RESEARCH ARTICLE

The cell cycle-related genes RHAMM, AURKA, TPX2, PLK1, and PLK4 are associated with the poor prognosis of breast cancer patients

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Abstract
Breast cancer is the third most common type of cancer diagnosed. Cell cycle is a complex but highly organized and controlled process, in which normal cells sense mitogenic growth signals that instruct them to enter and progress through their cell cycle. This process culminates in cell division generating two daughter cells with identical amounts of genetic material. Uncontrolled proliferation is one of the hallmarks of cancer. In this study, we analyzed the expression of the cell cycle-related genes receptor for hyaluronan (HA)-mediated motility (RHAMM), AURKA, TPX2, PLK1, and PLK4 and correlated them with the prognosis in a collective of 3952 breast cancer patients. A high messenger RNA expression of all studied genes correlated with a poor prognosis. Stratifying the patients according to the expression of hormonal receptors, we found that in patients with estrogen and progesterone receptor-positive and human epithelial growth factor receptor 2-negative tumors, and Luminal A and Luminal B tumors, the expression of the five analyzed genes correlates with worse survival. qPCR analysis of a panel of breast cancer cell lines representative of major molecular subtypes indicated a predominant expression in the luminal subtype. In vitro experiments showed that radiation influences the expression of the five analyzed genes both in luminal and triple-negative model cell lines. Functional analysis of MDA-MB-231 cells showed that small interfering RNA knockdown of PLK4 and TPX2 and pharmacological inhibition of PLK1 had an impact on the cell cycle and colony formation. Looking for a potential upstream regulation by microRNAs, we observed a differential expression of RHAMM, AURKA, TPX2, PLK1, and PLK4 after transfecting the MDA-MB-231 cells with three different microRNAs. Survival
1 | INTRODUCTION

Breast cancer is the most frequently diagnosed type of cancer in women, with high mortality mainly in developing countries. 1 Although established treatments consisting of surgery, chemotherapy, and radiotherapy have been efficient, there is an urgent need to design more personalized treatment because patients classified with the same subtype of cancer, respond differently to treatment and there remain significant clinical challenges that limit the survival of patients. 2 Diverse factors promote the progression of breast cancer including tumor heterogeneity, the communication with its microenvironment, and genomic instability associated with cell cycle progression. 3–5 Indeed, uncontrolled proliferation is one of the hallmarks of cancer. 6 The cell cycle is a complex but highly organized and controlled process, in which normal cells sense the growth (mitogenic) signals that instruct them to enter and progress through their cell cycle, culminating in cell division generating two daughter cells with the same amount of genetic material. 7 The normal course of the cell cycle, comprising the phases G0/G1, S, G2, and M, would not be possible without the participation of different proteins that are responsible for monitoring genetic integrity by sensing any genetic damage that occurs to ensure that healthy daughter cells are generated during cell division. These proteins are the cyclin-dependent kinases (CDKs) (CDK1, CDK2, CDK4, CDK6) that interact with their cyclin (Cyclin-A, -B, -D, and -E) partners to form a multiple complexes. However, in case of any damage that occurs during DNA synthesis and chromosome segregation the complex CDKs/Cyclin can be inhibited by the activation of cell cycle checkpoints that include the activation of the checkpoint kinases such as Aurora kinases (AURK) and Polo-like kinases (PLKs). 8 In the PLK family exist five members (PLK1–PLK5) that participate in different stages of the cell cycle including centrosome maturation, DNA checkpoint mitotic entry, spindle ensemble, and cytokinesis. 8 Of all PLK proteins, PLK1 is the most studied and is involved in the maturation of centrosomes by controlling the localization of Aurora-A (AURKA) and also, induce the activation of CDK1/Cyclin-

B also known as the M-phase promoting factor. During the metaphase-anaphase, PLK1 allow chromosome segregation and its expression is important for mitotic entry after G2 phase arrest due to DNA damage. 7,9 In cancer, overexpression of PLK1 has been observed in various types of cancer including lung, breast, colon, pancreas, prostate, and ovarian that correlates with highly aggressive tumors and poor prognosis. 10 PLK2, PLK3, and PLK5 have been less studied but it is known that Plk2 and Plk3 are expressed in response to mitogenic stimulation, while PLK5 protein has no kinase activity as it lacks almost the whole kinase domain. Therefore, it is believed that it does not have an important role during the cell cycle. However, it has been described that PLK2 and PLK5 participate in neuronal differentiation and synaptic homeostasis. 11 Finally, PLK4 is associated with centrosome separation and mitotic fidelity but also, it has been demonstrated that PLK4 can act as a tumor suppressor because studies in mouse models have shown that PLK4+/−/− heterozygous mice although they are viable have a high probability of developing spontaneous tumors in liver and lung. 12 However, there is evidence that PLK4 has an active role in different processes associated with tumor progression such as invasion and metastasis, proliferation, apoptosis, and inflammation. 13,14 Aurora kinases, that include Aurora A, B and C, are important regulators of cell division. During mitosis, Aurora A (AURKA) localizes to centrosomes and spindle poles, and its inhibition results in centrosome separation defects. On the other hand, after DNA damage, the cells are arrested in the G2 phase and AURKA phosphorylates and activates PLK1, promoting CDK1 activation and mitotic entry. Also, AURKA promotes G1-S progression by preventing the proteasomal degradation of NMYC (a transcription factor). 7 Furthermore, AURKA has been found to facilitate several non-mitotic functions like neurite extension, cell senescence and cell motility. 13 Both nonmitotic and mitotic functions of AURKA have been found to contribute to carcinogenesis and cancer growth. 14,15 AURKA is regulated by various proteins and one of them is TPX2. 16 TPX2 (target protein for Xenopus kinesin-like protein 2) binds to AURKA promoting its auto-phosphorylation and

analysis of miR-34c-5p, miR-375, and miR-142-3p showed a different impact on the prognosis of breast cancer patients. Our study suggests that RHAMM, AURKA, TPX2, PLK1, and PLK4 can be used as potential targets for treatment or as a prognostic value in breast cancer patients.

