

A Bioinformatics Approach to Predict Long non-coding RNA Targets of Shikonin in Apoptosis Induction of Breast Cancer Cells

Salome Dini^{1*}, Abozar Ghorbani², Neda Eskandarzade³ and Maryam Alibeiki⁴

¹ Young and Elite Researchers Club, Karaj Branch, Islamic Azad University, Karaj, Iran

² Department of Plant Protection, Nuclear Agriculture Research School, Nuclear Science and Technology Research Institute (NSTRI), Karaj, Iran

³ Department of Basic Sciences, School of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran

⁴ Department of Microbiology, Faculty of Basic Sciences, Shahrood Branch, Islamic Azad University, Shahrood, Iran

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ABSTRACT

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*Corresponding author

dinsa022@student.otago.ac.nz



Phytochemicals such as shikonin accomplish their pleiotropic anti-tumor activities by modulating the expression of non-coding RNAs. The role of shikonin in modulating long non-coding RNAs (lncRNAs) to induce apoptosis *in vitro* in breast cancer cells was investigated. An RNA-Seq dataset available in NCBI, in which shikonin induced apoptosis in different breast cancer cell lines, was analysed to examine lncRNA expression. We identified 88 differentially expressed lncRNAs in the shikonin-treated groups. After identifying the 10 up- and down-regulated lncRNAs, we found that dysregulation of putative lncRNAs is associated with cancer pathophysiology, particularly breast cancer. The significantly up- and down-regulated lncRNAs in shikonin-treated cell lines were NEAT1, ALDH1A3-AS1-201, MIR3142HG, AC079305.1, and DDR1-AS1. Furthermore, a network of interactions between key lncRNAs and miRNAs was constructed. Our comparative analysis of the lncRNA transcriptional landscape identified several novel lncRNA candidates that may regulate breast cancer cells' response to shikonin treatment. This may pave the way for the development of new molecular biomarkers with diagnostic, prognostic, and potentially therapeutic applications.

Keywords: Apoptosis, lncRNAs-miRNAs network, Shikonin, Breast Cancer, Phytochemicals

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INTRODUCTION

Non-coding RNAs (ncRNAs) are distinguished by their non-translational properties [1]. A subset of small non-coding RNAs (sncRNAs), microRNAs (miRNAs) with a length of ~22 nucleotides, exerts a regulatory effect on gene expression, typically by binding to the 3' untranslated region (3'UTR) and inducing their mRNA target. A substantial body of research has investigated the relationship between microRNAs (miRNAs) and a myriad of cellular processes, including cancers [2-5]. Moreover, compelling evidence shows that variations in miRNA expression can affect the expression of other ncRNAs, such as long non-coding RNAs (lncRNAs), thereby altering many cellular processes [6]. lncRNAs have been shown to modulate epigenetic modifications through diverse mechanisms, which include the following: (1) gene expression regulation, (2) post-translational modification as a regulatory factor for microRNAs (miRNAs), or (3) acting as a "sponge" for miRNAs (1). In the last scenario, lncRNAs compete with other endogenous RNAs (ceRNAs) to bind miRNAs, as part of epigenetic mechanisms that ultimately affect gene expression. lncRNAs generally possess intricate secondary structures, including single-stranded, double-stranded, and circular conformations. They function as decoys, guides, signals, and scaffolds to interact with DNA, RNA, and proteins, respectively, thereby influencing the initiation and progression of cancer [7]. Consequently, there is significant interest in modern oncology in investigating dysregulation of lncRNA transcripts in

response to various pathophysiological states, including cancer [8].

Breast cancer (BRCA) is a commonly diagnosed female malignancy with more than 2.3 M new cases and 670,000 deaths in 2022 [9]. Breast cancer that lacks expression of the receptors ER, PR, or HER2 is classified as "triple-negative breast cancer" (TNBC). This particular type of cancer has demonstrated a suboptimal response to chemotherapy and a high chance of recurrence, resulting in the aggressive clinical BRCA phenotype [10]. Although chemotherapy remains the primary line of medication for breast cancer treatment, the adverse health effects and chemoresistance remain two significant challenges [11-13]. To minimise the side effects of chemical anti-cancer drugs and target specific molecular pathways involved in the progression of BRCA subtypes, researchers have been investigating effective natural alternatives, such as thymol, carvacrol, geraniol, berberine, and flavonoids [14-20].

