Innovative targets of the lncRNA-miR-mRNA network in response to low-dose aspirin in breast cancer patients

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This study aimed to investigate innovative targets in breast cancer patients by considering the interaction of the lncRNA-miR-mRNA network in response to low-dose aspirin. The candidate miRs were first taken from the GEO and TCGA databases. Then, the candidate network was constructed using the high-throughput sequencing data. The expression levels of candidate targets were finally measured using Real-Time PCR in luminal A breast cancer patients undergoing aspirin (80 mg daily for three months) and non-aspirin groups during chemotherapy after surgery. The expression levels of TGFβ, IL-17, IFNγ, and IL-β proteins were measured using the ELISA technique. 5 lncRNAs, 12 miRs, and 10 genes were obtained in the bioinformatic phase. A significant expression increase of the candidate tumor suppressor lncRNAs, miRs, and genes and a substantial expression decrease of the candidate onco-lncRNAs, oncomiRs, and oncogenes were achieved after the aspirin consumption. Unlike the non-aspirin group, the expression levels of TGFβ, IL-17, IFNγ, and IL-β proteins were significantly decreased following aspirin consumption. The Kaplan–Meier analysis indicated a longer overall survival rate in the patients after aspirin consumption. Our results showed that the lncRNA-miR-mRNA network might be a significant target for aspirin; their expression changes may be a new strategy with potential efficacy for cancer therapy or prevention.

Inflammation predisposes to cancer development and promotes all stages of tumorigenesis. Its inhibition can hinder tumor growth and progress, increase the chances of early detection, and shed light on how metastatic seeds outgrow once distantly established1. Accordingly, anti-inflammatory drugs could reduce the risk of cancer and cancer-related deaths. The epidemiologic studies disclosed the inverse correlation between non-steroidal anti-inflammatory drugs and breast cancer incidences. Recent meta-analyses of observational studies have revealed that aspirin (acetylsalicylic acid; aspirin) could reduce the risk of breast cancer due to its anti-inflammatory effects, mainly in the hormone receptor-positive breast cancer subtype2,3. Early investigations have already established that aspirin could inhibit cyclooxygenase 2 (COX2) activity and reduce prostaglandin E2 (PGE2) production, both overexpressed in breast cancer. In this setting, aspirin may act, at least in part, by suppressing aberrant nuclear factor-xB (NF-xB) signaling that can promote tumor cell survival, proliferation, migration, invasion, angiogenesis, and resistance to therapy. Other anti-cancer mechanisms for aspirin have included inhibiting cyclooxygenase4, activating AMPK, mTOR, STAT3, and NF-xB pathways5, decreasing reactive oxygen species (ROS)6, inducing autophagy7, and changing tumor microenvironment8. Moreover, aspirin could meditate its anti-cancerous properties by changing the expression of non-coding RNAs, such as microRNAs (miRs)9. Accordingly, McDonald et al. (2018) showed that aspirin could alter the expression of miRs in endometrial cancer cells.
in a dose-dependent manner. Other studies demonstrated the increased expression of miR-340-5p and miR-137 by inhibiting cancer cells’ proliferation and decreasing cyclin D1 and miR-7-5p expression due to aspirin consumption.

Furthermore, the anti-proliferative effects of aspirin could also be through the regulation of the long non-coding RNAs (lncRNAs), inducing OLA1P2 expression through FOXD3 upregulation. Wang et al. (2019) reported that aspirin could reduce the P4AH2 expression via let-7g up-regulation, restraining the axis of NF-kB/P4HA2 and LMCDC1-AS1/let-7g/P4HA2. Therefore, lncRNAs can act by miR response elements or binding sites to bind to their target mRNAs, forming a ceRNA network. For instance, the pseudogene PTENP1 lncRNA has the miR sponge capacity to regulate the PTEN gene. In addition, H19 is a cytoplasmic lncRNA that has been shown to bind preference to let-7, promoting the cancer stem cells by making a reciprocal negative feedback loop with let-7 target. In this setting, Fan et al. (2018) constructed a lncRNA-miR-mRNA network and showed four lncRNAs, which have prognostic values in breast cancer patients. This issue necessitates in-depth analyses of these networks in breast cancer. Therefore, we aimed for a randomized trial with a systematic approach that introduced innovative targets in breast cancer by considering the interaction of the lncRNA-miR-mRNA network in response to low-dose aspirin.

Results

Patients and tumor characteristics. Figure 1 demonstrates the flow chart of the patients. Patients in the intervention groups, five in the Aspirin group and four in the non-aspirin group, were withdrawn from the study because the physicians decided only to prescribe endocrine therapy for their systemic adjuvant treatment. Three patients in the Aspirin group and one in the non-aspirin group did not follow the research. Therefore, 40 patients, including 20 in the Aspirin group and 20 in the non-aspirin group, were entered into the data analysis. In addition, the mean age of the patients in the Aspirin and non-aspirin groups and healthy individuals was 57 ± 11, 53 ± 8, and 49 ± 7, respectively. The mean tumor size was 1.6 ± 0.3 mm in the Aspirin group and 1.8 ± 0.3 in the non-aspirin group. According to the inclusion criteria, all tumors were positive for hormone receptors, negative for Her2, and their Ki-67 was less than 15.

Identification of differentially expressed genes (DEGs). FunRich_3.1.3 software made a Venn diagram and extracted the candidate datasets (Fig. 1). A total of 157 miRs (37 up-regulation and 120 down-regulation) (Fig. 1A), 2183 mRNAs (996 up-regulation and 1187 down-regulation) (Fig. 1B), and 169 IncRNAs (102 up-regulation and 67 down-regulation) (Fig. 1C) were obtained from the candidate datasets. The top up-regulated miRs included miR-21, miR-10b, miR-155, miR-20a, miR-20b, miR-141, and miR-200a. The top down-regulated miRs were miR-145, miR-224, miR-125a, and miR-205 (Table 1). Targetscan, miRmap, and mirwalk2 software identified the target genes, including TGFβR2, PIK3CD, AKT3, ERBB2 (HER2), MYC, NOTCH1, IGF1, PTEN, FOXO3, and SOCS5 (Tables 2, 3, and 4). Besides, the LncRNA2target, LncRNADisease v2.0, Lnc2cancer v3.0, and TANRIC datasets identified the candidate lncRNAs, including MALAT1, HOTAIR, XIST, GAS5, and ZFAS1 (Table 5).

