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# The Possible Role of The Long Non-Coding RNA NORAD in Mitomycin C-Related Chemoresistance

## Uzun Kodlamayan RNA NORAD'ın Mitomisin C İlişkili Kemorezistansta Olası Rolü

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**ABSTRACT**

**Aim:** Non-coding RNA activated by DNA damage (NORAD) is a long non-coding RNA activated during DNA damage response. Accumulating evidence suggest that NORAD is overexpressed in human cancers and associated with the drug-induced chemoresistance. The present study aims to explore the possible role of long non-coding RNA NORAD during mitomycin C-related chemoresistance and mitomycin C-induced DNA damage response.

**Materials and Methods:** In cell culture experiments, MDA-MB-231 breast cancer cells were used. MTT cell viability assay was used to determine the effects of mitomycin C on breast cancer cells and application dose was determined accordingly. For analysis of NORAD gene expression levels, quantitative real-time polymerase chain reaction method was used.

**Results:** Mitomycin C was found to suppress cell viability of breast cancer cells in a dose-dependent manner and half-maximal inhibitory concentration was determined as 1.12 µg/mL ($p<0.0001$). Notably, significant differential activation of NORAD was determined in breast cancer cells treated with the mitomycin C ($p<0.0001$).

**Conclusion:** Findings obtained here strongly suggest that NORAD is possibly involved in mitomycin C-related chemoresistance and mitomycin C-induced DNA damage response.

**Keywords:** DNA damage, breast cancer, mitomycin C, lncRNA, NORAD

**ÖZ**

**Amaç:** Non-coding RNA activated by DNA damage (NORAD), deoksiribo nükleik asit (DNA) hasarı cevabı sırasında aktive olan bir uzun kodlamayan ribonükleik asittir (RNA). Çalışmalar, NORAD'ın insan kanserlerinde aşırı eksprese edildiğini ve ilaca bağlı kemorezistans ile ilişkili olduğunu göstermektedir. Bu çalışma, mitomisin C ile ilişkili kemorezistans ve mitomisin C ile indüklenen DNA hasar yanıtı sırasında spesifik olarak aktive edilen bir uzun kodlamayan RNA NORAD'ın olası rolünü araştırmayı amaçlamaktadır.

**Gereç ve Yöntem:** Hücre kültürü deneylerinde MDA-MB-231 meme kanseri hücreleri kullanıldı. Mitomisin C'nin meme kanseri hücreleri üzerindeki etkilerini belirlemek için MTT hücre canlılığı testi kullanıldı ve uygulama dozu buna göre belirlendi. NORAD gen ekspresyon düzeylerinin analizi için kantitatif qPCR yöntemi kullanıldı.

**Bulgular:** Mitomisin C'nin meme kanseri hücrelerinin hücre canlılığını doza bağlı olarak baskıladığı ve yarı-maksimum inhibisyon konsantrasyonunun 1,12 µg/mL olduğu belirlendi ($p<0,0001$). Özellikle, Mitomisin C ile muamele edilen meme kanseri hücrelerinde NORAD'ın önemli bir farklı aktivasyonu belirlendi ($p<0,0001$).

**Sonuç:** Burada elde edilen bulgular, mitomisin C ile ilişkili kemoresistans ve mitomisin C ile indüklenen DNA hasar yanıtında NORAD'ın olası rolünün olabileceğini göstermektedir.

