

***RNF8* over-expression promotes genomic instability in aggressive breast cancer**

La sobre-expresión de *RNF8* promueve la inestabilidad genómica en el cáncer de mama agresivo

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ABSTRACT

Triple Negative Breast Cancer (TNBC) is associated with extensive genomic instability, where double-strand breaks (DSB) are the most consequential DNA lesions. Knockdown of the *RNF8* inhibits focus formation at DSB sites, suggesting that *RNF8* is an essential regulator of the DNA repair system. We are investigating whether *RNF8* acts as a regulator of *BRCA1* and *53BP1* proteins in human breast cancers (HBC). We analyzed 57 formalin-fixed and paraffin-embedded (FFPE) HBC taken from a population of Mexican women enriched for basal-like cancer if altered *RNF8*, *53BP1*, *CB1*, and *Ki-67* mRNA expression occurs compared to median expression as reference. *RNF8* transcript levels varied among HBC. The combined effect *RNF8* high/*53BP1* low was associated with high expression of *Ki67* ($p = 0.028$).

Conversely, *RNF8* low expression was associated with low *Ki67* ($p = 0.017$), and *CB1* ($p = 0.035$) expression, independent of the *53BP1* expression. Differential expression of *RNF8* and *53BP1* in a series of HBC and the association between *RNF8/53BP1* levels and the tumor phenotype was demonstrated. Our findings suggest that some breast tumors have an altered DNA repair pathway resulting in activation of *RNF8* and deregulation of *53BP1*, which may contribute to the genomic instability in more aggressive tumors.

Keywords: TNBC; DNA repair; Mexican women.

RESUMEN

TNBC se asocia con inestabilidad genómica, donde las roturas de doble cadena (DSB) son las lesiones más importantes del ADN. La eliminación de *RNF8* inhibe la formación de focos en los sitios DSB, lo que sugiere que *RNF8* es un regulador importante del sistema de reparación del ADN. Estamos investigando si *RNF8* funciona como regulador de las proteínas *BRCA1* y *53BP1* en tumores mamarios humanos. Analizamos 57 tumores mamarios FFPE tomados de una población de mujeres mexicanas, para evaluar la expresión de *RNF8*, *53BP1*, *CB1* y *Ki67*. Los niveles de *RNF8* variaron entre los tumores. El efecto de *RNF8* alto/*53BP1* bajo se asoció con una alta expresión de *Ki67* ($p = 0.028$). Por el contrario, *RNF8* bajo se asoció con *Ki67* bajo ($p = 0.017$) y *CB1* bajo ($p = 0.035$) y fue independiente de la expresión de *53BP1*. Se demostró la expresión diferencial de *RNF8* y *53BP1* en una serie de tumores mamarios humanos y la asociación entre los niveles

de *RNF8/53BP1* y el fenotipo tumoral. Nuestros hallazgos sugieren que algunos tumores mamarios tienen una vía de reparación del ADN alterada que resulta en la activación de *RNF8* y la desregulación de *53BP1*, lo que puede contribuir a la inestabilidad genómica en tumores más agresivos.

Palabras clave: TNBC; reparación del ADN; mujeres mexicanas.

INTRODUCTION

Breast cancer is the most common cancer in the world. Breast cancer cases in Mexico have an incidence of 27.64/per 100,000 habitants over 20 years old (INEGI, 2023). Ongoing research on breast cancer is trying to improve its treatment to assist in dealing with this issue.

Genomic instability is a hallmark of cancer, including triple-negative breast cancer, an aggressive subtype characterized by the absence of estrogen and progesterone receptors, and *HER2/neu* amplification. In this context, the role of *RNF8* in genomic instability is crucial. The RING finger protein 8-induced ubiquitination signaling cascade promotes DNA repair and maintains genomic stability (Krishnan *et al.*, 2023). Our study reveals a potential correlation between *RNF8* expression and genomic instability in breast cancer among Mexican women.

