Circ0120816 Acts As An Oncogene of Esophageal Squamous Cell Carcinoma via Inhibiting miR-1305 and Releasing TXNRD1

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Primary research

Keywords: circ0120816, miR-1305, TXNRD1, esophageal squamous cell carcinoma

DOI: https://doi.org/10.21203/rs.3.rs-47889/v1

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Abstract

Background: The morbidity and mortality of esophageal squamous cell carcinoma (ESCC) remains stubbornly high, in spite of emerging new diagnoses methods. The role of circular RNAs (circRNAs) in ESCC progression is still in need of more exploration.

Methods: In this research, we gathered 36 patients’ ESCC tissues to analyze the expression of circ0120816, miR-1305 and TXNRD1. KYSE450 and KYSE510 cells were used to conduct the transfection. Aiming to verify our hypothesis, qRT-PCR, RNase R treatment, nuclear extraction, luciferase reporter assay, RNA immunoprecipitation assay, CCK-8, BrdU, cell adhesion, caspase 3 activity assay were used.

Results: Circ0120816, upregulated in ESCC, acted as a sponge for miR-1305. Circ0120816 combined miR-1305 to enhance cell viability, proliferation adhesion and repress cell apoptosis in ESCC cell lines. On the contrary, miR-1305 exerted reversed effect in ESCC cells through decreasing TXNRD1.

Conclusions: This research demonstrated that circ0120816 promoted ESCC development by competitive binding miR-1305 which could increase the expression of TXNRD1.

Background

Esophageal cancer which was the most common malignant tumor all over the world leaded to 508,585 deaths in 2018 [1]. Esophageal squamous cell carcinoma (ESCC) was one of the histological types with high incidence of esophageal cancer in China [2, 3]. Compared to other cancers, ESCC had poor survival time in spite of the improving methods for ESCC in the recent 10 years [4, 5]. Due to the lack of early diagnosis as well as its diffuse and invasive nature, patients with ESCC had poor response to surgical operation or chemoradiotherapy [6, 7]. Consequently, there remained an instant need for effective molecular target approaches for ESCC therapy [4, 8].

Circular RNA (circRNA) was a newly non-coding RNA with a circular structure which was stably existed in cytoplasm [9–12]. Furthermore, circRNA was formed through back splice covalently joined 3′- and 5′- which was not to encode a protein [13–15]. Recently, researches have confirmed that circRNA regulated several cancer progressions containing oral carcinoma, ovarian carcinoma, pancreatic carcinoma, hepatocellular carcinoma and bladder carcinoma [16–22]. Moreover, circRNA affected the development of carcinoma acting as a sponge for microRNA (miRNA) [23–25]. For instance, circ0000515 acted as a ceRNA of miR-326, increasing the expression of ELK1 and promoting ovarian carcinoma [26]. It is worth noting that at present, there are few studies on how circRNA can participate in the occurrence and development of ESCC by regulating miRNA to further regulate genes related to encoding proteins, let alone the specific regulatory function of circ0120816 in ESCC via interacting miRNA.

In the past 10 years, miRNA had been demonstrated to take an essential part in tumorigenesis [27–30]. Take miR-1305 for example, it was reported to have lower expression in various cancers including lung
carcinoma, breast cancer and hepatocellular carcinoma, exhibiting a repressive role in cancer development [31–33]. On the contrary, there was no report confirming that miR-1305 was involved in ESCC acting as an upstream regulator of TXNRD1. Also, the interaction between circ0120816 and miR-1305 remains unclear.

TXNRD1 (thioredoxin reductase 1) encodes a protein that belongs to the pyridine nucleotide-disulfide oxidoreductase family. This protein is a member of the thioredoxin (Trx) system, playing a key role in redox homeostasis. [34, 35]. TXNRD1 was widely reported to participate in the positive regulation of hepatocellular carcinoma [36–38]. In addition, TXNRD1 was observed to contribute to development of lung cancer [39] and breast cancer [40, 41]. However, study explaining the affection of TXNRD1 in ESCC in detail is rare.

Here, we tried to explore the molecular mechanism underlying ESCC pathogenesis associated to the function and interaction of circ0120816, miR-1305 and TXNRD1 in ESCC cell lines. Our results might serve as new perspective for prognosis and therapeutic strategies for ESCC patients.