Enrichment analysis of DEGs. Gene Ontology (GO) analysis was performed by FunRich to evaluate the biological functions of DEGs. The pathways were specifically enriched by DEGs, including receptor binding, cell cycle, proliferation, transcription factor activity, serine/threonine kinase activity, growth factor activity, DNA repair protein, EGF receptor, glypican, ErbB, and Rap1 (Table 6). All candidate miRs could regulate the signaling pathways, including Wnt, PI3K-AKT, EGF, NOTCH, JAK/STAT, and apoptosis. The most modified routes were the PI3K/AKT and WNT pathways. Thus, candidate miRs could target the significant genes involved in these pathways (Table 6). As shown in Fig. 2, functional enrichment of DEG genes was also analyzed using gProfiler software.

Protein–protein interaction (PPI) network analysis of DEGs. PPI analysis of the 2183 DEGs was performed in the FunRich software (score ≥ 7). TGFβR2, PIK3CD, AKT3, ERBB2, MYC, NOTCH1, IGF1, PTEN, FOXO3, and SOCS5 were hub nodes with higher node degrees in up-regulated genes (Fig. 3A). PTEN, FOXO3, and SOCS5 were hub nodes in down-regulated genes (Fig. 3B). As a result, TGFβR2, PIK3CD, AKT3, ERBB2, MYC, NOTCH1, IGF1, PTEN, FOXO3, and SOCS5 were selected as hub genes for further analysis to the high degree of connectivity (Fig. 3C).

Construction of lncRNA-miR-mRNA network. Our results showed that 5 lncRNAs were involved in regulating 12 miRs. 10 target genes regulated by miRs were then identified. Thus, 5 lncRNAs, 11 miRs, and 10 mRNAs were directly related to the lncRNA-miR-mRNA network in breast cancer (Fig. 4). Moreover, we created a heat map of the expression of the candidate lncRNAs, miRs, and mRNAs using CiMminer (https://discover.nci.nih.gov/cimminer/home.do) (Fig. 5).

The expression levels of TGFβ, IL-17, IFNγ, and IL-β proteins. The expression levels of TGFβ, IL-17, IFNγ, and IL-β proteins were measured using the ELISA technique. TGFβ, IL-17, IFNγ, and IL-β expressions were significantly increased in the patients (pre-treatment) compared to the control group (P < 0.05). Unlike the non-aspirin group, the proteins’ expression levels were significantly decreased following aspirin consumption; however, their expressions were significantly lower in aspirin users than in non-aspirin users (Table 7).
The expression of onco-lncRNAs, oncomiRs, and oncogenes. The expression levels of the onco-lncRNAs (Fig. 6A–C), -miRs (Fig. 7A–H), and -mRNAs (Fig. 8A–G) were significantly increased in the patients (pre-treatment) compared to the control group (P < 0.05). Unlike the non-aspirin group, the expression levels of the onco-lncRNAs, -miRs, and -mRNAs were significantly decreased following aspirin consumption (P < 0.0001).
The expression of the tumor suppressor lncRNAs, miRs, and mRNAs. The expression levels of the tumor suppressor-lncRNAs (Fig. 6D,E), -miRs (Fig. 7I–L), and -mRNAs (Fig. 8H–J) were significantly decreased in the patients (pre-treatment) compared to the control group (P < 0.05). The tumor suppressor-lncRNAs, -miRs, and -mRNAs were significantly higher in aspirin users than non-aspirin users (P < 0.0001).

Table 2. Interaction analysis between selected miRs and target genes in breast cancer patients.

| miRs   | Target genes                                      |
|--------|---------------------------------------------------|
| miR-21 | AKT2, APC, APP1, BCL2, CCND1, CYCS, EGFR, IGF1, MSH2, MSH6, MYC, PIK3RI1, IGF1B1, IGF2B2, IGFBR2, PTEN |
| miR-20a| ACVR1B, BCL2, CCND1, CYCS, FZD9, MAPK1, MAPK9, MSH3, MYC, SMAD4, TCF7L2, TGFB2R2, TP53 |
| miR-20b| ACVR1B, CCND1, CYCS, FZD9, MAPK1, MAPK9, MSH3, SMAD4, TCF7L2, TGFB2R2 |

miR-125a

E2F2, HEYL, PIK3R3, JUN, GADD45A, SHC1, AKT3, NCOA1, CTNNB1, WNT5A, GSK3B, PIK3CB, DLL3, KIT, LIF, FGF2, PIK3R1, POLK, TCF7, EGFR, FGF1, WNT10B, IGF1R, PT53, BRCA1, WNT3, SHC2, AKT2, BAX, NCOA3, MAPK1, ERRB2

miR-141

NOTCH2, SOS1, WNT5A, GSK3B, PIK3R1, WNT8A, FGFR1, E2F3, HEY2, ER, FRAT1, DBD2, CDK4, IGF1R, WNT9B, RP56K1, API, HMGAI, STAT6, JAK1

miR-145

WNT4, E2F2, HEYL, JUN, WNT9A, AKT3, RAFL1, WNT5A, GSK3B, PIK3CB, PIK3CA, PIK3R1, FGF1, CDK6, WNT16, BRAF, MYC, PTEN, FRAT1, FRAT2, SP1, FGFR9, E2F2, IGF1R, GADD45B, JAG1, WNT7B

miR-155

FGF1, BRAF, SP1, IGF1R, FZD2, WNT3, WNT9B, E2F1

Table 3. The predicted candidate genes in breast cancer patients.

| Genes | Target miRs                                      |
|-------|---------------------------------------------------|
| AKT3  | miR-141, miR-17, miR-200a                         |
| TGFBR2| miR-17                                            |
| MYC   | miR-21, miR-20a, miR-125a, miR-145                |
| PIK3CD| miR-125a, miR-145, miR-124, miR-128               |
| AKT3  | miR-21, miR-125a, miR-145, miR-224                 |
| ERBB2 | miR-125a                                          |
| IGF1  | miR-21, miR-155, miR-10b, miR-20a, miR-224, miR-145 |
| SOCS5 | miR-106a, miR-141, miR-155, miR-200a, miR-342, miR-21, miR-20a, miR-20b, miR-224, miR-205 |
| PTEN  | miR-21, miR-145                                    |
| FOXO3 | miR-224, miR-155, miR-125a, miR-10b, miR-21        |

Table 4. The list of selected genes involved in breast cancer patients.
Aspirin could reduce the WNT activity, arresting the cell cycle via inhibition of the β-catenin pathway. In this setting, Khan et al. (2019) demonstrated that aspirin could inhibit the cell migration of breast cancer cells. In addition, one patient had a recurrence and underwent a new course of treatment in the non-aspirin group. The mean follow-up duration of the patients was 4.7 ± 1.3 years. Four patients were deceased due to breast cancer disease in the non-aspirin group. The overall survival rate after aspirin consumption was longer than the non-aspirin group (Fig. 9). The mean follow-up duration of the patients was 4.7 ± 1.3 years. Four patients were deceased due to breast cancer disease in the non-aspirin group. The overall survival rate after aspirin consumption was longer than the non-aspirin group (Fig. 9).