**Anahtar Kelimeler:** DNA hasarı, meme kanseri, mitomisin C, lncRNA, NORAD

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## INTRODUCTION

Although the human genome harbors hundreds of long non-coding ribonucleic acid (RNAs) (lncRNAs), only about a dozen with distinct biological activities and biochemical processes have been identified. Non-coding RNA activated by deoxyribonucleic acid (DNA) damage (NORAD, formerly known as LINC00657), a particular lncRNA transcript which is stimulated in response to DNA injury, has been reported to be necessary for the maintenance of genome integrity[1]. Deletion of NORAD leads to chromosomal instability and aneuploidy[2]. NORAD is abundantly expressed in various human cell lines and tissues. Compared to other lncRNAs in mammals, NORAD is highly conserved[2,3]. NORAD has been discovered to engage with the proteins that regulate DNA replication and repair mechanisms and translocate to the nucleus when stimulated by DNA damage or replication stress[2,3]. NORAD interacts with RBMX, a DNA damage response component, and prevents RNA-binding motif protein, X-linked (RBMX) from assembling at NORAD-activated ribonucleo protein complex 1. NORAD- or RBMX-depleted cells exhibited a higher incidence of chromosomal segregation abnormalities, slower replication fork speed and deregulation of cell cycle progression[2]. Moreover, studies suggest that NORAD is deregulated in breast, ovarian, cervical, gastric, colorectal, bladder, pancreatic, hepatocellular, esophageal, prostate, lung, thyroid, retinoblastoma, and neuroblastoma cancers[4]. Specifically, overexpression of NORAD stimulates proliferation, invasion, and metastasis of cancerous cells and prevents apoptosis[4]. In addition, several lines of evidence suggest that NORAD is associated with chemoresistance to currently available chemotherapeutic agents such as doxorubicin, gemcitabine, and 5-fluorouracil. In neuroblastoma, it has been shown that increased expression of NORAD is enhanced proliferation, metastasis, and doxorubicin resistance and while interfering with the apoptotic death of neuroblastoma cells by inducing HDAC8 expression through sponging miR-144-3p[5]. Also, NORAD has been reported to act as a competing endogenous RNA (ceRNA) to mediate gemcitabine chemoresistance in bladder cancer through regulating WEE1 expression via sponging miR-155-5p[6]. Also, differential activation of NORAD has shown to be associated with the 5-fluorouracil chemoresistance during hypoxia by acting as a ceRNA by sponging miR-495-3p/HIF-1$\alpha$ expression in colorectal cancer[7].

Mitomycin C is a naturally occurring anticancer antibiotic substance derived from streptomyces caespitosus and other streptomyces species. Mitomycin C was discovered in the 1950s by Japanese microbiologists. Mitomycin C is one of the few antibiotics known to react with DNA[8]. It induces DNA damage by introducing monofunctional and bifunctional DNA cross-links. As a prodrug, mitomycin C itself remains inactivated and does not react with DNA and requires bioactivation[9]. The reduction of the quinone ring, which transforms mitomycin c into a highly reactive unstable alkylating species, is required for DNA crosslinking and alkylating activities[10]. Mitomycin C is widely used for the treatment of several human malignancies because of its ability to suppress DNA replication and cell division. It has been shown to be an effective antitumor agent in several cancers, including breast cancer, cervical cancer, non-small cell lung cancer, pancreatic cancer, gastric cancer, prostate cancer, and bladder cancer. Mitomycin C is characterized by its high bio-reductive alkylation under hypoxic conditions. Inside solid tumors, oxygen-deprived cells form an environment in which mitomycin c is highly active[11].

Although the molecular mechanism underlying the biological and pharmacological properties of mitomycin C is well characterized, its effect on non-coding RNAs activated during the DNA damage response has not yet been described. Accordingly, in this study, we sought to explore the possible role of NORAD, a lncRNA specifically activated during DNA damage response, during mitomycin C-related chemoresistance and mitomycin C-induced DNA damage response.

## MATERIALS AND METHODS

### Cell Lines, Culture Conditions, and Chemicals

For the study, HTB-26 (MDA-MB-231) triple negative breast adenocarcinoma cells were obtained from ATCC and propagated using DMEM medium (Cat. No.: D6429, Sigma-Aldrich) complemented with the 1% penicillin/streptomycin and 10% fetal calf serum solution (Cat. No.: A4766801, Sigma-Aldrich). Cell cultures were maintained in moistened carbon dioxide incubator at 37 °C. Mitomycin C isolated from Streptomyces caespitosus was commercially obtained from Sigma-Aldrich (Cat. No.: M4287). 2 mg of mitomycin C was suspended in 10 mL $ddH_2O$ to obtain a stock solution of 200 $\mu$g/mL.

### MTT Analysis

Cells were seeded at a concentration of $3x10^4$ cells/mL in 96-well plates and incubated overnight to determine cell viability. Following overnight incubation, cells were treated with the various concentrations of mitomycin C ranging from 0.125 to 2 $\mu$g/mL and incubated for 24 hours. Then, supernatants were discarded, and cells were washed several times with the 1 X PBS solution and subsequently incubated with the 1 mg/mL of MTT (3- (4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide) solution for 40-60 minutes at 37 °C.