Genome integrity could be compromised without DNA repair or due to the ineffective control of the cell cycle (Chowdhury *et al.*, 2022). Therefore, after the assault of genotoxic agents or intrinsic instability, cooperative mechanisms for effective repair systems (Tan *et al.*, 2023) and appropriate regulation of checkpoints for the cell cycle play essential roles. Failure of DNA repair may result in different disorders and tumorigenesis. The DNA damage response (DDR) protects against genomic instability; tumorigenesis can progress if it fails (Vázquez-Romo *et al.*, 2023).

DNA double-strand breaks (DDSB) are recognized by a series of proteins related to DDR. These DDSB molecular damages promote a cascade of molecular signals that activate *RNF8* (Zhou *et al.*, 2019).

RNF8 acts as an E3 protein ubiquitin ligase and possesses the FHA amino-terminal domain and a RING finger carboxyl terminus domain (Kolas *et al.*, 2007). Proteins bearing a RING-finger domain promote ubiquitin transfer without forming a covalent intermediate (Branigan *et al.*, 2020). The FHA

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domain can interact in a phosphodependent manner with ATM-phosphorylated MDC1 (Huen *et al.*, 2007), mediating the polyubiquitination of repair nuclear proteins and promoting their ionizing radiation-induced foci (IRIF) (Mailand *et al.*, 2007). RNF8 works in the DNA repair system through different pathways, targeting 53BP1 and BRCA1 proteins and localizing them at damaged DNA sites (Kolas *et al.*, 2007). 53BP1 is involved in the error-prone DNA repair of the non-homologous end-joining process (NHEJ) by its relationship to the shieldin complex (Noordermeer *et al.*, 2018). BRCA1 is associated with the error-free homologous recombination mechanism (HR) (Toh and Ngeow, 2021).

53BP1 loss rescues homologous recombination in BRCA1-deficient breast cancers (Schochter *et al.*, 2020). However, the regulatory factor for selecting HR and NHEJ is still elusive. We hypothesized that RNF8 is a proliferation modulator and a novel regulator of BRCA1 and 53BP1 proteins in human breast cancers. The ultimate objective is to identify patterns of gene expression that can be used directly as markers for breast cancer aggressiveness.

MATERIAL AND METHODS

Ethical Considerations

The study was approved by the Local Health Research Committee 2602 of Hospital de Especialidades 2, a High Specialty Medical Unit of the Instituto Mexicano del Seguro Social at the Centro Médico Nacional del Noroeste, under registration number: R-2007-2601-43, with the title: Perfil inmunohistoquímico en recaída de pacientes con cancer de mama en el noroeste de México.

We take care of the safety and well-being of our patients. We fully respect the principles outlined in the Nuremberg Code, the Declaration of Helsinki and its amendments, the Belmont Report, and the United States Code of Federal Regulations.

Participants

We conducted a pilot study in 57 formalin-fixed-paraffin-embedded tissues from invasive breast carcinomas from participants of the "Binational Study of Breast Cancer, ELLA" in the Universidad de Sonora at Hermosillo, Mexico. Participants included in the study were females 18 years or older of Mexican descent diagnosed with invasive breast cancer within 24 months before informed consent.

Histology & Immunohistochemistry

For routine histology analysis, tissues were fixed in 10 % formalin, blocked in paraffin, sectioned, stained with hematoxylin and eosin, and subsequently examined by light microscopy. Antibodies for ER (1:35), PR (1:200), HER/2neu (1:200), and Ki67 (1:100) were employed for immunohistochemical analysis according to the manufacturer's instructions (BioSB, Santa Barbara, CA).

For RNF8 immunohistochemistry, 3 μ m tissue sections were stained using a rabbit polyclonal antibody (1:100; Sigma-Aldrich) anti-RNF8 utilizing a Discovery XT Automa-

ted Immunostainer [Ventana Medical Systems, Inc. (VMSI)]. VMSI validated reagents, including deparaffinization, cell conditioning (antigen retrieval with a borate-EDTA buffer), primary antibody staining, detection, and amplification using a biotinylated streptavidin-horseradish peroxidase and 3,3'-diaminobenzidine (DAB) system and hematoxylin counterstaining were performed. RNF8 was detected using a goat anti-rabbit secondary antibody and an UltraMap DAB detection kit (VMSI). Images were captured using an AperioScanScope with ImageScope management software (Aperio Technologies). The overall presence or absence and patterns of staining were recorded. The extent of staining was analyzed as a percentage of positively stained areas within the lesion. Each positive staining lesion was further quantified using an arbitrary scale of staining intensity (weak, moderate, or strong).

