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ORIGINAL ARTICLE

Is the regulation by miRNAs of NTPDase1 and ecto-5′-nucleotidase genes involved with the different profiles of breast cancer subtypes?

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Abstract

Breast cancer (BC) is a public health problem worldwide, causing suffering and premature death among women. As a heterogeneous disease, BC-specific diagnosis and treatment are challenging. Ectonucleotidases are related to tumor development and their expression may vary among BC. miRNAs may participate in epigenetic events and may regulate ectonucleotidases in BC. This study aimed to evaluate the expression of ectonucleotidases according to BC subtypes and to predict if there is post-transcriptional regulation of them by miRNAs. MCF 10A (non-tumorigenic), MCF7 (luminal BC), and MDA-MB-231 (triple-negative BC - TNBC) breast cell lines were used and *ENTPD1* (the gene encoding for NTPDase1) and *NT5E* (the gene encoding for ecto-5'-nucleotidase) gene expression was determined. Interestingly, the expression of *ENTPD1* was only observed in MCF7 and *NT5E* was lower in MCF7 compared to MDA-MB-231 cell line. ATP, ADP, and AMP hydrolysis were observed on the surface of all cell lines, being higher in MDA-MB-231. Like qPCR, the activity of AMP hydrolysis was also lower in the MCF7 cells, which may represent a striking feature of this BC subtype. In silico analyses confirmed that the miRNAs miR-101-3p, miR-141-3p, and miR-340-5p were higher expressed in MCF7 cells and targeted *NT5E* mRNA. Altogether, data suggest that the regulation of *NT5E* by miRNAs in MCF7 lineage may direct the molecular profile of luminal BC. Thus, we suggest that the roles of ecto-5'-nucleotidase and the aforementioned miRNAs must be unraveled in TNBC to be possibly defined as diagnostic and therapeutic targets.

Keywords Breast cancer · CD73 · Adenosine · miR-101-3p · miR-141-3p · miR340-5p

Introduction

Breast cancer (BC) is a result of the disordered proliferation of transformed cells and is characterized by a heterogeneous profile in terms of molecular and clinical behaviors. BC subtypes are defined according to the expression of hormonal receptors (estrogen and progesterone) and of the human epidermal growth factor receptor 2 (HER2), differing in prognosis and therapeutic response [1]. In this sense, research aiming to identify molecular changes that characterize the development and progression of BC is extremely important to improve clinical management [2–4].

The identification of BC biomarkers is mainly focused on the pathways involved in cell proliferation, invasiveness, metastasis, and inhibition of the antitumor response. From this perspective, purinergic signaling should be further investigated regarding its involvement in tumor development, and especially in BC [5, 6]. In the early 1970s, the

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signaling performed by purines and pyrimidines was firstly recognized and named purinergic signaling. It is now largely known and accepted that purinergic signaling is present in several organisms and in different biological systems, being involved in various metabolic and signaling pathways, both in normal and pathological states [7], including cancer [8, 9]. A hierarchical control system regulates the magnitude and duration of response to extracellular nucleotides by controlling their concentration in the extracellular environment. This efficient control is performed by ectonucleotidases [10] that are ectoenzymes located on the cell surface and are responsible for the hydrolysis of extracellular nucleotides [11]. In this study, we focused on two ectonucleotidases that have been already described as involved in tumor development: NTPDase1 (also called CD39 and is encoded by *ENTPD1*) and ecto-5'-nucleotidase (also called CD73 and is encoded by *NT5E*) [12].

NTPDase1 has been identified as one of the enzymes responsible for regulating the signaling activity of extracellular adenosine tri (ATP) and di (ADP) phosphate, considering that it performs the conversion of ATP into ADP and ADP into adenosine monophosphate (AMP). Ecto-5'-nucleotidase, in turn, is an enzyme that converts AMP into adenosine (Ado) [12], making possible the initiation of the signaling by this nucleoside [13]. These enzymes are thus involved in purinergic signaling and are pointed as essential for the development and resistance of tumors [14, 15].

The level of expression of these enzymes in tumors is pointed as a factor that alters the malignancy and invasiveness of tumor cells [16, 17]. This is because when it comes to cancer, ATP presents antitumor activity, while Ado activates proliferative and pro-metastatic pathways [12]. Regarding the expression profile of ectonucleotidases in BC, it is known that the presence of ecto-5'-nucleotidase is associated with poorer prognosis of TNBC, and also that its overexpression is related to the lower response rate to anthracycline treatment, since ecto-5'-nucleotidase expression suppresses antitumor functions of CD8+ T lymphocytes [18]. This factor is so relevant that it is already known that the anti-CD73 antibody prevents the phenomenon of immunosuppression in tumors, including BC, by blocking the adenosine pathway [19].

Post-transcriptional regulation of ectonucleotidase genes can be performed by microRNAs (miRNAs) [16] that are small molecules of endogenous RNAs of 21–25 nucleotides (nts) in length and are potent post-transcriptional regulators of gene expression in plants and animals [20]. miRNAs act by binding to the mRNA causing the cleavage or non-translation of it resulting in the reduction of protein levels of their target genes [20].

New alternatives to improve BC treatment may arise from purinergic signaling via the post-transcriptional modulation of ectonucleotidases by miRNAs that may regulate tumor microenvironment [21]. In fact, the miRNAs acting as a tumor suppressor or activator have been already discussed in the scientific community [22, 23]. Ectonucleotidases may modulate the antitumor immune response through the levels of ATP and Ado. ATP and Ado in low and high levels, respectively, present anti-inflammatory properties facilitating tumoral progress, being reasonable to regulate ectonucleotidases activities [17]. Therefore, it is reasonable to hypothesize whether the expression profile of ectonucleotidases is different between BC subtypes and if they are regulated by miRNAs.