### Gene Expression Analysis

To investigate the effects of mitomycin C on NORAD expression levels, cells were plated to 12-well culture dishes and incubated overnight to allow attachment. Subsequently, cells were treated with the mitomycin C and incubated for

24 hours. Cells were then harvested using Trypsin-EDTA solution, and RNA purifications were made using GeneJET RNA Purification Kit (Cat. No.: K0702, Thermo Scientific, Waltham, MA, USA) according to the recommendations provided by the manufacturer. RNA quality and concentrations were determined with the help of Thermo Scientific Multiskan GO instrument. The RevertAid First Strand cDNA Synthesis Kit (Cat. No.: FERK1622, Thermo Scientific, Waltham, MA, USA) was used for reverse transcription of RNA samples into cDNA samples. GAPDH and NORAD-specific gene expression PCR primers were designed with the help of NCBI primer blast. Primer pairs for GAPDH were Forward: 5'-AGACCACAGTCCATGCCATCAC-3', Reverse: 5'-GGTCCACCACCCTGTTGCTGT-3' and primer pairs for NORAD were Forward: 5'-CAGAGGAGGTATGCAGGGAG-3', Reverse: 5'-CCACCATCCCCGTGACTAAG-3'. Lastly, to assess gene expression levels of NORAD and GAPDH genes, RealQ Plus 2x Master Mix Green (Cat. No.: A323402, Ampliqon) was used and Rotor-Gene Q real-time PCR instrument was used to perform reactions. Briefly, 12.5 μl RealQ Plus 2x Master Mix, 0.5 μl from each primer (10 μM), 2 μl cDNA and 9.5 μl PCR-grade $H_2O$ were mixed and subjected to following thermal conditions; 15 minutes at 95 °C, 40 cycles of 15 s at 94 °C, 30 s at 60 °C, and 30 s at 70 °C. At the end of each reaction, a melting curve analysis was performed between 72 °C and 95 °C.

### Statistical Analysis

For the calculation of relative gene expression levels, $2^{-\Delta Ct}$ formula was used. GAPDH was used as a reference gene for calculations. All experiments were performed in triplicate. Statistical examination of the obtained data was achieved using GraphPad Prism 9 software, with p values less than 0.05 accepted as significant. For comparisons between two independent groups, the Student's t-test was used whereas variance analysis was used for comparisons of more than two groups.

## RESULTS

### Cell Viability of Cells Upon Mitomycin C Treatments

Triple negative breast cancer (TNBC) is an aggressive subtype of breast cancer with no effective therapy at present. To explore mitomycin C-related chemoresistance in breast cancer, MDA-MB-231 TNBC was used. Accordingly, MTT assay was used to examine the effect of mitomycin C on viability of breast cancer cells, and cells were treated with the varying concentrations of mitomycin C. Remarkably, the viability of breast cancer cells was found to be dramatically decreased in a dose-dependent manner (Figure 1). Half-maximal inhibitory concentration ($IC_{50}$) of mitomycin C against MDA-MB-231 breast cancer cells was calculated as 1.12 μg/mL. Treatment with 0.125 μg/mL, 0.25 μg/mL, 0.5 μg/mL, 1 μg/mL and 2 μg/mL mitomycin C for 24 h resulted in 68.72%, 60.86%, 53.20%, 49.27%, and 35.20% cell viability, respectively, compared to the control group (Figure 1). Significant inhibition of cell viability was observed at 1 μg/mL ($p<0.0001$) and 2 μg/mL ($p<0.0001$) doses of mitomycin C compared to the untreated group.

### Differential Activation of NORAD Upon Mitomycin C Treatments

To further test the influence of mitomycin C on the expression of NORAD lncRNA, we have performed gene expression analysis upon mitomycin C treatments in breast cancer cells. Notably, NORAD expression levels were determined to be significantly enhanced in cells treated with the 1 μg/mL and 2 μg/mL mitomycin C ($p<0.0001$). Fold-change analysis revealed that expression of NORAD was increased 5.7-fold and 5.2-fold in cells treated with the 1 μg/mL and 2 μg/mL mitomycin C, respectively (Figure 2), strongly indicating that treatment with mitomycin C induces DNA damage response involving NORAD lncRNA.

