

Functional Analysis of *BRCA1* 3'UTR Variants Predisposing to Breast Cancer

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Purpose: Breast Cancer (BC) is the main female cancer diagnosed worldwide, and it has been described that few genes, such as *BRCA1*, have a high penetrance for this type of cancer. In this manuscript, we were interested in evaluating the effect of 3'UTR variants on *BRCA1* expression.

Patients and Methods: To accomplish this objective, Whole Exome Sequencing (WES) data of 400 patients with unselected BC was used to filter variants located in the region of interest of *BRCA1* gene, finding two of them (c.*36C>G and c.*369_373del). miRGate and miRanda in silico tools were used to predict microRNA (miRNA) interaction.

Results: The two variants (c.*36C>G, c.*369_373del) were predicted to affect miRNA interaction. After cloning of *BRCA1* 3'UTR into pMIR-Report vector, the construct was transfected into two BC cell lines (MDA-MB-231 and MCF-7), and the variant c.*36C>G evidenced overexpression of reporter gene luciferase, showing that the transcript was not being degraded by the miRNA in MDA-MB-231 cells.

Conclusion: The variant seems to protect against Triple Negative BC probably due to the expression level of miRNA in this particular cell line (MDA-MB-231). This is consistent with the clinical history of the patients who harbor BC Hormone Receptors positive (HR+).

Keywords: miRNA, 3'UTR variant, breast cancer, *BRCA1*

Introduction

Breast cancer (BC) is the first cancer in the world in terms of incidence and mortality for women. It has been estimated that 2,261,419 were newly diagnosed and 684,996 died of BC in 2020¹ and is predicted an increase of approximately 10% of both rates for 2025 (www.gco.iarc.fr). The majority of BC is sporadic (93–95%) and approximately 5–7% of BC is hereditary and associated with family history.²

BC is a disease with genetic and environmental risk factors.³ The main environmental factors are age, alcohol consumption, obesity, and smoking among others.^{4–7} In the last three decades, 12 genes have been associated with the risk of developing BC, *ATM*, *BRCA1*, *BRCA2*, *BRIPI*, *CDHI*, *CHEK2*, *PALB2*, *PTEN*, *RAD51C*, *RAD51D*, *STK11* and *TP53*. Some of them are high penetrance genes such as *BRCA1* and *BRCA2*, and others are moderate penetrance genes, for example *CHEK2* and *BRIPI*.⁸

The two major genes associated with BC are *BRCA1* and *BRCA2* discovered in 1990 and 1994, respectively. These two genes are located on chromosomes 17 and 13 and are involved in the maintenance of genome stability, especially in the double-strand DNA break repair through homologous recombination. These genes show an autosomal dominant inheritance pattern.⁹ In fact, the cumulative risk of BC increases with aging among people having *BRCA1* and *BRCA2* pathogenic variants, reaching a maximum of 72% and 69% at the age of 80 years, respectively.¹⁰ *BRCA1/2* genes account

for the cause of 25% of hereditary BC cases and in the general population, the prevalence of pathogenic variants in *BRCA1/2* genes has been estimated as 1 in 300 and 1 in 800, respectively.¹¹ In the Ashkenazi Jewish ethnic group, the prevalence is higher than the general population, due to endogamic behavior, and estimated as 1 in 40.¹² It has been demonstrated that *BRCA1* pathogenic variants are more frequent in Triple Negative BC (TNBC) compared to *BRCA2* gene which are more prevalent in less aggressive BC subtypes.¹³ Nowadays, it has been described around 4900 pathogenic variants in *BRCA1/2* genes (BRCA Exchange <https://brcaexchange.org/>) and the majority of them are located in coding regions. Few pathogenic variants are reported in non-coding regions including splicing regions (70 pathogenic variants) or UTR regions (0 pathogenic variant).

Non-coding regions have paramount importance in gene expression regulation. In particular, 3'UTR interacts with several types of miRNAs, which are a type of non-coding RNAs (~22 nucleotides) that interact with 3'UTR and can inhibit the translation or/and promote mRNA degradation. Briefly, the miRNAs are transcribed by RNA pol II or III in pri-miRNA, then they are processed into short 70-nt stem-loop RNA, pre-miRNAs by the Microprocessor complex (Drosha enzyme – DiGeorge syndrome critical region 8 gene (DGCR8)). These RNAs are then exported by exportin-5 from nucleus to cytoplasm and processed into double-strand miRNAs by the RNase II enzyme, Dicer. The duplex is loaded onto Argonaute protein (AGO) and RISC complex (RNA inducing silencing complex). The strand less thermodynamically stable is removed and the complex RISC-miRNA is able to recognize 3'UTR of target mRNA. The seed region, a sequence spanning from position 2 to 8 at the 5' end of the miRNA, recognizes the miRNA response element (MRE) in the target mRNA.^{14,15} A variant in the 3'UTR or in the miRNA can inhibit the mRNA-miRNA interaction or create a new interaction with a different miRNA that has been previously described in breast and ovarian cancer.^{16–19}

In this study, we performed the functional analysis of germline variants located on 3'UTR of *BRCA1* gene. This study is the first analysis of these type of variants described in Colombian patients diagnosed with unselected BC.