Shikonin, a naphthoquinone pigment, has a long history in Chinese herbal medicine and has recently garnered significant interest due to its ability to inhibit tumorigenesis at multiple levels, such as initiation, promotion, and progression, as well as restoring cancer cells' drug sensitisation [21-24]. The potential application of shikonin and its derivatives as an anti-cancer chemotherapeutic was previously approved against breast, gastric, thyroid, and lung cancers, as well as hepatocellular carcinoma, melanoma, and glioblastoma [21]. A study by Xu et al. (2019) found that shikonin has an anti-proliferative effect on cancer cells

in a caspase-dependent manner [25]. Shikonin induced the final stage of apoptosis in MDA-MB-231 human breast cancer cells *in vitro* by activating caspase-3/7 via the p38 and JNK pathways [25]. Moreover, the anticancer effects of shikonin are mediated by epigenetic regulation, including modulation of ncRNA expression profiles and their downstream targets [21, 26]. For instance, *in vitro* and *in vivo* studies show that shikonin reduces tamoxifen resistance in the breast cancer MCF-7R cell line by inducing the ultra-conserved lncRNA 57, leading to downregulation of its mRNA target (BCL11A) and inhibition of PI3K/AKT or MAPK signaling pathways [27].

As cumulative studies have described the miRNA-regulatory function of lncRNAs [28], identifying lncRNAs induced by shikonin will help us better understand the molecular mechanisms underlying their anti-cancer effects for therapeutic applications. In this study, we attempted to analyse the changes in lncRNAs expression in human breast cancer cell lines treated with shikonin. Novel lncRNAs and their miRNA targets are of great value for further experimental functional studies.

MATERIALS AND METHODS

RNA-Seq data of three types of breast cancer cell lines: MCF-7, SK-BR-3, and MDA-MB-231, treated with shikonin, were downloaded from the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under Bioproject number PRJNA392672 [29]. The details of all samples are summarized in Supplementary File 1. The RNA-Seq raw data, including the untreated and treated groups were imported into QIAGEN CLC Genomics Workbench (version 20) and trimmed to remove both low-quality sequences and adaptors based on the following parameters (i) quality scores limit: 0.05; (ii) removal of the short reads: 50 nt cut-off value, and (iii) maximum number of ambiguities: 2.

Mapping RNA-Seq Reads to the Human Reference Genome

The trimmed reads were mapped to the human reference genome GRCh38 (NCBI annotation release 110). Mapped reads were filtered based on all annotations on the reference genome: Ref_mRNA, Ref_CDs, Ref_Exon, Ref_rRNA, Ref_tRNA, Ref_Centromer, Ref_Mobile elements, Ref_Gene, Ref_Precursor RNA, and Ref_Replication using the default parameters: mismatch cost: 2; insertion cost: 3; deletion cost: 3; length fraction: 0.8; and similarity fraction: 0.8. The unmapped read collections were assembled using the *de novo* assembly option with a minimum contig length of 250 for further analysis.

Identification of Putative and Novel lncRNAs

To eliminate transcripts with protein-coding potential, a series of rigorous filtering steps was performed. First, obtained contigs from the previous step were blasted against all human protein sequences downloaded from NCBI and UniProt using BLASTX with the following parameters: Query genetic code: standard; the number of threats: 20; P-Value: 0.0001; word size: 3; Maximum number of hit sequence: 10. The detected protein-coding transcripts were discarded and non-coding transcripts were imported into Coding Potential Calculator (CPC) tool to assess the protein-coding potential of transcripts based on six biologically meaningful sequence features. Transcripts with a CPC score < 0 were defined as non-coding RNAs and then blasted against the Pfam database to discard transcripts with high similarity to known protein domains (E-value < 0.001). For the detection of novel lncRNAs, predicted lncRNAs from the

previous step were blasted (BLASTn) against the ncRNAs (>250 nt long) downloaded from Ensembl using the following parameters: the number of threats: 50; P-Value: 0.0001; word size: 11; Maximum number of hit sequences: 1. Our identified lncRNAs were selected as novel lncRNAs, and the rest transcripts were putative lncRNAs.

Analysis of lncRNAs Expression

The final list of lncRNAs was used as a reference for the RNA-seq analysis tool using QIAGEN CLC Genomics Workbench (version 20). All trimmed reads from our original RNA-seq data (accession number: PRJNA392672) were mapped to the final list of detected lncRNAs by RNA-seq analysis method: mismatch cost: 2; insertion cost: 3; deletion cost: 3; length fraction: 0.8; similarity fraction: 0.8; the maximum number of hits for a read: 10. The lncRNAs expression of the control group in compare to shikonin-treated groups was analyzed by selecting the GE results of all samples and setting up experiments. Baggerley's test was used to detect statistically significant differences in lncRNA expression between groups, and then transcripts with fold change values greater than 2 and less than -2 were highlighted. An FDR of < 0.01 was considered statistically significant. Expression values were estimated as RPKM and normalised against the overall set, based on the number of reads per 1,000,000. These expression values were used to create a volcano plot.