## DISCUSSION

The main goal of cancer treatment is surgical removal of tumor cells or their destruction by radiation or chemotherapy. Therefore, understanding drug-induced genetic alterations and chemoresistance is of great importance for improving patient outcomes in cancer chemotherapy. The poor success of cancer chemotherapy is often due to adverse effects and resistance to chemotherapeutic agents[12]. There is increasing evidence that an optimal level of DNA repair is essential for the elimination of mutations and DNA lesions to prevent malignant transformation[12]. Thus, DNA repair function emerges as a double-edged sword in cancer treatment. While a low level of DNA repair activity sensitizes cancer cells to chemo-mediated killing, it also allows a second primary tumor to develop[12]. In contrast, overactivity of DNA repair functions promotes cancer progression. Maintenance of the genome integrity by DNA damage response and repair mechanisms ensures genomic stability and keeps chromosome number constant. Moreover, lncRNA NORAD is a specific non-coding RNA molecule triggered in response to DNA injury. More recently, NORAD has shown to interact with proteins of DNA replication and repair machinery and migrates to the nucleus in case of stress conditions. NORAD has also been shown to be essential for the assembly of a topoisomerase complex, called NARC1, which is critical for the maintenance of genome stability. NORAD was also shown to sequester PUMILIO proteins to preserve genome integrity[1,13,14]. Although NORAD is critical for genomic stability, it has been reported to be a crucial oncogenic driver in human cancers. High activity of NORAD was associated with increased cell proliferation and aggressiveness of human cancers. NORAD was reported to be markedly overexpressed in gastric cancer tissue compared to adjacent healthy tissue.

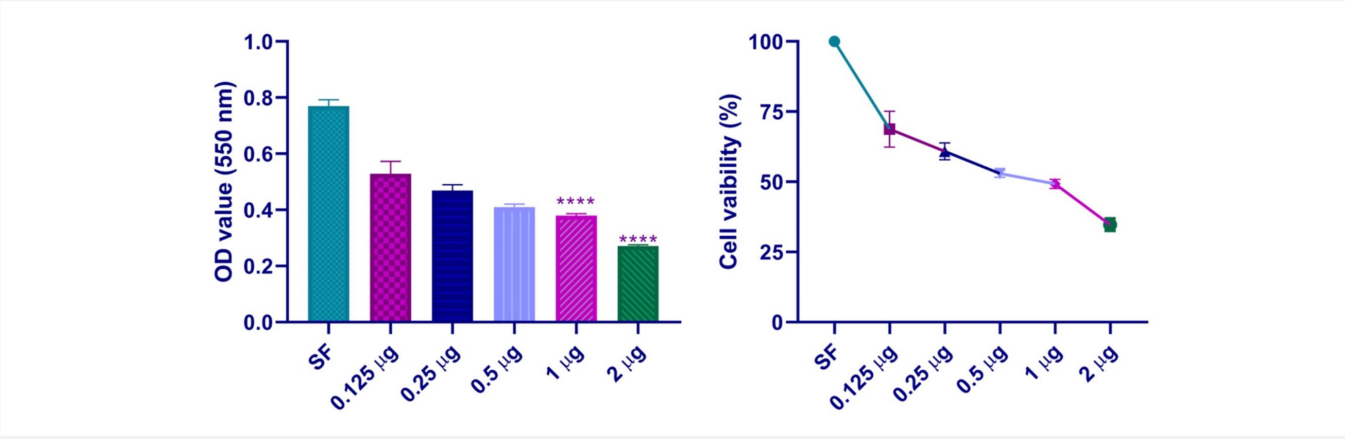


**Figure 1.** Dose-dependent inhibition of cell viability of MDA-MB-231 breast cancer cells treated with different concentrations of mitomycin C