Materials And Methods

Bioinformatics analysis

GSE131969 was downloaded from GEO DataSets for screening the upregulated circRNAs using limma 3.26.8 with adj.P value <0.05 and log fold change (logFC) >1.5. GSE33810 downloaded from GEO DataSets was used to screen the upregulated differentially expressed genes (DEGs) with P value <0.05 and logFC >1.5, and GSE20347 also downloaded from GEO DataSets was used to screen the upregulated DEGs with adj.P value <0.05 and logFC >1.5. STRING was performed to analysis the key biological processes for DEGs. TargetScan and circInteractome were applied to predict the miRNAs binding to TXNRD1 and circ0120816, respectively.

Patients collection

36 patients from Wuhan Asia Heart Hospital Affiliated to Wuhan University of Science and Technology have been diagnosed with ESCC. ESCC tissues and corresponding healthy adjacent tissues were used to demonstrate our assumption. The collection and usage of tissue samples according to the ethical standards set out in the Helsinki Declaration. The clinical characteristics of 36 patients were list in Table 1.

RNA extraction, reverse transcription and real-time quantification PCR

Total RNA was dissociated by TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa, Japan), and RNA reverse transcription was performed by PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). The 7500 fast instrument (ABI, USA) was used to measure the expression of circ0139153, circ0096710, circ0095414, circ0085539, circ0120816, miR-1305 and TXNRD1 in ESCC tissues or ESCC cells using SYBR Green PCR kit (Takara, Japan). GAPDH was the reference gene for circRNA and mRNA, while U6
was the reference miRNA for miRNA. All the primers used in this study (Table 2) were designed and synthesized from Tiangen Biochemical Technology (Beijing, China).

**Cell culture**

The human ESCC cell lines (KYSE30, KYSE180, KYSE450 and KYSE510) and the normal esophageal epithelial cell line Het-1A were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Germany). ESCC cell lines were cultured in RPMI 1640 which had been supplemented with 10% FBS and 1% streptomycin/penicillin in a humified incubator with 5% CO₂ at 37 °C.

**Cell transfection**

Si-circ0120816, miR-1305 mimic, miR-1305 inhibitor, si-TXNRD1 and their negative control (NC) were designed by GeneCopoeia (Guangzhou, China). Lipofectamine 2000 reagent (Thermo Fisher Scientific, USA) was used to conduct cell transfection. In brief, cell transfection was carried out at the point that KYSE450 or KYSE510 cells grew into a density of 70%-80%. As described in the protocol, 50nM Si-circ0120816, miR-1305 mimic, miR-1305 inhibitor, si-TXNRD1 or NC was transfected KYSE450 and KYSE510 cells. The transfection efficiency was analyzed by qRT-PCR after 48-h transfection.

**RNase R treatment**

Total RNA was inactivated by RNase inhibitor (Beijing Tiangen Biochemical Technology, China) at 37 °C for 15 min. Next, equal RNA was used to reverse transcription into cDNA and to analyze the expression of circ0120816 by qRT-PCR which assessed the stability of circ0120816 and its linear isoform.

**Nuclear extraction**

This performance was carried out by using Nuclear Extraction Kit (Millipore, USA). In brief, 1×10⁷ cells were added warmed trypsin cell detachment buffer for approximately 2 min. Ice cold 1 × Cytoplasmic Lysis Buffer containing 0.5 mM DTT and 1/1000 dilution was added to the sample for 15-min incubation on ice. For nuclear extraction, the nuclear pellet was resuspended in cell pellet volume in ice cold Nuclear Extraction Buffer. A rotator was used to gently agitate the nuclear suspension at 4 °C for 60 min. The nuclear suspension centrifuged at 16,000 × g for 5 min at 4 °C. The supernatant was the nuclear extract. The cytoplasmic fraction or nuclear lysate was used in subsequent RNA extraction.

**Luciferase reporter assay**

The wild and mutant circ0120816 sequences or wild and mutant TXNRD1 3'UTR sequences synthesized from GeneCopoeia (Guangzhou, China) were subcloned into pmiR-GLO reporter vector (circ-WT, TXNRD1-WT or circ-Mut, TXNRD1-Mut). KYSE450 or KYSE510 cells were seeded in a 96-well plate and then co-transfected with 100 ng of circ-WT or circ-Mut (TXNRD1-WT or TXNRD1-Mut), and 50 nM of miR-1305 mimic or mimic NC. After 48-h co-transfection, Dual-GLO® Luciferase Assay System Kit (Promega, USA) and Fluorescence/Multi-Detection Microplate Reader (BioTek, USA) were used to detect luciferase activity.
RNA immunoprecipitation assay (RIP assay)

RNA immunoprecipitation assay was used to identify the relationship between circ0120816 and miR-1305 by Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) in accordance with the protocols of manufacture. KYSE450 or KYSE510 cells after transfection of miR-1305 were lysed in RIP lysis buffer containing magnetic beads conjugate with human anti-Argonaute2 (Ago2) antibody or negative control IgG. Then, the cell lysates were incubated with Proteinase K and the immunoprecipitation RNA was isolated. Circ0120816 expression was detected by qRT-PCR.