Materials and Methods

Patients

Four hundred unselected BC, regarding cancer family history, female patients were aged over 18 years old, with a diagnosis of invasive BC confirmed histopathologically, without personal and family history of germline BC risk pathogenic variants. These women were attended to Fundación CardioInfantil, Hospital Universitario de Mederi, Bogota, Colombia and different cancer centers located throughout Colombia (Bucaramanga, Cali, Medellin, Manizales, Pereira).

This study was performed in compliance with the Helsinki Declaration and was approved by both, Fundación Cardioinfantil–Instituto de Cardiología and Universidad del Rosario Ethics Committee (approval numbers: 402,018 7-11-2018, DVO005 1805-CV1469 3-12-2021, Pfizer: WI241988 – Investigator initiate research, independent review board: 28–08-2018, GF1147 2018). All patients approved the informed consent and were part of a previously described study <https://doi.org/10.1158/1538-7445.SABCS21-P3-07-05>. The study was financially supported by the grant “Capital Semilla” of the University of Rosario, IV-FCS036.

Variants Analysis

Germline variants located on 3'UTR of *BRCA1* gene (~500pb) were filtered by population frequencies, taking into consideration a Minor Allele Frequency (MAF) $\leq 1\%$ exomes in the gnomAD database (<https://gnomad.broadinstitute.org> v2.1.1). Levels of expression of miRNAs in breast and/or BC were used to use in silico tools miRGate (<http://mirgate.bioinfo.cnio.es>) and to filter germline variants ([supplementary Tables 1–3](#)). miRanda package v1.9 on Linux (<https://cbio.mskcc.org/miRNA2003/miranda.html>) was used to predict the miRNA-3'UTR interaction.

None of the women, harboring the c.*36C>G variant, have pathogenic/likely pathogenic germline variants in the ORF of 10 genes with known penetrance for BC (*ATM*, *BARD1*, *BRCA1*, *BRCA2*, *CHEK2*, *CDH1*, *PALB2*, *TP53*, *RAD51C*, and *RAD51D*).

pMIR-Report Construction

The 3'UTR of *BRCA1* was amplified from patient DNA using GoTaq Master Mix (Promega, cat#M7122) with the following primers (huBRCA1_3UTR_For:CCCACTAGTCTGCAGCCAGCCACAGGTAC, huBRCA1_3UTR_Rev:

CCCAAGCTTCGATCCCAAGCACTCTCCTTC), PCR products were cloned into pCR4-TOPO vector (Invitrogen, cat# 45-0030), and then cloned into the pMIR-ReportTM Luciferase vector (Invitrogen, cat#AM5795) through enzyme digestion (SpeI, HindIII, New England Biolabs, cat# R0133S and # R0104S).

Cell Culture

MDA-MB-231 and MCF-7 cells (ATCC, cat#HTB-26, cat#HTB-22) were maintained in culture with DMEM-F12 (10% FBS, 1%P/S) at 37°C and 5% of CO₂.

Luciferase Assay

Cells were transfected through Fugene 6 (Promega, cat#E2692) with 1µg of vector and co-transfected with 30ng of Renilla reporter vector and incubated for 48h. Cells were lysed with Passive Buffer of Dual-Glo luciferase assay (cat#E2980) among the manufacturer's recommendations. Briefly, 20µL of cell extracts were used to the read of the luminescence by the GloMax system (Promega). Luciferase was read at 570nm through LARII reagent and Renilla at 480nm through STOP&Glo reagent. Three experiments were realized, and RLU's were normalized by experiment and compared.

GEO Database

The GSE146477 reports an array of miRNA expression and the miR-99a expression was compared in MDA-MB-231 and MCF-7 cells.

Statistical Tests

The replicates were compared by Mann–Whitney test using Prism 10 (<https://www.graphpad.com/features>).