Discussion

In the present study, we measured the effects of aspirin consumption on the expression profiles of lncRNAs, miRs, and mRNAs in patients with non-metastatic early luminal A breast cancer. Using computational approaches, we first constructed a network of the lncRNA-miR-mRNA based on our multi-level methodology. In this study, we focused on aspirin effects on the constructed network expression. Aspirin could reduce the WNT activity, arresting the cell cycle via the WNT/β-catenin axis. In this regard, Zhang et al. (2017) constructed a massive network of lncRNA-miR-mRNA in breast cancer. They showed that aspirin selectively inhibited the growth of mutant PIK3CA breast cells. Besides, it also shown that aspirin could increase the PI3K pathway inhibitors. According to Henry et al. (2017), PIK3 pathway inhibitors had limited clinical response despite the high incidence of PIK3CA mutations in breast cancer patients. To our knowledge, the constructed network between these molecules has not been previously reported. It has been established that the cross-talk between lncRNAs and miRs can be a principal component of cancer pathophysiology. In this regard, Zhang et al. (2017) constructed a massive network of lncRNA-miR-mRNA in breast cancer. Accordingly, miR-510 was the most potent miRNA controller and regulator of numerous target genes.

We showed a group of lncRNAs, including PTCP1, CCAT1, and linco0861, that interacted with particular clinical biomarkers such as estrogen and progesterone receptors. In this study, we focused on aspirin effects on the constructed network expression. Aspirin could reduce the WNT activity, arresting the cell cycle via the WNT/β-catenin axis. In this setting, Khan et al. (2019) demonstrated that aspirin could inhibit the cell migration and invasion via the down-regulation of WNT/β-catenin, consequently reducing fibromodulin expression. Besides, Tang et al. (2016) used aspirin and ursolic acid to co-treatment against breast cancer. This combination could reduce the metastatic feature of breast cancer via regulating EGFR mediating signaling pathways. Aspirin could increase the sensitivity of the tamoxifen-resistant breast cancer cells to tamoxifen by inhibiting c-Myc and cyclin D1 proteins. Consistent results have also been reported in other cancers. Xie et al. (2018) showed that aspirin could increase the sensitivity of hepatocellular carcinoma cells to doxorubicin via modulation of miR-491/ABCG2 expression. Altogether, the findings of this study and those reported in the literature can support the idea of aspirin co-administration with chemotherapy regimens in breast cancer patients. However, these findings should be interpreted cautiously, and large-scale clinical trials should be conducted to assess the co-administration effects of aspirin with different chemotherapy agents in breast cancer patients.

As the well-known aspirin effects are anti-inflammatory, we measured a group of cytokines closely related to the constructed ceRNA network. In this respect, aspirin users had decreased levels of TGFβ, IFNγ, IL-1β, and IL-17. Similar to our findings, Ma et al. (2021) observed that low-dose aspirin administration would reduce the COX2 and TGFβ intensity in breast cancer patients previously irradiated. In the early stages of tumorigenesis, TGFβ acts as a tumor suppressor by inhibiting cell proliferation, inducing apoptosis, and suppressing growth factors, cytokine, and chemokine production. TGFβ overexpression can impair immune surveillance and promote angiogenesis, tumor invasion, and metastasis. Besides, aspirin can inflate the anti-tumoral effects of IFN-α. From a closer look, aspirin could enhance the IFN-α-induced apoptosis via the JAK1/STAT1 pathway. Moreover, earlier studies revealed that the intra-tumoral levels of IL-17 were increased and correlated with the expansion of breast cancer. Cochaud et al. (2013) demonstrated that recombinant IL-17A could activate the

### Table 5. Interaction analysis between selected miRs and lncRNAs in breast cancer patients.

| LncRNAs    | Target miRNAs                                                                 |
|------------|-------------------------------------------------------------------------------|
| MALAT1     | miR-10b, miR-125a, miR-141, miR-145, miR-155, miR-17, miR-200a, miR-205, miR-20a, miR-20b, miR-21, and miR-224 |
| GAS5       | miR-10b, miR-141, miR-155, miR-205, miR-20a, miR-20b, miR-21, and miR-224       |
| XIST       | miR-10b, miR-125a, miR-141, miR-145, miR-155, miR-17, miR-200a, miR-205, miR-20a, miR-20b, miR-21, and miR-224 |
| HOTAIR     | miR-10b, miR-145, miR-17, miR-205, miR-20a, miR-20b, and miR-21                |
| ZFAS1      | miR-10b, miR-145, miR-17, and miR-21                                           |