CCK-8 assay

Cell Counting Kit-8 (Vazyme, China) was used to detect cell viability. Briefly, KYSE450 or KYSE510 cells were seeded in a 96-well plate at a count of 1×10^5 cells/well. At different time points, 10 μL of CCK-8 solution was added to each well of the plate using a repeating pipettor. Then, the plate was incubated in dark for 1 hour. The absorbance at 450 nm was measured using a microplate reader (BioTek, USA).

BrdU assay

The proliferation of ESCC cells was analyzed using 5-bromo-2'-deoxyuridine (BrdU) Kit (YEASEN, China). Cell culture was carried out on 96-well plates with cell density at 1×10^5 cells/mL according to standard procedures. According to the dilution at a ratio of 1:30, 1 mM BrdU working liquid was prepared and then added into cells (100 μL for each well) for 2-h incubation. Finally, the absorbance at 450 nm was measured in a microplate reader (BioTek, USA).

Cell adhesion assay

KYSE450 or KYSE510 cells were seeded in a 24-well plate. After 48-h transfection, cells were suspended using serum-free culture medium and added into the 96-well plate precoated with collagen I solution at 5×10^4 cells per well for 1-h incubation at 37 °C. Then, PBS was added to wash away the cells that were not attached. Subsequently, 100 μL 10% ethanol was added to each well for 5-min incubation at 25 °C. The absorbance was determined using a microplate reader (BioTek, USA) at 570 nm.

Caspase 3 activity assay

KYSE450 and KYSE510 cells were seeded in a 96-well plate at 5×10^4 concentration. Caspase-3 Activity Assay Kit (Cell Signaling, USA) was applied to carry out the detection. In brief, 30 μL cell lysis buffer was added to each well and the culture plate was placed on the ice for 5 min. Then, the lysates were treated by ultrasound on ice and separated by microcentrifugation at 4 °C for 10 min, and the supernatant was transferred to a new tube. 200 μL substrate solution B and 25 μL lysis solution were mixed together on a black culture plate suitable for fluorescence detection. The plate was incubated in the dark. Finally, we read the RFU on the fluorescent plate reader using an excitation wavelength of 380 nm and an emission wavelength of 420 nm.
RNA pull-down

MiR-1305 mimic-biotin and its negative control (Bio-NC) were synthesized from RiboBio (Guangzhou, China). RNA pulldown Kit (BersinBio, China) was used to perform RNA pull-down. Briefly, cell lysis buffer was blended with probe-bead complex. The tubes were put on the magnetic stand for the collection of the beads, and then protein K and DNase A were used to remove protein and DNA, respectively. The RNA separated from RNA-bead complex was reversed transcript to cDNA. Finally, TXNRD1 mRNA expression level was measured by qRT-PCR.

Statistical analyses

We analyzed our results by SPSS 23.0 (SPSS, USA). All results had three experiments independently. The data were shown as mean ± SD. Student’s t-test was used for analyzing differences between two groups, while ANOVA was used for analyzing differences in multiple groups. P <0.05 were regarded as statistically significant.

Results

Circ0120816/miR-1305/TXNRD1 axis was identified as the key regulator in ESCC

To confirm the key circRNA participating in ESCC progression, GSE131969 downloaded from GEO DateSets was used. The top 5 upregulated circRNAs were list in Fig. 1A. Then, we detected the expression of top 5 upregulated circRNAs in our 36 paired clinical ESCC tissue samples. The results showed that circ0120816 with the highest expression in tumor samples was observed compared with other circRNAs (Fig. 1B-F). Therefore, circ0120816 was identified as our interested circRNA to be explored in ESCC, and the structure of circ0120816 was shown in Fig. 1G. Next, GSE33810 and GSE20347 also downloaded from GEO DateSets was used to screen the key genes. By Venny.2.1.0 analysis, 72 upregulated DEGs were overlapped from GSE33810 and GSE20347 (Fig. 1H). Uploading the 72 common genes to STRING, TXNRD1 participating in cell population proliferation attracted our attention (Fig. 1I). For confirming the key miRNA, TargetScan and circInteractome were used to predict the miRNAs binding to TXNRD1 and circ0120816, respectively. Finally, eight miRNAs were overlapped from TargetScan and circInteractome (Fig. 1J). After literature review, miR-1305 was identified as our interested miRNA due to its tumor suppressor effect on multiple cancers [31, 33, 42, 43]

Circ0120816 was upregulated in ESCC cells.

