NR2F2-AS1 Suppressed Trastuzumab Effects on Esophageal Cancer By Inhibiting miR-4429 and miR-425-5p Expression Through Targeting IGF1R

Zhibin Zhang  
The First Affiliated Hospital of Xian Jiaotong University

Xu Zhao  
The First Affiliated Hospital of Xian Jiaotong University

Rui Zhu  
The First Affiliated Hospital of Xian Jiaotong University

Hong Ren (renhong@xjtu.edu.cn)  
The First Affiliated Hospital of Xian Jiaotong University

Research Article

Keywords: NR2F2-AS1, miR-4429, miR-425-5p, IGF1R, Trastuzumab

DOI: https://doi.org/10.21203/rs.3.rs-401449/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. 
Read Full License
Abstract

Aim: In this manuscript, we aimed to investigate the involvement of non-coding RNAs in mediating trastuzumab effects in EAC. Background: Scarce evidences supported that targeted drugs, like Trastuzumab, can be applied to esophageal adenocarcinoma patients (EAC). Objective: Evaluating the role and mechanism of NR2F2-AS1 in regulating Trastuzumab effects in EAC patients.

Method: RNA sequencing to screen IGF1R related IncRNAs. qRT-PCR and western blot were used to evaluate the expression level of genes. CCK-8 was used to test the cell proliferation ability. Dual luciferase reporter gene assay and RNA pull-down were used for crosstalk evaluation.

Results: NR2F2-AS1 was identified to be associated with HER2 expression by RNA sequencing and its expression related to worse prognosis and advanced T and N stage. NR2F2-AS1 expression induced by Trastuzumab through mediating H3K27ac. Furtherly, miR-4429 and miR-425-5p, which were predicted and proved to interact with NR2F2-AS1 and IGF1R, expressed lowly in esophageal cancer both in vivo and in vitro and suppressed cell viability. Most importantly, miR-4429 and miR-425-5p overexpression could increase trastuzumab’s inhibitory effect on cell viability.

Conclusion: Trastuzumab has the potential to suppress EAC progression mainly in the presence of miR-4429 and miR-425-5p overexpression targeting HER2. However, Trastuzumab induces exosomal NR2F2-AS1 expression, which binds to miR-4429 and miR-425-5p to suppress their expression, resulting in the failure of trastuzumab treatment. Therefore, targeting exosomes might be a novel way to develop auxiliary drugs for trastuzumab in EAC.

Introduction

HER2(Epidermal Growth Factor Receptor 2), usually activated by HER1, 3 and 4 by forming dimers, is proved to mediate malignant behaviors, for example, promoting cell proliferation, migration, invasion and angiogenesis[1]. Therefore, HER2 is a feasible therapeutic target and widely applicable in treating breast cancer[1]. Numerous studies have elucidated the distinguished effect of Trastuzumab in prolonging HER2 positive breast cancer by suppressing the HER2 activation[2]. Accumulating studies have shown that HER2 overexpressing esophageal cancer patients have shorter overall survival months than HER2 negative patients with more than 70% HER2 positive rate[3]. Besides, around 30% esophageal cancer patients had overexpressed HER2, indicating the possibility of applying Trastuzumab[4]. A phase II study showed that Trastuzumab did not increase the toxicity and side effects, suggesting the accepted safety[5]. Under this circumstance, successfully applying trastuzumab to esophageal cancer will significantly improve the prognosis[6, 7]. However, a substantial proportion of HER2 positive EAC patients did not well respond to trastuzumab, partially due to acquired resistance[8, 9]. Loss of PTEN is one of the most important mechanisms that contributed to the trastuzumab resistance[10, 11]. Apart from PTEN, IGF1R (insulin-like growth factor-I receptor) activation induces HER2 phosphorylation and impairs the HER2 activation in trastuzumab resistant cell lines[12]. IGF1R knock down will block the
heterodimerization with HER2 and improve cancer cells’ response to trastuzumab\textsuperscript{[13]}. Here, we have assumed that NR2F2-AS1 might regulate trastuzumab sensitivity by targeting IGF1R.