KEYWORDS
breast cancer, cell cycle check points, KM-plotter, miRNAs, radiation, survival analysis
activation.\textsuperscript{17,18} Besides, the phosphorylation of TPX2 by Plx1 is necessary to activate AURKA.\textsuperscript{19} Like the other cell cycle-related proteins, TPX2 is overexpressed in different types of cancer including breast,\textsuperscript{20} prostate,\textsuperscript{21} bladder,\textsuperscript{22} and liver.\textsuperscript{23} Even though the receptor for hyaluronan (HA)-mediated motility (RHAMM) has been described as an extracellular protein, RHAMM also has an important role in the cell cycle. Intracellular RHAMM is required for spindle formation and progression through G2/M. High expression or downregulation of RHAMM result in multipole spindles and wrong chromosome segregation that could result in genomic instability, carcinogenesis, and tumor progression.\textsuperscript{24} RHAMM is expressed in different types of cancer including carcinomas of the breast, prostate, as well as in hematological cancer such as multiple myeloma, leukemias, and lymphomas.\textsuperscript{25–30} Due to its little-known role in the prognosis of patients with breast cancer stratified according to different characteristics, in this study, the Kaplan–Meier plotter online database was used to evaluate the association between the expression of RHAMM, AURKA, TPX2, PLK1 and PLK4 and the prognosis for breast cancer patients. Furthermore, the influence of irradiation on the expression of RHAMM, AURKA, TPX2, PLK1, and PLK4 in the triple-negative MDA-MB-231 and Luminal MCF-7 breast cancer cell lines was investigated. Functional in vitro assays were performed to study the influence of TPX2, PLK1, and PLK4 on the cell cycle and colony formation of MDA-MB-231 cells, in which TPX2 and PLK4 were downregulated by small interfering RNA (siRNA), and PLK1 was inhibited using a pharmacological inhibitor.

2 | MATERIALS AND METHODS

2.1 | Kaplan–Meier plots

To study how the cell cycle-related genes influence the relapse-free survival (RFS) and overall survival (OS) of breast cancer patients, we used the Kaplan–Meier plotter online tool.\textsuperscript{31} The database contains RFS information from 3951 patients and OS data of 1402 patients and the patient set was split into a low and high expression cohort using the “median” setting.\textsuperscript{31} All data originates from the Gene Expression Omnibus (GEO)\textsuperscript{31} and European Genome-Phenome Archive (EGA).\textsuperscript{31,32} All plots were created using the web interface available at https://www.kmplot.com and the messenger RNA (mRNA) (gene chip) option for breast cancer. The probe set with the highest Jetset score\textsuperscript{33} was used for each gene to ensure the probe with the highest specificity and coverage was selected. Patients were not filtered by follow-up timespan. Plots were created once utilizing the whole dataset and with several restricted datasets. Restricted datasets included patients filtered by estrogen receptor (ER) status (both experimentally determined and derived from genomic data,\textsuperscript{34} progesterone receptor (PR), human epithelial growth factor receptor 2 (HER2) status, intrinsic subtype, lymph node (LN) status, tumor grade, and TP53 status.\textsuperscript{31} The patient dataset was not restricted based on treatment or SEER (Surveillance Epidemiology and End Results) prevalence. The KM-Plotter was also used for the prognosis based on microRNA expression with the tool miRpower. This database of microRNA expression in breast cancer patients contains 2,178 breast cancer patients. The database is established using microRNA (miRNA) expression data from GEO, The Cancer Genome Atlas, EGA, and Pubmed.\textsuperscript{35} The clinicopathological characteristics of the patients were previously described and are presented in Table 1.\textsuperscript{31,36,37} The following Affymetrix IDs were used for each studied gene: 208079_s_at (AURKA), 207165_at (RHAMM), 210052_s_at (TPX2), 223500_at (PLK1), 204886_at (PLK4).

2.2 | Protein interaction network analysis (STRING)

To predict protein–protein interaction the STRING v11.0 database and corresponding web-based prediction tool were used (https://string-db.org).\textsuperscript{38} RHAMM (HMMR), AURKA, TPX2, PLK1, and PLK4 were analyzed using an interaction score of 0.400 (medium confidence), and the first and second shell with no more than 10 interactions. The protein interactions were defined by neighborhood, gene fusion, co-occurrence, co-expression, experimental evidence, databases, and textmining. Functional enrichments (biological process and KEGG pathways) analysis was obtained using STRING.\textsuperscript{38} The terms with the highest false discovery rate (FDR) values resulting from the analysis platform were showed.

2.3 | Cell culture

To determine the expression levels of the five target genes in breast cancer cell lines representative of the major molecular classifications, the following breast cancer cell lines were cultured: MCF-7, T47D, BT474, SKBR3, MDA-MB-453, MDA-MB-468, and MDA-MB-231. All seven cell lines were purchased from ATCC/LGC Promochem. Cell lines T47D, MDA-MB-453, MDA-MB-468, MDA-MB-231, and SKBR3 were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% FCS, 1% glutamine, and 1% penicillin/streptomycin under a humidified atmosphere of 7.5% CO\textsubscript{2} at 37°C. MCF-7 cells were maintained in RPMI containing 10% FCS, 1% glutamine, and 1% penicillin/streptomycin under a
humidified atmosphere of 5% CO₂ at 37°C. BT474 cells were maintained in RPMI containing 20% FCS, 1% glutamine and 1% penicillin/streptomycin, and 0.01 mg/ml insulin under a humidified atmosphere of 5% CO₂ at 37°C.

### 2.4 | Cell irradiation

MCF-7 and MDA-MB-231 cells were irradiated with a TrueBeam linear accelerator (Varian) using energy of 6MeV. An energy dose of 2 Gy or 4 Gy was applied. The following RNA isolation was performed 24, 48, and 72 h after irradiation.

### 2.5 | Quantitative real-time PCR

RNA was extracted from cells using the innuPREP RNAMini Kit 2.0 (Analytik Jena AG) according to manufacturer instructions. Extracted RNA was reverse-transcribed into complementary DNA (cDNA) using the high Capacity cDNA Reverse Transcription Kit (applied biosystem by Thermo Fisher Scientific). Quantitative real-time PCR was performed in triplicates for each gene using Tayon ROX SYBR 2x MasterMix blue dTTP (Eurogentec). Gene expression levels were measured with a peQSTAR 96Q System (PeQlab a VWR company). The RT-Products were quantified using relative quantification and a single fluorescent dye. Gene expression measurements were normalized based on the cycle threshold (Ct) values from each sample and its corresponding β-actin Ct value (ΔΔCt method). To confirm specific product amplification a melting curve analysis was conducted. Primer sequences were furthermore verified by NCBI BLAST analysis (For details see Table S1).