To observe the difference of circ0120816 expression between ESCC cells (including KYSE30, KYSE180, KYSE450, KYSE510) and normal human esophageal epithelial cell line (Het-1A) (Fig. 2A). What’s more, circ0120816 expression in KYSE450 and KYSE510 was obviously higher in KYSE450 and KYSE510 than other ESCC cells, thus we chose KYSE450 and KYSE510 cells for further study. Next, RNase R degradation assay was
used to determine the stability of circ0120816 in ESCC cells while the result showed that circ0120816 having the circular transcription was resisted to RNase R treatment while linear 0120816 was dramatically degraded by around 70% (Fig. 2B). In order to ascertain the specific role of circ0120816 in ESCC cells, sub-localization of circ0120816 in KYSE450 and KYSE510 cells was performed. Furthermore, qRT-PCR was used to confirm the location of circ0120816 in ESCC cells with the result of circ0120816 was most existed in the cytoplasm of ESCC cells (Fig. 2C). Specifically, circ0120816 expression in cytoplasm was 5-fold as high as in nuclear and linear0120816 expression in cytoplasm was almost 1.5-fold as high as in nuclear in KYSE450 cells, which exhibited a similar trend in KYSE510 cells (Fig. 2C).

**MiR-1305 was the downstream gene of circ0120816.**

It is well established that circRNA exerts its function by sponging miRNA. Thus, to better understand the molecular mechanism of circ0120816 in ESCC progression, we tried to explain this from the point of its interaction with miRNA. CircInteractome was used to predict the binding site of circ0120816 with miR-1305 with the sequences showed in Fig. 3A. Next, we carried out Luciferase reporter assay to observe the relationship between circ0120816 and miR-1305. The luciferase activity was significantly decreased by about 60% in circ-WT and miR-1305 co-transfection while there was no difference between mutant co-transfection groups (Fig. 3B). Furthermore, RIP assay found that circ0120816 significantly enriched in miR-1305 mimic which could be pulled down by anti-Ago2 antibody (Fig. 3C). From Fig. 3D, miR-1305 had a lower expression in tumor tissues. In addition, miR-1305 had a negative correlation with circ0120816 in ESCC tissues (Fig. 3E). These results collectively confirmed that circ0120816 was a sponge for miR-1305 in ESCC, which might play a critical role in ESCC development.

**Circ0120816 facilitated ESCC progression via sponging miR-1305.**

How circ0120816 played in ESCC progression via sponging miR-1305 was determined by the following experiments. We set five groups named blank control, negative control, si-circ0120816, miR-1305 inhibitor and si-circ0120816 + miR-1305 inhibitor by transfecting them into ESCC cells. To determine the effect of transfection, we used qRT-PCR to measure the expression of circ0120816 and miR-1305. The result showed that the circ0120816 expression was decreased in si-circ0120816 group by 70% compared to blank control group, but the circ0120816 expression was not regulated by miR-1305 inhibitor (Fig. 4A). In addition, the expression of miR-1305 was reduced in miR-1305 inhibitor group by about 70% and increased by more than 1.5-fold in si-circ0120816 group compared to blank control group, as well as the inhibitory effect of miR-1305 inhibitor on miR-1305 expression could be relieved by co-transfection of si-circ0120816 and miR-1305 inhibitor (Fig. 4A). Cell viability analysis by CCK-8 assay showed that si-circ0120816 inhibited the cell viability while miR-1305 inhibitor restored the role of si-circ0120816 in ESCC cells (Fig. 4B). However, the cell viability in the ESCC cells with co-transfection of si-circ0120816 and miR-1305 inhibitor did not show any significant differences compared with control group (Fig. 4B). Equally, in BrdU assay we observed that si-circ0120816 could decrease cell proliferation by 40% and miR-1305 inhibitor could increase cell proliferation by about 40%, while these variables could be compromised by si-circ0120816 + miR-1305 inhibitor treatment in both KYSE450 and KYSE510 cells (Fig. 4C). We also
performed cell adhesion assay which showed that si-circ0120816 could repress cell adhesion by about 40% and miR-1305 inhibitor facilitated cell adhesion by about 30% compared to blank control in KYSE450 cells, which showed a similar trend in KYSE510 cells (Fig. 4D). Furthermore, the change caused by si-circ0120816 or miR-1305 inhibitor was reversed by si-circ0120816 + miR-1305 inhibitor treatment (Fig. 4D). Next, we designed caspase 3 activity assay to further explore the effect of circ0120816 on cell apoptosis of ESCC cells. The result confirmed that si-circ0120816 facilitated cell apoptosis by about 5-fold and miR-1305 inhibitor restrained cell apoptosis by about 60%, while these variables could be compromised by si-circ0120816 + miR-1305 inhibitor treatment in both KYSE450 and KYSE510 cells (Fig. 4E). Finally, we reached a conclusion that circ0120816 strengthened the viability, proliferation and adhesion while lightened the apoptosis of ESCC cells in vitro via miR-1305.