The objective of this study was to investigate the ectonucleotidases expression in BC molecular subtypes by evaluating the mRNA levels of *ENTPD1* and *NT5E* in human breast cell lines MCF 10A (non-tumor mammary cell line), MCF7 (tumorigenic, luminal BC), and MDA-MB-231 (metastatic, triple-negative BC - TNBC). Besides, we also aimed to identify putative miRNAs involved in the post-transcriptional regulation of these genes as potential targets to be considered in BC prognosis and therapies.

Material and methods

Cell culture

MCF 10A, MCF7, and MDA-MB-231 cell lines were obtained from the *American Type Culture Collection* (ATCC®). Cells were grown in culture bottles in specific media as follows: MCF 10A cells were cultured in DMEM-F12 medium (Cultilab, São Paulo, Brazil) supplemented with 10% fetal bovine serum, 20 ng/mL of epidermal growth factor (EGF), 500 ng/mL of hydrocortisone, 10 µg/mL of insulin, and 50 µg/L of gentamycin.; MCF7 and MDA-MB-231 were cultured in IMDM medium (Cultilab, São Paulo, Brazil) supplemented with 10% fetal bovine serum and 50 µg/ L gentamicin antibiotic. Cells were kept at 37 °C with 5% CO_2 and the culture medium was replaced on alternate days until they reached 80% confluence for further use.

Reverse transcription and qPCR assays

Total RNA from cells was extracted with the aid of the Trizol® reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using random primers and the M-MLV Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) as previously described (BRAGA et al., 2018). Each aliquot of 2 µL of cDNA was amplified with 5 pmol of specific primer for the study of *ENTPD1* (5′ TGTGGT GGAGAGGAGCCTCA 3′ and 5′ GCTGAACCACCTTGT TTTCTGAC 3′) and *NT5E* (5′ CTCATCGCTCAGAAAGTG AGGG 3′ and 5′ TTGGAAGGTGGATTGCCTGT 3′) genes.

qPCR was then performed using SYBR Green PCR blend (Invitrogen, Carlsbad, CA, USA) in a thermocycler (StepOne Plus, Thermo Scientific, USA). Three experimental replicates were run in duplicates and data were normalized with beta-2 Microglobulin (*β2M*) as the reference gene [19]. Standard relative curves for all primers were constructed and expression of each gene was quantified through the comparative Cq method.

Measurement of ATP, ADP, and AMP hydrolysis

Cells adhered to 24-well culture plates were washed three times with a reaction mix containing [final concentration, in mM] $CaCl_2$ 2.0, NaCl 120.0, KCl 5.0, Glucose 10.0, and Hepes 20.0, pH 7.4, at 37°C. Incubation was started with the addition of nucleotides (ATP, ADP, or 2.0 mM AMP) prepared in the reaction mix. For AMP hydrolysis, $CaCl_2$ was replaced by 2.0 mM $MgCl_2$ [24]. After 20 min, the reaction was terminated by transferring a 0.2 mL aliquot to a tube containing 0.2 mL of 10% trichloroacetic acid (TCA) previously kept on ice, and then 1.0 mL of malachite reagent was added to each tube. Inorganic phosphate (Pi) released was measured at 630 nm [25]. After stopping the reaction, cells were solubilized with the aid of a scraper in 100 μL of NaOH (1.0 M), and subsequently frozen. The next day, an aliquot was collected, and the protein was determined by the method of Bradford (1976), using bovine serum albumin as a standard [26]. Enzyme activity was expressed in nmol of Pi released/ minute/milligram of protein.

Statistical analyses

Results are presented as mean ± standard deviation (S.D.) of each of the measures performed in each of the groups studied. Sample number (n) represents the number of experiments performed with different cultures of the breast cell lines. ANOVA test followed by *Bonferroni* multiple comparison tests was used to analyze the expression results of each enzyme transcript and the hydrolysis of ATP, ADP, and AMP in different cell lines. For the comparative analysis of the expression profile of the two enzymes transcripts, the t test was used. Differences between samples were considered significant when the statistical analysis showed p value ≤ 0.05. GraphPad Prism software, version 7.04 for Windows, was used as a computational tool.

In silico analyses of miRNAs

The software TargetScanHuman 7.2 (http://www.targetscan.org/vert_72/), DIANA TOOLS (DIANA tarBAse v.8, DIANA microT v5.0) (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=site/index), miRTarBase 8.0 (http://mirtarbase.mbc.nctu.edu.tw), PolymiRTS Database 3.0 (https://compbio.uthsc.edu/miRSNP/) ENCORI (http://starbase.sysu.edu.cn/), MIRNAMAP (http://mirnamap.mbc.nctu.edu.tw/) and miRGator v3.0 (http://mirgator.kobic.re.kr/miRTargetNExpression.html), were used to find and describe miRNAs that possibly target the mRNA of *ENTPD1* and *NT5E* genes.

- TargetScanHuman 7.2 software: allows the identification of miRNAs that target the genes of interest and classifies these miRNAs according to their score and degree of conservation in human.
- DIANA TOOLS software: also allows the identification of miRNAs that act on genes of interest but offering other information, such as experimental validation.
- miRTarBase allows the identification of miRNAs that act on genes of interest, organizing them by validation experiments. In addition, it allows the identification of miRNAs that are involved in physiological and pathological processes, such as BC.
- PolymiRTS Database 3.0 tool: is a database containing polymorphisms in the DNA that can be target of different types of miRNA, allowing the identification of different types of miRNA that target the genes of the enzymes studied here, considering polymorphisms and SNPs [20].
- ENCORI tool: allows the identification of miRNAs by interacting with target genes.
- MIRNAMAP tool: a database of microRNAs that have already been experimentally verified and their interactions with target miRNA genes in humans and some other species [21].
- miRGator v3.0 tool: considered a miRNA portal, encompassing a diversity of miRNAs, as well as their expression profiles, relationships, and events involved [22].