The candidate miRs and mRNAs as predictive targets. Our results have exhibited a longer overall survival rate after aspirin consumption than the non-aspirin group (Fig. 9). The mean follow-up duration of the patients was 4.7 ± 1.3 years. Four patients were deceased due to breast cancer disease in the non-aspirin group despite the Aspirin group. In addition, one patient had a recurrence and underwent a new course of treatment in the non-aspirin group.
| Term_ID         | Term_name                                         | adj_P_value  |
|-----------------|---------------------------------------------------|--------------|
| GO: BP          |                                                   |              |
| positive regulation of protein phosphorylation | GO:0001934  | 5.773 × 10⁻⁶ |
| positive regulation of phosphate metabolic process | GO:0045937  | 1.554 × 10⁻⁵ |
| positive regulation of the cellular metabolic process | GO:0031325  | 3.100 × 10⁻⁵ |
| mammary gland development                             | GO:0030879  | 5.701 × 10⁻⁵ |
| protein phosphorylation                                | GO:006468   | 1.006 × 10⁻⁴ |
| cellular response to organic substance                 | GO:0071310  | 1.155 × 10⁻⁴ |
| regulation of transferase activity                    | GO:0051338  | 1.234 × 10⁻⁴ |
| regulation of protein phosphorylation                 | GO:001932   | 1.419 × 10⁻⁴ |
| positive regulation of macromolecule metabolic process | GO:0010604  | 3.616 × 10⁻⁵ |
| growth                                                   | GO:0040007  | 1.536 × 10⁻⁴ |
| positive regulation of transferase activity            | GO:0051347  | 1.882 × 10⁻⁴ |
| cell development                                         | GO:0048468  | 2.377 × 10⁻⁴ |
| regulation of growth                                    | GO:0040008  | 2.632 × 10⁻⁴ |
| regulation of molecular function                        | GO:0065009  | 2.644 × 10⁻⁴ |
| cellular response to growth factor stimulus             | GO:0071363  | 3.138 × 10⁻⁴ |
| positive regulation of cellular protein metabolic process | GO:0032270  | 3.251 × 10⁻⁴ |
| regulation of phosphorylation                           | GO:0042325  | 3.269 × 10⁻⁴ |
| response to growth factor                               | GO:0070848  | 4.126 × 10⁻⁴ |
| embryonic organ development                             | GO:0048568  | 4.374 × 10⁻⁴ |
| regulation of signal transduction                       | GO:0009966  | 5.169 × 10⁻⁴ |
| circulatory system development                          | GO:0072359  | 5.191 × 10⁻⁴ |
| positive regulation of protein metabolic process        | GO:0051247  | 5.589 × 10⁻⁴ |
| blood vessel development                                 | GO:0001568  | 5.608 × 10⁻⁴ |
| response to endogenous stimulus                         | GO:0009719  | 5.759 × 10⁻⁴ |
| regulation of protein kinase activity                   | GO:0045859  | 7.087 × 10⁻⁴ |
| phosphorylation                                          | GO:0016310  | 7.136 × 10⁻⁴ |
| positive regulation of molecular function               | GO:0044093  | 7.358 × 10⁻⁴ |
| vasculature development                                  | GO:0001944  | 7.583 × 10⁻⁴ |
| cardiovascular system development                       | GO:0072358  | 8.177 × 10⁻⁴ |
| positive regulation of MAP kinase activity              | GO:0043406  | 1.119 × 10⁻³ |
| regulation of protein modification process              | GO:0031399  | 1.187 × 10⁻³ |
| regulation of kinase activity                           | GO:0043549  | 1.354 × 10⁻³ |
| positive regulation of protein kinase activity          | GO:0045860  | 1.534 × 10⁻³ |
| regulation of cell communication                         | GO:0010646  | 1.722 × 10⁻³ |
| positive regulation of MAPK cascade                     | GO:0043410  | 1.841 × 10⁻³ |
| positive regulation of the biological process           | GO:0048518  | 1.946 × 10⁻³ |
| regulation of signaling                                 | GO:0023051  | 1.947 × 10⁻³ |
| positive regulation of catalytic activity               | GO:0043085  | 2.219 × 10⁻³ |
| MAPK cascade                                             | GO:0000165  | 2.405 × 10⁻³ |
| cell population proliferation                            | GO:0008283  | 2.426 × 10⁻³ |
| positive regulation of kinase activity                  | GO:0033674  | 2.432 × 10⁻³ |
| GO: MF                                                    |                                                   |              |
| protein kinase activity                                  | GO:004672   | 4.844 × 10⁻⁴ |
| signaling receptor binding                              | GO:005102   | 8.392 × 10⁻⁴ |
| phosphatase binding                                     | GO:0019902  | 1.702 × 10⁻³ |
| kinase activity                                          | GO:0016301  | 2.682 × 10⁻³ |
| ATP binding                                              | GO:0005524  | 6.829 × 10⁻³ |
| transferase activity, transferring phosphorus-containing groups | GO:0016772  | 7.224 × 10⁻³ |
| adenyl ribonucleotide binding                           | GO:0032559  | 8.755 × 10⁻³ |
| enzyme binding                                           | GO:0019899  | 8.948 × 10⁻³ |
| protein kinase binding                                   | GO:0019901  | 1.443 × 10⁻² |
| drug binding                                             | GO:0081444  | 1.906 × 10⁻² |
| mitogen-activated protein kinase kinase binding          | GO:0031435  | 1.917 × 10⁻² |
| kinase binding                                           | GO:0019900  | 2.499 × 10⁻² |
| purine ribonucleoside triphosphate binding              | GO:0035639  | 2.641 × 10⁻² |