The lncRNA-miRNA Interaction

All human miRNAs were downloaded from the miRBase 22 database (<http://www.mirbase.org/>), and all differentially expressed lncRNAs were used for the prediction of target mimicry by the psRNATarget web server (<https://www.zhaolab.org/psRNATarget/>) with a maximum expectation of 3. Differentially expressed lncRNAs and their miRNA targets were used to construct interactive networks in Cytoscape.

Gene Ontology and Pathway Analysis of Transcriptomic Data

Differentially expressed lncRNAs were annotated to the reference genome (maximum evaluation: 0.0001 and maximum identity: 95) to find the location of lncRNAs on each chromosome. The nearest neighbouring genes to lncRNAs (located 10 kbp upstream) were extracted using *Homo sapiens* (assembly GRCh38.p14) in <https://www.ensembl.org/index.html>, and their expression values were calculated by QIAGEN CLC Genomic Workbench (version 20). Gene ontology (GO) enrichment was computed for all neighbouring protein-coding genes, and more abundant terms were identified for each category of molecular function, biological process, and cellular component using <https://string-db.org/>.

RESULTS

Identification of Putative and Novel lncRNAs

Error! Reference source not found. depicts the workflow for employing bioinformatics tools to assess how shikonin, via the action of long non-coding RNAs (lncRNAs), might influence breast cancer at the molecular level. At the first step, RNA-Seq data from human breast cancer cell lines were used to analyse the lncRNA expression in response to shikonin treatment. The reads were retrieved from previously deposited data in the NCBI under accession numbers PRJNA392672 (Supplementary File 1). After removing protein-coding transcripts, we identified 3054 lncRNAs, among which were 1,735 putative lncRNAs and 1,319 novel lncRNAs (Supplementary File 2).

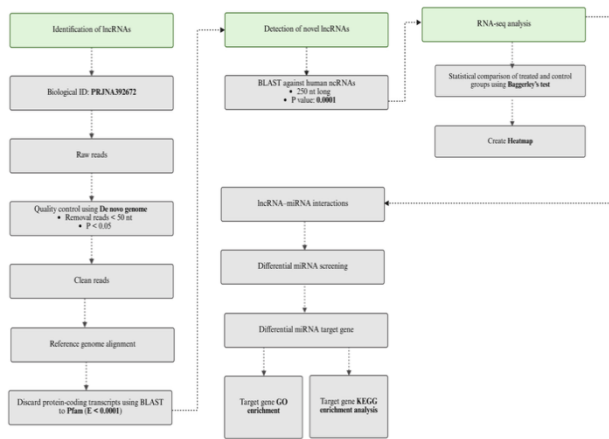


Fig. 1 A schematic representation of a workflow for bioinformatics analysis

Differentially Expressed lncRNAs in Shikonin-treated Cells

In the shikonin-treated groups, a total of 88 lncRNAs were differentially expressed between the 134 control and treatment groups, with 48 upregulated and 40 downregulated (**Error! Reference source not found.** and 3, Supplementary File 2). The top 10 differentially expressed lncRNAs are listed in Table 1. Among the up-regulated putative lncRNAs, the maximum fold change was ~21.5, belonging to ENST00000428541.1. Among the down-regulated putative lncRNAs, the largest fold change was -12.55, observed for ENST00000415917.2. The main up-regulated novel lncRNAs showed a ~54-fold increase in abundance (Table 1).

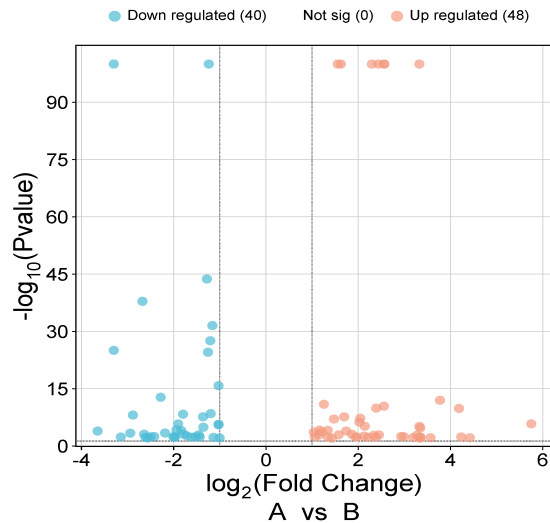


Fig. 2 Volcano plots showing the distribution of the gene expression fold changes in the group treated with shikonin.