### 2.6 | siRNA transfection

A total of $2.5 \times 10^5$ cells/well MDA-MB-231 were seeded with DMEM medium in a 6-well plate for 24 h. The cells are grown semi-confluent (60%–70%) on the day of transfection and transfection is performed with the Dharmafect reagent (Cat. No. T-2001-03; Dharmacon™) according to the manufacturer’s instructions. In total 1 ml is applied to the cells containing 840 µl Opti-MEM® medium (Gibco®, Cat. No. 31985-070; Thermo Fisher Scientific), 80 µl 2.5% Dharmafect/ Opti-MEM® solution, and 80 µl 20 nM TPX2 siRNA/Opti-MEM® (ID s22747; Ambion® Life Technologies), or 80 µl 20 nM PLK4 siRNA/Opti-MEM® (ID 103348; Ambion® Life Technologies), or negative control siRNA (Ambion® Life Technologies, cat. no. 4390844). The cells were incubated at 37°C and 7.5% CO₂, after 24 h the medium is changed into DMEM medium with FCS. Messenger RNA (mRNA) and Protein extraction was performed 48 h after transfection, colony-assay 24 h after transfection.

| Table 1 | Clinicopathological characteristics of the patients |
|---------|-----------------------------------------------|
| Parameter | Cohort | Proportion of patients |
| Array platform | HGU133A | 52.1% |
| | HGU133A plus 2.0 | 47.9% |
| ER status | ER-positive | 76.4% |
| | ER-negative | 23.6% |
| HER2 status | HER2 positive | 17.7% |
| | HER2 negative | 82.3% |
| Lymph node status | Node positive | 39.2% |
| | Node negative | 60.8% |
| Grade | Grade 1 | 14.8% |
| | Grade 2 | 42.3% |
| | Grade 3 | 42.8% |
| Molecular subtype (StGallen) | TNBC | 17.1% |
| | Luminal A | 48.6% |
| | Luminal B | 27.7% |
| | HER2 positive | 6.5% |
| Molecular subtype (Pietenpol) | Basal-like 1 | 19.2% (within TNBC) |
| | Basal-like 2 | 7.8% (within TNBC) |
| | Immunomodulatory | 23.4% (within TNBC) |
| | Mesenchymal | 18.4% (within TNBC) |
| | Mesenchymal stem-like | 9.2% (within TNBC) |
| | Luminal androgen-receptor | 22% (within TNBC) |
| Age | Mean | 53.6 years |
| | Median | 53 years |
| | Range | 24–93 years |
| Relapse-free survival | Follow-up time (months) | 72.8+/-46.6 |
| | The proportion of events (relapse) | 32% |
| Overall survival | Follow-up time (months) | 84.8+/-47.8 |
| | The proportion of events (death) | 25% |

Abbreviation: TNBC, triple negative breast cancer.
2.7  |  miRNA transfection

The miRNA transfection was performed like the siRNA transfection, but with a concentration of 10 nM has-miR-375 (ID PM10327; Ambion® Life Technologies), has-miR-34c-3p (ID PM11939; Ambion® Life Technologies), has-miR-142-3p (ID AM10398; Ambion® Life Technologies) and Pre-miR™ (Ambion® Life Technologies) as a negative control. In addition, mRNA and protein extraction was performed after 72 h.

2.8  |  Colony formation assay

For the colony formation assay, defined cell counts of MDA-MB-231 were plated out in petri dishes after transfection with siRNA. The cells are cultivated with DMEM medium with 20% FCS for 10 days. The cells were subsequently fixed and stained with methylene blue staining solution after washing with PBS. All colonies with more than 30 cells were counted. For the analysis, the survival fraction (%SF) was calculated using the following formula. %SF = PE (irradiated)/PE (control) × 100, with the plating efficiency (PE) calculated using the following formula.

2.9  |  Cell cycle analysis

MDA-MB-231 cells were seeded in 6-well-plates and after 24 h the cells were treated with a concentration of 150, 300, 750, and 1500 nM of the PLK1 inhibitor Ro3280, as a control vehicle, we used dimethyl sulfoxide (DMSO). The cell cycle status was analyzed via flow cytometry after 24 h of Ro3280 treatment. MCF-7 cells were seeded in 6-well-plates and transfected after 24 h as we previously described. 24 h after transfection, the transfection medium was changed back to cell line-specific medium and another 24 h later the cell cycle status was analyzed via flow cytometry. For this, cellular DNA was stained with 4',6-diamidino-2-phenylindole DAPI (Cystain, Sysmex/Partec), and fluorescence intensity was measured as previously described. Cell cycle distribution was calculated using FloMax software (Quantum Analysis).

2.10  |  Statistical analysis

For survival analysis, the Kaplan–Meier plotter web tool was used to calculate the hazard ratio (with 95% confidence intervals) and log-rank p values in addition to the graphical survival overview. To assess the association of proteins found in the STRING network with GO terms and KEGG pathways we used the FDR calculated by the STRING analysis tool and plotted the -log10(FDR) for each GO term and KEGG pathway. To assess the association of proteins found in the STRING network with GO terms and KEGG pathways a p value is first calculated for each GO term and KEGG pathway using a Hypergeometric test as explained in. All experiments were conducted at least three times as triplicates. Gene expressions are presented as means values ±SD. In all figures and tables, significant p values (≤.05) are either written in bold text or marked with an asterisk *.

3  |  RESULTS

3.1  |  High expression of RHAMM, AURKA, TPX2, PLK1, and PLK4 is associated with a poor prognosis of breast cancer patients

We analyzed the prognosis of breast cancer patients according to the expression of the genes related to the cell cycle including RHAMM, AURKA, TPX2, PLK1, and PLK4, which are also associated with the initiation and cancer progression. The prognostic values were determined based on the RFS and OS of the breast cancer patients. In total, 3952 patients were examined for RSF and 1402 for OS, by using the online database Kaplan–Meier Plotter (kmplot.com/breastcancer). Table 1 shows the clinicopathological characteristics of the analyzed patients. The difference between a high and low expression of the five genes was analyzed without any restriction into subtypes. We found that high expression of the five genes were associated with poor prognosis for RSF and OS when considering the total collective (Figure 1). AURKA showed the poorest prognosis with an hazard ratio (HR) of 1.92 (p = <1e-16), followed by TPX2, RHAMM, PLK4 and PLK1 has the lowest HR with 1.42 (p = 2.5e-10) for RSF. For OS the ranking was different, with TPX2 providing the worst prognosis with an HR of 1.8 (p = 7.8e-08), followed by AURKA, RHAMM, PLK4, and PLK1 (HR = 1.42, p = .0012) (Figure 1). Altogether these results showed that high expression of RHAMM, AURKA, TPX2, PLK1, and PLK4 is associated with the poor outcome of breast cancer patients.