**TXNRD1 was a target gene of miR-1305.**

To find the downstream target gene of miR-1305, we used TargetScan to predict the possible gene named TXNRD1 and its 3’UTR sequences paired with miR-1305 (Fig. 5A). Luciferase reporter assay demonstrated that miR-1305 obviously decreased the activity in TXNRD1-WT by around 60% while there was no effect in TXNRD1-Mut (Fig. 5B). Besides, RNA pull-down was also conducted to verify the relationship between miR-1305 and TXNRD1. We observed that TXNRD1 expression in Bio-miR-1305 was 6 times as high as Bio-NC in ESCC cells (Fig. 5C). qRT-PCR was used to measure the expression of TXNRD1 in ESCC tissues. The result determined that TXNRD1 was dramatically upregulated in tumor tissues compared to healthy adjacent tissues (Fig. 5D). What’s more, TXNRD1 expression had a negative association with miR-1305 expression (Fig. 5E). Totally, TXNRD1 was a target gene of miR-1305 in ESCC.

**MiR-1305 suppressed ESCC progression by targeting TXNRD1.**

To explore whether miR-1305 intensified the viability, proliferation and adhesion while inhibited the apoptosis of ESCC cells through TXNRD1, we performed a rescue experiment. qRT-PCR was used to observe the expression of TXNRD1 after transfecting si-TXNRD1, miR-1305 inhibitor or si-TXNRD1 + miR-1305 inhibitor in KYSE450 and KYSE510 cells. We observed about 60% down-regulation of TXNRD1 expression in si-TXNRD1 group while TXNRD1 expression level was twice as high as blank control group in miR-1305 inhibitor group (Fig. 6A). What’s more, TXNRD1 expression level was reversed by si-TXNRD1 + miR-1305 inhibitor (Fig. 6A). CCK-8 assay confirmed that si-TXNRD1 could inhibit cell viability, opposite to the positive effect of miR-1305 inhibitor (Fig. 6B). At the same time, co-transfection of si-TXNRD1 and miR-1305 inhibitor could attenuate the positive effect of miR-1305 inhibitor (Fig. 6B). BrdU assay demonstrated that si-TXNRD1 blocked cell proliferation by 40% and miR-1305 inhibitor enhanced it by about 35% compared to blank control group in KYSE450 cells (Fig. 6C). We also observed a similar trend in KYSE510 cells (Fig. 6C). What’s more, the changes all could be restored by si-TXNRD1 + miR-1305 inhibitor (Fig. 6C). Next, we designed cell adhesion assay to further explore the effect of TXNRD1 on ESCC cells. The result confirmed that si-TXNRD1 repressed cell adhesion by 40% and miR-1305 inhibitor enhanced it by 40% compared to blank control group in ESCC cells, and these changes were all compromised by si-TXNRD1 + miR-1305 inhibitor (Fig. 6D). Caspase 3 activity assay demonstrated si-
TXNRD1 accelerated cell apoptosis to a 7-fold level as blank control group and miR-1305 inhibitor repressed it by 50% in ESCC cells, and these effects were restored by si-TXNRD1 + miR-1305 inhibitor treatment (Fig. 6E). Together, we confirmed that miR-1305 promoted the viability, proliferation and adhesion while inhibited the apoptosis of ESCC cells in vitro by suppressing TXNRD1.

**Discussion**

Although the treatment of ESCC has been improved, ESCC still has poor prognosis due to the lack of early diagnosis as well as its diffuse and invasive nature. In our study, we confirmed the existence of circ0120816 upregulated in ESCC, and explored its specific effect on ESCC development. Our results determined that circ0120816 facilitated ESCC progression via miR-1305/ TXNRD1 axis.