Furthermore, the miRNAs identified were searched in the Genome Browser to identify regions of clusters or intragenic miRNAs relevant to the regulation of enzymes targeted or related to BC. Finally, in silico analyses were performed to verify the differential expression of the miRNAs identified in cell lines MCF 10A, MCF7, and MDA-MB-231. For this purpose, three samples of each lineage were obtained from the *SRA* public database (SRR2582220, SRR2582218, SRR2582217, SRR7155470, SRR7155471, SRR5329396, SRR7155472, SRR7155473, SRR6389813). The miRNAs alignment and counts were performed with *mapper.pl* and *miRDeep2* tools, respectively. The differential expression of the miRNAs was performed with *edgeR* (<http://bioconductor.org/packages/3.12/bioc/html/edgeR.html>) and *DESeq2* (<http://bioconductor.org/packages/release/bioc/html/DESeq2. html>). All scripts used to generate the results were developed by the authors of this study. Only those genes with *logFoldChange* < -1 or > 1 and *padj-value* < 0.05 were considered statistically significant.

In silico search of ectonucleotidases

In silico analyses also were performed to verify the differential expression of the *ENTPD1* and *NT5E* mRNAs in cell lines MCF 10A, MCF7, and MDA-MB-231. For this purpose, three samples of each lineage were obtained from the *SRA* public database (SRR12228622, SRR12228623, SRR12228624, SRR1345063, SRR1345064, SRR1345065, SRR14685262, SRR14685263, SRR14685264). The mRNAs alignment and counts were performed with *STAR software*. The differential expression of the mRNAs was performed with *DESeq2* (<http://bioconductor.org/packages/release/bioc/html/DESeq2. html>). All scripts used to generate the results were developed by the authors of this study. Only those genes with *logFoldChange* < -1 or > 1 and *padj-value* < 0.05 were considered statistically significant.

Correlation analysis

Correlation analyses were performed with *R* statistical software (<https://www.r-project.org/>) using three samples of each lineage, being each correlation performed between samples of the same number and the same lineage.

Results

Expression of ectonucleotidases is altered in breast tumor cell lines

Relative mRNA levels of *ENTPD1* were mostly detected in MCF7 cells (relative levels of mRNA expression ≅ 0.00265, $p<0.01$) (Fig. 1A). Regarding *NT5E*, the relative levels of mRNA in the MDA-MB-231 lineage (Fig. 1B) were significantly higher when compared to MCF7 cells (relative levels of mRNA expression ≅ 0.023515 and 0.0022 in MDA-MB-231 and MC7, respectively, $p<0.01$). When comparing