Results

On the 400 women diagnosed with BC, 2 germline variants were observed on 3'UTR of *BRCA1* gene with a MAF $\leq 1\%$. The variants c.*36C>G and c.*369_373del were tested on miRGate and were located on two miRNA interaction sites, miR-99a-3p and miR-26a-2-3p, respectively ([supplementary Tables 1 and 2](#)). The first variant was observed in three patients and the second in only one patient, all in heterozygote state. The c.*36C>G variant is located on the complementary interaction site of miR-99a-3p ([Figure 1A](#)) and the c.*369_373del variant is located on the miR-26a-2-3p interaction seed sequence ([Figure 1B](#)). These variants were then tested on two cell models of breast cancer, MDA-MB-231 (TNBC cells) and MCF7 cells (human breast cancer cell line with estrogen, progesterone and glucocorticoid receptors) ([Figure 1C and D](#), respectively). One variant, which has not been detected by WES, c.*421G>T was found and included in the experiments. Higher (Relative Luciferase Units) RLU were observed for the c.*36C>G variant compared to the WT *BRCA1* 3'UTR in the MDA-MB-231 cells (p -value 0.0286) ([Figure 1C](#)) but not in the MCF-7 cell line ([Figure 1D](#)). The other variants had no difference in the RLU compared to WT. The expression level of the miR-99a-3p was verified and compared between both BC cell lines from the GEO database (GSE146477) and the expression level of miR99a-3p is higher in MDA-MB-231 in comparison with MCF-7 ([supplementary Figure 1](#)).

Discussion

BC is the cancer most frequently diagnosed in women and causes 15% of cancer-related deaths in the same population. BC is a multifactorial disease, with a high percentage of unexplained heritability. It has been well described that approximately 10% of BC are hereditary and only the 5.6% of all BC cases are caused by germline pathogenic variants on 12 BC predisposition genes.^{8,20} At present, it is important to explore new mechanisms capable of explaining the cancer development and the heritability of this disease.

miRNAs are small non-coding RNA with an important role in the regulation of gene expression. It has been described that these miRNAs can be involved in different molecular processes in BC, such as apoptosis, epithelial-mesenchymal transition, and angiogenesis, among others.^{21–23} The miRNAs can exert their function via their union on the 3'UTR of the gene. It has been proposed that some miRNAs can be biomarkers of breast, prostate and ovarian cancer.^{24,25} It has been described that some miRNAs have an oncogenic and tumor suppressive function.^{26,27} The dysregulation of miRNAs expression could explain in part the dysregulation of some genes involved in BC.

A

Score: 126.000000 Q:1 to 22 R:28 to 48 Align Len (22) (72.73%) (77.27%)

```

3' UTR BRCA1 5' CAGGACCCCAAG-AAUGAGCUUA 3'
                || |||||  || | :|||
hsa-mir-99a-3p 3' GU-CUGGGUAUCUUCGCUCGAAC 5'

```

Energy: -24.370001 kCal/Mol

B

Score: 138.000000 Q:1 to 22 R:344 to 377 Align Len (33) (54.55%) (63.64%)

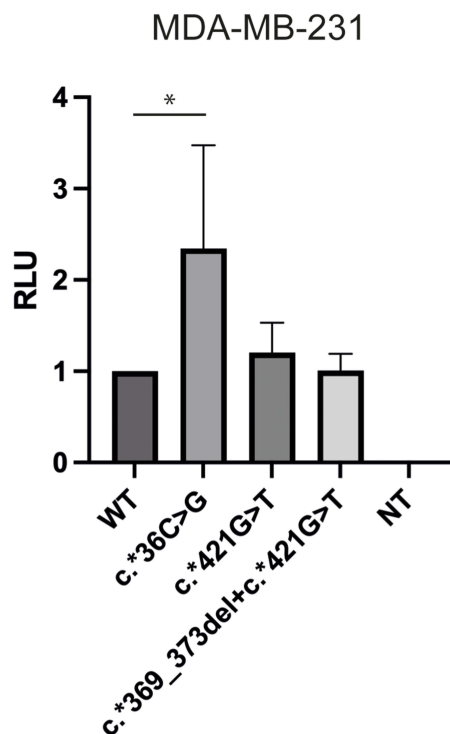
```

3' UTR BRCA1      5' GAAGCACAGAGTGGCUUGGCCUCAAGAGAAUAGC 3'
                  |||:|  |||:  |||  |||||
hsa-mir-26a-2-3p 3' CUUUGU---UCAUU-----AGU--UCUUAUCC 5'