Determination of the molecular subtype of breast cancer

The breast cancer molecular subtypes were defined as:

- Luminal (ER $\geq$ 5 % and/or PR $\geq$ 5 %).
- HER2 positive (HER2 +++).
- TNBC (ER < 5 % and/or PR < 5 %) and HER2 0/+.

Nucleic acids isolation

Total RNA was isolated from ten tissue sections of 10 μ m thickness each of a formalin-fixed and paraffin-embedded (FFPE) breast tissue sample using the RNeasy FFPE kit (Qiagen, Chatsworth, CA). The genomic DNA (gDNA) was isolated from FFPE tissue using the QIAmp DNA FFPE Tissue Kit (Qiagen, Chatsworth, CA), following the manufacturer's instructions.

RNF8 Genotyping

RNF8 (*rs195382*) was analyzed using the TaqMan[®] SNP Genotyping Assay from Applied Biosystems. The reaction components included TaqMan Genotyping Master Mix (12.5 μ L), 40X TaqManRNF8 SNP Genotyping Assay Mix (probe) (0.63 μ L), and genomic DNA (20 ng in 11.87 μ L dH₂O) for 25 μ L reactions. Thermal cycling conditions were an initial step at 95°C for 10 min, followed by 40 cycles of denaturation at 92°C for 15 sec and annealing/extension at 58°C for 1 min. The plate was read and analyzed using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Genotypes were determined by calculating the allele 1 to allele 2 amplification ratio. Samples with ratios greater than 5 were considered homozygous for allele 1 (AA), ratios less than 0.1 were considered homozygous for allele 2 (TT), and ratios between 0.1 and 5 were deemed heterozygous (AT).

RNF8 Gene Expression

Reverse transcription was performed using the RT2HT First Strand Kit (SA Biosciences, Frederick, MD), reactions give a final cDNA concentration of 6.25 ng/ μ L following the manufacturer's instructions. We preamplified probes Hs00187634_m1(RNF8), Hs01032443_m1 (Ki67), Hs99999188_m1 (CB1), and Hs00996818_m1 (53BP1) as target genes, and RPLPO as a housekeeping gene by Mul-

tiplex PCR (duplex) using the TaqManPreAmp Master Mix (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. The reactions contained 12.5 μ L of 2X Applied Biosystems TaqManPreAmp Master Mix, 3.125 μ L of probe, 6.25 μ L of cDNA, and water up to 25 μ L final volume. Amplification conditions included an initial heat up at 95 °C for 10 min, followed by 14 cycles of denaturation at 95 °C for 15 sec, and alignment/extension at 60 °C for 4 min. The reactions were run in a conventional thermocycler (Vapo-protect, Eppendorf, US). Finally, relative quantification was performed by polymerase chain reaction (RQ-PCR) using 12.5 μ L of 2X QuantiFast Probe PCR Master Mix (Qiagen, Chatsworth, CA), 1.25 μ L of probe (20X), 6.25 μ L of preamp product and 5 μ L of dH₂O. Amplification conditions included an initial heat up at 95 °C for 10 min, and then 40 cycles of denaturation at 92 °C for 15 sec, alignment /extension at 58 °C for 1min. The reaction plate was run using a 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Data were analyzed using the 2 $^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

RNF8 expression on GEO datasets

Geo datasets GSE1456 (Pawitan *et al.*, 2005), GSE1561 (Kastan *et al.*, 2005), and GSE3494 (Miller *et al.*, 2004) were investigated for grade-specific differential *RNF8* expression within invasive breast tumor tissue samples. Raw data and associated clinical annotation for these Affymetrix array experiments were downloaded from GEO.