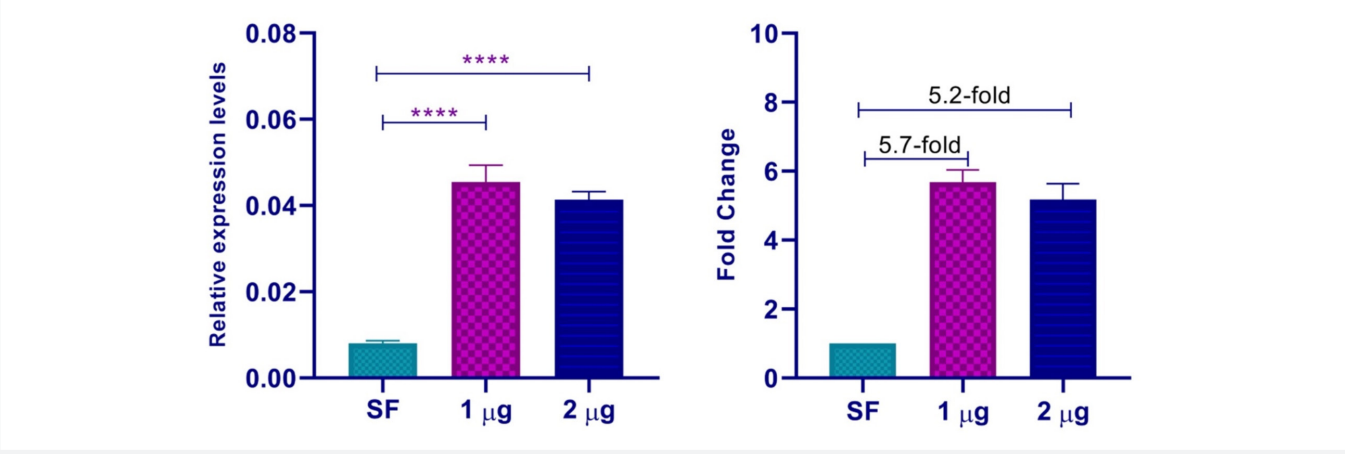


**Figure 2.** The effect of mitomycin C treatments on NORAD expression levels. NORAD was determined to be differentially activated in response to mitomycin C treatments

*NORAD: Non-coding RNA activated by DNA damage*

Inhibition of NORAD expression reduced cell viability and migration and promoted apoptotic death of gastric cancer cells by inducing the expression of E-cadherin Bax, and PTEN, and downregulating Bcl-2 proteins[15]. NORAD was also determined to be overexpressed in oral squamous cell carcinoma tissues and cell lines, and it functions as a tumor promoter to enhance the advancement of oral squamous cell carcinoma by sponging miR-577[16]. Similarly, higher expression of NORAD was found in cancerous tissues of thyroid carcinoma patients, and ectopic overexpression of NORAD was associated with increased cell proliferation, invasion, and migration and activated epithelial-to-mesenchymal transition (EMT) by inhibiting miR-202-5p[17]. Knockdown of NORAD was also discovered to be associated with the inhibition of EMT of head and neck cancer stem cells through regulating miR-26a-5p[18]. Significant overexpression of NORAD was also found in osteosarcoma tumors and was associated with the clinic and pathological findings of patients, such as tumor size, grade and metastasis[19]. Although limited, a relationship between NORAD and resistance to certain types of chemotherapeutic agents has also been demonstrated. Particularly, NORAD has been linked to gemcitabine resistance in bladder cancer by acting as a competitive endogenous RNA that inhibits miR-155-5p activity. Silencing of NORAD in gemcitabine-resistant bladder cancer cells impairs proliferation and cell cycle progression and induces apoptosis via regulation of the miR-155-5p/WEE1 axis[6]. In addition, NORAD was shown to be associated with the hypoxia-induced 5-fluorouracil chemoresistance in colorectal cancer[7]. Consistent with all these observations, in the present study, it has been shown that NORAD was significantly activated in response to mitomycin

C treatment in breast cancer cells, suggesting that NORAD may be related to mitomycin C resistance at the organism level. NORAD inhibitory therapy in combination with mitomycin C chemotherapy could be a good treatment method for triple negative breast cancer. Overall, the strong tumor-promoting function and association with chemoresistance to certain drugs also support a role for NORAD in chemoresistance to mitomycin C.

### Study Limitations

While this study successfully evaluated the role of NORAD in mitomycin C-related chemoresistance and mitomycin C-induced DNA damage response, there were some limitations. In the future studies, gain of function and loss of function experiments of NORAD along with the mitomycin treatments are of great interest to understand more about the role of NORAD during DNA damage response and repair.

## CONCLUSION

In conclusion, we describe here that lncRNA NORAD was activated in response to mitomycin C treatments in TNBC cells, strongly indicating that NORAD may relate to the mitomycin C-related chemoresistance. However, these findings need to be supported by additional comprehensive studies.

### Ethics

**Ethics Committee Approval:** The study was performed in cancer cell lines; therefore, ethical approval was not required.