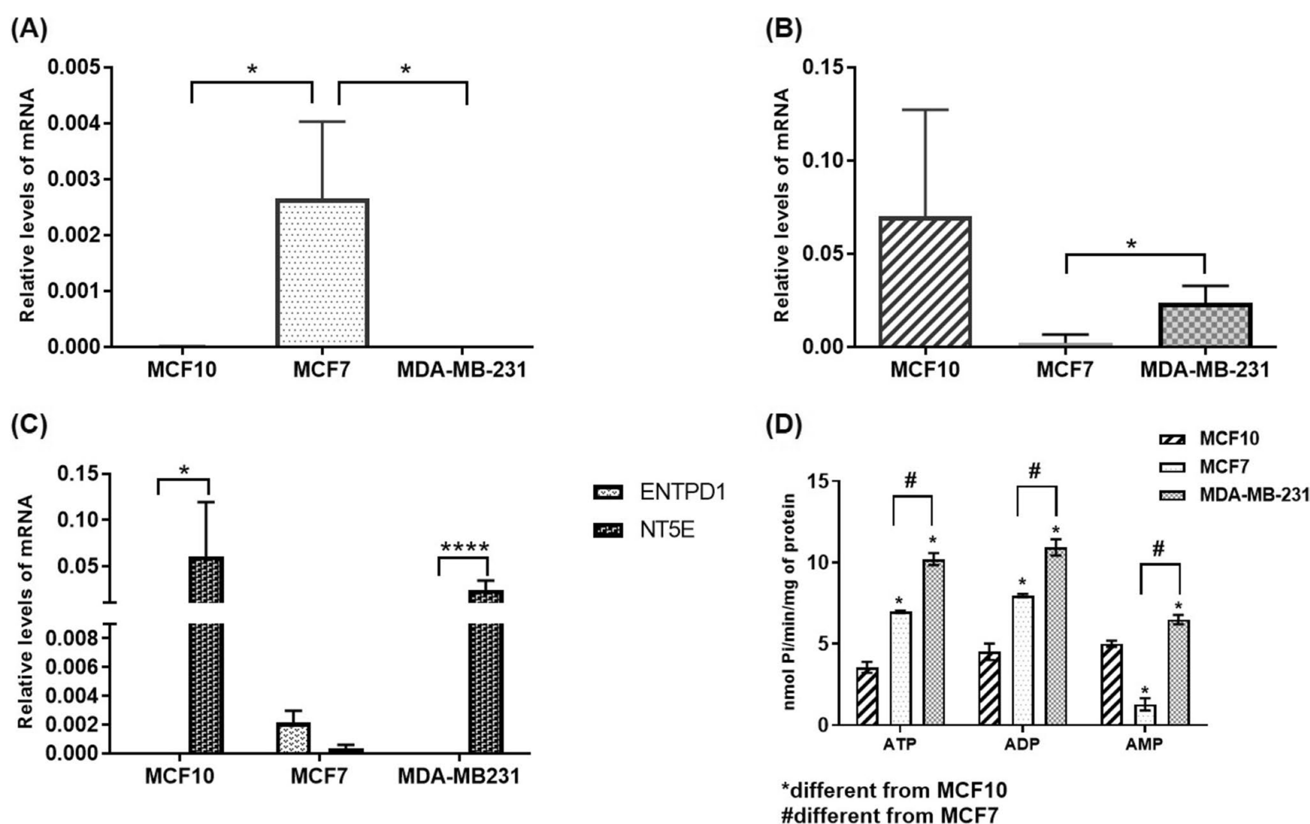


Fig. 1 NTPDase1 (*ENTPD1*) and ecto-5'-nucleotidase (*NT5E*) relative mRNA levels and nucleotides hydrolysis in breast cell lines (MCF 10A, MCF7, and MDA-MB-231). **A** NTPDase1 expression is significantly increased MCF7 compared to other cells (*$p < 0.05$). **B** Ecto-5'-nucleotidase expression is significantly increased in MDA-MB-231 compared to MCF7 (*$p < 0.05$). **C** Ecto-5'-nucleotidase expression is significantly increased in MCF 10A and MDA-MB-231 compared to NTPDase1 expression, while there is no significant difference between the expression of both enzymes in MCF7 (****$p < 0.0001$; *$p < 0.05$). Reference gene: *Beta-2 Microglobulin* (*β2M*). **D** ATP, ADP, and AMP hydrolysis on the surface of MCF 10A, MCF7, and MDA-MB-231 cell lines in nmol Pi/min/mg of protein (mean ± SD, n=6, *different from MCF 10A, #different from MCF7)

the levels of both ectonucleotidases by cell line, it was observed that both MCF 10A (p <0.05) and MDA-MB-231 (p <0.0001) presented higher *NT5E* than *ENTPD1* mRNA levels (Fig. 1C). Altogether, qPCR results point that *NT5E* is related to a more aggressive BC subtype, since *ENTPD1* mRNA levels were generally lower when compared to *NT5E*. We thus suggest that there is a coordinated action of both ectonucleotidases NTPDase1 and ecto-5'-nucleotidase, regulating the availability of purines and the tumorigenic action of these molecules. The dysregulation of these enzymes and other components of the purinergic signaling cascade may be related to the different BC phenotypes. From these data, we hypothesize that the expression of *NT5E* in the MCF7 lineage may be post-transcriptionally regulated by miRNAs and this could be a signature of luminal breast tumors.

Determination of ATP, ADP, and AMP hydrolysis

Ectonucleotidase mRNA relative levels were determined by qPCR. We further determined nucleotide hydrolysis to check if NTPDase 1 and ecto-5'-nucleotidase were functionally active on cell surface. In general, the hydrolysis of ATP, ADP, and AMP were significantly higher in the MDA-MB-231 lineage (p<0.0001 for ATP and ADP, when compared to the MCF7 and MCF 10A; p<0.01 for AMP, when compared to the MCF 10A; and p<0.0001 for AMP, when compared to the MCF7) (Fig. 1D). Similar to what was observed in qPCR, AMP hydrolysis was significantly lower in MCF7 cells than in other cell lines (5.003±0.184, 1.277±0.374, and 6.49 ±0.287 nmoles Pi/min/ mg of protein, n=6, in MCF10, MCF7, and MDA-MB-231, respectively), indicating a lower expression and activity of ecto-5'-nucleotidase in the MCF7, which may signal a striking feature and possibly a biomarker in this lineage. It should also be noted that the highest ecto-5'-nucleotidase activity was observed in MDA-MB-231 cells, strongly suggesting the relationship of the presence of this enzyme in the most aggressive BC subtype.

Ectonucleotidase genes are targeted by miRNAs

From in silico analyses, it was possible to observe that there were 1473 distinct miRNAs targeting *ENTPD1* gene and 806 distinct miRNAs targeting *NT5E* gene. The miRNAs identified by each tool are presented in the Supplementary Material, as well as their location in the human genome and the genes in clusters with them (Supplementary Tables). After the identification of miRNAs, their differential expression was then observed between each subtype of breast cell lineages evaluated (MCF 10A, MCF7, and MDA-MB-231). The results are presented in heatmaps for easier observation. All heatmaps generated are available in the Supplementary Material (Supplementary Figures 1-4).

Our analyses are concentrated on the heatmaps obtained by the intersection of *edgeR* and *DESeq2* results comparing the cell lines in pairs (Fig. 2) and on the heatmaps showing the comparison of miRNA expression between MCF7 and MDA-MB-231 with both software separately (Fig. 3). Together with gene expression results, in silico analyses for *ENTPD1* were not conclusive to ensure that this gene is regulated by miRNAs in a differential way between BC subtypes. On the other hand, *NT5E* expression was greatly diminished in MCF7 compared to the MDA-MB-231 cell line, suggesting a possible involvement of this enzyme in the progression, invasiveness, chemoresistance, and aggressiveness of the BC. In this context, it is worth mentioning the importance of miRNAs miR-101, miR-141, and miR-340 in the regulation of *NT5E*, which are upregulated in the MCF7 and downregulated in the MDA-MB-231 cells as obtained in *DESeq2* (Fig. 3B).

