Evaluation of Expression Levels of Linc-ROR and HULC Genes in Breast Cancer Cells (MCF7) Following Treatment with Nanocurcumin

Mahboobe Seyed Hosseyni [1], Amirali Gholamin [1], Fateme Roohollah [2] and Majid Sadeghizadeh [3, *]

[1]Department of Molecular Genetics, Faculty of Advance Sciences and Technology, Medical Sciences Branch, Islamic Azad University, Tehran, Iran
[2]Department of Cellular and Molecular Biology, Faculty of Advance Sciences and Technology, Medical Sciences Branch, Islamic Azad University, Tehran, Iran
[3]Department of Molecular Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

*Corresponding author: Department of Molecular Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Jalal Ahmad St, P.O. Box: 14115154, Tehran, Iran. Tel: +98-2182884409, Fax: +98-2182884717, Email: sadeghma@modares.ac.ir

Abstract

Background: Breast cancer is one of the most common types of cancers and the leading cause of death, especially in women throughout the world. Various genetics and environmental factors have been identified as a risk factor for development of breast cancer. Long non-coding RNAs (lncRNAs) have gained significant attention in recent years as new and crucial players in cancer development. Curcumin is a plant compound that has been shown to inhibit various aspects of cancer including apoptosis, inhibition of cell proliferation, invasion and angiogenesis.

Objectives: Elevated expression level of two lncRNAs, HULC and Linc-ROR, has been reported as contributing factor to the development of breast cancer. Therefore, we aimed to investigate the effect of nanocurcumin on expression levels of these genes. Besides, apoptotic effect of nanocurcumin on MCF7 cells was investigated.

Methods: MCF7 cells were exposed to various concentrations of nanocurcumin and their metabolic activity was measured by MTT assay. The level of HULC and Linc-ROR genes was evaluated by real-time PCR. Apoptosis was also determined by Annexin test.

Results: Twenty micromollar (μm) concentrations of nanocurcumin significantly decreased the expression of HULC and Linc-ROR genes (P < 0.05). These concentrations also led to significantly higher apoptosis rate in MCF7 cells (97%) compared to cell treated with curcumin.

Conclusions: Our findings suggested that nanocurcumin exhibits apoptotic effects on breast cancer MCF7 cells by reducing the expression level of two proto-oncogene lncRNAs, HULC and Linc-ROR, which are both involved in downregulation of p53.

Keywords: Breast Cancer, Nanocurcumin, Apoptosis, Long-Non Coding RNA

1. Background

Cancer is an uncontrolled proliferation of abnormal cells. In normal cells, the proliferation is restricted by an appropriate balance of stimulatory and inhibitory factors. Dysregulation of genes involved in cell division control can lead to uncontrolled proliferation and cancer formation (1). Breast cancer is one of the most common types of cancer in women, accounting for 25% of all cases of cancer (2-4). Epidemiological studies in Iran have reported eight thousand people develop breast cancer every year. The incidence of breast cancer among Iranian women is reported to be about 30 to 35 per 100,000 people (5, 6).

The role of LncRNAs as tumor suppressor or oncogene has been proven in several types of cancers. Apoptosis is a planned cell death, which has many determinants in launching and promoting its messaging cascades (7). It has been reported that LncRNAs play an important role in con-

trolling cell growth by regulating the cell cycle and apoptosis. Linc-ROR is a LncRNA that maintains important regulatory roles in interacting with miRNAs. It is well-known that during stress, DNA damage, the expression of tumor suppressor P53 is significantly increased by various translational and transcriptional mechanisms (8, 9). Linc-ROR is reported to be controlled by P53; thus, its level increases when P53 is up regulated. Given the several important roles of Linc-ROR, including inhibition of apoptosis in cellular stress, its importance in breast cancer is reported in various studies.

HULC is another LncRNA, playing important biological functions in cell proliferation and apoptosis (10). It is reported that downregulation of HULC leads to activation of p53 and induced the increased expression of p21, finally leading to cell cycle arrest and apoptosis. Thus, it indicates that HULC acts as an oncogene, metastatic indicator and is

a potential therapeutic target ([11]).

Curcumin is a natural component of turmeric, which, due to its specific chemical structure, has unique biological and medicinal properties. Several cellular and molecular mechanisms involved in cancer formation have been reported to be target of curcumin. Considering that no specific toxic effects of this natural product have been reported, the use of this product as a supplement or treatment regimen in cancer patients is considered. Among various mechanisms, induction of apoptosis plays an important role in the anticancer effect of this substance ([12]).