The data were analyzed using Bioconductor modules (<http://www.bioconductor.org>). BioConductor Affymetrix® module was used to perform background subtraction and quantile normalization using the Robust MultiChip Algorithm (RMA). QC analysis on the chips included correlation plots, density plots, box plots, and RNA degradation analysis. Multiple probe values were averaged for every gene. ANOVA was used to estimate differential expression between samples and groups. This was done using the BioConductorLimma module.

Since grade 3 samples show within-group variance in the three datasets, the samples were divided into "regular" and "high" expression groups. For the cutoff, any samples in grade 3 with higher expression than those in grades 1 and 2 were put into higher expression groups. For this, samples were sorted by value, and dot plots were used to decide the cutoff (Figure 1). ANOVA was done on these two groups for each dataset to determine statistically significant changes in *RNF8* expression between these two groups. We searched among these groups to determine whether other genes co-express with *RNF8*. Only *RNF8* and one other gene were statistically significant amongst these comparisons across the datasets.

Statistical Analysis

Gene expression was analyzed and compared against tumor markers, the molecular subtype of breast cancer [for this purpose, three molecular subtypes were considered Luminal

(ER+ and/or PR+), HER2 positive (HER2 +++), and TNBC (ER-, PR- and HER2 0/+)], age at diagnosis, age at menopause onset, genotype, nuclear grade, and TNM (Tumor, Node, Metastasis) stage. Gene expression was categorized as low and high in reference to the expression median for each gene analyzed. Expression equal to or lower than the median was considered high, and expression higher than the median was considered low. Fisher's exact test analyzed differences in proportions. All analyses were carried out using STATA software, version 11.0.

RESULTS AND DISCUSSION

Distribution of breast cancer subtypes

Samples of 57 Mexican cases of invasive breast carcinomas of the "Binational Study of Breast Cancer, ELLA", were analyzed for the distribution of "intrinsic" breast cancer subtype. Thirty (52.63 %) of the cases were luminal, 9 (15.79 %) were HER2+, 10 (17.54 %) were Triple Negative Breast Cancer (TNBC), and 8 (14.04 %) were unclassified.

High proliferative tumor phenotype

Our gene expression experiments show that breast tumors exhibiting the combined effect **High *RNF8*/Low *53BP1*** were associated with high expression of *Ki67* ($p = 0.028$), indicative of increased proliferation (Romero *et al.*, 2011). Additionally, the high *Ki67*-containing tumors were associated with high expression of *CB1* ($p = 0.003$), as shown in Table 1.

Low proliferative tumor phenotype

RNF8 low expression was associated with low expression of *Ki67* ($p = 0.017$) and low *CB1* ($p = 0.035$) and was independent of the *53BP1* expression ($p = 0.115$). Additionally, the low *53BP1* tumors were associated ($p = 0.031$) with a luminal subtype of breast cancer. Moreover, those tumors with low *RNF8*/low *53BP1* show an association ($p = 0.037$) with low expression of *cyclin B1*. Supporting previous results, there was a strong association between tumors expressing low *53BP1* and those expressing *Ki67* low ($p = 0.003$) and *CB1* low ($p = 0.035$), as shown in (Table 1).

Screening for somatic mutations in *RNF8*

To examine whether the heterogeneous expression in breast cancer was due to somatic mutations in *RNF8*, the allelic

Table 1. Association between gene expression and tumor markers.

Tabla 1. Asociación entre la expresión genética y los marcadores tumorales.

High proliferative breast cancer
RNF8↑/TP53BP1 ↓: MKi67↑ ($p = 0.028$), Cyclin B1↑ ($p = 0.003$)
Low proliferative breast cancer
RNF8↓/TP53BP1↓: MKi67↓ ($p = 0.028$), CyclinB1↓ ($p = 0.037$), ER+ ($p = 0.020$), PR+ ($p = 0.039$)
TP53BP1↓: MKi67↓ (0.003), Cyclin B1↓ ($p = 0.035$), PR+ ($p = 0.035$), Luminal ($p = 0.031$)
RNF8↓: MKi67↓ ($p = 0.017$), Cyclin B1↓ (0.035), ER+ ($p = 0.048$)

Arrow up (↑) denotes gene overexpression; arrow down (↓) denotes gene underexpression; RNF8 ring finger number 8; 53BP1 tumor protein p53 binding protein 1; MKi67 marker of proliferation Ki-67; ER estrogen receptor; PR progesterone receptor.

variation of *rs195382* of saliva samples from participants of the ELLA were analyzed. From a group of 72 gDNA samples, 67 (93.06 %) were homozygous for allele A, and 5 (6.94 %) were heterozygous (AT). We did not find the mutant genotype (TT) among participants. Additionally, at the Hapmap Project Tool (<http://hapmap.ncbi.nlm.nih.gov/>), there was no identification of any candidate haplotype associated with breast cancer related to the genetic variation on *RNF8*.