Increasing evidence have shown the neglectable impacts of non-coding RNAs in the process of developing trastuzumab resistance\textsuperscript{[14]}. For example, linc-ATB functioned as a microRNA sponge to suppress the expression of miR-200c to up-regulate the ZEB1 and ZNF-27 to activate EMT process, resulting in the trastuzumab resistance\textsuperscript{[15]}. In this manuscript, IncRNA NR2F2-AS1 was identified using RNA-sequencing in vivo as candidate trastuzumab resistance associated genes. IncRNA NR2F2-AS1 is involved in the cancer cell proliferation, apoptosis, cell cycle arrest, cancer stemness and etc. as an oncogene\textsuperscript{[16-19]}. Lin et al provided valuable evidence that NR2F2-AS1 enhanced Rac1 expression to increase cancer stemness in clear cell renal cell carcinoma; therefore, it can be inferred that NR2F2-AS1 might be engaged in developing drug resistance\textsuperscript{[16]}. Current studies reported that NR2F2-AS1 mainly promotes tumorigenesis by means of acting as a microRNA sponge. For instance, NR2F2-AS1 sponges miR-230b and miR-4429 to regulate their regulation of downstream genes\textsuperscript{[16, 20]}. Therefore, we assume that NR2F2-AS1 might regulate IGF1R expression through sponging potential microRNAs. In this manuscript, we predicted that miR-4429 and miR-425-5p could interact with both NR2F2-AS1 and IGF1R. miR-4429 and miR-425-5p were found to be tumor suppressors in various cancer\textsuperscript{[21-26]}. However, evidence about their biological functions in esophageal cancer is lacking.

**Methods**

Esophageal adenocarcinoma patients

In total 201 esophageal cancer patients (Her2 positive) were enrolled in first affiliated hospital of Xi’an Jiaotong University between March 2011 and March 2013. Every enrolled patient has received and signed the written consent. Protein and RNA were extracted from esophageal cancer and corresponding normal tissue samples obtained from each patient immediately; and remnant samples were paraffin-embedded. The main inclusion criteria were as follows: 1. Pathologically diagnosed as esophageal adenocarcinoma; 2. Receive surgery plus chemotherapy; 3. Did not receive any other treatment related to EAC elsewhere; 4. Older than 18-year-old and ability to take care of herself/himself. The main exclusion criteria were: 1. Diagnosed with other primary malignancies; 2. IV stage (based on 7th edition of UICC tumor staging system); 3. Refuse to sing the informed consent. Two employees were in charge of documenting clinical and follow-up records of each patient. Besides, the corresponding author quality controlled every data and validated the data integrity. Our study has gained approval from the Ethics Committee of first affiliated hospital of Xi’an Jiaotong University. This study was performed in accordance with the Declaration of Helsinki.

RNA sequencing

RNA sequencing process was guided and supported by Genechen (Shanghai, China)
Total RNA was extracted from 4 EAC (Her2-positive) patients’ cancer tissues and corresponding normal tissues by means of TRIzol (Invitrogen, Carlsbad, CA). NA Clean XP Kit (Beckman Coulter, Kraemer Boulevard Brea, CA) and the RNase-Free DNase Set (QIAGEN, GmbH, Germany) were used for the RNA purification. Illumina HiSeq 2000/2500 (Illumina Inc, San Diego, CA) was used for RNA-sequencing.

Cell culture

Normal esophageal epithelial cell line HEEC and adenocarcinoma cell lines OE-33 and SK-GT-4 were purchased from American Type Culture Collection (Virginia, USA). All kinds of cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a 37°C and 5% CO₂ incubator. Culture medium were changed every day.

Cell transfection

NR2F2-AS1 and miR-4429 knock down and overexpression plasmids were purchased from Genechem (Shanghai, China); miR-425-5p knock down and overexpression plasmids were purchased from GenePharma (Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used to assist in transfecting above plasmids into BIC-1 and SK-GT-4 cell lines.

qRT-PCR

Total RNA was extracted from cell lines, EAC patients’ cancer tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Cytoplasmic & Nuclear RNA Purification Kit (Norgen, Canada) was used to isolate cytoplasmic and nuclear RNA. First-strand cDNA synthesis kit (Tiangen Biotech, Beijing, China) was used for reverse transcription to generate cDNA. SYBR® Premix Dimer Eraser kit (Takara Shiga, Japan) was used to perform qRT-PCR and test the expression of NR2F2-AS1 and IGF1R. β-actin was used as internal control. miScript microRNA RT PCR kit (Qiagen, Toronto, ON, Canada) was used for cDNA synthesis and qRT-PCR process for miR-4429 and miR-425-5p expression. U6 was used as the internal control. ABI 7500 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) was used to perform the qRT-PCR process. The expression level was calculated by $2^{-\Delta\Delta Ct}$ method.