The cytotoxicity and apoptotic effects of curcumin in cancer setting are both dose-dependent and time-dependent. Apoptosis induction by curcumin basically involves mitochondrial pathways ([13]). Curcumin is known to induce the expression of bax, cytochrome C, P53, P21, and suppresses Bcl-2 expression, reducing the mitochondrial membrane potential, releasing cytochrome C in cytosol, and exposing phosphatidyl serine residues to cellular levels. Reduced mitochondrial membrane potential by curcumin promotes caspase3 activity. This leads to release of cytochrome C and production of active oxygen species, which act on the P53 protein ([14]).

On the basis of this knowledge, this study was conducted to investigate the ability of nanocurcumin to induce apoptosis on MCF7 breast cancer cell. Our findings showed that concentrations 15 μM and 20 μM of nanocurcumin are capable to induce apoptosis in MCF7 breast cancer cell line. This anticancer effect seems to be partially the result of down regulation of two oncogenic LncRNAs, Linc-ROR and HULC.

3. Methods

3.1. Cell Culture

MCF7 cell line was purchased from Pasteur Institute (Tehran, Iran). Cells were cultured in T-25 flasks or 96-well plates in 10% FBS DMEM/F12, containing 1% penicillin-streptomycin antibiotic.

3.2. Survival and Cellular Growth Study

Considering the importance of the number of cells per well in MTT test results, a standard curve was first drawn to select the optimal number of cells. In this way, the number of cells was determined for 48 hours to be 6,000 cells. After reaching the standard number of the cells, 10 μL of MTT (5 mg/mL) was added to each well. The insoluble color in the water was solved by DMSO (200 μL/well), and then the color intensity was evaluated by the ELISA reader (BioTekU.SA). In the next step, the specified number of cells

according to the previous step in each of the wells was cultured, but this time, different concentrations of nanocurcumin were added to each well. Cells were incubated with nanocurcumin for 48 hours. After this time course, MTT solution was added in the same manner and the result was determined by the ELISA reader.

3.3. Real Time PCR

For reverse transcription reactions, total RNA was isolated from cells, using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. cDNA was synthesized from 1 μg total RNA using PrimeScriptTM 1 st strand cDNA Synthesis (Takara, Japan) with oligo dT (Invitrogen, USA). Primer sequences used for PCR reactions were as follows:

Ampliqon Taq DNA polymerase 2$\times$ Master Mix Red (Denmark) was used for RT-PCR, according manufacturer's protocol. By gradient PCR the optimal temperature for primer annealing was determined. Real time PCR was performed for target genes and GAPDH as internal control. The sequences of primers were as fallowed:

Linc-ROR forward: GCTTGAATTATCTGAAGTTGTGGAC
Linc-ROR reverse: AAGAGAAGATTGGAGTGGAAGTG
HULC forward: CAAGGCAGGAAGAGTCGTC
HULC reverse: TGTGCTTAGTTTATTGCCAGGA
GAPDH forward: GTCTCCTCTGACTTCAACAGCG

3.4. Apoptosis Examination Using the Annexin V/FITC Test

Apoptosis assay was performed using the Annexin-V-FluosStaining kit (Roche, Germany) according to kit instructions and the data was analyzed using FlowMax software.

3.5. Treatment with Nanocurcumin

MCF7 cells were cultured in the 6-well plates and different concentrations of nanocurcumin were added to the wells. In parallel, untreated MCF7 cells were cultured in identical conditions without adding nanocurcumin.