***RNF8* gene expression in public data sets**

To confirm if *RNF8* over-expression was associated with more aggressive breast cancer, levels of *RNF8* mRNA in three available databases of gene expression array from 104, 45, and 241 invasive breast cancer specimens were analyzed (Miller *et al.*, 2004; Kastan *et al.*, 2005; Pawitan *et al.*, 2005). Cases were divided by nuclear grades. ANOVA between nuclear grades does not show *RNF8* as a significantly changing gene, perhaps because of the tiny proportion of nuclear grade 3 samples (but not evident in grades 1 and 2) with increased *RNF8* expression. However, over-expression of *RNF8* mRNA was detected consistently among grade 3 samples for each data set (Figure 1).

Once the former relationship was confirmed, a search for genes commonly overexpressed among nuclear grade 3 breast carcinomas began. There was only one additional gene, *CDC42*, whose increased expression was statistically significant, and it was common in 2 of the 3 analyzed data sets (Table 2).

***RNF8* protein level in breast cancer**

After confirmation of the association between breast cancer aggressiveness and *RNF8* expression at the mRNA level, the detection of protein in tumor tissues was done by immunohistochemistry to correlate with gene expression. Although staining was unspecific, the approach was excellent in identifying aberrant mitotic events, such as cells with multipolar nuclei and peripheral chromatin, suggesting further damage to the DNA repair system among TNBC (Table 3).

Analysis from the GTEx Portal indicates that under normal conditions, the expression of *RNF8* and its DNA Damage Response (DDR) partners is low, which is consistent with the quiescent, non-proliferative state of healthy breast tissue (GTEx Portal, 2025). The low baseline expression on *RNF8* underscores the dysregulation of these pathways in tumorigenesis.

Our results suggest that *RNF8* plays a role in the balance of aggressiveness of breast tumors. In breast cancer, *RNF8* appears to play a dual role. On one hand, it can promote EMT and metastasis, likely by facilitating the DNA damage repair necessary for cancer cell survival during dissemination (Kuang *et al.*, 2016).

On the other hand, our findings and those of Li *et al.* (2018) indicate that *RNF8* ubiquitinates and degrades components of the oncogenic Notch signaling pathway. In this scenario, *RNF8* acts as a crucial brake on a potent driver of cell proliferation and stemness, demonstrating that low *RNF8* mRNA expression is a marker of poor prognosis. We can