Recently, Emerging circRNAs were discovered to play an irreplaceable role in the progression of ESCC [44–47]. A reporter covered that circ-TTC17 was apparently upregulated in ESCC cells, which enhanced cell proliferation, migration and invasion [46]. On the contrary, in Zhang Y’s study, they discovered decreased circ-SMAD7 in ESCC patients’ plasma could block tumor proliferation and migration [47]. Our experiment identified a novel circRNA in the regulation of ESCC progression, circ0120816, which acted as a tumor promoter, strengthening cell viability, proliferation and adhesion, and arresting apoptosis. What’s more, it is well accepted that a bunch of circRNAs mediate ly influenced protein-coding gene expression via competitive sponging for miRNAs. Take the study of Rui-chao Li (2018) for example, it demonstrated that ciRS-7 overexpression abolished the tumor-suppressive effects of miR-7 in the facilitation of ESCC malignant progression [45]. Consistent with this biological mechanism, circ0120816 was observed here to accelerate ESCC progression by sponging miR-1305. Together, circ0120816, a sponge for miR-1305, was capable of facilitating cell viability, proliferation and adhesion, while suppressing cell apoptosis in the regulation of ESCC pathogenesis.

MiR-1305 was found by Ng TK et al. in 2015 for the first time and was obviously overexpressed in human periodontal ligament-derived stem cells of people who usually smoked, which exerted deteriorative effects on stem cells [48]. Soon afterwards, it was reported decreased miR-1305 not only accelerated the metastasis but also aggravated the poor prognosis of NSCLC patients by inhibiting the expression of MDM2 [31]. There was a report illustrating reduction of miR-1305 in triple negative breast cancer could enhance the expression of RUNX2 and gave rise to the cancer aggressiveness [32]. What’s more, miR-1305 was found to be a destruction for the activation of AKT signaling pathway through competitively binding UBE2T in order to suppress the tumorigenicity of hepatocellular carcinoma cells [33]. All these results above consistently revealed that miR-1305 was able to block carcinogenesis. In our study, we also determined the repressive role of miR-1305 in ESCC progression by suppressing cell viability, proliferation and adhesion and facilitating cell apoptosis. What’s more, we were the first to determine TXNRD1 as a downstream target gene of miR-1305 in the suppression of ESCC. Generally speaking, miR-1305 acted as an inhibitory regulator in ESCC cells, decreasing the expression of TXNRD1.
TXNRD1 is a key enzyme implicated in detoxification of reactive oxygen species (ROS) and redox signaling [49]. Interestingly, ROS has been discovered in various cancers, leading to activation of tumor signal as well as facilitation of cell proliferation [50]. The corresponding reaction is that cancer cells will rise levels of antioxidant proteins (such as TXNRD1), which can detoxify the enhancive ROS to maintain redox balance and anti-apoptosis [50]. Indeed, as described in some hepatocellular carcinoma cases, TXNRD1 was elevated to control ROS level and maintain the tumorigenesis [51, 52]. These previous studies suggested that reduced TXNRD1 strengthened the anticancer treatment, which showed a consistent trend in our research. Specifically, TXNRD1 here exhibited a higher expression in ESCC tissues and exerted a promotional effect on ESCC formation. Furthermore, we presented solid evidence that the post-transcriptional regulation of TXNRD1 was partly controlled by circ0120816 in ESCC progression through competing for miR-1305 in cytoplasm.

However, there are several deficiencies in our experiment. We didn’t design experiment in depth to verify the change of ROS in ESCC cells. Furthermore, we did not use TXNRD1-inhibitor to observe how ROS changed in ESCC. What’s more, we have not designed experiment to measure the distant metastasis in mice.

**Conclusions**

In summary, our data shed light on comprehensive mechanism that circ0120816 acts as a novel prognostic biomarker and therapeutic target in ESCC. Circ0120816 could impair antitumor effect of miR-1305 on ESCC cells. Moreover, miR-1305 was able to target and reduce TXNRD1 expression in ESCC. Strikingly, the circ0120816/miR-1305/TXBRD1 axis was activated in ESCC and provided a promising therapeutic target in ESCC treatment.

**List Of Abbreviations**

ESCC: esophageal squamous cell carcinoma; circRNAs: circular RNAs; microRNA miRNA; TXNRD1: thioredoxin reductase 1; Trx: thioredoxin; RIP assay: RNA immunoprecipitation assay; Ago2: anti-Argonaute2; ROS: reactive oxygen species.

**Declarations**

**Ethics approval and consent to participate**

The study was approved by Wuhan Asia Heart Hospital Affiliated to Wuhan University of Science and Technology. The collection and usage of tissue samples in the study is according to the ethical standards set out in the Helsinki Declaration. All patients signed written informed consent.