3.2  |  The outcome of the patients related to the expression of the cell cycle-related genes is determined by the hormonal receptor status of the tumor

Subsequently, the patients were stratified according to the expression of the hormonal receptors, ER, PR, and...
FIGURE 1  Prognostic value of the expression of the five cell cycle-related genes in patients with breast cancer without any restriction according to subtypes or receptor status. Kaplan–Meier plots based on relapse-free survival (RFS) and overall survival (OS) show the impact of high (red curves) or low (black curves) expression of (A) RHAMM, (B) AURKA, (C) TPX2, (D) PLK1, and (E) PLK4. \( n = 3951 \) for RFS and 1402 for OS. Hazard ratio (HR) and \( p \) value are shown for each gene.
HER2. Therefore, a total of 2061 ER-positive and 801 ER-negative patients tumors for the RSF and 548 ER-positive and 251 ER-negative for OS breast cancer patients tumors were analyzed. We observed that when the patients have ER-positive tumors the high expression of all the five genes (RHAMM, AURKA, TPX2, PLK1, and PLK4) showed a significant HR value for RSF and OS which were associated with poor prognosis (Table 2). AURKA showed the worst prognosis with an HR of 1.95 (p = 2.7e-15), followed by TPX2, RHAMM, PLK1, and the lowest HR value is seen for PLK4 with 1.38 (p = .0012) for RSF. However, in the case of ER-negative breast cancer patients, high expression of TPX2 and PLK1 were associated with poor RFS (HR = 1.52, p = .0012) and OS (HR = 3.01, p = .024) in HER2-negative tumors (Table 4). All these results suggest that high expression of all the five cell cycle-related genes are associated with the poor outcome of the patients with ER-positive, PR-positive, and HER2-negative tumors.

### 3.3 Patients with tumors of the intrinsic subtypes luminal A and luminal B show a poor prognosis for RSF when RHAMM, AURKA, TPX2, PLK1, or PLK4 are overexpressed

The patients were also filtered according to the intrinsic subtypes: basal (ER-PR-/HER2−), luminal A (ER+/HER2−/MKI67 low), luminal B (ER+/HER2−/MKI67 high; ER+/HER2+), and HER2 positive (ER-/PR−/HER2+). A total of 1933 breast cancer patients for RSF and 611 for OS for the Luminal A and 1149 breast cancer patients for RSF and 433 for OS for the Luminal B were analyzed. We observed that high expression of RHAMM, AURKA, TPX2, PLK1, and PLK4 was associated with poor RFS and OS in the Luminal A subtype, while in Luminal B tumors they only presented a significantly poor prognosis in the RFS (Figure 2 and Table S2 and S3). AURKA (RFS, HR = 2.3, p = <1.000e-16; OS, HR = 2.39, p = 2.90e-06) and TPX2 (HR = 1.96, p = 2.9e-14; OS, HR = 2.4, p = .0000037) showed the highest HR for luminal A (Figure 2 and Table S2 and S3). All the genes had no association with RFS or OS in the HER2+ and basal subtype (data not shown).

| Genes | ER status | Relapse-free survival | Overall survival |
|-------|-----------|-----------------------|-----------------|
| RHAMM | Positive  | 2061                  | 548             |
|       | Negative  | 801                   | 251             |
| AURKA | Positive  | 2061                  | 548             |
|       | Negative  | 801                   | 251             |
| TPX2  | Positive  | 2061                  | 548             |
|       | Negative  | 801                   | 251             |
| PLK1  | Positive  | 2061                  | 548             |
|       | Negative  | 801                   | 251             |
| PLK4  | Positive  | 2061                  | 548             |
|       | Negative  | 801                   | 251             |

| Relapse-free survival | Cases | HR 95% CI | p value | Overall survival | Cases | HR 95% CI | p value |
|-----------------------|-------|-----------|---------|-----------------|-------|-----------|---------|
| RHAMM                 | Positive | 2061 | 1.57 (1.33–1.85) | .000000078 | 548 | 2.01 (1.4–2.89) | .00013 |
|                       | Negative | 801 | 1.07 (0.86–1.34) | .55 | 251 | 1.16 (0.74–1.8) | .52 |
| AURKA                 | Positive | 2061 | 1.95 (1.65–2.31) | 2.7E-15 | 548 | 1.92 (1.33–2.76) | 3.40E-04 |
|                       | Negative | 801 | 0.96 (0.77–1.2) | .73 | 251 | 1.01 (0.64–1.58) | .98 |
| TPX2                  | Positive | 2061 | 1.72 (1.46–2.01) | 8.9E-11 | 548 | 2.47 (1.7–3.6) | .00000097 |
|                       | Negative | 801 | 0.77 (0.51–0.97) | .024 | 251 | 0.85 (0.54–1.34) | .48 |
| PLK1                  | Positive | 2061 | 1.5 (1.27–1.77) | .000013 | 548 | 1.94 (1.35–2.79) | .00026 |
|                       | Negative | 801 | 0.74 (0.59–0.93) | .01 | 251 | 0.82 (0.52–1.3) | .39 |
| PLK4                  | Positive | 2061 | 1.38 (1.17–1.62) | .00012 | 548 | 1.63 (1.14–2.33) | .00072 |
|                       | Negative | 801 | 1.1 (0.88–1.37) | .42 | 251 | 1.46 (0.92–2.32) | .1 |

Note: The case numbers, HR values, and p values are given. Bold p values indicate the significances (p < .05).

Abbreviations: CI, confidence interval; HR, hazard ratio.
3.4 The high expression of PLK4 correlates with poor prognosis in Grade 3 tumors

The prognostic value of the expression of the cell cycle-related genes was also examined in the breast cancer patients depending on tumor grades. We found that the high expression of RHAMM, AURKA, TPX2, and PLK1 showed a significant poor RFS in the patients with tumor Grades 1 and 2 (Table S4). AURKA also showed a poorer OS in the patients with tumor grade 1, while RHAMM, TPX2, and PLK1 have correlated with worse OS in patients with Grade 2 tumors (Table S4). Interestingly, the only gene that showed poor RFS and OS in the patients with Grade 3 tumors was PLK4 (Table S4). These results suggest that PLK4 could be an important target for Grade 3 tumors which are associated with the most aggressive characteristics of cancer cells such as invasion and metastasis. The other four genes are more important for tumors Grades 1 and 2. Finally, all the genes were analyzed according to LN status and the expression of all genes correlated with poor RFS in both LN-positive and LN-negative patients. In the case of the OS, all the genes were associated with worse...
prognosis in the patients with LN-negative tumors (Table S5).