Western blot

Total protein was extracted from cells using RIPA buffer (Sigma-Aldrich, Darmstadt, Germany) and was quantified by BCA Protein Assay Kit (Beyotime, Shanghai, China). Proteins were transferred to PVDF after separated by SDS-PAGE. 5% skim milk was used for transferred PVDF membranes block for 1 hour at room temperature. IGF1Rβ (1:1000, CST, Shanghai, China) was incubated overnight at 4°C, following secondary antibody (1:10000, Beyotime, Shanghai, China) Enhanced chemiluminescence (ECL, Beyotime, Shanghai, China) was used to quantify the protein expression level.

Dual luciferase reporter gene assay
Plasmids of pGL3-NR2F2-AS1 wild type (WT) or NR2F2-AS1 mutant type were co-transfected with the miR-425-5p/NC or miR-4429/NC mimic into SK-GT-4 cells for 48 hours with the assistance of Lipofectamine 2000 (Beyotime, Shanghai, China). Plasmids of pGL3-IGF1R wild type (WT) or IGF1R mutant type were co-transfected with the miR-425-5p/NC or miR-4429/NC mimic into SK-GT-4 cells as well. Then Dual-luciferase reporter system (Promega, Madison, WI, USA) was used to measure the firefly luciferase activity, which was normalized to renilla luciferase activity.

RNA pull-down

Biotin labeled miR-4429-WT/miR-4429-MUT or miR-425-5p-WT/miR-425-5p-MUT were synthesized by GeneCreate (Wuhan, China) and were transfected into SK-GT-4 cells, which were incubated with lysis buffer (Ambion, Austin, Texas, USA) for 15 minutes. Then, M-280 streptavidin beads (S3762, Sigma-Aldrich, St Louis, MO, USA) precoated with RNase-free bovine serum albumin (BSA) and yeast tRNA (TRNBAK-RO, Sigma-Aldrich, St Louis, MO, USA) were used to incubate with the cell lysates overnight at 4 °C. Trizol was then used to purify the bound RNA and qRT-PCR was used to test the NR2F2-AS1 enrichment.

Statistics

GraphPad Prism 8.2 was used for plotting statistical graphs. R 3.3.1 was used for statistic analysis: WGCNA and Limma package were used for RNA sequencing analysis. t-test was used for two groups comparison; one-way ANOVA for multiple groups comparison and repeated measurement ANOVA for CCK-8 results analysis. P < 0.05 was indicative of statistically significant difference.

Results

1. RNA sequencing to identify the potential involvement of NR2F2-AS1 in regulating IGF1R expression

RNA sequencing was used to identify differential expressed IncRNAs between IGF1R positive and IGF1R negative esophageal adenocarcinoma patients (4 Vs 4): we have plotted heatmap to visualized the top 30 differentially expressed IncRNAs (Figure 1A). Further in vivo experiments showed that NR2F2-AS1 was highly expressed in 201 Her2 positive esophageal squamous cell carcinoma patients rather than that in normal adjacent esophageal tissues (Figure 1B). Moreover, we noticed that NR2F2-AS1 expression was related to advanced T (Figure 1C) and N stages (Figure 1D); and NR2F2-AS1 expressed higher in IGF1R positive patients (Figure 1E).

2. NR2F2-AS1 promotes migration and inhibits apoptosis of EAC in vitro by regulating IGF1R

We found that NR2F2-AS1 expressed higher in OE-33 and SK-GT-4 cell line (Figure 2A), besides IGF1R was aberrantly up regulated in OE-33 and SK-GT-4 cell line both in mRNA and protein level (Figure 2B and 2C). Then we found that NR2F2-AS1 resided in the cytoplasm of OE-33 and SK-GT-4(Figure 2D and 2E). Furthermore. NR2F2-AS1 knock down (Figure 2F) leads to decreased IGF1R expression (Figure 2G and
Further experiments showed that NR2F2-AS1 knock down leads to impaired cell viability of OE-33 and SK-GT-4 cell line (Figure 2I and 2J). Then, transwell assay showed decreased migration ability led by NR2F2-AS1 knock down (Figure 2K). In addition, flow cell cytometry showed that NR2F2-AS1 knock down resulted in increased apoptosis rate (Figure 2L).