Differential expression analysis of ectonucleotidase mRNAs

In differential expression analysis of ectonucleotidase mRNAs, three *ENTPD* genes were downregulated in MCF 10A and three other *ENTPD* genes were downregulated in MCF7. None of those differential expressions for *ENTPD* genes was *ENTPD1* gene. Furthermore, the *NT5E* gene was upregulated in MCF 10A compared to MCF7 samples (Supplementary figure 5A). In differential expression analysis between MCF7 and MDA-MB-231 lineages, *ENTPD2* and *ENTPD8* but not *ENTPD1* genes were upregulated in MCF7 comparing to MDA-MB-231 (Supplementary figure 5B). Additionally, the *NT5E* was overexpressed in MDA-MB-231 comparing to MCF7 cells (Supplementary figure 5B). Finally, between MDA-MB-231 and MCF 10A cells, the same increased levels of the *NT5E* mRNAs occurred in MDA-MB-231 while four different *ENTPD* genes were overexpressed in MCF 10 A (Supplementary figure 5C).

Correlation between ectonucleotidase mRNAs and miRNA levels

It was revealed a negative correlation between hsa-miR-101-3p and its target gene *NT5E* in MCF7 cells and between hsa-miR-340-5p and its target gene *NT5E* (Supplementary figure 6A). Curiously, the same results were found in MCF 10A non-tumorigenic cells while in MDA-MB-231 there were no correlations (Supplementary figures 6B e 6C). Once *ENTPD1* genes were not differentially expressed, they were not included in correlation analysis.

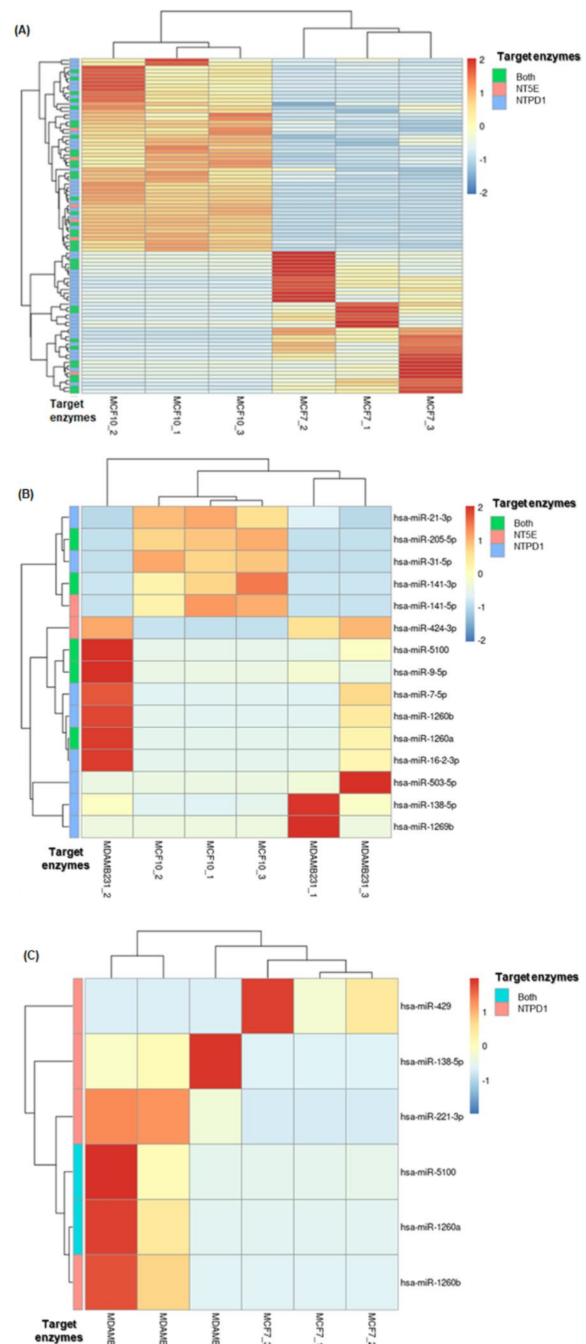


Fig. 2 Heatmap of the differential expression of miRNAs that target NTPDase1 (*ENTPD1*) and/or ecto-5'-(*NT5E*) nucleotidase in MCF 10A and MCF7 lines (**A**), MCF-7 and MDA-MB-231 lines (**B**), and MCF7 and MDA-MB-231 lines (**C**) obtained by the *edgeR* and *DESeq2* tools. **A** Top-down miRNA sequence: hsa-miR-221-5p; hsa-miR-7974; hsa-miR-188-5p; hsa-miR-411-5p; hsa-miR-509-3p; hsa-miR-410-3p; hsa-miR-508-3p; hsa-miR-409-3p; hsa-miR-892a; hsa-miR-577; hsa-miR-381-3p; hsa-miR-654-3p; hsa-miR-98-3p; hsa-miR-22-5p; hsa-miR-574-5p; hsa-miR-138-5p; hsa-miR-5690; hsa-miR-16-5p; hsa-miR-590-3p; hsa-miR-28-5p; hsa-miR-873-3p; hsa-miR-576-5p; hsa-miR-24-2-5p; hsa-miR-4664-3p; hsa-miR-4326; hsa-miR-573; hsa-miR-944; hsa-miR-141-5p; hsa-miR-1293; hsa-miR-3176; hsa-miR-423-5p; hsa-miR-3605-5p; hsa-miR-4775; hsa-miR-708-3p; hsa-let-7-b-3p; hsa-miR-31-5p; hsa-miR-873-5p; hsa-miR-6842-3p; hsa-miR-22-3p; hsa-miR-221-3p; hsa-miR-582-3p; hsa-miR-205-3p; hsa-miR-205-5p; hsa-miR-222-3p; hsa-miR-224-5p; hsa-miR-452-5p; hsa-miR-222-5p; hsa-miR-582-5p; hsa-miR-1910-5p; hsa-miR-708-5p; hsa-miR-584-5p; hsa-miR-1304-3p; hsa-miR-330-5p, hsa-miR-653-3p; hsa-miR-4714-3p; hsa-miR-5691; hsa-miR-338-3p; hsa-miR-184; hsa-miR-3200-3p; hsa-miR-5699-5p; hsa-miR-542-3p; hsa-miR-4758-3p; hsa-miR-4713-5p; hsa-miR-339-5p; hsa-miR-195-5p; hsa-miR-345-3p; hsa-miR-622; hsa-miR-96-5p; hsa-miR-1301-3p; hsa-miR-152-3p; hsa-miR-5094; hsa-miR-653-5p; hsa-miR-338-5p; hsa-miR-544b; hsa-miR-9-3p; hsa-miR-2276-3p; hsa-miR-195-3p; hsa-miR-1251-5p; hsa-miR-3664-3p; hsa-miR-455-5p; hsa-miR-503-5p; hsa-miR-429; hsa-miR-4654; hsa-miR-1290; hsa-miR-1246; hsa-miR-7-5p; hsa-miR-3127-3p; hsa-miR-618; hsa-miR-185-5p; hsa-miR-629-5p; hsa-miR-185-3p; hsa-miR-9-5p. **B** Top-down miRNA sequence: hsa-miR-21-3p, hsa-miR-205-5p, hsa-miR-31-5p, hsa-miR-141-3p, hsa-miR-141-5p, hsa-miR-424-5p, hsa-miR-424-3p, hsa-miR-5100, hsa-miR-9-5p, hsa-miR-7-5p, hsa-miR-1260b, hsa-miR-1260a, hsa-miR-16-2-3p, hsa-miR-503-5p, hsa-miR-138-5p, hsa-miR-1269b. **C** Top-down miRNA sequence: hsa-miR-429, hsa-miR-138-5p, hsa-miR-221-3p, hsa-miR-5100, hsa-miR-1260a, hsa-miR-1260b