### 3.5 KEGG enrichment pathways associated with the cell cycle-related genes

Normal development is regulated by different pathways that control cell cycle and differentiation, a process that is dysregulated in cancer. In this study, we showed that RHAMM, AURKA, TPX2, PLK1, and PLK4 were associated with a poor prognosis in patients with breast cancer. Then, we evaluated which pathways have related to these genes by using the online bioinformatics tool STRING. As we expected, the protein–protein network showed that the five analyzed proteins (in red boxes) are highly connected and other proteins associated with the cell cycle (Figure 3A). The KEGG enrichment pathway analysis revealed pathways associated with Oocyte meiosis, cell cycle, progesterone-mediated oocyte maturation, FoxO, p53 signaling pathways, ubiquitin-mediated proteolysis, cellular senescence, viral carcinogenesis, and HTLV-I infection (Figure 3B). The GO analysis was used to assess the molecular function, cellular component, and biological process associated with the analyzed proteins. In Table S6, we showed the 10 most significantly enriched terms ($p < .05$) in each category. In the case of the molecular function, the proteins in the network were related to protein serine/threonine kinase, ubiquitin-protein transferase regulator and catalytic activity, ATP, anaphase-promoting complex, drug, carbohydrate, and small molecule binding. In the cellular component category, the proteins were related to the microtubule cytoskeleton and organizing center, centrosome, spindle, cytosol, intracellular non-membrane-bounded organelle, and condensed chromosome centromeric region. Finally, the biological process analysis identified mitotic cell process, cell cycle, regulation of cell cycle, nuclear division, and microtubule-based process as relevant processes associated with the analyzed cell-cycle related factors (Table S6). In conclusion, the analyzed proteins were not only associated with the cell cycle but also with other important signaling pathways related to carcinogenesis such as FoxO, p53, and HTLV infection.

### 3.6 Radiation affects the expression of the cell cycle-related genes as well as the colony formation of the triple-negative MDA-MB-231 breast cancer cell line

Radiation therapy continues to be one of the main methods for treating cancer. However, despite advances in the study of cancer, it is still not clear why some cells are sensitive and others resistant to radiation. Importantly, radiation has been shown to affect the cell cycle of tumor cells and, as a consequence, their response to treatment. In this work, we wanted to know how radiation affects the expression of previously described cell cycle-associated genes in different breast cancer cell lines. To this purpose, using qRT-PCR we analyzed the expression of RHAMM, AURKA, TPX2, PLK1, and PLK4 in two Luminal (MCF-7 and T47D), two HER2-positive (BT474 and SKBR3), and three triple-negative (MDA-MB-231, MDA-MB-468, and MDA-MB-453) breast cancer cell lines. The results showed that all the genes were basally expressed for all the cell lines but at different levels (Figure 4A). Compared to the other cell subtypes, RHAMM, AURKA, TPX2, PLK1, and PLK4 were mainly expressed by Luminal cells. In the case of the HER2-positive cells, BT474 overexpress AURKA and TPX2 while, RHAMM was expressed by both HER2-positive cell lines at the same levels as the Luminal cells. Finally, of the three triple-negative cell lines that we used, MDA-MB-231 were the ones that expressed AURKA, TPX2, and

![FIGURE 2](image-url) Kaplan–Meier plots based on the molecular subtype Luminal. Graphic of the prognostic value of the expression of RHAMM, AURKA, TPX2, PLK1, and PLK4 in patients with breast cancer according to the molecular classification Luminal A (A) and Luminal B (B). Hazard ratio (HR) and $p$ value (*) are shown for each gene.
FIGURE 3  Protein–protein interaction network of the cell cycle-related genes. (A) STRING database output depicting functional and physical interactors of RHAMM, AURKA, TPX2, PLK1, and PLK4 obtained from http://string-db.org/. (B) KEGG pathway analysis. All the adjusted statistically significant values of the terms were negative 10-base log-transformed. For each pathway presented in the bar graph, the KEGG identifier was shown, the term “hsa” corresponds to the organism which in this case is Homo sapiens (human) and the numbers indicate the pathway ID. RHAMM, receptor for hyaluronan (HA)-mediated motility.

FIGURE 4  The influence of radiation on the relative gene expression of RHAMM, AURKA, TPX2, PLK1, and PLK4, colony formation and cell cycle in MDA-MB-231 cells. (A) Basal expression levels of the five analyzed genes in Luminal (MCF-7 and T47D), HER2-positive (BT474 and SKBR3), and triple-negative (MDA-MB-231, −453, and −468) breast cancer cell lines. Relative expression of the genes compared to control, after 24, 48, and 72-h postirradiation with 4 Gy in the Luminal MCF-7 (B) and MDA-MB-231 triple-negative breast cancer cell lines (C). (D) Colony formation capacity of MDA-MB-231 cells after the transfection with siRNA control (Ctrl) or PLK4 and TPX2 siRNA and after radiation at 2 Gy dose. (D) Changes in cell cycle progression after pharmacological inhibition (Ro3280) of PLK1 as quantified by DNA staining followed by flow cytometry, compared to respective controls (ctrl). Different concentrations of Ro3280 were used and DMSO as the vehicle. Data represent the mean ± SD from the experiment in triplicates. *p < .05, **p < .01, and ***p < .001. DMSO, dimethyl sulfoxide.
PLK4 at the highest level (Figure 4A). To know how radiation affects the expression of the genes in the cell lines, we irradiated the MCF-7 and MDA-MB-231 cells with 2 and 4 Gy and evaluated the expression of RHAMM, AURKA, TPX2, PLK1, and PLK4 after 24, 48, and 72 h of radiation. We chose to work with one luminal cell line (MCF-7) and one triple-negative cell line (MDA-MB-231) because the two luminal cell lines expressed the genes at a comparable level and of the triple-negative cells, MDA-MB-231 have the highest expression in three of the five analyzed genes compared to the other triple-negative cell lines. According to our results, we did not find any significant changes in the expression of RHAMM, AURKA, TPX2, PLK1, and PLK4 in both cell lines after 24, 48 h of radiation with 2 Gy (Figure S1A,B). After 72-h postradiation with 2 Gy, the five genes were significantly downregulated only in the MCF-7 cell line (Figure S1B). We observed that after 24-h postradiation with 4 Gy, the MDA-MB-231 cells overexpressed significantly RHAMM, while after 48- and 72-h postradiation, we found no significant changes in the expression of all the genes (Figure 4B). In the case of the MCF-7 cells, only after 72-h postradiation the expression of AURKA, TPX2, PLK1, and PLK4 were significantly downregulated (Figure 4C). Unlike RHAMM, AURKA, and PLK1, little is known about the role of TPX2 and PLK4 in the cytotoxic effects of radiation. Colony formation was then used to show effects on clonogenic cell survival after TPX2 and PLK4 inhibition and irradiation with doses of 2 Gy, the most commonly used dose for radiation and also, we previously showed that after 2 Gy of radiation MDA-MB-231 cells showed less colony formation, demonstrating the suitability of the selected dose in this assay and model system. Using a siRNA we inhibited the expression of TPX2 and PLK4 in MDA-MB-231 cells (Figure S2). We observed that after TPX2 knockdown MDA-MB-231 cells had less colony formation capacity compared to control. In contrast, we did not see any difference in the colony formation of MDA-MB-231 cells after PLK4 inhibition (Figure 4D, left). When the cells were irradiated, they were significantly less likely to form colonies under all treatment conditions (Figure 4D, right). These results indicate that radiation affected colony formation in control cells and PLK4 knockdown cells, while the decrease in the number of colonies under TPX2 knockdown condition was primarily due to a decrease in TPX2 expression and not a radiation-specific effect. Finally, we pharmacologically inhibited PLK1 using the selective inhibitor Ro3280. Flow cytometry cell cycle analysis was done to understand the effects of PLK1 on MDA-MB-231 cell proliferation. We cultivated the cells with different concentrations of Ro3280 and DMSO as vehicle control. We observed that after the inhibition of PLK1 the cells decline in the G1 phase and switched to the G2/M phase when compared to the controls (Figure 4E). We did not observe changes in the cells cultivated with DMSO compared to control. Also, we checked the cell cycle in MCF-7 control or PLK4 and TPX2 knockdown cells. We found that the knockdown of TPX2 induces arrest in the G2/M phase while control or PLK4 knockdown cells mainly entered into G1 phase (Figure S3). All these data suggest that radiation influences the expression of the cell cycle-related genes, and in consequence the clonogenic survival and the cell cycle.