**Consent for publication**

Not applicable.
Availability of data and materials

The data used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interests.

Funding

This study was supported by the Hubei Province Health and Family Planning Scientific Research Project (Grant Number WJ2019H238), the Health and Family Planning Commission of Wuhan Municipality Scientific Research Project (Grant Number WX18Q33 and WX17B19), and Wuhan Young & Middle-aged Medical Backbone Training Program.

Authors’ contributions

XYL performed the experiments and data analysis. LCS formed the methodology and conducted research. BW analyzed and interpreted the data and collected experimental materials. CT did data visualization and literature analysis. LS wrote the paper and analyzed literature. MX conceived and designed the study and collected the funding. All authors read and approved the manuscript.

Acknowledgments

Not applicable.

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Table 1
The clinical characteristics of the 36 patients with esophageal squamous cell carcinoma.

| Characteristics              | N (%)   |
|------------------------------|---------|
| Age (years)                  |         |
| ≤ 60                         | 17 (47.2%) |
| > 60                         | 19 (52.8%) |
| Gender                       |         |
| Female                       | 16 (44.4%) |
| Male                         | 20 (55.6%) |
| Tumor location               |         |
| Upper esophagus              | 8 (22.2%) |
| Middle esophagus             | 17 (47.2%) |
| Lower esophagus              | 11 (30.6%) |
| Pathological T stage         |         |
| T1                           | 10 (27.8%) |
| T2                           | 16 (44.4%) |
| T3                           | 10 (27.8%) |
| Lymph node metastasis        |         |
| No                           | 21 (58.3%) |
| Yes                          | 15 (41.7%) |
| Tumor differentiation        |         |
| Well                         | 7 (19.4%) |
| Moderately                   | 20 (55.6%) |
| Poorly                       | 9 (25.0%) |
| Name           | Primer sequences (5’-3’)               |
|----------------|----------------------------------------|
| Circ_0120816   | AGCCAGAGTCTGTCGTGAAC                   |
|               | TCCCACACCAGCAGAATCAT                   |
| Circ_0139153   | TGGGATTTTGCCTTTTGGTA                   |
|               | CAGTGAATGGAATGCACCAG                   |
| Circ_0096710   | AGCAACATTGCGGTTTCATC                   |
|               | GTTCGGCACATGGGTAAAAG                   |
| Circ_0095414   | TTATGATCACCAGGGAGGA                    |
|               | CTCCATTTCACCCTCCAGAA                   |
| Circ_0085539   | TAGATCCTGCCCTGTGGTCT                 |
|               | CCACAGTGACAGCAGGACTC                   |
| miR-1305       | ACAGCACCAGGACAAGTGAATA                 |
|               | GCTGTCAACGATACGCTACGTAACG             |
| U6             | CTCGCTTCGGCAGCACA                       |
|               | AACGCTTCACGAAATTGCGT                   |
| TXNRD1         | AAATTTCTAGGACGGTACGGG                 |
|               | AGTCTGCCTTCCTGATAAGC                   |
| GAPDH          | GTCAAGGCTGAGAACGGGAA                    |
|               | AAATGAGCCCAGCCTTCTC                   |
Figures
Figure 1

The identification of Circ0120816/miR-1305/TXNRD1 axis in ESCC. A The top 5 upregulated circRNAs in circRNA microarray GSE131969. B-F The expression of top 5 upregulated circRNAs in our clinical samples. G The structure of circ0120816. H A total of 72 upregulated DEGs were overlapped from GSE33810 and GSE20347. I TXNRD1 was the key gene involving cell population proliferation by STRING anlaysis. J Eight miRNAs were overlapped from TargetScan and circInteractome. TargetScan was the online tool for the prediction of miRNAs binding to TXNRD1. CircInteractome was the online tool for the prediction of miRNAs binding to circ0120816.
Circ0120816 was upregulated in ESCC cells. A The expression of circ0120816 was higher in ESCC cell lines than in normal cells (Het-1A) by qRT-PCR. *P<0.05, **P<0.001, compared with Het-1A cells. B RNase R assay confirmed the stability of circ0120816. **P<0.001, the comparison between RNase R- and RNase R+. C The distribution of circ0120816 and linear 0120816 in ESCC cells. **P<0.001, the comparison between Cytoplasm and Nuclear.
Figure 3