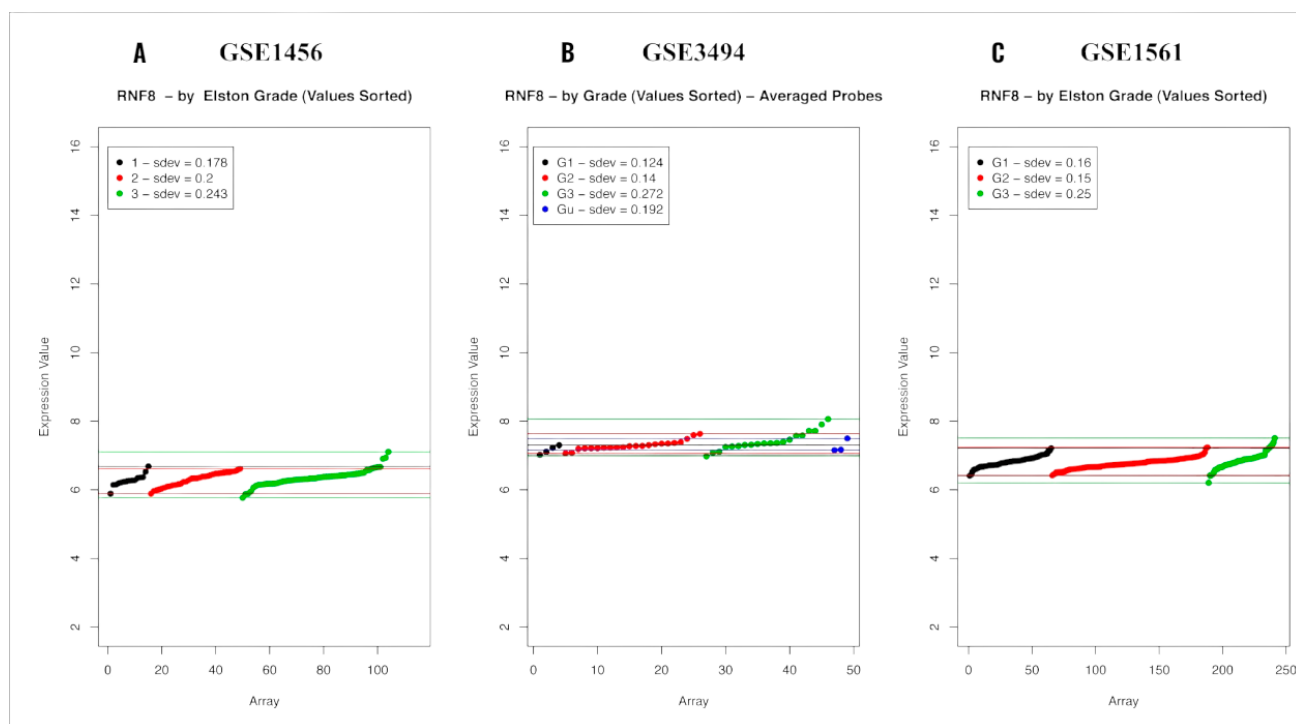


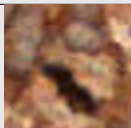
Figure 1. Sample distribution among Nuclear Grades. 1, 2 & 3 and/or G1, G2 & G3 were used to indicate Nuclear Grade; Gu was used to indicate Grade undetermined. All cases (A. Dataset GSE1456, B. Dataset GSE3494, C. Dataset GSE1561) present Grade 3 with higher expression for *RNF8*.

Figura 1. Distribución de muestras entre los Grados Nucleares. 1, 2 & 3 y/o G1, G2 & G3 fueron utilizados para indicar el Grado Nuclear; Gu fue utilizado para indicar Grado indeterminado. Todos los casos A. Datos GSE1456, B. Datos GSE3494, C. Datos GSE1561) exhibieron el Grado 3 con la mayor expresión de *RNF8*.

Table 2. Gene expression by nuclear grades.**Tabla 2.** Expresión genética por grados nucleares.