4. Results

4.1. Effect of Nanocurcumin on MCF7 Cells Viability

The results of cell survival rate (active metabolic cells) were evaluated by MTT assay. Our findings showed that IC50 concentration for MCF7 cells was 20 μM for 48 hours treatment with nanocurcumin. However, the treatment of these cells with the same concentrations of curcumin did not reach IC50, even at 45 μm. This was the same case for nanocarrier as well ([Figure 1]).
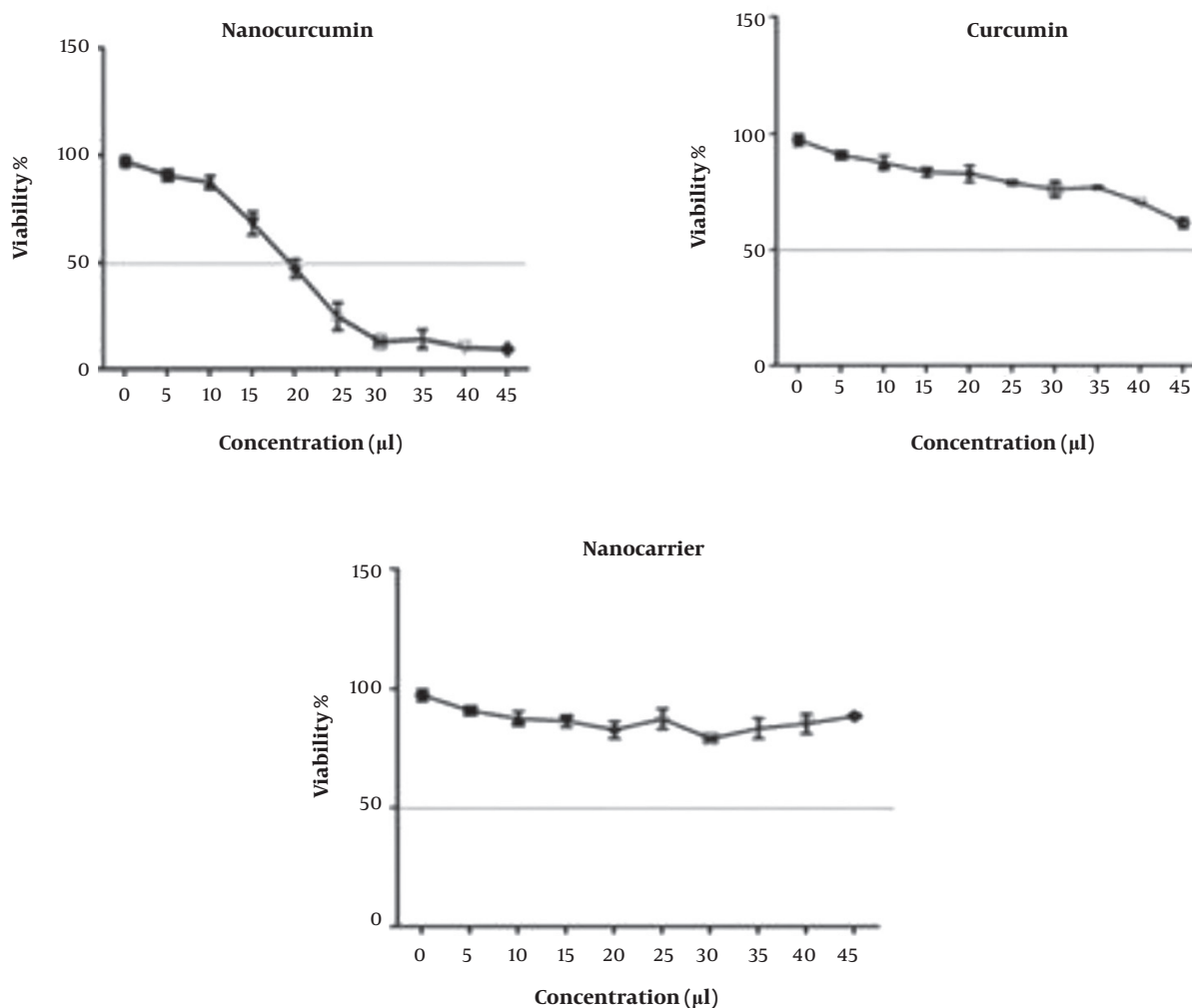
Figure 1. The toxic effect of different concentrations of nanocurcumin on MCF7 cells after 48 hours. The vertical axis shows vitality of cells and horizontal axis shows concentration of the nanocurcumin in μm. IC50 value for nanocurcumin treated MCF7 was 20 μM (for 48 hours treatment). Cells treated with either nanocarrier or curcumin did not reach IC50 even in highest concentration (45 μM).

4.2. Effect of Nanocurcumin on Expression Level of HULC and Linc-ROR

After cDNA synthesis, real time PCR was used to compare the expression level of HULC and Linc-ROR genes in MCF7 treated with nanocurcumin or that received no treatment. Results showed that treatment with nanocurcumin led to a significant reduction in expression of HULC and Linc-ROR in the MCF7 cells. P value was 0.0189 and 0.0315 for HULC and Linc-ROR, respectively (Figure 2).

4.3. Effect of Nanocurcumin on Cell Apoptosis

The rate of apoptotic cells was evaluated by Annex-inV/FITC method. The results showed that nanocurcumin treatment at 15 μM and 20 μM doses induced apoptosis, up to 97% of cells. However, no apoptotic cells was observed in curcumin treated cells (Figure 3).