3. NR2F2-AS1 knock down sensitizes EAC patients to Trastuzumab

Her-2 was expressed higher in OE-33 cell line, however SK-GT-4 barely expressed Her-2(Figure 3A and 3B); therefore, we have chosen OE-33 to further construct Trastuzumab resistance cell line. The IC50 of trastuzumab resistant OE-33(OE-33-TR) was 143.6 μg/ml and the IC50 of OE33 was 36.3 μg/ml; therefore, the resistance index was 3.95 (Figure 3C).

Furtherly, NR2F2-AS1 and IGF1R aberrantly high expressed in OE-33-TR cell than those in OE-33(Figure 3D to 3F). After knocking down NR2F2-AS1, we found that the IC50 of OE-33-TR to trastuzumab was decreased (141.4 vs 58.1μg/ml) (Figure 3G). Then, we overexpressed IGF1R in NR2F2-AS1 knock down OE-33-TR found that IC50 was increased (Figure 3H).

4. miR-4429 and miR-425-5p expression profile in esophageal cancer patients

Starbase V3.0 was used to predict the potential crosstalk microRNAs for NR2F2-AS1 and IGF1R; miR-4429 and miR-425-5p were identified to be both interacted with NR2F2 and IGF1R (Figure 4A). Further in vivo experiments showed that miR-4429 and miR-425-5p were both suppressed in Her2 positive esophageal adenocarcinoma patients (Figure 4B and 4C). Besides, miR-4429 and miR-425-5p expressed higher in IGF1R positive patients (Figure 4D and 4E). miR-4429 was negatively associated with advanced T stage and N stages (Figure 4F and 4G). However, miR-425-5p seems not to be relevant with T or N stage (Figure 4H and 4I).

5. miR-4429 and miR-425-5p could sensitize esophageal cancer cells to Trastuzumab by targeting IGF1R. miR-4429 and miR-425-5p both lowly expressed in OE-33-TR cell line (Figure 5A and 5B). Therefore, miR-4429 and miR-425-5p overexpression (Figure 5C and 5D) could decrease the IC50 of OE-33-TR (Figure 5E and 5F). In addition, miR-4429 overexpression led to decreased expression of IGF1R by qRT-PCR and western blot (Figure 5G and 5H); same result was detected after miR-425-5p overexpression (Figure 5I and 5J). Furthermore, Dual luciferase reporter gene assay showed that miR-4429 and miR-425-5p could bind to the 3’UTR of IGF1R and suppress its expression (Figure 5K and 5L).

6. NR2F2-AS1 targets miR-4429 and miR-425-5p to regulate IGF1R expression

In OE-33-TR, we have found that NR2F2-AS1 resided mostly in the cytoplasm (Figure 6A). Therefore, we assumed that NR2F2-AS1 might target miR-4429 and miR-425-5p.

miR-4429 and miR-425-5p expression were significantly increased after NR2F2-AS1 knock down in OE-33-TR (Figure 6B and 6C). Dual luciferase reporter gene assay showed that relative luciferase activity of
NR2F2-AS1-WT can be decreased by miR-4429 and miR-425-5p, however, NR2F2-AS1-Mut`s luciferase activity was not significantly influenced (Figure 6D and 6E). Further RNA pull-down assay showed that NR2F2-AS1 was abundantly enriched in miR-4429-WT and miR-425-5p-WT group (Figure 6F and 6G).

Moreover, we found that miR-4429 knock down could reverse the inhibitory effect of NR2F2-AS1 knock down on the IGF1R expression (Figure 6H to 6I). Same results were detected for miR-425-5p knock down (Figure 6J to 6K). In conclusion, NR2F2-AS1 binds to miR-4429 and miR-425-5p to regulate IGF1R expression to exert biological functions.

**Discussion**

Trastuzumab contained regimens have made great progress in improving the prognosis of breast cancer\[10\]; recent clinical trials implies the possibility of applying trastuzumab in esophageal cancer\[2, 5\]. There is a growing consensus on this issue that HER2 overexpression EAC patients are eligible for receiving trastuzumab\[2, 5\]. However, lessons from breast cancer application show that a large proportion of patients would experience acquired trastuzumab resistance even though they are well responsive to the initial therapy\[10\]. IGF1R signaling pathway activation mainly accounts for trastuzumab resistance by interacting with both HER2 and EGFR to suppress the activation of downstream pathways\[13\]. Therefore, we have utilized RNA sequencing to identify that NR2F2-AS1 that was associated with IGF1R.