Table 1 The top-10 up- and down-regulated lncRNAs in the different cell lines of breast cancer treated by shikonin

No.	Ensemble Gene ID	Ensemble Transcript ID	Gene Symbol	Fold change	FDR	Status
1	-	-	Novel	53.83	1.58784E-06	Up
2	ENSG00000222043.2	ENST00000428541.1	AC079305	21.40	0.00	Up
3	-	-	Novel	18.79	0.00	Up
4	-	-	Novel	18.18	1.45864520462169E-10	Up
5	ENSG00000227422.1	ENST00000453209.1	DDR1-DT-203	13.66	1.0480101634998E-12	Up
6	ENSG00000272140	ENST00000415917.2	lnc-AGAP5-1:10	-12.55	0.00	Down
7	ENSG00000245532.13	ENST00000501122.2	NEAT1	-9.84	0.00	Down
8	ENSG00000253522	ENST00000517927.1	MIR3142HG (PTTG1-1:1)	-9.84	9.01E-26	Down
9	ENSG00000245275.10	ENST00000524264.6	SAP30L AS RNA 1	-8.86	0.00	Down
10	ENSG00000259583.5	ENST00000560068.1	ALDH1A3-AS1-201	-7.68	0.00	Down

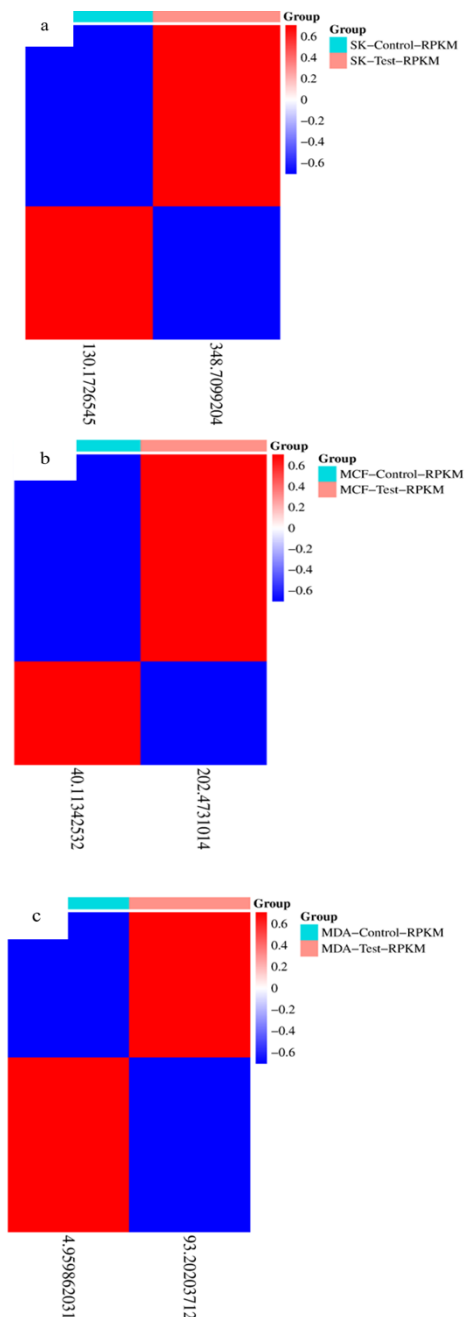


Fig. 3 lncRNAs transcriptional modulation of breast cancer cell lines treated with shikonin and resveratrol. Heatmap plot displaying the differentially expressed lncRNAs in the shikonin-treated SK (A), MCF (B), and MDA (C) cell lines.

The lncRNA-miRNA Interactions

All human miRNAs were downloaded from the miRBase 22 database (<http://www.mirbase.org/>), and all differentially expressed lncRNAs were used for the prediction of target mimicry by psRNATarget (<https://www.zhaolab.org/psRNATarget/>). Top-10 differentially expressed lncRNAs and their miRNA targets were used for interactive networks using Cytoscape (Fig. 4).