Discussion

BC is the most frequent tumor among women with 2.1 million cases registered in 2018 worldwide, corresponding to about 12% of the total incidence of cancers [3, 27]. In Brazil, in 2021 there were 18,295 deaths from BC and an estimated 66,280 new cases of the disease for each year of the 2020–2022 triennium [28]. There are different types of BC and a single tumor can be the combination of several characteristics, which difficult specific diagnosis and efficient treatment [29]. In addition, several pathways are involved in the onset and development of this disease, such as purinergic signaling and miRNAs [30, 31]. Our work sought to analyze these two mechanisms by acting in consonance in the context of BC in different subtypes.

Briefly, our results indicate that the detection of relative levels of *ENTPD1* mRNA was higher in MCF7 cell line and that the higher expression of *NT5E* was found in MDA-MB-231. Studies have shown that NTPDase1 has significantly higher rates of protein expression in tumor tissues when compared to normal tissues [14, 32–36]. NTPDase1 is described as the enzyme that plays a dominant role in the purinergic regulation of inflammation and immune response [37]. In addition, it is associated with increased tumor recurrence, reduced overall survival, advanced stages of the disease [35], and increased tumor invasiveness and metastasis [36]. However, studies show that not only the expression of NTPDase1 but also of the other ectonucleotidases and purinergic receptors through a synergistic effect regulate the action of ATP and Ado [38]. Thus, other enzymes that also hydrolyze ATP and ADP may provide substrate for ecto-5'-nucleotidase [39] and increase the production of Ado, worsening the prognosis of a patient with BC [40].

The expression of ecto-5'-nucleotidase is also often associated with a poorer prognosis, increased risk of metastasis, and resistance to chemotherapy and it is known that this enzyme promotes the growth and metastasis of BC [41–43]. Studies demonstrate that NTPDase1 and ecto-5'-nucleotidase are key modulators of the biochemical composition of the tumor microenvironment and may be associated with antitumor immunity and of patient's survival [40]. Acting in concert, NTPDase1 and ecto-5'-nucleotidase produce Ado that, in turn, promotes immunosuppression. Even though some studies report that Ado can generate antitumor effect via A1 activation, this molecule is predominantly associated with a pro-tumorigenic effect [40]. Considering the role of ectonucleotidases, especially ecto-5'-nucleotidase, in the progression and metastasis of BC, the need to find molecules or methods that regulate these enzymes for the treatment or diagnosis of the disease has been noted. Jin et al. performed tests involving the combination of anti-CD73 nanoparticles with chemotherapeutics and observed that the action is synergistic and leads to tumor destruction, as well as preventing metastasis [19].

We also detected ATP, ADP, and AMP hydrolysis on the surface of MCF 10A, MCF7, and MDA-MB-231. Thus, the ectoenzyme activities are functionally active in all cell lines analyzed. In agreement with the qPCR results, the AMP hydrolysis activity performed by ecto-5'-nucleotidase is significantly lower in MCF7 cells than in other cell lines. It should also be noted that the highest ecto-5'-nucleotidase activity was observed in MDA-MB-231 cells, strongly suggesting the relationship of the presence of this enzyme in the most aggressive BC subtype. The high rate of AMP hydrolysis in the blood has been discussed as associated with a poorer prognosis in BC and therapies that reduce this hydrolysis are pointed out as promising [44].

In agreement with the analysis of RNA transcript levels, the ATP and ADP hydrolysis activity performed by NTPDase1 and other ectonucleotidases is also significantly higher than the AMP hydrolysis activity in MCF7 cells, reinforcing the hypothesis of post-translational regulation of ecto-5'-nucleotidase by miRNAs. Even though low relative levels of *ENTPD1* mRNA were detected in MCF 10A and MDA-MB-231 cells, the hydrolysis of ATP and ADP were found in all cell lines. Thus, the mRNA levels of *ENTPD1* were enough to ensure protein translation and the hydrolysis of NTPDase1 substrates. However, it must be considered that enzyme activities were determined from the release of inorganic phosphate (Pi) and we thus cannot rule out the presence of other ectonucleotidases together with NTPDase1 performing this reaction on the cells surface. Therefore, the results regarding the involvement of NTPDase1 with BC are still unclear, suggesting that other regulatory mechanisms in addition to miRNAs may be involved in this process [14].