Continued
| Term_ID  | Term_name                                      | adj_P_value   |
|----------|-----------------------------------------------|---------------|
| GO: CC cytoplasmic part                        | GO:0044444    | 2.581 x 10^-2 |
| KEGG     | Breast cancer                                 | KEGG:05224    | 9.079 x 10^-12 |
|          | Pathways in cancer                            | KEGG:05200    | 5.859 x 10^-12 |
|          | EGFR tyrosine kinase inhibitor resistance      | KEGG:01521    | 7.808 x 10^-10 |
|          | Endocrine resistance                          | KEGG:01522    | 3.173 x 10^-9  |
|          | Proteoglycans in cancer                       | KEGG:05205    | 1.214 x 10^-8  |
|          | Endometrial cancer                            | KEGG:05213    | 1.220 x 10^-8  |
|          | Central carbon metabolism in cancer           | KEGG:05230    | 3.240 x 10^-6  |
|          | Cellular senescence                           | KEGG:04218    | 5.472 x 10^-6  |
|          | MicroRNAs in cancer                           | KEGG:05206    | 6.265 x 10^-6  |
|          | MAPK signaling pathway                        | KEGG:04010    | 8.334 x 10^-6  |
|          | PI3K-Akt signaling pathway                    | KEGG:04151    | 2.790 x 10^-5  |
|          | FoxO signaling pathway                        | KEGG:04068    | 7.248 x 10^-5  |
|          | ErbB signaling pathway                        | KEGG:04012    | 4.710 x 10^-4  |
|          | PD-L1 expression and PD-1 checkpoint pathway  | KEGG:05235    | 5.932 x 10^-4  |
|          | Focal adhesion                                | KEGG:04510    | 6.213 x 10^-4  |
|          | HIF-1 signaling pathway                       | KEGG:04066    | 1.327 x 10^-3  |
|          | Sphingolipid signaling pathway                | KEGG:04071    | 1.876 x 10^-3  |
|          | Osteoclast differentiation                    | KEGG:04380    | 2.277 x 10^-3  |
|          | Relaxin signaling pathway                     | KEGG:04926    | 2.499 x 10^-3  |
|          | mTOR signaling pathway                        | KEGG:04150    | 4.896 x 10^-3  |
|          | Adherens junction                             | KEGG:04520    | 1.093 x 10^-2  |
|          | Progesterone-mediated oocyte maturation       | KEGG:04914    | 2.750 x 10^-2  |
|          | Th17 cell differentiation                     | KEGG:04859    | 3.374 x 10^-2  |
| REACTUM  | Negative regulation of the PI3K/AKT network   | REAC: R-HSA-199418 | 1.167 x 10^-5 |
|          | Diseases of signal transduction               | REAC: R-HSA-5663202 | 1.221 x 10^-5 |
|          | Signal Transduction                           | REAC: R-HSA-162582 | 1.980 x 10^-5 |
|          | PIP3 activates AKT signaling                  | REAC: R-HSA-1257604 | 3.592 x 10^-5 |
|          | Intrinsic Pathway for Apoptosis               | REAC: R-HSA-109606 | 6.437 x 10^-5 |
|          | Intracellular signaling by second messengers  | REAC: R-HSA-9006925 | 8.534 x 10^-5 |
|          | Signaling by Interleukins                     | REAC: R-HSA-449147 | 3.810 x 10^-4 |
|          | PI3K/AKT Signaling in Cancer                  | REAC: R-HSA-2219528 | 6.145 x 10^-4 |
|          | Activation of BH3-only proteins               | REAC: R-HSA-1114452 | 1.241 x 10^-3 |
|          | FLT3 Signaling                                | REAC: R-HSA-9607240 | 1.396 x 10^-3 |
|          | Oncogene Induced Senescence                  | REAC: R-HSA-2559585 | 1.665 x 10^-3 |
|          | Other interleukin signaling                   | REAC: R-HSA-449836 | 2.144 x 10^-3 |
|          | Generic Transcription Pathway                 | REAC: R-HSA-212436 | 2.415 x 10^-3 |
|          | Cytokine Signaling in Immune system           | REAC: R-HSA-1280215 | 2.836 x 10^-3 |
|          | Activation of NOXA and translocation to mitochondria | REAC: R-HSA-111448 | 3.279 x 10^-3 |
|          | PTEN Regulation                               | REAC: R-HSA-6807070 | 3.433 x 10^-3 |
|          | RNA Polymerase II Transcription               | REAC: R-HSA-73857 | 4.995 x 10^-3 |
|          | Apoptosis                                     | REAC: R-HSA-109581 | 8.618 x 10^-3 |
|          | MAPK1 (ERK2) activation                      | REAC: R-HSA-112411 | 9.164 x 10^-3 |
|          | Programmed Cell Death                        | REAC: R-HSA-5357801 | 9.220 x 10^-3 |
|          | Gene expression (Transcription)              | REAC: R-HSA-74160 | 1.032 x 10^-2 |
|          | Activation of PUMA and translocation to mitochondria | REAC: R-HSA-1359915 | 1.177 x 10^-2 |
|          | Disease                                      | REAC: R-HSA-1643685 | 1.294 x 10^-2 |
|          | Extra-nuclear estrogen signaling              | REAC: R-HSA-9009391 | 1.927 x 10^-2 |
|          | Downregulation of ERBB2-ERBB3 signaling      | REAC: R-HSA-1358803 | 2.155 x 10^-2 |
|          | TP53 Regulates Metabolic Genes                | REAC: R-HSA-5628897 | 2.619 x 10^-2 |
|          | RAF/MAP kinase cascade                        | REAC: R-HSA-5673001 | 2.758 x 10^-2 |
|          | TP53 Regulates Transcription of Genes Involved in G1 Cell Cycle Arrest | REAC: R-HSA-6804116 | 2.967 x 10^-2 |
|          | Regulation of TP53 Activity through Association with Co-factors | REAC: R-HSA-6804759 | 2.967 x 10^-2 |
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ERK1/2 pathway and thus promote resistance to docetaxel-based chemotherapy in various cell lines. Altogether, the current literature showed the unintended effects of IL-17 on breast cancer progression49. Further investigations are needed to validate the impact of aspirin on particular cytokines in breast cancer patients.

Clinical applications. Aspirin is one of the most widely used NSAIDs globally; remarkably, it is still one of the most attractive medicines globally, with an extent of use much beyond its primary usage in controlling fever, pain, and inflammation51. For example, the co-prescription of the drugs, such as clopidogrel and oral anticoagulation, with aspirin in populations with coronary artery diseases could reduce the risk of death and myocardial

| Term_ID                      | Term_name                                                                 | adj_P_value   |
|------------------------------|---------------------------------------------------------------------------|---------------|
| MAPK1/MAPK3 signaling        | REAC: R-HSA-5684996                                                       | 2.995 × 10−2  |
| PI3P, PP2A, and IER3 Regulate PI3K/AKT Signaling | REAC: R-HSA-6811558                                                       | 3.343 × 10−2  |
| TFAP2 (AP-2) family regulates transcription of growth factors and their receptors | REAC: R-HSA-8866910                                                       | 3.421 × 10−2  |
| WP                          | Breast cancer pathway                                                    | 2.981 × 10−11 |
|                             | Integrated Breast Cancer Pathway                                          | 2.633 × 10−9  |
|                             | Endometrial cancer                                                        | 4.450 × 10−8  |
|                             | DNA Damage Response (only ATM dependent)                                  | 1.351 × 10−6  |
|                             | ErbB Signaling Pathway                                                    | 2.591 × 10−5  |
|                             | Integrated Cancer Pathway                                                 | 6.752 × 10−5  |
|                             | EGF/EGFR Signaling Pathway                                               | 4.824 × 10−4  |
|                             | Leptin signaling pathway                                                  | 6.182 × 10−4  |
|                             | PI3K-Akt Signaling Pathway                                               | 1.074 × 10−3  |
|                             | Focal Adhesion                                                           | 1.288 × 10−3  |
|                             | Senescence and Autophagy in Cancer                                         | 2.411 × 10−3  |
|                             | TCA Cycle Nutrient Utilization and Invasiveness of Ovarian Cancer         | 2.425 × 10−3  |
|                             | MAPK Signaling Pathway                                                   | 3.757 × 10−3  |
|                             | Focal Adhesion-PI3K-Akt-mTOR-signaling pathway                           | 9.986 × 10−3  |
|                             | RAC1/PAK1/p38/MMP2 Pathway                                               | 1.751 × 10−2  |

Table 6. Integrative pathway enrichment analysis for DEGs.

Figure 2. Functional enrichment by the g: Profiler software. (A) the X-axis shows the functional terms grouped and color-coded by the data source. (B, C, F) the position of terms in the plots fixed and terms from the same branch of Gene Ontology. (D) p-values in the table outputs are color-coded from yellow (insignificant) to blue (highly significant). (E) in a multi-query case, the same term is highlighted on other plots. (G) a click allows for pinning the circles to the plot with a numeric ID that creates a more detailed result in the table below the image.

ERK1/2 pathway and thus promote resistance to docetaxel-based chemotherapy in various cell lines. Altogether, the current literature showed the unintended effects of IL-17 on breast cancer progression49. Further investigations are needed to validate the impact of aspirin on particular cytokines in breast cancer patients.