3.7 | miRNAs have a differential impact on the expression of some cell cycle-related genes

We next wanted to identify potential physiological regulatory mechanisms determining the expression of the five cell-cycle-related genes. For this purpose, we focused on posttranscriptional regulation by microRNAs, known to be dysregulated in breast cancer cancer. To identify suitable microRNAs predicted to target RHAMM, AURKA, TPX2, PLK1, and/or PLK4, we performed an in-silico analysis using the online tool TargetScan, a miRNA target prediction algorithm (www.targetscan.org/database). We identified PLK4 as a potential target of miR-375 and RHAMM (HMMR) as potential target of miR-34c-5p (Figure S4). We did not find information about other miRNAs that target the other genes related to the cell cycle. As we had previously shown that miR-142-3p has an impact on the cancer stem cell (CSC) characteristics, invasive properties and the radioresistance of breast cancer cells, we included this microRNA as a control with relevance to the radioresistance of breast cancer cells. To analyze the regulatory impact of miR-375, miR-34c-5p, and miR-142-3p on RHAMM, AURKA, TPX2, PLK1, and PLK4, MDA-MB-231 breast cancer cells were transfected with control pre-miR or pre-miR-375, pre-miR-34c-5p, and pre-miR-142-3p and we analyzed the expression of the previously mentioned genes by qRT-PCR 72 h after transfection. PLK1 was statistically significantly upregulated after the transfection with hsa-miR-375 and hsa-miR-142-3p. RHAMM and PLK4 were downregulated after transfection with hsa-miR-34c-5p. AURKA was upregulated by hsa-miR-142-3p (Figure 5A). According to these results, there is differential regulation of genes depending on the type of miRNA that targeted them. Moreover, hsa-miR-142-3p had a regulatory impact on AURKA although it is not a predicted target, suggesting possible indirect regulation. Finally, we analyzed the prognostic value of miR-375, miR-34c-5p, and miR-142-3p on breast cancer
Using the miRPower tool of kmplot (https://kmplot.com/analysis/index.php?p=service%26cancer=breast_mirna) we found that the high expression of hsa-miR-375 was associated with poor OS while the high expression of hsa-miR-34c-5p correlated with better OS (Figure 5B,C). The expression of hsa-miR-142-3p was not predictive for patient survival (Figure 5D).

**FIGURE 5** miRNAs have a differential impact on the expression of RHAMM, AURKA, TPX2, PLK1, and PLK4 and breast cancer patient’s survival. (A) MDA-MB-231 cells were transfected with control pre-miR or pre-miR-375, −34c-5p, or −142-3p and assayed for the expression of RHAMM, AURKA, TPX2, PLK1, and PLK4 by qRT-PCR after 72 h of transfection. Data represent the mean ± SD from the experiment in triplicates. *p < .05. Kaplan–Meier plots based on overall survival (OS) show the expression of (B) hsa-miR-375, (C) hsa-miR-34c-5p, and (D) hsa-miR-142-3p. Hazard ratio (HR) and p value (*) are shown for each gene. miRNA, microRNA

4 | DISCUSSION

One of the hallmarks of malignancy is the deregulation of the cell cycle, leading to aberrant cell proliferation. To avoid this dysregulation, cells have sophisticated control mechanisms and checkpoints that involves growth-regulatory signals and detect mistakes during DNA synthesis and chromosome segregation, maintaining the genetic integrity. This cell cycle regulation is controlled by cyclins, CDKs, CDKs inhibitors and the checkpoint kinase proteins such as Aurora kinases and PLKs. It is well known that perturbations in the expression and/or the function in those proteins are pivotal carcinogenic events, so targeting these proteins could be an effective strategy for cancer treatment. In breast cancer, there are different clinical trials in which the efficacy and safety of inhibitors for some proteins that control the cell cycle are being studied, including CDK4/6 and other CDKs inhibitors such as flavopiridol, roscovitine, SNS032, palbociclib, and others. Previously, Xing et al. showed that the high expression of CDK1 and the cyclins A2 and B1 correlates with worse OS, recurrence-free probability, and postprogression survival of breast cancer patients. Therefore, having specific inhibitors against these molecules could have a positive impact on the treatment of patients with breast cancer. However, despite these promising results, we know that many
other proteins are involved in the control of the cell cycle and could also be important targets in the treatment of not only breast cancer but also other types of cancer. In this study using a public database, we demonstrated that in a large cohort of breast cancer patients, the expression of RHAMM, AURKA, TPX2, PLK1, and PLK4 were associated with poor survival. Unlike Xiang who focused more on cyclins and we on checkpoints and regulatory proteins of the cell cycle, they found that CDK1, CCNA2, and CCNB1 correlates with poor survival in terms of OS, PFS, PFFS, while we found differences in the survival of the patients depending on the gene expression, the classification of the patient and if the RFS or the OS was analyzed. For example, we observed that AURKA expression correlated with the worst prognosis in terms of RFS for patients with ER-positive tumors, while for the OS, TPX2 was the gene that was most associated with survival in patients with ER-positive tumors. It is important to mention that in cancer, the RFS refers to the period of time after primary surgical treatment for a cancer up to the first relapse, while the OS refers to the duration of patient survival from this time point. Our results suggest that AURKA has an influence in the response to primary treatment while TPX2 participates in the evolution of the patient from the diagnosis of breast cancer patients with ER-positive tumors. Of note, another important observation that we can mention regarding the differences between the RFS and the OS that we found in patients when we classified them by the expression of the PR, could stem from differences in sample numbers analyzed in different cohorts. In an ideal scenario where all data would be available cohorts with similar sizes could enable to match the number of samples analyzed for the RFS and OS.