**Informed Consent:** Not applicable for the present study.

**Peer-review:** Externally and internally peer-reviewed.

**Financial Disclosure:** The author declared that this study received no financial support.

## REFERENCES

1. Lee S, Kopp F, Chang TC, Sataluri A, Chen B, Sivakumar S, et al. Noncoding RNA NORAD Regulates Genomic Stability by Sequestering PUMILIO Proteins. Cell. 2016;164:69-80.
2. Munschauer M, Nguyen CT, Sirokman K, Hartigan CR, Hogstrom L, Engreitz JM, et al. The NORAD lncRNA assembles a topoisomerase complex critical for genome stability. Nature. 2018;561:132-6.
3. Yang Z, Zhao Y, Lin G, Zhou X, Jiang X, Zhao H. Noncoding RNA activated by DNA damage (NORAD): Biologic function and mechanisms in human cancers. Clin Chim Acta. 2019;489:5-9.
4. Soghli N, Yousefi T, Abolghasemi M, Qujeq D. NORAD, a critical long non-coding RNA in human cancers. Life Sci. 2021;264:118665.
5. Wang B, Xu L, Zhang J, Cheng X, Xu Q, Wang J, et al. LncRNA NORAD accelerates the progression and doxorubicin resistance of neuroblastoma through up-regulating HDAC8 via sponging miR-144-3p. Biomed Pharmacother. 2020;129:110268.
6. Yang Y, Zhang G, Li J, Gong R, Wang Y, Qin Y, et al. Long noncoding RNA NORAD acts as a ceRNA mediates gemcitabine resistance in bladder cancer by sponging miR-155-5p to regulate WEE1 expression. Pathol Res Pract. 2021;228:153676.
7. Zhang L, Wu H, Zhang Y, Xiao X, Chu F, Zhang L. Induction of lncRNA NORAD accounts for hypoxia-induced chemoresistance and vasculogenic mimicry in colorectal cancer by sponging the miR-495-3p/ hypoxia-inducible factor-1α (HIF-1α). Bioengineered. 2022;13:950-62.
8. Tomasz M, Lipman R, Chowdary D, Pawlak J, Verdine GL, Nakanishi K. Isolation and structure of a covalent cross-link adduct between mitomycin C and DNA. Science. 1987;235:1204-8.
9. Tomasz M. Mitomycin C: small, fast and deadly (but very selective). Chem Biol. 1995;2:575-9.
10. Seow HA, Penketh PG, Baumann RP, Sartorelli AC. Bioactivation and resistance to mitomycin C. Methods Enzymol. 2004;382:221-33.
11. Gad SE. Mitomycin C. In: Wexler P, ed. Encyclopedia of Toxicology (Third Edition). Oxford: Academic Press. 2014:354-6.
12. Kiwerska K, Szyfter K. DNA repair in cancer initiation, progression, and therapy-a double-edged sword. J Appl Genet. 2019;60:329-34.
13. Elguindy MM, Kopp F, Goodarzi M, Rehfeld F, Thomas A, Chang TC, et al PUMILIO, but not RBMX, binding is required for regulation of genomic stability by noncoding RNA NORAD. Elife. 2019;8:e48625.
14. Tichon A, Gil N, Lubelsky Y, Havkin Solomon T, Lemze D, Itzkovitz S, et al. A conserved abundant cytoplasmic long noncoding RNA modulates repression by Pumilio proteins in human cells. Nat Commun. 2016;7:12209.
15. Raei N, Safaralizadeh R, Hosseinpourfeizi M, Latifi-Navid S, Yazdanbod A. Suppression of lncRNA NORAD may affect cell migration and apoptosis in gastric cancer cells. Mol Biol Rep. 2022;49:3289-96.
16. Qi C, Liu J, Guo P, Xu Y, Hu J, Han X. LncRNA NORAD facilitates oral squamous cell carcinoma progression by sponging miR-577 to enhance TPM4. Biol Direct. 2022;17:1.
17. He H, Yang H, Liu D, Pei R. LncRNA NORAD promotes thyroid carcinoma progression through targeting miR-202-5p. Am J Transl Res. 2019;11:290-9.
18. Hu W, Zhao Y, Su L, Wu Z, Jiang W, Jiang X, et al. Silencing the lncRNA NORAD inhibits EMT of head and neck squamous cell carcinoma stem cells via miR 26a 5p. Mol Med Rep. 2021;24:743.
19. Wang Y, Zhou B, Yan L, Wu J, Xing Z, Zhang S, et al. lncRNA NORAD promotes the progression of osteosarcoma via targeting of miR-155-5p. Exp Ther Med. 2021;21:645.