Expression of lncRNAs Neighboring Protein-coding Genes

To determine the functions of the identified putative and novel lncRNAs, we extracted the GO enrichment of protein-coding genes located in 10 kb upstream loci of our discovered lncRNAs. The essential functions of the differentially expressed genes were classified by GO analysis.

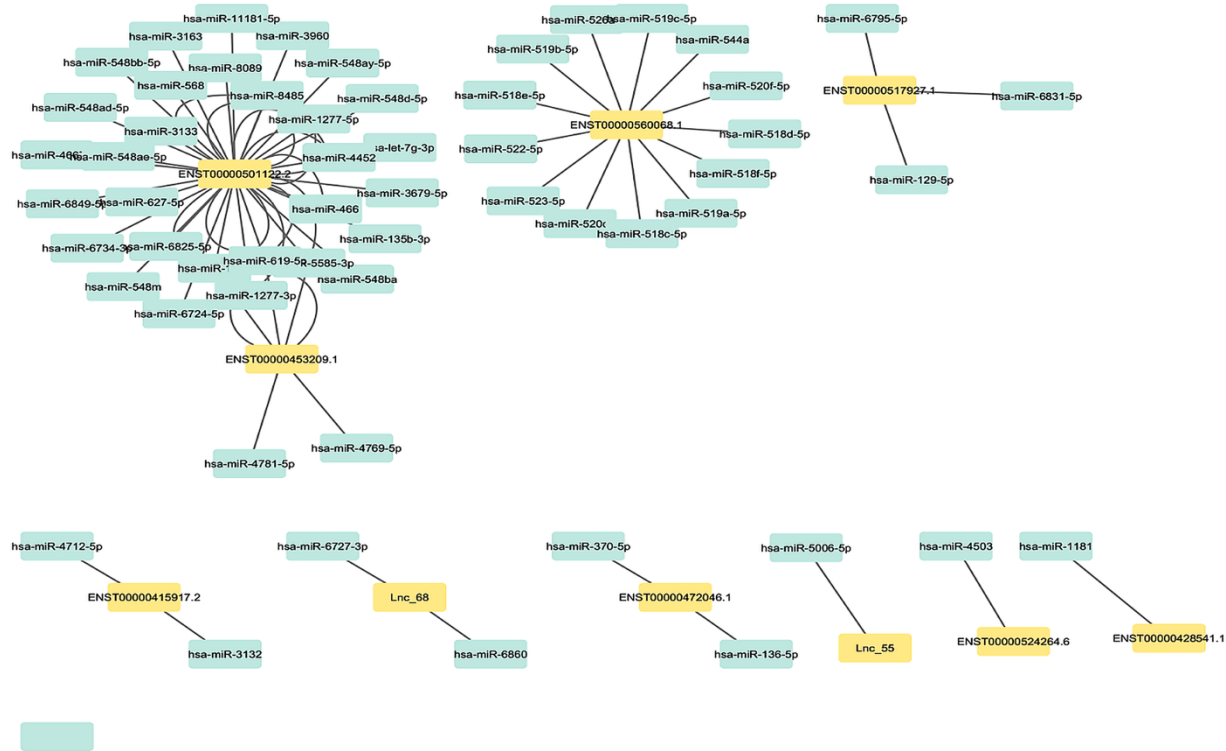


Fig. 4 The differentially expressed lncRNAs-miRNAs interactions in shikonin-treated breast.

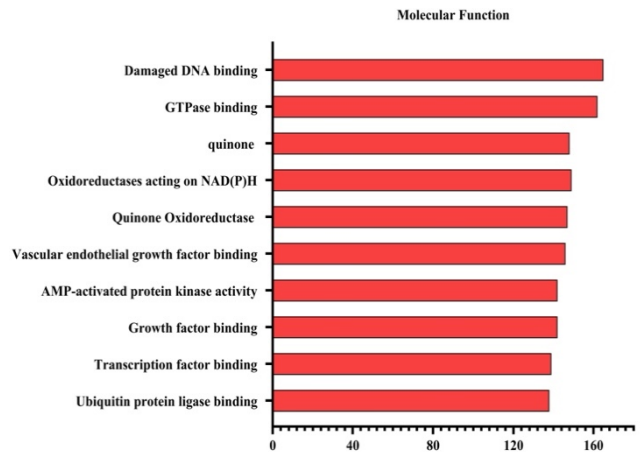


Fig. 5 Gene ontology categories were performed on the up- and downregulated lncRNA transcripts in breast cancer cell lines affected by shikonin. GO terms for biological processes were obtained from the STRING database for analysis using the BiNGO tool, a Cytoscape plugin. Significant GO terms (FDR < 0.01) were identified and further refined to select non-redundant terms. The top-10 molecular functions annotated by GO analysis with comparison of RNA-Seq from shikonin-treated cell lines versus untreated cell lines.