According to our results, Shütze et al showed that high expression of RHAMM in biopsies of breast cancer patients was associated with a decrease in their survival. Unlike our study, they not found a significant association between the expression of RHAMM and the survival of the patients according to ER and PR status. The authors also observed in the aggressive breast cancer cell line MDA-MB-231 that after the inhibition of RHAMM the cells had less migratory capacity. Interestingly, contrary to what we observed (Figure S1), the same authors showed that the expression of RHAMM in MCF-7 cells was downregulated while in MDA-MB-231 cells it did not change after irradiating the cells with 2 Gy. The inhibition of some RHAMM splice forms and radiation increases the radiosensitivity of the luminal breast cancer cell line MCF-7. The upregulation of RHAMM might lead to radioresistance, as genes that are associated with chromatin assembly might probably drive the activation of radioresistance. It was reported that the expression of RHAMM isoforms, such as RHAMMb, correlates with poor survival of patients with pancreatic ductal adenocarcinoma. In agreement with this study but nonsmall cell lung carcinoma (NSCLC), Wang and collaborators showed poorer survival rates in patients with high expression of RHAMM and that the downregulation of RHAMM diminishes the migratory capacity on adenocarcinoma cell lines. Also, the authors observed that the splice variant of RHAMM RHAMMb was highly expressed in NSCLC compared to the other variants. On the other hand, in multiple myeloma, the expression of RHAMM was also associated with poor survival and osteolytic bone lesions. In patients with acute leukemia, the expression of RHAMM correlates with minimal residual disease and worse survival. According to the results of Schütze and Wang, it is suggested that RHAMM has an important role in the invasiveness of tumor cells. Suggesting that due to its role in invasion RHAMM is a poor prognostic factor for patients.

To date, many studies predict AURKA gene expression and mutation to be a reliant predictive and prognostic marker for breast cancer patients. In one study, by gene expression data array was showed that the expression of AURKA correlates with shorter metastasis-free survival in the ER-positive/HER2-negative subtypes. In our study, we also observed that the expression of AURKA correlates with worse survival in patients with ER-positive and HER2-negative tumors. By immunofluorescence tissue microarrays AURKA and AURKB were also associated with poorer survival. In other solid tumors such as NSCLC, colorectal, ovarian, bladder, gastric cancer, and others, AURKA has an important prognostic value. Therefore, efforts have been made to find targeted inhibitors for AURKA. The best-studied inhibitor is the Alisertib (MLN8237) with several clinical studies confirming its applicability in the treatment of different malignant diseases and for treating breast and lung cancer. The inhibition of AURKA by alisertib in gastric and colon cancer cell lines resulted in less proliferation and more apoptotic cells and also, gastric cancer cells became more sensitive to cisplatin treatment, and in NSCLC the cells become more radiosensitive. In accordance with these data, we have observed a higher expression of AURKA after irradiation, but only with 4 Gy in the triple-negative cell line MDA-MB-231 (Figure 4B). Although (pre)-clinical trials demonstrated that the inhibition of AURKA using Alisertib improved the disease progression and progression-free survival, its administration was associated with severe haematological disorders. These data emphasize that further knowledge about the modes of action and protein–protein interactions of AURKA are essential for effective therapeutic application.
new AURKA inhibitors are being evaluated in different clinical trials. In conclusion, AURKA is a potential target in cancer therapy and can provide an important prognostic value.

Like RHAMM and AURKA, TPX2 also has an important role in the progression of cancer. It is well known that TPX2 mediates the localization of AURKA to microtubules in the mitotic spindle. Therefore, the interaction between these two proteins could play an important role in the progression and treatment of cancer. In this context, van Gijn et al. showed that the inhibition of TPX2 and AURKA reduce the cell viability in BRCA2-deficient breast cancer cells.47 In our study, in the triple-negative breast cancer cell line MDA-MB-231 the knockdown of TPX2 by a siRNA shows a decrease in the colony formation capacity (Figure 4D). Elango et al. showed a decrease in the colony formation capacity of triple-negative breast cancer cells after the inhibition of TPX2. Also, we showed that high expression of TPX2 was associated with worse survival in ER-positive and PR-positive and HER2-negative tumors. In agreement, previously was demonstrated that TPX2 was overexpressed in ER-positive metastatic breast cancer.83 However, in other work, the expression of TPX2 correlates with low survival of patients with HER2-positive tumors.84 In prostate cancer tissues the expression of TPX2 was related to worse survival, this is probably because in vitro studies show that TPX2 expression induces the capacity of proliferation, migration, and invasion of prostate cancer cells.21 While in colon cancer, TPX2 expression was associated with metastasis. The authors also showed that after inhibition of this gene, the proteins levels of pAKT and MMP2 were also diminished and the cells were less migratory and invasive.85 Interestingly, Blanco et al. found that the interaction of AURKA/RHAMM/TPX2 has an impact on the survival of breast cancer patients with BRCA1/2 mutations. In gastric cancer, ovarian, esophageal, pancreas cancer the high expression of TPX2 was associated with poor prognosis of the patients.87-90 Unlike AURKA at the moment, there are no specific inhibitors against TPX2 that are being tested in clinical trials, however, it has been shown that inhibiting TPX2 through siRNAs significantly reduces the proliferation capacity of tumor cells and the formation of tumors in immunodeficient mice, inducing apoptosis.80 Another strategy that is being followed to inhibit TPX2 is by blocking the AURKA/TPX2 complex. Astereti et al identified two small molecules (C20 and C23) that block the formation of the AURKA/TPX2 complex in osteosarcoma cells.91 Also, AURKA inhibitors have a direct/indirect effect on TPX2 turning them into a powerful strategy to target TPX2.16,92

In this study, the Kaplan–Meier plots also showed that the high expression of PLK1 and PLK4 was associated with a worse prognosis in breast cancer patients. This supports previous findings in the literature that a high PLK1 or PLK4 expression often correlates with aggressiveness and poor prognosis in many cancers. In accordance to our results, it has been shown that PLK1 expression was associated with poor prognosis in the ER-positive/HER2-negative subtypes and in tumors with p53 mutations. In the case of PLK4, it was observed that high expression of this gene is higher in the HER2-positive subtype that correlates with drug resistance. PLKs are typically known for their role during the progress of mitosis. Nevertheless, it has been described that PLKs have also an important role in other processes associated with the progression of cancer such as invasion and the epithelial-mesenchymal transition. However, some studies have shown that under certain conditions PLK1 could function as a tumor suppressor. In colon cancer cells with germinal mutations in adenomatous polyposis coli the expression of PLK1 reduces cell survival and prevents the development of intestinal carcinogenesis in ApcMin/+ mouse models. In Kaplan–Meier survival analysis, the high expression of PLK1 correlates with better survival of colon cancer patients. On the other hand, in an in vivo model, the overexpression of PLK1 inhibit the formation of Kras-and Her2-induced breast tumors accompanied by chromosome instability. Interestingly, in breast cancer patients’ tissues the overexpression of PLK1 was associated with better survival. Even though more studies indicate that PLK1 and PLK4 function more as a protumor than as an antitumor, it is important to study these factors in different contexts and to know more about their function in different cancers. However, for the time being, it has been observed that treatment with inhibitors of PLK1 and PLK4 has had a positive impact on cancer patients. Clinical trials using PLK1 inhibitors have shown that PLK1 is an important target factor in the treatment not only of solid tumors but also in hematological tumors. But also, it has been demonstrated that the combination of PLK1 and other inhibitors targeting other proteins such as VEGF, KRAS, MEK, PI3K/AKT, improve their antitumor activity. In a recent review, Zhang et al described not only the role of PLK4 in cancer but also the types of inhibitors that currently exist against PLK4 and those that are being investigated in clinical trials.12