GSE1456								
Symbol	Genbank	ID	LogFC	Expression average	t	P-value	Adjusted P-value	B
NPR3	NM_024563	219054_at	2.03516	7.100573	5.929014	2.06E-07	0.000504	6.791356
RNF8	AK022075	203160_s_at	0.666723	6.351411	5.805324	3.25E-07	0.000684	6.379113
LEFTY2	NM_003240	206012_at	0.637766	6.54045	5.754404	3.93E-07	0.000715	6.209827
NPR3	AI628360	219789_at	1.79899	5.69822	5.725219	4.38E-07	0.000715	6.11292
ARHGEF9	AI625739	203263_s_at	1.017352	6.199438	5.396486	1.46E-06	0.002153	5.028509
LOC100288413	NM_024534	219987_at	0.984704	5.393065	5.329812	1.87E-06	0.002495	4.810399
GSE1561								
Symbol	Genbank	ID	LogFC	Expression average	t	P-value	Adjusted P-value	B
ARHGEF9	AI625739	203263_s_at	1.047828	7.300862	7.155729	6.43E-07	0.009464	4.964772
NA	AL050040	215172_at	0.876194	4.80918	6.048259	6.66E-06	0.048991	3.250659
RNF8	AK022075	203160_s_at	0.536941	7.421806	5.82088	1.10E-05	0.05263	2.871869
PHLPP1	AF130097	210919_at	0.688053	4.800312	5.701018	1.43E-05	0.05263	2.66867
FAHD2A	NM_016044	218504_at	1.055671	8.777061	5.443019	2.55E-05	0.074984	2.223344
UGT8	NM_003360	208358_s_at	1.322088	5.87878	5.266085	3.80E-05	0.093202	1.911941
GSE3494								
Symbol	Genbank	ID	LogFC	Expression average	t	P-value	Adjusted P-value	B
IL17RB	NM_018725	219255_x_at	1.653993	7.545471	6.303732	5.82E-08	0.00082	7.779465
TMEM100	NM_018286	219230_at	1.449564	4.824955	6.127711	1.11E-07	0.00082	7.210729
KLK10	BC002710	209792_s_at	1.266715	6.198886	5.760468	4.28E-07	0.002101	6.028788
CCHCR1	AF216493	209698_at	0.608897	6.482418	5.636222	6.73E-07	0.002477	5.631097
CTAG2	NM_020994	207337_at	0.751017	5.504585	5.306623	2.21E-06	0.006246	4.583942
RNF8	AK022075	203160_s_at	0.558517	6.851999	5.267207	2.55E-06	0.006246	4.459632

Table 3. Mitotic figures by breast cancer molecular subtype.**Tabla 3.** Figuras mitóticas por subtipo molecular de cáncer de mama.

*Antibody RNF8 staining				
				
Subtype	Mitosis (X)	Cytokinesis (X)	Multipolar (X)	Chromatin package (X)
Luminal A	NE	NE	NE	NE
Luminal B	55.75	14.75	1.5	13
TNBC	185.33	24.33	7	50.33

NE: No events detected; *Preliminary results

Luminal A [(ER ≥ 5% and/or PR ≥ 5%), Ki67 < 13%, HER2 0/+]; Luminal B [(ER ≥ 5% and/or PR ≥ 5%), Ki67 > 13%, HER2 +++]

reconcile this paradox by considering RNF8's multifaceted functions as a ubiquitin ligase, whose effects are determined by its protein substrates and the cellular context in which it operates.

Our results suggest that RNF8 functions through its interactions with key players, such as 53BP1. First, the decrease in *RNF8* expression independent of *53BP1* status was associated with low proliferative tumors (Table 1). Second, tumors showing upregulation of *RNF8*, along with suppression of *53BP1*, are associated with highly proliferative tumors (Table 1). The reduction in 53BP1 expression among some human TNBC and BRCA-deficient tumors is associated with positive selection for 53BP1 loss of function, which could be a strategy to promote survival in BRCA1-deficient cells after DNA damage (Bouwman *et al.*, 2010), through depletion of 53BP1

radiosensitizing tumors *in vivo* (Yang *et al.*, 2021). The 53BP1 protein is often limited in TNBC tumors (Bouwman *et al.*, 2010). Unfortunately, 53BP1-negative breast cancer patients are more likely to metastasize and present significantly lower survival (Lowndes, 2010).

Due to its molecular characteristics, location in the DDR pathway, and involvement in DNA repair, RNF8 is a suitable candidate for evaluating potential mutational effects on DNA restoration. We designed a pair of mutations in conserved residues in the RING finger domain (C403S) and FHA (R42A). These mutations demonstrate that RNF8 serves as a molecular link, connecting protein phosphorylation through its FHA domain and protein ubiquitination through its RING finger domain. Such domains are essential for assembling and maintaining DNA damage response (Kolas *et al.*, 2007;

Huen *et al.*, 2008; Peuscher and Jacobs, 2011). However, none of these mutations in RNF8 have been detected in humans.

We determined the frequency of the “rs195382” SNP in our study population and did not detect any individuals with the mutant phenotype (TT). Recent information suggests that this SNP does not cause any changes in the amino acid residues or structure of RNF8 (Applied Biosystems, Oster City, CA), indicating that it would not affect RNF8 function. A Finnish study of breast cancer-affected families seeks mutations in the *RNF8*, *UBC13*, and *MMS2* genes. The analysis revealed several alterations in *RNF8* and *UBC13*, but none were associated with breast cancer risk (Vuorela *et al.*, 2011). These results indicate that somatic mutations are not causally associated with *RNF8* in breast cancer.