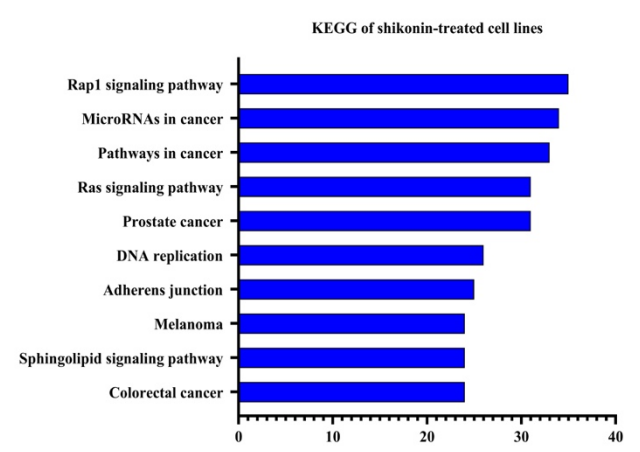


Fig. 6 Gene ontology categories were performed on the up- and downregulated lncRNA transcripts in breast cancer cell lines affected by shikonin. GO terms for biological processes were obtained from the STRING database for analysis using the BiNGO tool, a Cytoscape plugin. Significant GO terms (FDR < 0.01) were identified and further refined to select non-redundant terms. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis. The CytoKEGG plugin was used to import the pathways into Cytoscape 3.8. C: The top-10 biological processes annotated by GO analysis with comparison of RNA-Seq from shikonin-treated cell lines versus untreated cell lines.

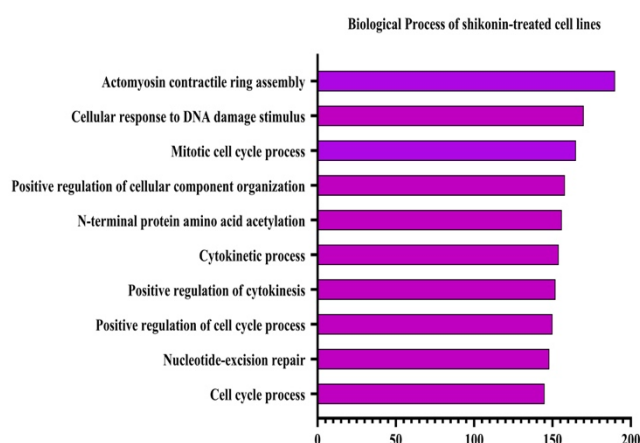


Fig. 7 Gene ontology categories were performed on the up- and downregulated lncRNA transcripts in breast cancer cell lines affected by shikonin. GO terms for biological processes were obtained from the STRING database for analysis using the BiNGO tool, a Cytoscape plugin. Significant GO terms ($FDR < 0.01$) were identified and further refined to select non-redundant terms. The top-10 biological processes annotated by GO analysis with comparison of RNA-Seq from shikonin-treated cell lines versus untreated cell lines.

The top 10 molecular functions, biological processes, and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways identified by target genes of the lncRNAs in each group were shown in Figure 5. In terms of biological processes, the detected lncRNAs' target genes in shikonin-treated cells were enriched in “Actomyosin contractile ring assembly” and related events, such as “Cytokinetic process”, “Positive regulation of cytokinesis”, and “Mitotic cell cycle process”. The major KEGG signalling pathway was the “Rap1 signalling pathway” (Figs. 5-7).

DISCUSSION

Shikonin modulates cancer biology, including tumour cell growth, migration, and survival, by regulating the expression of non-coding RNAs (ncRNAs), such as microRNAs (miRNAs) [21]. However, few studies have investigated the potential of shikonin to modulate long non-coding RNA (lncRNA) expression and induce apoptosis in cancer cells. Compared to mRNAs, lncRNAs display a lower level of expression, but their expression is tissue- and condition-specific. The specific expression patterns of functional lncRNAs across different stages of cancer biology or in tumour cells' response to therapy have the potential to serve as optimal disease biomarkers and to inform therapeutic strategies [1, 28]. We utilised an existing RNA-Seq dataset to identify putative and novel lncRNAs in response to shikonin treatment in breast cancer cell lines [29]. We identified 88 differentially expressed lncRNAs between breast cancer cells and breast cancer cells treated with shikonin. Focusing on the functional characteristics of these novel lncRNAs could yield valuable insights into the development of breast cancer therapy.