```

Energy: -16.370001 kCal/Mol

C



D

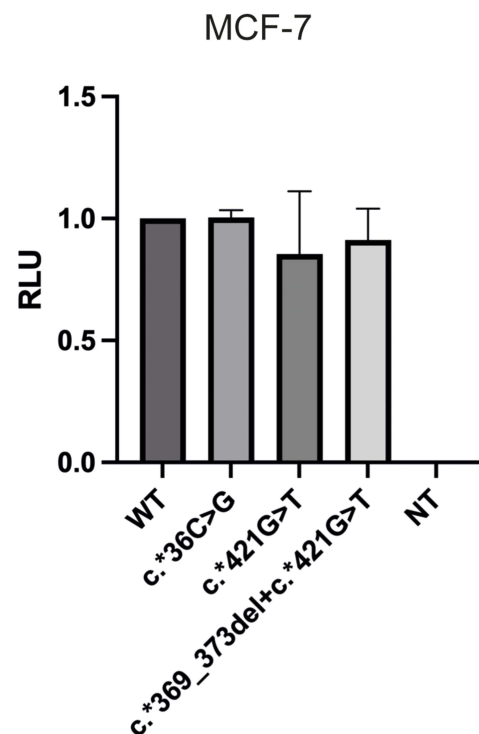


Figure 1 Involvement of variants on 3'UTR on *BRCA1* expression. (A) Interaction of miR-99a-3p with *BRCA1* 3'UTR in presence of c.*36C>G variant (red) (B) Interaction of miR-26a-2-3p with *BRCA1* 3'UTR in presence of c.*369_373del variant (red) (C and D) Luciferase assay representing the transfection of pMIR-Report with the different variants in MDA-MB-231 and MCF-7 cell lines, respectively. Statistical significance *p-value<0.05.

A few years ago, different studies have shown the impact of 3'UTR variants on the expression and susceptibility to cancer development.^{18,28,29} In this study, we were interested in exploring the impact of 3'UTR variants on the gene expression of *BRCA1*. In fact, *BRCA1*, as well as *BRCA2*, have an important role in the biology of the cell, especially in tumor microenvironment in BC.

The function of BRCA1/2 consists essentially in the maintenance of genome stability through participation in DNA repair processes including homologous recombination.³⁰ Pathogenic variants on this gene have a penetrance of approximately 69% up to the age of 80 years and seem to exhibit an autosomal dominant inheritance.¹⁰ On the BRCA Exchange database (brcaexchange.org) there are reported more than 2000 pathogenic variants located on coding regions and splicing sites of *BRCA1/2* genes. All 3'UTR variants are probably considered non-pathogenic, according to the current ACMG/AMP guidelines, but these variants could be considered risk factors as described by Zhang and collaborators.¹⁸ In this study, we observed that the c.*36C>G variant evidenced an overexpression of the reporter gene luciferase in MDA-MB-231 cells, a TNBC cell line. This finding is consistent with the clinicopathological characteristics of the BC in the three patients harboring this variant, who present a positive hormone receptor (HR+) subtype (supplementary Table 4). This variant seems to protect against the development of TNBC, which is a subtype of BC with a worse prognosis compared with HR+ BC. Expression of the miR-99a was validated in the two cell lines, by the GEO database (GSE146477), and we observed that the miR-99a-3p is overexpressed in MDA-MB-231 cell line in comparison with MCF-7 cell line (1 vs 0.73 fold-change). The difference in miRNA expression could explain the lack of RLU difference between the variants and WT in MCF-7 cells.

It has been demonstrated that miR-99a correlates negatively with inflammation in adipose tissue in humans. It has been well described that obesity/overweight is a pro-inflammatory status, and a BC risk factor. The mechanism, involved in obesity and hormonal changes is related to adipokines, such as leptin, adiponectin, and resistin among others. The hormonal changes in BC patients with obesity/overweight also have oxidative stress in the tumoral microenvironment regulated by the adipokines, supporting carcinogenesis in breast tissue.^{31–34} The MCF-7 cell model could explain this situation since it expresses HR and a lower level of miR-99a. 2/4 of the patients harboring the 3'UTR variant (c.*36C>G) have a BMI >25 kg/m² (supplementary Table 4) this finding could reinforce the hypothesis of obesity/overweight role in breast carcinogenesis.

In summary, the c.*36C>G variant does not allow the degradation of BRCA1, as a protector effect in TNBC model, and not in the HR+ BC model, probably due to differential miRNA expression levels.

We suggest that the scientific community should analyze the impact of 3'UTR variants on gene expression, given that in a model of multifactorial disease, each variants have a little additive effect that, when added, can permit the development of the phenotype. Furthermore, 3'UTR variants allow gene expression dysregulation in different diseases with a Mendelian inheritance pattern.^{35,36} Evolution of technologies with bioinformatics algorithm like miRGate or miRanda could help to expand the knowledge in genetics and allow us to propose new molecular mechanisms involved in different diseases including BC.

Data Sharing Statement

Data supporting this study are available under request.

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Disclosure

The authors report no conflicts of interest in this work.

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