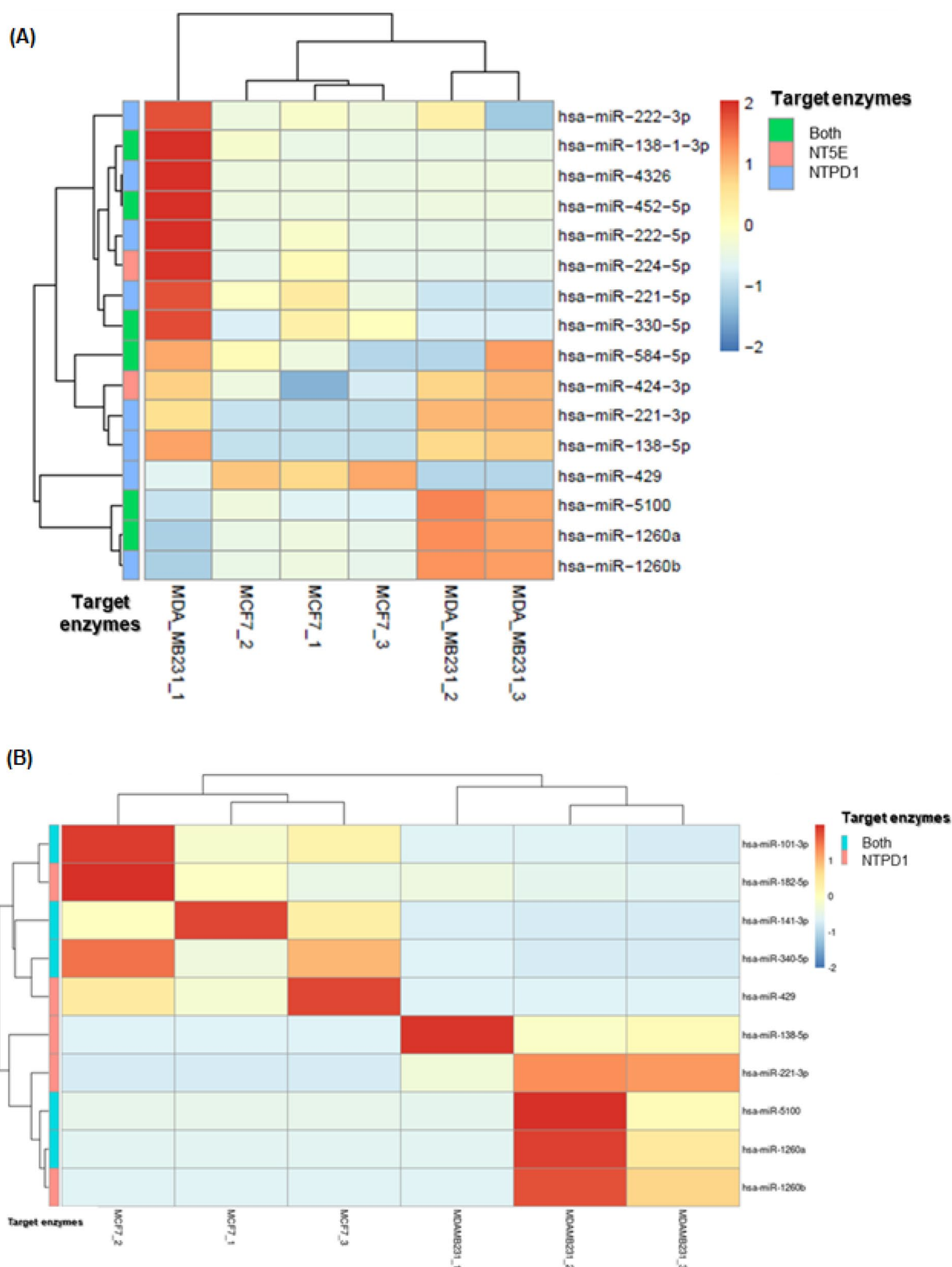
From in silico analyses, we identified several miRNAs that target *ENTPD1* and *NT5E* genes and are expressed differently in these three breast cell lines (Figs. 2 and 3). They should be the target of further research as they may be related to the development and progression of the different BC subtypes. Particularly, we identified hsa-miR-101-3p, hsa-miR-141-3p, and hsa-miR-340-5p as regulators of *NT5E*, which deserve special attention. They are more over expressed in MCF-7 cells when compared to MDA-MB-231 cells (Fig. 3B).

MiR-101 is identified in the literature as an inhibitor of migration, metastasis, and growth of the mammary tumor [45–48], and its inhibitory potential has already been explored in vitro as a therapy for BC [49]. Although the ecto-5'-nucleotidase mRNA has not yet been described as a target for this miRNA, our data indicate that such regulation may be considered a therapeutic approach to BC cells. Besides, other results are in line with our hypothesis showing that the low expression of miR-101 in MDA-MB-231 is associated with the more aggressive profile of this tumor, considering that the administration of such miRNA inhibits cell proliferation and migration [50].

Regarding miR-141, data presented in the literature are still contradictory, but it is known that the dysregulation in miR-141 blood levels in BC cancer patients can be used as a metastasis biomarker [51, 52]. It is also worth mentioning that miR-141 was downregulated in BC cells resistant to the chemotherapy drug trastuzumab, which shows the association between the low expression of such miRNA with chemoresistance [53]. However, in differentially expressed miRNAs (DEmiRNAs) analysis comparing lineages tamoxifen-sensitive MCF7 with tamoxifen-resistant LY2, deposited in GEO database as GSE28267, the hsa-miR-141 was not differentially expressed [54].