5. Discussion

Extensive studies have been conducted on therapeutic effects of nanocurcumin, especially in different types of cancers. It has been shown that this compound can induce apoptosis in various types of cancer cells (15, 16).

Here we showed that nano-formulation of curcumin can induce apoptotic death of MCF7 cells, in a dose dependent manner (Figure 3). Moreover, nanocurcumin concentrations at 15 μM and 20 μM doses led to 97% apoptosis in this cell line.
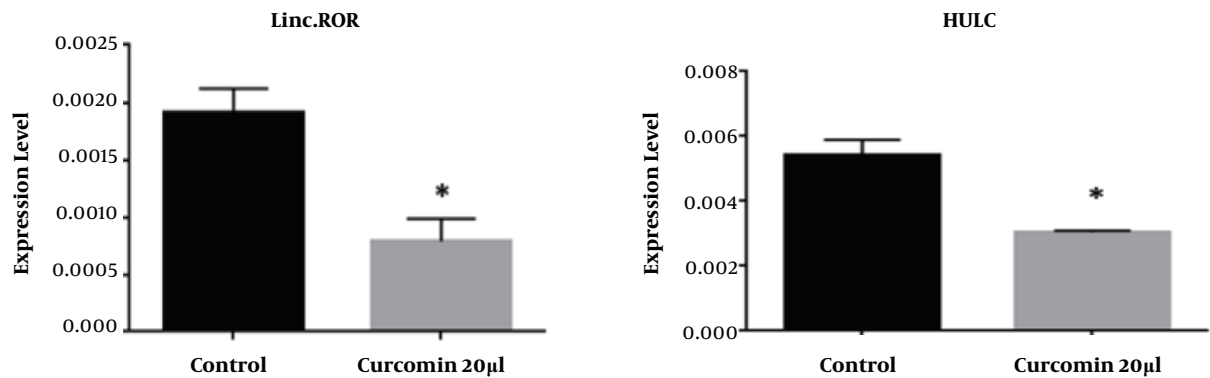
Figure 2. Comparison of Linc-ROR and HULC gene expression in MCF7 cells treated with nanocurcumin and those that received no treatments showed a significant decrease in the expression of both of these oncogenes after treatment with nanocurcumin, especially at 20 μM concentration.
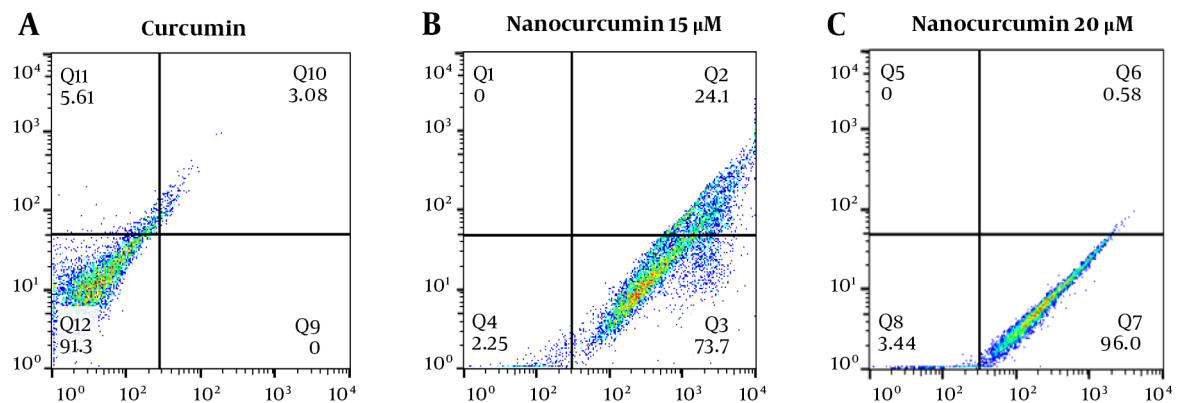

Figure 3. The result of flowcytometry followed by Annexin test showed that nanocurcumin in concentrations 15 μM and 20 μM induced cell apoptosis in 97 % of MCF7 cells.

In order to evaluate the effect of nanocurcumin on two lncRNAs, involved in cancer development, we treated MCF7 cells with different concentration of this compound. Our results showed that nanocurcumin treatment significantly decreased the expression level of HULC and Linc-ROR genes.

Accumulating evidence has demonstrated that linc-ROR and HULC are highly expressed in a variety of cancers, including breast cancer. Increased expression of these lncRNAs is thought to enhance different aspects of cancer development including, tumor growth, migration and invasion, and promotion of epithelial–mesenchymal transition.