We analyzed the expression of the aforementioned genes in different breast cancer cell lines representing the luminal, Her2-positive and basal subtypes. Interestingly, we found that the expression of RHAMM, AURKA, TPX2, PLK1, and PLK4 was different in each cell line, even when they are classified in the same molecular subgroup (Figure 4A). This could be explained by the high
heterogeneity within the cell lines considering the influence of the CSCs, the clonal evolution, and the tumor cell plasticity. Even with these results and interestingly, we could observe that mainly genes associated with cell cycle were prominently expressed in the cell lines with the Luminal phenotype. It has been described that the expression of the ER promotes the proliferation of the luminal cells lines MCF-7 and T47D. This could explain why these cells have the higher expression of the cell cycle-related genes. Also, luminal cells have a smaller population of stem cells and a greater population of differentiated cells that are in constant proliferation. On the other hand, we observed that AURKA, TPX2 and PLK4 expression were higher expressed in the triple-negative MDA-MB-231 cell line. It has been shown that the inhibition of AURKA and TPX2, and PLK4 in triple negative cells decreased their invasion, proliferation and tumor growth capacity. As well, the inhibition of AURKA limits the growth of HER2+ mammary xenograft tumors. In our study, using TargetScan we observed that miR-375 targets PLK4 while miR-34c-5p targets RHAMM (HMMR), and these miRNAs affect the expression of the cell cycle-related genes. It has been demonstrated that multiple cell-cycle proteins are controlled by miRNAs. Interestingly, different studies have shown that the miR-34 family members are frequently downregulated in different types human cancers, which suggest this miRNA family as potential tumor suppressors. Agreement with our results, we observed that the high expression correlates with better survival in the breast cancer patients. Downregulation of miR-34c could increase the expression of RHAMM which would result in the worse survival of the breast cancer patients. Although PLK4 is not a direct target of miR-34c, we could speculate this miRNA could also regulate PLK4 since in our analysis of String we observed a direct interaction between RHAMM and PLK4 (Figure 3). On the other hand, although in our TargetScan analysis predicted that PLK4 is miR-375 target, we did not observe downregulation of this gene after transfecting the triple-negative cells with miR-375 (Figure 5A). However, an unexpected upregulation of PLK1 was observed suggesting that miR-375 has an influence in the PLKs pathway and indirectly affects the expression of PLK1. Interestingly, we observed that the high expression miR-375 in breast cancer patients correlates with poor prognosis which could be related with an increase of PLK1. Previously was observed that miR-375 was overexpressed in breast cancer samples compared to no-tumor tissues. Also, the authors showed that this miRNA was associated with pathways related to proteoglycans in cancer and focal adhesion. In a different study, the overexpression or miR-375 through the activation of STAT3 by enhancer of zeste homologue 2 (EZH2) leads the downregulation forkhead box protein O1 and p53, an important antitumor proteins. Interestingly, in a mouse model, Chiba et al. observed significantly increased serum levels of miR-375-3p after the exposition to radiation. Moreover, miR-375 desensitizes gastric cancer cells to radiation by targeting p53. In a different study, the expression of miR-375 increased the radiosensitivity of nonsmall lung cancer cells, suggesting an association between the expression of miR-375 and radiotherapy response. On the other hand, our group has demonstrated that overexpression of miR-142-3p downregulates the stem cells and the radioresistance characteristics of breast cancer cells. Here, we observed that miR-142-3p upregulates the expression of AURKA and PLK1 in the triple-negative cells which may be associated with a decrease in the stem cell population and an increase in the population with greater proliferative capacity. Despite this evidence, it is necessary to increase the studies to identified miRNAs as potential biomarkers for human cancer diagnosis, prognosis, and therapeutic cell cycle-related targets, which needs further investigation and validation.

In conclusion, the expression of the five analyzed genes RHAMM, AURKA, TPX2, PLK1, and PLK4 were associated with poor RFS in all the patients. Interestingly, in patients with estrogen and PR-positive that are considered to have a good prognosis due to the availability of treatment, the expression of the five cell cycle-related genes correlates with worse RFS. Also, in HER2-negative tumors, the expression of the genes was associated with poor RFS. The String analysis showed that the cell cycle-related genes not only were associated with the cell cycle pathway but also with other pathways related to cancer progressions such as FoxO, p53, cellular senescence, and viral infection. It is known that some breast tumor cells have resistance to radiotherapy, however, the resistance mechanisms are not fully known. In this study, we show that radiation affects the expression of some of the genes we analyzed and that the inhibition of TPX2 and PLK4 alone or in combination with radiation affects the colony-forming capacity of breast cancer cells. Finally, although we observe that some miRNAs have a differential impact on the expression of the genes that we study associated with the cell cycle, we do not know the impact on some cellular functions, so it is important in future studies to analyze the role of these miRNAs in the characteristics of aggressiveness of the cells. This is also because, for example, miR-375 expression was associated with a poor prognosis in breast cancer patients while miR-34c-5p correlated with a good prognosis. Our study suggests that RHAM, AURKA, TPX2, PLK1, and PLK4 could have significant
therapeutic potential in breast cancer patients with ER-positive, PR-positive, and HER2-negative tumors.

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CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS
Iris Kahl and Julian Mense: performed Kaplan-Meier, transfection, cell cycle, and colony formation analyses. Christopher Finke, Anna-Lena Boller, and Clara Lorber: performed the Real Time-PCR data analysis. Burkhard Greve and Martin Götte: co-supervised Kahl I, Mense J, Finke C, Boller AL, and Lorber C and provided expertise in gene expression, cell cycle, and colony formation analysis. Balázs Győrfy: provided essential resources and bioinformatics expertise. Iris Kahl, Julian Mense, and Nancy Adriana Espinoza-Sánchez: prepared the figures and wrote the main manuscript and all authors reviewed and commented on the manuscript. Martin Götte, Burkhard Greve, and Nancy Adriana Espinoza-Sánchez: conceived, coordinated, and supervised the study.

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