Finally, miR-340 [55] is also considered a BC suppressor, as it inhibits the migration of tumor cells and tissue invasion, considering that the deletion of the gene of such miRNA is directly associated with an increase in the migration and invasion of other tissues by mammary tumor cells [56–59]. Also, the stimulus to overexpression of miR-340 in MDA-MB-231 cell lines was able to significantly reduce the proliferation and migration of these cells [60–62].

Additionally, in DEmiRNAs analysis between spheroid-enriched cells with cancer stem cells (CSC) properties and parental cells, both from MDA-MB-231 lineages, none of these miRNAs was found differentially expressed [63]. Contrary, in studies comparing spheroid-enriched cells and parental cells of MCF7 lineages, the miR-101-1/101-2 and miR-340 were downregulated [64]. So, as CSC have chemoresistance and pro-metastatic roles, the miR-101 and miR-340 are BC suppressors [65]. Thus, these findings corroborated with our results, because these miRNAs increased in parental MCF7 cells but not in spheroid MCF7 cells and



neither MDA-MB-231 cells. These studies were deposited in GEO database with GSE75396 and GSE68246 accession numbers.

In our results of differential expression analysis, the *NT5E* mRNA levels increased in MDA-MB-231 compared to MCF7 cells (Supplementary Figure 5B) corroborating with

◂**Fig. 3** Heatmap of the differential expression of miRNAs that target NTPDase1 (*ENTPD1*) and/or ecto-5'-nucleotidase (*NT5E*) in the MCF7 and MDA-MB-231 lines obtained by the *edgeR* tool (**A**) and by the DESeq2 tool (**B**). **A** Top-down miRNA sequence: hsa-miR-222-3p, hsa-miR-138-1-3p, hsa-miR-4326, hsa-miR-452-5p, hsa-miR-222-5p, hsa-miR-224-5p, hsa-miR-221-5p, hsa-miR-330-5p, hsa-miR-584-5p, hsa-miR-424-3p, hsa-miR-221-3p, hsa-miR-138-5p, hsa-miR-429, hsa-miR-5100, hsa-miR-1260a, hsa-miR-1260b. **B** Top-down miRNA sequence: hsa-miR-101-3p, hsa-miR-182-5p, hsa-miR-141-3p, hsa-miR-340-5p, hsa-miR-429, hsa-miR-138-5p, hsa-miR-221-3p, hsa-miR-5100, hsa-miR-1260a, hsa-miR-1260b

the results of qPCR (Fig. 1B). Furthermore, the hsa-miR-101-3p, hsa-miR-141-3p, and hsa-miR-340-5p were down-regulated in MDA-MB-231 (Fig. 3B). Thus, the regulation of the *NT5E* gene by these miRNAs and their suppressor tumoral aggressiveness role in BC could be confirmed. Besides, to our knowledge, this is the first time the analysis between MCF7 and MDA-MB-231 lineages, describing the differential expression of miR-101, miR-141, and miR-340 and their regulation upon ecto-5'-nucleotidase mRNAs, is reported in the literature and deposited in public databases.

Finally, according to the literature, the 3′UTR region of the FMR1 mRNA is a target of miR-101, the 3′UTR of the KLF12 and AR genes are targets of the miR-141, and the same region of the REV3L mRNA is a target of the hsa-miR-340-5p [66–69]. Our data show that miRNAs hsa-miR-101-3p and hsa-miR-340-5p target *NT5E* and *ENTPD1* mRNAs, as verified by miRTarBase and Diana tools, respectively. Therefore, we suggest that these miRNAs likely regulate the *ENTPD1* and *NT5E* mRNAs through their 3′UTR regions. Also, as the mRNA levels were downregulated at the same time these miRNAs were upregulated, the mechanism of miRNAs control on ectonucleotidases expression is likely by cleavage of the mRNAs.

Conclusion

Considering the results presented, we suggested a central role of regulation of *NT5E* by miRNAs in BC. We highlighted the relevance of miRNAs miR-101, miR-141, and miR-340, which target the transcripts of this enzyme and have been already presented in the literature as associated with tumor suppression, consolidating our hypothesis. Therefore, we believe that both the enzyme ecto-5'-nucleotidase and the miRNAs presented here should be considered strong candidates to be targeted in new diagnostic techniques and therapies for BC. Finally, further studies are needed to better identify the involvement of ecto-5'-nucleotidase and these miRNAs in the aggressiveness and invasiveness of BC.

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Author contribution F.C.S., T.G.A., C.R.F., and M.S.G. designed the study. F.C.S. and C.A.M. performed the qPCR analyses of ectonucleotidases gene expression. F.C.S., A.B.M.N., and T.C.S.C conducted all in silico analyses. F.C.S., M.S.G., T.G.A., and C.R.F. designed and supervised experiments. F.C.S., A.B.M.N., C.A.M., and T.C.S.C. analyzed data. F.C.S. and C.R.F. drafted the manuscript and all authors reviewed and approved the final version sent for publication.

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Code availability Not applicable.

Declarations

Conflicts of interest The authors declare no competing interests.

Ethical approval Not applicable.

Informed consent
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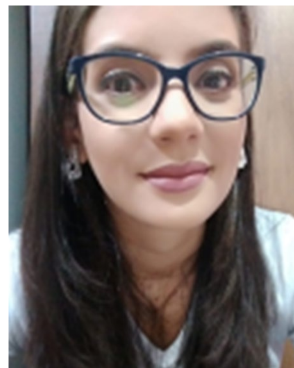

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