NR2F2-AS1 has been identified to be oncogenic in various cancers. In this manuscript, we further investigated the role of NR2F2-AS1 in trastuzumab treatment. We found that trastuzumab can significantly induce the expression of NR2F2-AS1, especially exosomal NR2F2-AS1. Further experiments indicate the unneglectable role of NR2F2-AS1 in promoting proliferation of esophageal cancer cell. Then, our result showed that exosomal NR2F2-AS1 induced by trastuzumab played an important role in promoting migration and inhibiting apoptosis of EAC cell lines. Most importantly, exosomal NR2F2-AS1 could suppress the anti-cancer function of trastuzumab treatment. Therefore, we assume that trastuzumab could induce the exosomal NR2F2-AS1 expression and in verse inhibit trastuzumab’s anti-cancer effects.

IGF1R regulated receptor tyrosine kinase signaling, which might play significant role in trastuzumab resistance; and its overexpression is associated with trastuzumab resistance in breast cancer cells\[13\]. With the assistance of online bioinformatics tools, we have noticed that NR2F2-AS1 might sponge miR-4429 and miR-425-5p to regulate IGF1R expression, resulting in impaired trastuzumab effects. Dual luciferase reporter gene assay and RNA pull down further confirmed that NR2F2-AS1 could interact with miR-4429 and miR-425-5p; and miR-4429 and miR-425-5p could bind to the 3`UTR of IGF1R to suppress its expression.

In conclusion, we have identified the key regulator NR2F2-AS1 for IGF1R based on RNA sequencing analysis. Furthermore, exosomal NR2F2-AS1, induced by trastuzumab treatment, could inhibit the anti-cancer effect of trastuzumab by regulating IGF1R through sponging miR-4429 and miR-425-5p.
Declarations

The data that support the findings of this study are available from the corresponding author on request.

Competing interests

The authors declare no conflicts of interest.

Funding information

This work was supported by the Chinese National Science Foundation Projects (NSFC 30740022).

Consents

All patients involved in this study has signed the written informed consent to participate in our study. Our study was approved to be published by Ethics Committee of the First Affiliated Hospital of Xi’an Jiaotong University.

References

1. Maximiano, S. et al. Trastuzumab in the Treatment of Breast Cancer. *BioDrugs*. **30** (2), 75–86 (2016).
2. Gerson, J. N. et al. Perspectives of HER2-targeting in gastric and esophageal cancer. *Expert Opin. Investig. Drugs*. **26** (5), 531–540 (2017).
3. Barsouk, A. et al. Targeted Therapies and Immunotherapies in the Treatment of Esophageal Cancers. Med Sci (Basel), 2019, 7(10)
4. Safran, H. et al. Trastuzumab, paclitaxel, cisplatin, and radiation for adenocarcinoma of the esophagus: a phase I study. *Cancer Invest*. **22** (5), 670–677 (2004).
5. Gravalos, C. et al. Phase II study of trastuzumab and cisplatin as first-line therapy in patients with HER2-positive advanced gastric or gastroesophageal junction cancer. *Clin Transl Oncol*. **13** (3), 179–184 (2011).
6. Ko, B. K. et al. Combination of novel HER2-targeting antibody 1E11 with trastuzumab shows synergistic antitumor activity in HER2-positive gastric cancer. *Mol Oncol*. **9** (2), 398–408 (2015).
7. Shin, S. H. et al. Establishment of a Patient-derived Xenograft for Development of Personalized HER2-targeting Therapy in Gastric Cancer. *Anticancer Res*. **38** (1), 287–293 (2018).
8. Espelin, C. W. et al. Dual HER2 Targeting with Trastuzumab and Liposomal-Encapsulated Doxorubicin (MM-302) Demonstrates Synergistic Antitumor Activity in Breast and Gastric Cancer. *Cancer Res*. **76** (6), 1517–1527 (2016).
9. Tanizaki, J. et al. Synergistic antitumor effect of S-1 and HER2-targeting agents in gastric cancer with HER2 amplification. *Mol Cancer Ther*. **9** (5), 1198–1207 (2010).
10. Kim, C. et al. PTEN loss and level of HER2 amplification is associated with trastuzumab resistance and prognosis in HER2-positive gastric cancer. *Oncotarget*. **8** (69), 113494–113501 (2017).
11. Rimawi, M. F. et al. Low PTEN levels and PIK3CA mutations predict resistance to neoadjuvant lapatinib and trastuzumab without chemotherapy in patients with HER2 over-expressing breast cancer. *Breast Cancer Res Treat.* **167** (3), 731–740 (2018).

