggesting nuclear localization is not a function of misfolding. Furthermore, localization does not appear to be related to expression levels, as SKBR3, MCF-7, and MDA-MB-468 cells all showed clear nuclear staining despite their variability in relative TAAR1 protein levels by IF, while T47D cells had primarily cytoplasmic staining even though TAAR1 was abundant.

An increasing number of GPCRs have been reported to exhibit nuclear localization (Boivin et al. 2008). In fact, members of all major classes of GPCRs have been detected in the nucleus of species from *Caenorhabditis elegans* to mammals which indicates that nuclear GPCR signaling may be evolutionarily preserved (Boivin et al. 2008), although in this context it should be noted that TAAR1 is only found in vertebrate species (Gainetdinov et al. 2018). Several physiological functions of nuclear GPCRs have been suggested including roles in cell proliferation, survival, inflammatory responses, tumourigenesis, DNA synthesis, and transcription (Boivin et al. 2008). The observation that three BC cell lines show pronounced localization of TAAR1 to the nucleus may also be indicative of a failure of intracellular trafficking in these cell lines. In either case, our confirmation of nuclear localization in a subset of breast cancer cell lines, using a thoroughly validated, highly selective antibody, indicates that the role of TAAR1 in the nucleus should be a subject of further investigation.

In the brain TAAR1 regulates dopaminergic neurones to prevent dopaminergic hyperactivity (Bradaia et al. 2009; Revel et al. 2011, 2013; Harmeier et al. 2015; Leo and Espinoza 2016). In other cell types, the dopamine D_1-like receptors (D_1 and D_5) are involved in the amplification of proliferation (Vallone et al. 2000), while D_2-like receptors (D_2, D_3, and D_4) can inhibit proliferation (Missale et al. 1998; Vallone et al. 2000; Pisick et al. 2003). As such, D_2-like receptor agonists have recently been proposed as potential BC therapeutic agents (Pornour et al. 2015). Interestingly, TAAR1 mediates its modulation of central dopaminergic activity through regulation of D_2-like receptor signaling (Bradaia et al. 2009; Revel et al. 2011, 2013; Harmeier et al. 2015; Leo and Espinoza 2016; Berry et al. 2017). As such, TAAR1 directed ligands may provide an alternate strategy for the treatment of breast cancer through the modulation of D_2-like receptor signaling. Furthermore, TAAR1 levels could be an important factor with respect to cell sensitivity to D_2-like receptor agonists, as, in other cell types, there is evidence for either constitutive TAAR1 activity or tonic activation of TAAR1 by endogenous ligands (Berry et al. 2017).

In conclusion, we have shown that TAAR1 mRNA and protein are expressed at varying levels in BC cell lines with mRNA and protein levels correlating in all the BC cell lines tested. Although the level of TAAR1 expression does not appear to correlate with known molecular phenotypes of BC, there was generally greater TAAR1 mRNA levels in normal breast tissue than in BC cell lines, however, this did not correlate with TAAR1 protein levels in the one normal cell line tested. Notably, we were not able to replicate the previously reported correlation between TAAR1 and HER2 (Vattai et al. 2017) at either the mRNA or protein levels, but found that TAAR1 expression levels do not correlate with any molecular breast cancer phenotype; with no clear relationship present between ER/PR positivity, TNBC status, or epithelial versus mesenchymal cell line origin (Table 1). Finally, our data indicates that caution should be exercised in the use of currently available TAAR1 antibodies, and independent validation of the selectivity of these commercially available reagents is required. Although the role of TAAR1 in normal breast tissue and in the development of BC is still unclear, our data clearly indicates variability in both the expression levels and intracellular localization of the presumably mature TAAR1 protein, and that future studies of the role of TAAR1 in normal and cancerous breast tissue are warranted.

Acknowledgements We thank Drs. Kensuke Hirasawa (Memorial University), Sheila Drover (Memorial University), and Alicia Viloria-Petit (University of Guelph) for their generous gifts of cell lines, Nicole Smith for expert technical assistance with flow cytometry, and Dr. Nikitha Pallegar for critical discussions.

Funding Funding provided by Natural Sciences and Engineering Research Council (MDB, SLC, JNM) and Memorial University (MSP, JNM, and MDB). Parts of this work were completed in partial fulfillment of Memorial University of Newfoundland B.Sc. honours theses (MSP and JNM).

Compliance with ethical standards

Conflict of interest MSP, JNM, SLC, and MDB declare no conflict of interest. MCH is an employee of F. Hoffmann-La Roche.

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