AC079305.1 (ENST00000428541.1) was among the most up-regulated lncRNAs in shikonin-treated breast cancer cell lines (Table 1). The RNA-seq analysis by Lin et al. (2013) using the same database (PRJNA392672) indicated that shikonin inhibits the JNK and p38 MAPK pathways by upregulating DUSP1 and DUSP2, leading to cell cycle arrest and apoptosis in breast cancer cells [29]. A recent study reported that the AC079305/DUSP1 axis is linked to oxidative stress and immune cell infiltration in ischemic stroke [30]. In shikonin-treated breast cancer cell lines, both AC079305.1 and DUSP1 were upregulated, suggesting that

this axis may play a role in shikonin-induced apoptosis. Experimental studies are required to confirm these assumptions. Table 1 displays that BCX111D4.7 (DDR1-AS1; ENST00000453209.1) was among the top five upregulated lncRNAs in shikonin-treated breast cancer cell lines. This lncRNA is produced from the antisense transcription of the DDR1 gene, a protein-coding gene involved in cancer biology. In breast cancer, DDR1 (Discoidin Domain Receptor 1) plays a complex and sometimes conflicting role: some studies report that it promotes tumour progression, while others suggest it can suppress tumour growth and invasion. DDR1 is a collagen-activated receptor tyrosine kinase, and its activity is strongly influenced by the tumour microenvironment. In the present study, lnc-DDR1-AS1 was the most upregulated lncRNA, yet Lin et al. (2018) reported no changes in DDR1 gene expression in the same dataset [29]. This indicates that lnc-DDR1-AS1 transcription is independent of its host gene and that its upregulation may be one of the pathways through which shikonin induces apoptosis.

The lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1; ENST00000501122.2) was downregulated in shikonin-treated breast cancer cell lines (Table 1). NEAT1 acts as a competitive endogenous RNA (ceRNA) to regulate the transcription of target genes [31]. For example, previous studies have shown that NEAT1 can competitively bind to miR-146b-5p, increasing TRAF6 expression and promoting the proliferation, migration, and invasion of pancreatic cancer cells [32]. Similarly, NEAT1 can target miR-152-3p to enhance cell viability, cell cycle progression, and apoptosis in ovarian cancer cells [33]. Multiple reports have linked NEAT1 expression to clinical features such as tumour size, lymph node involvement, and distant metastasis. Generally, higher NEAT1 levels are associated with poor prognosis and decreased survival in cancer patients [34]. Therefore, NEAT1 dysregulation is considered a strong and reliable indicator of adverse outcomes in human cancers, offering potential for better diagnostic, therapeutic, and prognostic strategies. NEAT1 has also been implicated in chemoresistance by influencing apoptosis, cell cycle regulation, drug transport and metabolism, DNA damage repair, autophagy, cancer stem cell properties, and metabolic reprogramming [13]. These findings suggest that NEAT1 is a promising therapeutic target for overcoming chemotherapy resistance and a potential biomarker for predicting treatment response. In our study, shikonin treatment reduced NEAT1 expression (Supplementary File 2). This aligns with the findings of Zang et al. (2020), who reported that shikonin induced apoptosis in paclitaxel-resistant lung cancer cells by downregulating NEAT1 [35]. Moreover, Jin et al. (2021) reviewed miRNA-lncRNA interactions contributing to breast cancer cell proliferation and identified a four-lncRNA signature for predicting breast cancer survival [36], in which NEAT1—also detected in our study—was included (Supplementary File 2).

In our study, treating SK-BR-3 cells with shikonin downregulated lncRNA-ALDH1A3-AS1-201 (ENST00000560068.1). This lncRNA is an antisense transcript of the Aldehyde Dehydrogenase 1A3 (ALDH1A3) gene. Antisense lncRNAs are produced from the opposite strand of a protein-coding or non-coding gene and can regulate the expression of their corresponding ‘sense’ genes. They participate in many cellular functions and are associated with diseases such as cancer and neurological disorders. ALDH1A3 serves as a marker of cancer stem cells (CSCs), and its overexpression is often associated with higher tumour grade, poor prognosis, and chemotherapy resistance in various cancers by altering gene expression,