12. Hubalek, M. et al. Resistance to HER2-targeted therapy: mechanisms of trastuzumab resistance and possible strategies to overcome unresponsiveness to treatment. *Wien Med Wochenschr,* 2010, 160(19–20): 506 – 12

13. Chakraborty, A. K. et al. In vitro and in vivo studies of the combination of IGF1R inhibitor figitumumab (CP-751,871) with HER2 inhibitors trastuzumab and neratinib. *Breast Cancer Res Treat.* **152** (3), 533–544 (2015).

14. Nagini, S. Breast Cancer: Current Molecular Therapeutic Targets and New Players. *Anticancer Agents Med Chem.* **17** (2), 152–163 (2017).

15. Shi, S. J. et al. LncRNA-ATB promotes trastuzumab resistance and invasion-metastasis cascade in breast cancer. *Oncotarget.* **6** (13), 11652–11663 (2015).

16. Chen, L. et al. LncRNA NR2F2-AS1 Upregulates Rac1 to Increase Cancer Stemness in Clear Cell Renal Cell Carcinoma. *Cancer Biother Radiopharm.* **35** (4), 301–306 (2020).

17. Liu, J. et al. LncRNA NR2F2-AS1 Silencing Induces Cell Cycle Arrest in G0/G1 Phase via Downregulating Cyclin D1 in Colorectal Cancer. *Cancer Manag Res.* **12**, 1835–1843 (2020).

18. Qin, H. & Qin, C. Downregulation of long non-coding RNA NR2F2-AS1 inhibits proliferation and induces apoptosis of nasopharyngeal carcinoma cells by upregulating the expression of PTEN. *Oncol Lett.* **19** (2), 1145–1150 (2020).

19. Zhang, S. et al. LncRNA NR2F2-AS1 promotes tumourigenesis through modulating BMI1 expression by targeting miR-320b in non-small cell lung cancer. *J Cell Mol Med.* **23** (3), 2001–2011 (2019).

20. Liu, D. et al. NR2F2-AS1 accelerates cell proliferation through regulating miR-4429/MBD1 axis in cervical cancer. *Biosci Rep,* 2020, 40(6)

21. Cristobal, I. et al. Potential therapeutic value of miR-425-5p in metastatic colorectal cancer. *J Cell Mol Med.* **20** (11), 2213–2214 (2016).

22. Fu, Y. et al. Overexpression of miR-425-5p is associated with poor prognosis and tumor progression in non-small cell lung cancer. *Cancer Biomark.* **27** (2), 147–156 (2020).

23. He, H. et al. MiR-4429 prevented gastric cancer progression through targeting METTL3 to inhibit m(6)A-caused stabilization of SEC62. *Biochem Biophys Res Commun.* **517** (4), 581–587 (2019).

24. Liang, L. et al. miR-4429 Regulates the Proliferation, Migration, Invasion, and Epithelial-Mesenchymal Transition of Cervical Cancer by Targeting FOXM1. *Cancer Manag Res.* **12**, 5301–5312 (2020).

25. Liu, D. et al. Hsa-miR-425-5p promotes tumor growth and metastasis by activating the CTNND1-mediated beta-catenin pathway and EMT in colorectal cancer. *Cell Cycle.* **19** (15), 1917–1927 (2020).

26. Liu, S. et al. MiR-425-5p suppresses tumorigenesis and DDP resistance in human-prostate cancer by targeting GSK3beta and inactivating the Wnt/beta-catenin signaling pathway. *J Biosci,* 2019, 44(4)
**Figures**

A. Heatmap for LncRNA sequencing between IGF1R positive and IGF1R negative patients.

B. NR2F2-AS1 expression in esophageal cancer patients.

C. NR2F2-AS1 expression in esophageal cancer patients stratified by T stage.

D. NR2F2-AS1 expression in esophageal cancer patients stratified by N stage.

E. NR2F2-AS1 expression in IGF1R positive and IGF1R negative esophageal cancer patients.

**Figure 1**

A Heatmap for LncRNA sequencing between IGF1R positive and IGF1R negative patients. B NR2F2-AS1 expression in esophageal cancer patients. C NR2F2-AS1 expression in esophageal cancer patients stratified by T stage. D NR2F2-AS1 expression in esophageal cancer patients stratified by N stage. E NR2F2-AS1 expression in IGF1R positive and IGF1R negative esophageal cancer patients.
Figure 2