It is known that linc-ROR and HULC both are involved in regulation of tumor suppressor, p53. Linc-ROR regulates p53 in a feedback regulatory loop. While, induction of p53 increases the linc-ROR level, linc-ROR can significantly suppress p53 in a translation repression mechanism by interrupting the interaction of heterogeneous nuclear ribonucleoprotein I (hnRNP I) with p53 mRNA. The suppressive effect of HULC on activity of p53 and p21 has been indicated as well.

Thus, our findings suggest that nanocurcumin induces apoptosis through promoting the expression of p53, via down regulation of linc-ROR and HULC.

5.1. Conclusions

In the present study based on the results of the MTT test, cell apoptosis, real-time PCR analysis it is suggested that nanocurcumin induces breast cancer cell apoptosis by reduction of expression of HULC and Linc-ROR genes. As both of these genes play significant roles in breast cancer development, these data suggests a potential beneficial effects for nanocurcumin in breast cancer.

Acknowledgments

Footnotes

References

1. Sharma RA, Gescher AJ, Steward WP. Curcumin: the story so far. *Eur J Cancer*. 2005;**41**(13):1955–68. doi: 10.1016/j.ejca.2005.05.009. [PubMed: 16081279].

2. National Cancer Institute. *Male breast cancer treatment*. 2014. Report No.: 2014 Jun 29.

3. *World cancer report*. World Health Organization; 2014. Report No.: ISBN 92- 832-0429.

4. *World cancer report*. International Agency for Research on Cancer; 2008.

5. *SEER stat fact sheets: Breast cancer*. NCI; 2014.

6. Nelson HD, Tyne K, Naik A, Bougatsos C, Chan BK, Humphrey L, et al. Screening for breast cancer: an update for the U.S. Preventive Services Task Force. *Ann Intern Med*. 2009;**151**(10):727–37. W237-42. doi: 10.7326/0003-4819-151-10-200911170-00009. [PubMed: 19920273]. [PubMed Central: PMC2972726].

7. Noori Daloii MR, Yaghubi MM. Apoptosis or programmed cell death and its relation to cancer. *Razi J*. 1999;**11**(1):7–27.

8. Levine AJ, Oren M. The first 30 years of p53: Growing ever more complex. *Nat Rev Cancer*. 2009;**9**(10):749–58. doi: 10.1038/nrc2723. [PubMed: 19776744]. [PubMed Central: PMC2771725].

9. Vousden KH, Prives C. Blinded by the light: The growing complexity of p53. *Cell*. 2009;**137**(3):413–31. doi: 10.1016/j.cell.2009.04.037. [PubMed: 19410540].

10. Yu X, Zheng H, Chan MTV, Wu WKK. HULC: An oncogenic long noncoding RNA in human cancer. *J Cell Mol Med*. 2017;**21**(2):410–7.

11. Peng W, Wu J, Feng J. Long noncoding RNA HULC predicts poor clinical outcome and represents pro-oncogenic activity in diffuse large B-cell lymphoma. *Biomed Pharmacother*. 2016;**79**:188–93. doi: 10.1016/j.biopha.2016.02.032. [PubMed: 27044827].

12. Sharma RA, Euden SA, Platton SL, Cooke DN, Shafayat A, Hewitt HR, et al. Phase I clinical trial of oral curcumin: Biomarkers of systemic activity and compliance. *Clin Cancer Res*. 2004;**10**(20):6847–54. doi: 10.1158/1078-0432.CCR-04-0744. [PubMed: 15501961].

13. Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, et al. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res*. 2001;**21**(4B):2895–900. [PubMed: 11712783].

14. Zhou H, Beevers CS, Huang S. The targets of curcumin. *Curr Drug Targets*. 2011;**12**(3):332–47. doi: 10.2174/138945011794815356. [PubMed: 20955148]. [PubMed Central: PMC3025067].

15. Calabrese V, Bates TE, Mancuso C, Cornelius C, Ventimiglia B, Cambria MT, et al. Curcumin and the cellular stress response in free radical-related diseases. *Mol Nutr Food Res*. 2008;**52**(9):1062–73. doi: 10.1002/mnfr.200700316. [PubMed: 18792015].

16. Lao CD, Ruffin M, Normolle D, Heath DD, Murray SI, Bailey JM, et al. Dose escalation of a curcuminoid formulation. *BMC Complement Altern Med*. 2006;**6**:10. doi: 10.1186/1472-6882-6-10. [PubMed: 16545122]. [PubMed Central: PMC1434783].