signalling pathways, and glycometabolism [37]. Research has demonstrated ALDH1A3's role in cancers such as breast, glioblastoma, bladder, prostate, melanoma, and colorectal cancers [37, 38]. Studies that knocked down the 19 ALDH isoforms in breast cancer indicated that the A3 isoform was the primary driver of cancer progression in both patient tumours and cell lines [38, 39]. Due to its involvement in cancer growth and drug resistance, ALDH1A3 is considered a potential target for cancer therapy. Elevated levels in tumours can result from genetic amplification, epigenetic modifications, or post-translational changes. ALDH1A3 is also frequently regulated at the post-transcriptional level by non-coding RNAs, including both lncRNAs and microRNAs [38]. For example, the lncRNA MIR600HG binds to a sequence in the 3'UTR of ALDH1A3, thereby reducing metastasis and increasing chemosensitivity [40]. lncRNAPTGT1-1:1, also known as MIR3142HG or ENST00000517927, was one of the most downregulated lncRNAs in shikonin-treated breast cancer cell lines (Table 1). Previous research showed that lncRNA PTTG1-1:1 was overexpressed during inflammation following TNF α stimulation in erythroleukemic cells [41]. High serum levels of tumour necrosis factor (TNF) α are considered a poor prognostic factor for survival in patients with acute myeloid leukemia (AML). TNF α , produced by different immune cells, can act both directly and indirectly as a pro-proliferative autocrine tumour growth factor. Evidence suggests that TNF α may influence the expression of the pleiotropic cytokine IL-2 through lncRNA PTTG1-1:1 in the regulation of inflammation [41]. In our study, shikonin treatment appeared to induce apoptotic cell death in cancer cell lines by downregulating lncRNA PTTG1-1:1.

The gene ontology analysis of our identified lncRNAs revealed that the most significantly overrepresented KEGG signalling pathway was the "Rap1 signaling pathway". The Ras-associated protein-1 (Rap1) activates integrin receptors to sense cell adhesion to the extracellular matrix (ECM), which is required for regulating the actomyosin cytoskeleton and ensuring faithful cytokinesis [42-44]. Silencing the Rap1 signaling pathway and its downstream mediators restored contact inhibition of proliferation, potentially halting tumour growth and cell migration. For example, inhibition of Rap1-GTP has been shown to reduce metastasis in breast cancer [45-46]. Rap1 is a small GTPase; therefore, it exerts its function through several Rap GTPase-activating proteins [45]. The most important molecular function was "GTPase binding". It is not surprising that "GTPase binding" was one of the most activated molecular functions, as shown in Fig. 5.

Two downstream mediator pathways of Rap1 signaling: MAPK and VEGF, which are necessary for angiogenesis [45], are both enriched in our study (Fig. 5). Switching off the MAPK pathway is also confirmed to be related to cell cycle arrest and apoptosis effects of shikonin treatment in breast cancer cells [46]. Mitotic stress activates DNA damage signaling and impairs ubiquitin-mediated degradation of anti-apoptotic proteins, thereby initiating apoptosis [47, 48]. "Ubiquitin protein ligase binding" and "Cellular response to DNA damage stimulus" were two pathways enriched in the KEGG analysis of our study (Fig. 5).

CONCLUSION

The number of lncRNAs and their downstream targets implicated in breast cancer biology or response to a medication is continuously growing. The current study revealed that lncRNAs whose expression levels were altered after shikonin treatment

were associated with breast cancer. The identified potential clinically relevant lncRNAs might correlate with breast cancer apoptotic cell death. deserve further attention, particularly at the molecular level in terms of functional relations. Although precise molecular targets behind the anticancer effect of shikonin have not been identified yet, it seems that shikonin induces cancer cell death by dysregulation of some lncRNAs such as AC079305.1 or NEAT1. As breast cancer-related lncRNAs are detectable in body fluids as well as tissue biopsy samples, such data may be useful for developing non-invasive biomarkers for clinical analysis of breast cancer, and they could also delineate the molecular pathophysiology of the disease.

Furthermore, novel molecular techniques such as antisense oligonucleotides (ASOs), RNA interference (RNAi), and exosome-transmitting ncRNAs) can be used to target lncRNAs, which play key roles in a specific tumour cell apoptosis. Further experimental validation will be required to confirm the functional roles of these lncRNAs.

Author Contributions

Salome Dini: software; validation, investigation; writing - original draft; formal Analysis. Abozar Ghorbani: methodology; conceptualization; supervision. Neda Eskandarzade: software; validation; writing - original draft; data curation; and formal analysis. Maryam Alibeiki: writing - review & editing; data curation.

Conflicts of Interest

The authors declare no conflicts of interest.

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