A NR2F2-AS1 expression in esophageal cancer cell line. B IGF1R expression in esophageal cancer cell line by qRT-PCR. C IGF1R expression in esophageal cancer cell line by western blot. D NR2F2-AS1 resides mostly in cytoplasm of OE-33. E NR2F2-AS1 resides mostly in cytoplasm of SK-GT-4. F NR2F2-AS1 expression in OE-33 and SK-GT-4 after NR2F2-AS1 knocking down. G IGF1R expression in OE-33 and SK-GT-4 after NR2F2-AS1 knocking down by qRT-PCR. H GF1R expression in OE-33 and SK-GT-4 after NR2F2-
AS1 knocking down by western blot. I CCK-8 results for NR2F2-AS1 knocked down OE-33 cells. J CCK-8 results for NR2F2-AS1 knocked down SK-GT-4 cells. K Transwell results for NR2F2-AS1 knocked down OE-33 and SK-GT-4 cells. L Apoptosis results for NR2F2-AS1 knocked down OE-33 and SK-GT-4 cells.

Figure 3

A Her-2 expression in OE-33 and SK-GT-4 by western blot. B Her-2 expression in OE-33 and SK-GT-4 by qRT-PCR. C CCK8 to test IC50 for OE-33 and OE-33-TR. D NR2F2-AS1 expression in OE-33-TR. E IGF1R expression in OE-33-TR by qRT-PCR. F IGF1R expression in OE-33-TR by western blot. G CCK8 to test IC50
Figure 4

Predicted binding site for miR-4429(Up) and miR-425-5p(Down) to NR2F2-AS1. B miR-4429 expression in esophageal cancer patients. C miR-425-5p expression in esophageal cancer patients. D miR-4429 expression in IGF1R positive and IGF1R negative esophageal cancer patients. E miR-425-5p expression in IGF1R positive and IGF1R negative esophageal cancer patients.
IGF1R positive and IGF1R negative esophageal cancer patients. F miR-4429 expression in esophageal cancer patients stratified by T stage. G miR-4429 expression in esophageal cancer patients stratified by N stage. H miR-425-5p expression in esophageal cancer patients stratified by T stage. I miR-425-5p expression in esophageal cancer patients stratified by N stage.

Figure 5
A miR-4429 expression in OE-33 and OE-33-TR. B miR-425-5p expression in OE-33 and OE-33-TR. C miR-4429 expression in miR-4429 overexpressed OE-33-TR. D miR-425-5p expression in miR-425-5p overexpressed OE-33-TR. E CCK8 to test IC50 of miR-4429 overexpressed OE-33-TR. F CCK8 to test IC50 of miR-425-5p overexpressed OE-33-TR. G IGF1R expression in miR-4429 overexpressed OE-33-TR by qRT-PCR. H IGF1R expression in miR-4429 overexpressed OE-33-TR by western blot. I IGF1R expression in miR-425-5p overexpressed OE-33-TR by qRT-PCR. J IGF1R expression in miR-425-5p overexpressed OE-33-TR by western blot. K Dual luciferase to evaluate the crosstalk between IGF1R and miR-4429. L Dual luciferase to evaluate the crosstalk between IGF1R and miR-425-5p.
Figure 6

A NR2F2-AS1 resides mostly in cytoplasm of OE-33-TR. B miR-4429 expression in NR2F2-AS1 knocked down OE-33-TR. C miR-425-5p expression in NR2F2-AS1 knocked down OE-33-TR. D Dual luciferase to evaluate the crosstalk between NR2F2-AS1 and miR-425-5p. E Dual luciferase to evaluate the crosstalk between NR2F2-AS1 and miR-4429. F RNA pull down for miR-425-5p and NR2F2-AS1 in OE-33-TR. G RNA pull down for miR-4429 and NR2F2-AS1 in OE-33-TR. H IGF1R expression in miR-4429 and NR2F2-AS1

**Page 16/17**
knock down OE-33-TR by qRT-PCR. I IGF1R expression in miR-4429 and NR2F2-AS1 knock down OE-33-TR by western blot. J IGF1R expression in miR-425-5p and NR2F2-AS1 knock down OE-33-TR by qRT-PCR. K IGF1R expression in miR-425-5p and NR2F2-AS1 knock down OE-33-TR by western blot.