Circ0120816 acted as a sponge for miR-1305. A The target prediction between circ0120816 and miR-1305. B Luciferase reporter assay was used to confirm the relationship between circ0120816 and miR-1305. **P<0.001, compared with miR-NC. miR-NC: miR-1305 negative control, circ-WT: circ0120816 wild type.
type, circ-Mut: circ0120816 mutant type. C RIP analysis showed that circ0120816 was abundantly pulled down by anti-Ago2 antibodies when transfected with miR-1305 mimics in ESCC cells compared with miR-1305 NC and IgG group. **P<0.001, compared with miR-NC. miR-NC: miR-1305 negative control, IgG: negative control, Ago2: anti-Argonaute2 antibody. D The expression of miR-1305 was decreased in ESCC tissues. Normal: healthy adjacent tissues, Tumor: ESCC tissues. E miR-1305 was negatively associated with circ0120816.
Circ0120816 facilitated ESCC progression via sponging miR-1305. A The expression of circ0120816 and miR-1305 after transfection. B CCK-8 assay was performed for cell viability in ESCC cells after transfection. C BrdU assay showed that circ0120816 promoted proliferation of ESCC cells while miR-1305 could rescue it. D Cell adhesion assay showed that circ0120816 promoted cell adhesion of ESCC cells while miR-1305 could rescue it. E Caspase 3 activity assay discovered that circ0120816 decreased the apoptosis of ESCC cells while miR-1305 could rescue it. *P<0.05, **P<0.001, compared with blank control group. #P<0.05, ##P<0.001, compared with co-transfection group of si-circ0120816 plus miR-1305 inhibitor. NC: negative control, inhibitor: miR-1305 inhibitor, si-circ: si-circ0120816, si-circ+inhibitor: si-circ0120816 plus miR-1305 inhibitor.
**Figure 5**

A. Position 2126-2133 of TXNRD1 (5’...3’)

| hsa-miR-1305 (3’...5’) |
|-------------------------|
| GAUCUCUGAUAAUGUGUUGAAAAA |
| AGAGAGGAUAACUCACAAUUUU |

B. KYSE450

- **miR-NC**
- **miR-1305**

| Relative luciferase activity | WT | Mut |
|-----------------------------|----|-----|
| miR-NC                      | 1.0|     |
| miR-1305                    | 0.5|     |

C. KYSE510

- **miR-NC**
- **miR-1305**

| Relative luciferase activity | WT | Mut |
|-----------------------------|----|-----|
| miR-NC                      | 1.0|     |
| miR-1305                    | 0.5|     |

D. The relative mRNA expression of TXNRD1

| The relative mRNA expression of TXNRD1 |
|----------------------------------------|
| Bio-NC                                 |
| KYSE450                                |
| KYSE510                                |

C. Bio-NC

D. Bio-miR-1305

P<0.0001

E. The relative expression of miR-1305

- Y= -0.1001X + 0.989
- R²= 0.6397
- P<0.0001

The relative mRNA expression of TXNRD1
TXNRD1 was a target gene of miR-1305. A The target prediction between TXNRD1 3’UTR and miR-1305. B Luciferase reporter assay was used to observe the relationship between miR-1305 and TXNRD1. **P<0.001, compared with miR-NC. miR-NC: miR-1305 negative control, WT: TXNRD1 wild type, Mut: TXNRD1 mutant type. C RNA pull-down assay was used to determine the relationship between miR-1305 and TXNRD1. **P<0.001, compared with Bio-NC. Bio-NC: Bio-miR-1305 negative control. Bio-miR-1305: miR-1305 mimic-biotin. D The expression of TXNRD1 was increased in ESCC tissues. E MiR-1305 had a negative relationship with TXNRD1.
Figure 6
MiR-1305 suppressed ESCC progression by targeting TXNRD1. A The expression of TXNRD1 after transfection by qRT-PCR. B CCK-8 assay was performed for cell viability in ESCC cells after transfection. C BrdU assay showed that miR-1305 inhibited proliferation of ESCC cells while TXNRD1 could rescue it. D Cell adhesion assay showed that miR-1305 blocked cell adhesion of ESCC cells while TXNRD1 could rescue it. E Caspase 3 activity assay discovered that miR-1305 induced the apoptosis of ESCC cells while TXNRD1 could rescue it. *P<0.05, **P<0.001, compared with blank control group. #P<0.05, ##P<0.001, compared with co-transfection group of si-TXNRD1 plus miR-1305 inhibitor. NC: negative control, inhibitor: miR-1305 inhibitor, si: si-TXNRD1, si+inhibitor: si-TXNRD1 plus miR-1305 inhibitor.