RNF8 can promote Non-Homologous End Joining through 53BP1 at the ends of unprotected telomeres (Peuscher and Jacobs, 2011), where *RNF8* does not develop any direct function on DNA via non-homologous repair. This function is attributed to 53BP1, which increases the mobility of damaged chromatin, thereby promoting random encounters between two damaged telomeres (Dimitrova *et al.*, 2008). It promotes DNA repair by NHEJ, a highly error-prone mechanism; this feature may be related to cancer development due to its concomitant induction of genomic instability (Ellison *et al.*, 2024).

On the other hand, 53BP1 binding to broken chromatids in BRCA1-deficient cells interferes with HR, not by NHEJ *per se*, but by partially blocking the ATM-dependent DNA end resection necessary for the HR repair pathway (Bunting *et al.*, 2010). Consistent with this idea, another study showed that (Embryonic Stem) ES cells BRCA1-depleted accumulate in the G2 phase of the cell cycle, reflecting a response induced by the accumulation of unrepaired DNA. This cell cycle arrest in G2 disappears in cells lacking 53BP1 but not in those lacking p53, suggesting that ATM-dependent checkpoint activation requires 53BP1 (Bouwman *et al.*, 2010). There was also a higher expression of *53BP1* in cells lacking BRCA1, possibly reflecting the cell's attempt to repair DNA defects, which generated greater genomic instability in the process.

The role of RNF8 in DNA repair does not take place during mitosis, but during the G2/M phase of the cell cycle (Kolas *et al.*, 2007; Huen *et al.*, 2008; Plans *et al.*, 2008; Giunta and Jackson, 2011), which must be tightly regulated to prevent the accumulation of genomic damage. Recent evidence suggests that RNF8 might be involved in functions unrelated to DNA repair during mitosis.

Reduction of RNF8 causes a delay in mitotic exit, and its overexpression promotes the formation of aberrant cytokinetic events (Plans *et al.*, 2008), resulting from the cytokinesis collapse that predisposes to tumorigenesis and chromosomal instability (Valente *et al.*, 2015), suggesting a homeostatic role for RNF8 in late mitosis (Plans *et al.*, 2008). Our analysis of RNF8 protein expression consistently revealed a higher proportion of aberrant mitotic events (multipolar nuclei cells and peripheral chromatin) among TNBC, which is not observed in less aggressive tumors (Table 3).

RNF8 might develop a structural role in chromatin reorganization, as observed in the centrosome activity of MDA-MB-231 cells (Giunta and Jackson, 2011). Serial experiments in different cell lines provided insight into the role of RNF8 in CHD4-mediated chromatin remodeling, which facilitates local chromatin decondensation and enhances ubiquitin conjugation of RNF168 and BRCA1 (Luijsterburg *et al.*, 2012). Additionally, the relationship between RNF8 and kinetochores and centrosomes has been reported, suggesting a role for RNF8 in controlling mitotic exit (Tuttle *et al.*, 2007; Plans *et al.*, 2008), which could be independent of its role in DNA repair (Giunta and Jackson, 2011). These features are consistent with the overexpression of *RNF8* associated with breast tumors of nuclear grade 3, within which *CDC42* gene overexpression was detected. This gene regulates centrosome function by controlling spindle formation (Zasadzińska and Foltz, 2017); such characteristics extend the field of involvement for RNF8.

CONCLUSIONS

Our results suggest that among some breast tumors, the upregulation of this altered DNA repair pathway includes activation of RNF8, which contributes to genomic instability in highly proliferative breast tumors. In addition, RNF8 modulates the balance in tumor aggressiveness between the error-free HR repair and the error-prone NHEJ mechanisms. Additional studies will be needed, but this molecule could be exploited as a potential therapeutic drug target.

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CONFLICTS OF INTEREST

The author declares no conflicts of interest.

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