Clinical applications. Aspirin is one of the most widely used NSAIDs globally; remarkably, it is still one of the most attractive medicines globally, with an extent of use much beyond its primary usage in controlling fever, pain, and inflammation51. For example, the co-prescription of the drugs, such as clopidogrel and oral anticoagulation, with aspirin in populations with coronary artery diseases could reduce the risk of death and myocardial
Moreover, a low dose of aspirin was recommended for pregnant women at high risk of preeclampsia and obstetric antiphospholipid syndrome to prevent diverse pathologies of gestation. Nonetheless, aspirin administration for breast cancer prevention or its treatment is still debatable. So far, plenty of clinical trials have explored the effects of aspirin in breast cancer patients. A recent updated meta-analysis of 38 observational studies yielded that aspirin could reduce the risk of breast cancer patients, such as postmenopausal, hormone receptor-positive tumors, or in situ tumors. Although pooled observational studies have shown that long-term aspirin usage is associated with a low risk of breast cancer incidence, a recent meta-analysis of clinical trials showed that aspirin did not necessarily reduce cancer risk (RR = 1.01, 95% CI: 0.97–1.04). The discrepancies between the clinical and observational studies may reduce the potential clinical practicality of aspirin in breast cancer management. Notwithstanding this dispute, recent clinical trials where aspirin was used as adjuvant therapy or as an add-on strategy showed promising results. According to Joharatnam-Hogan et al. (2019), the regular use of aspirin after standard treatments could prevent recurrence and prolong survival in breast cancer patients. Our results showed that the aspirin users had better expressional biomarkers than the non-aspirin users. Although both groups showed significant improvements in their expressional profiles, these changes were more prominent in aspirin users. These findings suggest that aspirin can increase the efficacy of current chemo-therapies by increasing the sensitivity of the cancer cells to chemotherapy.

Interestingly, we could demonstrate this benefit of aspirin in the clinical outcomes such as 5-year overall survival. We followed our patients for a long time and showed that the patients exhibited a longer overall survival rate after aspirin consumption than the non-aspirin group. Similar to our findings, Liue et al. (2021) showed that aspirin reduced breast-cancer-specific death by 31%, and the risk of recurrence/metastasis decreased by 9%. In this respect, aspirin may improve all-cause mortality, specific mortality, and risk of recurrence/metastasis in patients with breast cancer. Sendur et al. (2014) also showed that despite the contradictory results regarding aspirin and breast cancer incidence, its use in breast cancer was associated with improved disease-free survival. Aspirin users had a significantly lower incidence of histological grade II–III tumors, but no effect was found on other clinicopathological properties.

**Figure 3.** Protein–protein interaction (PPI) network. PPI network was constructed with the DEGs from the GEO and TCGA datasets. (A,B) The significant interactions were identified from the PPI network using the STRING database with a score of ≥ 7. (A) the interaction of up-regulated genes, (B) the interaction of down-regulated genes, and (C) the interaction between up-and-downregulation genes.
Conclusion
We demonstrated that adding aspirin to the treatment of breast cancer patients could reduce the expression of oncologicRNAs, oncomiRs, and oncogenes and simultaneously increase the levels of tumor-suppressor lncRNAs, miRs, and mRNAs.

Methods
Breast cancer datasets. The expression profiles of miRs (GSE81000) and mRNAs (GSE86374) of luminal A breast cancer patients were downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) and analyzed by the GEO2R tools\textsuperscript{18,59}. Their differential expression between tumor and standard samples was collected according to the following parameters: with $|\log_2 FC| > 0.075$ and P-value $< 0.05$. Moreover, the GEPIA2 (http://gepia2.cancer-pku.cn), the cBioPortal (https://www.cbioportal.org), and the Broad Institute’s FireBrowse (http://firebrowse.org) are websites for analyzing the DEGs from the TCGA and Genotype-Tissue Expression projects\textsuperscript{18,59}. The platforms for miRs of the TCGA dataset included the OncomiR (http://www.oncomir.umn.edu/oncmiR/), miRGator 3.0 (https://tools4mir.org), and miRCancerdb (http://mir cancercancerdb.uc.edu) databases\textsuperscript{60}. The databases for lncRNAs of the TCGA dataset included LncRNADisease (http://www.rnanut.net/lncrnадisease), Lnc2Cancer v3.0 (http://bio-bigdata.hrbmu.edu.cn/lnc2cancer), and TANRIC datasets\textsuperscript{61}. Figure 10 shows a flowchart diagram for used bioinformatics analysis.

The analysis of GO term pathways by the FunRich software. The pathway enrichment analyses of the GO database were executed through the FunRich (http://www.funrich.org) software\textsuperscript{85}. Likewise, the same genes were explored for pathway enrichment using the g: Profiler tool (http://biit.cs.ut.ee/gprofiler)\textsuperscript{59}. At last, the miR/target gene regulatory network was built using the Cytoscape (https://cytoscape.org) software\textsuperscript{89}.

LncRNA–miR–mRNA network construction. The lncRNA–miR–mRNA network was constructed and visualized using Cytoscape software based on the ceRNA theory\textsuperscript{96}. Here, the nodes and edges represent extensive biological data described previously\textsuperscript{96}. A network analysis was performed using a Cytoscape plug-in to explore the structure and feature of the lncRNA–miR–mRNA competing triplets\textsuperscript{99}.
Correlation analysis among lncRNAs, miRs, and mRNAs. The correlation coefficient was calculated among lncRNAs, miRs, and mRNAs. The absolute value of the correlation coefficient equal to or more than 0.5 represented a significant correlation.

Sample collection. This study is part of an ongoing randomized clinical trial registered in the Iranian randomized control trial (IRCT2016080818745N11). All participants were informed of the current research objectives, study protocol, and informed consent to participate in the study. The proposal was approved by the Ethics
Committee of Tehran University of Medical Sciences and followed the Helsinki Declaration’s ethical principles. Forty patients with luminal A breast cancer referred to the Cancer Institute and Arash Women’s Hospitals (two centers affiliated with Tehran University of Medical Sciences, Tehran, Iran) were entered into the study between April 2016 and March 2018. Besides, ten normal-risk women who had attended the breast clinic for screening purposes and had healthy breasts were entered as the controls. The aim and protocol of the study were explained to all participants, and they all provided written informed consent. The right to withdraw from the survey was reserved for all patients at any time.

Figure 6. The relative expression of the candidate lncRNAs in the breast cancer patients. The relative expression levels of the lncRNAs were normalized by a reference RNA. The oncolncRNAs included: (A) MALAT1, (B) HOTAIR, and (C) XIST. Tumor suppressor lncRNAs included: (D) GAS5 and (E) ZFAS1. ASA−: Non-aspirin group, ASA+: Aspirin group. The expression levels of the lncRNAs were calculated using the –ΔCT method.
Figure 7. The relative expression of the candidate miRs in the breast cancer patients. The relative expression levels of the miRs were normalized by a reference RNA. The oncomiRs included: (A) miR-21, (B) miR-10b, (C) miR-155, (D) miR-17, (E) miR-141, (F) miR-200a, (G) miR-20a, and (H) miR-20b. Tumor suppressor miRs included: (I) miR-145, (J) miR-224, (K) miR-125a, and (L) miR-203. ASA−: Non-aspirin group, ASA+: Aspirin group. The expression levels of the miRs were calculated using the –ΔCT method.
In all patients with breast cancer, 10 cc of blood was withdrawn twice at a three-month interval at the point of entry and at the end of the study period for each participant (defined below). The blood was centrifuged at 3000 g for 5 min, and the plasma was preserved at −80 °C. The patients' characteristics included age, tumor size, nodal status, histologic type, Her2, Ki-67, and hormone receptor status.

**Inclusion criteria for patients with breast cancer.**

1. Desire to participate
2. Age 20–70 years
3. Invasive ductal carcinoma of the breast
4. Luminal A breast cancer (ER+, PR+, Her2−, and ki-67 < 15%)
5. Early breast cancer confined to the breast
6. Tumor size larger than 10 mm
7. Undergoing adjuvant chemotherapy

**Exclusion criteria for patients with breast cancer.**

- Regional lymph node involvement
- Evidence of distant metastasis
- Pregnancy or breastfeeding
- Prior long-term aspirin use
- History of sensitivity to aspirin
- Platelet count < 100,000/µL
- History of coagulopathy or use of anti-coagulative agents

**Inclusion criteria for healthy women.**

1. Female
2. Desire to participate
3. Age 20–70 years
4. No family history of breast cancer in first and second-degree relatives
5. No history of breast cancer
6. No history of benign breast lump
7. Normal breast exam
8. Normal mammography for those 40 years of age or above

**Figure 7.** (continued)
Figure 8. The relative expression of the candidate mRNAs in the breast cancer patients. The relative expression levels of the genes were normalized by a reference gene. The oncogenes included: (A) TGFβR2, (B) PIK3CD, (C) AKT3, (D) ERBB2, (E) MYC, (F) NOTCH1, and (G) IGF1. Tumor suppressor genes included: (H) PTEN, (I) FOXO3, and (J) SOCS5. ASA−: Non-aspirin group, ASA+: Aspirin group. The expression levels of the mRNAs were calculated using the −ΔCT method.
Figure 8. (continued)

Figure 9. A Kaplan–Meier analysis of 5-year overall survival between the Aspirin and non-aspirin groups. A longer overall survival rate was seen after aspirin consumption.
History of other cancers, peptic or duodenal ulcers, diabetes, hypertension, acquired immunodeficiency syndrome (AIDS), liver and cardiovascular diseases

Exclusion criteria for healthy women.

- Pregnancy or breastfeeding
- Prior long-term aspirin use
- Platelet count < 100,000/µL
- History of coagulopathy or use of anti-coagulative agents
- History of other cancers, diabetes, hypertension, AIDS, liver and cardiovascular diseases

Randomization, allocation, and blinding. Eligible patients with breast cancer were randomly divided into the Aspirin and non-aspirin groups. Randomization was performed according to a table of random num-
bers. The allocation of treatments was performed in a 1:1 ratio, and the treatments were assigned using a sealed envelope. The oncologist in charge of the chemotherapy and the patients themselves knew about the groupings (except for the surgeons responsible for the patient and the researchers who collected the blood samples and performed the molecular and cellular tests) were blind to it. According to histologic results of the surgical specimens after the operation and the oncologist’s decision, several patients did not need chemotherapy and only underwent endocrine therapy as their systemic adjuvant treatment; these patients were withdrawn from the study. The other patients received their chemotherapy regimen of adriamycin, cyclophosphamide, and a taxane.

**Interventions.** The patients in the Aspirin group received an oral daily dose of 80 mg over three months (Fig. 11). Aspirin administration was initiated after the operation during chemotherapy and continued for three months throughout chemotherapy in all patients. The non-aspirin group received no aspirin or other NSAID during the first three months of the chemotherapy.
Primary outcomes. We measured the expression of the lncRNAs (Table 5), miRs (Table 1), and mRNAs (Table 4) as the primary outcomes before (baseline) and after three months of the intervention in the Aspirin and non-aspirin groups.

Secondary outcomes. We evaluated the protein levels of TGFβ, IFNγ, IL-17, and IL-1β pre-and-post intervention (Table 7) as the secondary outcomes before (baseline) and after three months of the intervention in the Aspirin and non-aspirin groups.

Evaluation of TGFβ, IFNγ, IL-17, and IL-1β proteins. ELISA test was used to evaluate the protein levels of TGFβ, IFNγ, IL-17, and IL-1β. The samples were first lysed using a lysis buffer. The protein levels of anti-TGFβ (ab193715, Sensitivity: 1.5 pg/ml, Range: 1.5–500 pg/ml), anti-IFNγ (ab174443, Sensitivity: 470 pg/ml, Range: 0.468–30 ng/ml), anti-IL-17 (ab119535, Sensitivity: 0.5 pg/ml, Range: 1.6–100 pg/ml), and anti-IL-1β (ab46052, Sensitivity: 6.5 pg/ml, Range: 15.6–500 pg/ml) were determined by a sandwich ELISA as follows: aliquots of 100 μl/well (5–10 μg/ml; monoclonal antibody) of anti-TGFβ, anti-IFNγ, anti-IL-17, and anti-IL-1β were used to coat 96-well plates and incubated overnight at 4 °C. Plates were blocked with PBS containing 1% bovine serum albumin (BSA) for one hour at room temperature, followed by washing with washing buffer (PBS) containing 0.1% BSA plus 0.05% Tween 20. Supernatants were diluted at 1:4 or 1:2 with PBS and dispensed into the wells. To generate a standard curve, TGFβ, IFNγ, IL-17, and IL-1β, were used at a concentration range of 25 ng/ml diluted to 100 pg/ml in 10 serial dilutions in PBS plus 1% BSA. After 2-h incubation at room temperature, plates were washed, and 100 μl of a 0.5 μg/ml of biotin anti-TGFβ, anti-IFNγ, anti-IL-17, and anti-IL-1β antibodies were added to each well. After two-hour incubation at room temperature, plates were thoroughly washed, and 100 μl of a 1:10,000 dilution of peroxidase-streptavidin conjugate was added to each well. Plates were incubated at room temperature for one hour. After washing off the unbound antibody, 100 μl of TMB-peroxidase substrate/chromogen solution was added to each well and incubated for 10–20 min. The reaction was stopped with 100 μl of 1 M H₃PO₄. An automated ELISA reader determined absorbance at 450 nm.

Real-time PCR analysis. The RNA was extracted from the plasma samples. Plasma (250 μl) was added to 750 μl TRIzol (Beijing Tiangen Biotech Co., Ltd.). RNA extraction was then carried out according to the manu-

| Genes/miRNAs | Forward primer | Reverse primer |
|-------------|----------------|----------------|
| TGFBR2     | GCTTTGCTGAGGGTCATATAAGGCG | GGTACTCTCTGAGGGTCTGAGG |
| PIK3CD     | TGCCGGGATAGACATATGCCG | ACCAGTAGGCAACCCTGAGAG |
| AKT3       | TGAAGTTGGACACACTCTAATCT | CGGCCTCTGTGACAAATGAAT |
| ERB2       | CAGGGGGTGTATGTCAGCCG | GGGAAAACCTGGAACCTCAGG |
| SOCS5      | TGAAGCTTCACCAAGGGTATATTAGT | GATTTCTATCTACATGACCT |
| TGF1       | GCTTTCTCAGTGTGCGTGTGAGA | GCCCTTTAGATCAGCAGTCG |
| MYC        | GACCCAGAAGATAGCTGCGG | GCCGCGAGTAGCTGACTAAAT |
| NOTCH1     | ACGAGTGTGTCGCTATGAAACC | TGCGAAGCTGATGCTCAGGAG |
| PTEN       | GGCTGGTTTGATGCTACTTCAAGAG | TGGATGCAGCTTAGCTAGCAC |
| FOXO3      | CAGCGCTGCTTCTGAGGAG | TACCCGACAACTTCTAACG |
| B-actin    | CACCATTGCAATAGGGGCTT | AGGTCTTTGGGAGTGTCACG |
| miR-17     | GCCGAGAAGAGCACTTGAGGCGA | TGGTGACAGCTGCTGGAGGA |
| miR-200a   | GGCTGGGAAAACCCGATGAT | CGGGGAGGCTTGTATTACCC |
| miR-205    | CCTGACTTCTTCTTACACC | GTTCGGCTGTTCATATGGCA |
| miR-114    | CCCCCATCAGAGGGTGAGG | GGCCTCCGGGTCAGTTCCT |
| miR-21     | CGCCATGTAAGAGTGCTTTATGAGC | CGATCTATTGAGGAGGGG |
| miR-10b    | TGGTATGCTACAGTAGAACC | TAAAGCAAGAGACTTAGGAGGA |
| miR-125a   | GTGTAGTCTCCGAGACCTTTTTA | GTCTCACAAAGATTTCCACAG |
| miR-155    | GCCCATGTTATAGCTCAAGTGTG | TTCCCAGACCAAGCATAGG |
| miR-20a    | CGCCATTGAAAGTGGTATAGTCG | CAGATTCTTAGTGAGGAGG |
| miR-20b    | GCCCTTAATGCCCCCTGCAGTCA | ACAGTCGACATCCGACCATCT |
| miR-224    | GCTTGGCAATGCTACTAGTGTG | TTTGAAGACGCTACAATGGGA |
| miR-145    | GTAGGAGACCTGATTTTCCACG | TGAATCTGGAACATACGGTTG |
| U6         | ATTCAGCTTTCGTTCACCAT | CCAATGCAGGAGATGGTTT |
| MALAT1     | GACTCTAGCTCTGCAGTCTTCT | CAAAGATCAGTATCTGAGG |
| XIST       | CTCCTCAGATAGCTGGAACC | AGCTTGCTGAACATGCTAA |
| GAS5       | CTCTGGGCTCAAGTGAATGCTT | TTGTGCAAGACATCCGATA |
| HOTAIR     | GCTTCTAAATCGTTT | CTCCACGGTAAATCCGGAC |
| ZFAS1      | AACCCGGTCCCGAGTAGAAC | ATCCCATGCAGGTCCTTCT |
facturer’s instructions. The absorbance ratio (A260/280) of total RNA, between 1.8 and 2.2, was determined using an ultraviolet (UV) spectrophotometer. According to the manufacturer’s recommendations, the miRcute miRNA cDNA First-Strand Synthesis kit (Beijing Tiangen Biotech Co., Ltd.) for miRs quantification and the cDNA Synthesis Kit Manual (TAKARA BIO INC. Cat. 6 30 v.0708) for mRNAs and lncRNAs quantification were used. Then, cDNA was used in each real-time PCR assay with the miRcute miR Fluorescence Quantitative Detection kit (Tiangen Biotech Co., Ltd.) for miRs. The cycling conditions were the pre-denaturation at 94 °C for 2 min, followed by 40 cycles of 94 °C for 20 s and 60 °C for 34 s. The SYBR Green method (AccuPower Green Star qPCR Master Mix; Bioneer, Korea) was used for genes and lncRNAs. PCR cycling was performed as follows: one cycle at 95 °C for 10 min, 40 cycles at 95 °C for 20 s, and 60 °C for 45 s. The melting curve analysis was run from 60 to 95 °C to confirm specific amplification.18,59 The expression of U6 and B-actin was used to normalize miRs, lncRNAs, and genes as the Internal Reference Gene. The list of primers has shown in Table 8. The qRT-PCR reactions were performed using an ABI StepOne plus System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The expression level of the genes was calculated using the − ΔCT method. ΔCT was calculated by subtracting the CT values of U6 and B-actin from the targets63,64.

Clinical outcomes. We evaluated the association between the intervention groups with the clinicopathological feature of patients, such as the 5-year overall survival.

Data analysis. The sample size was calculated based on a study by Chen et al. (2016) and the differences in miR-21 expression in healthy subjects and patients with breast cancer.5 The sample size was 23 in each arm, considering the alpha error less than 0.05 (α) and the research power of 95% (1-β). The data analyses were performed by GraphPad Prism 7.0 (https://www.graphpad.com). We used the t-test and the Mann–Whitney to analyze the parametric and non-parametric data in two groups. The 5-year overall survival rate was evaluated using the Kaplan–Meier method. All data were presented as mean ± SD. P-value < 0.05 was considered to be statistically significant.

Ethical approval. The experimental procedures and care protocols were approved by a review board committee of Tehran University of Medical Sciences (No: IR.TUMS.VCR.REC.1397.606) and registered by the Iranian Randomized Control Trial (IRCT) ethical board (No: IRCT2016080818745N11). Written informed consent was obtained from each participant before the sample collection.

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S.A., S.K., and V.K: sample collection, sample processing, data analysis, and manuscript preparation. T.A., M.T., and M.D.: study conception and manuscript preparation. S.S.: manuscript revision. R.O.: sample processing. G.V. and B.E.: manuscript preparation. A.M.A.: study conception and design, manuscript revision, and data analysis.

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