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MYC-binding lncRNA EPIC1 promotes AKT-mTORC1 signaling and Rapamycin resistance in breast and ovarian cancer

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Abstract

AKT-mTORC1 (mammalian target of rapamycin complex 1) signaling pathway plays a critical role in tumorigenesis and can be targeted by rapamycin. However, the underlying mechanism of how lncRNAs (long noncoding RNA) regulate the AKT-mTORC1 pathway remains unclear. *EPIC1* is a Myc-binding lncRNA, which has been previously demonstrated to be overexpressed in multiple cancer types. In a pathway analysis including 4962 cancer patients, we observed that lncRNA *EPIC1* expression was positively correlated with the AKT-mTORC1 signaling pathway in more than 10 cancer types, including breast and ovarian cancers. RNA-seq analysis of breast and ovarian cancer cells demonstrated that *EPIC1*-knockdown led to the downregulation of genes in the AKT-mTORC1 signaling pathway. In MCF-7, OVCAR4 and A2780cis cell lines, *EPIC1* knockdown and overexpression respectively inhibited and activated phosphorylated AKT and the downstream phosphorylation levels of 4EBP1 and S6K. Further knockdown of Myc abolished the *EPIC1*'s regulation of AKT-mTORC1 signaling; suggested that the regulation of phosphorylation level of AKT, ERK, 4EBP1 and S6K by *EPIC1* depended on the expression of Myc. Moreover, *EPIC1* overexpressed MCF-7, A2780cis, and OVCAR4 cells treated with rapamycin showed significant decreasing in rapamycin mediated inhibition of p-S6K and p-S6 comparing with the control group. In addition, Colony Formation assay and MTT assay indicated that *EPIC1* overexpression led to rapamycin resistance in breast and ovarian cancer cell lines. Our results demonstrated the lncRNA *EPIC1* expression activated the AKT-mTORC1 signaling pathway through Myc and led to rapamycin resistance in breast and ovarian cancer.

Keywords

lncRNA *EPIC1*; Myc; AKT-mTORC1; rapamycin resistance

Introduction:

AKT-mTORC1 (mammalian target of rapamycin) has been identified as a critical signaling pathway contributing to cancer initiation and progression. Two major downstream targets of mTORC1, 4EBP1 (eukaryotic initiation factor 4E binding protein 1) and S6K (ribosomal protein S6 kinase1) play major roles in protein synthesis, ribosome biogenesis and lipid synthesis which are all related to cancer cell proliferation.¹⁻³ Besides AKT, ERK (extracellular-regulated kinase) has been demonstrated as another upstream regulator of mTORC1 and regulated its expressions and functions.⁴⁻⁷ Recent studies showed that Myc, as a transcription factor, is a potential regulator of mTORC1 in multiple cancer types⁸⁻¹¹. Moreover, the upregulation of Myc leads to drug resistance of mTORC1 inhibitors.¹² There are different types of AKT-mTORC1 inhibitors have been developed, and among these inhibitors, rapamycin has been known as a specific inhibitor of mTORC1.¹³ Currently, rapamycin is a known drug targeting mTORC1 and showing anticancer effect in different cancer types;¹⁴⁻¹⁷ including breast cancer.¹⁸

Long noncoding RNAs (lncRNAs) are RNA transcripts that are more than 200 nucleotides and do not translate into proteins.¹⁹ Recent studies have shown that lncRNAs are involved in tumorigenesis of different cancer progression include cell proliferation, anti-apoptosis, motility by interacting with chromatin, RNAs, and proteins.^{20,21} Moreover, several lncRNAs have been reported as potential regulators of mTORC1, which correlated with cancer cell metabolism and energy stress.^{22,23} The nuclear lncRNA *EPIC1* (epigenetically-induced lncRNA1) has been shown to regulate cell proliferation through the cell cycle by interacting with transcription factor Myc in breast cancer.²⁴ However, the role of *EPIC1* in regulating the AKT-mTORC1 pathway and rapamycin response is not clear.

In this study, we performed a comprehensive investigation into *EPIC1*'s regulation of the AKT-mTORC1 pathway using the genomics data from 4,962 tumor patients. The *EPIC1*'s regulation of the AKT-mTORC1 pathway in tumor samples was further confirmed by RNA-seq analysis in *EPIC1* knockdown cancer cell lines. We concluded that *EPIC1* activate AKT-mTORC1 signaling pathway in breast and ovarian cancer cells through increasing the phosphorylation level of phospho-S6K, phospho-S6, and phospho-4EBP1. Further silencing Myc rescued *EPIC1*'s activation of S6K, S6, and 4EBP1, suggested *EPIC1*'s regulation of the AKT-mTORC1 pathway was dependent on Myc. Moreover, we have demonstrated that *EPIC1* overexpression led to rapamycin resistance in both breast and ovarian cancer. In conclusion, our study revealed that *EPIC1* played an essential role in the rapamycin resistance and AKT-mTORC1 signaling pathway by interacting with Myc in breast and ovarian cancers.

Materials and Method

2.1 Cell culture, stable cell line generation

Human breast cancer cell line MCF-7 was purchased from ATCC (American Type Culture Collection) and cultured with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin. Human ovarian cancer cell line OVCAR4 was purchased from NIH/NCI and cultured with RPMI-1640 medium supplemented with 2

mM glutamine, 10% FBS, and 1% penicillin-streptomycin. Human ovarian cancer cell line A2780cis was purchased from ECACC (European Collection of Cell Cultures), supplied by Sigma-Aldrich and cultured with RPMI-1640 medium supplemented with 2 mM glutamine, 10% FBS and 1% penicillin-streptomycin. Stable cell lines were generated according to our previous publication.²⁴ Basically, 293T cells were seeded in 6-cm dish with DMEM medium with only 10% FBS, without any penicillin or streptomycin. After overnight, transfect 293T cells with 3 µg of control or EPIC1 plasmid, 2.25 µg of psPAX2 (Addgene, #12260), and 0.75 µg of pVSV-G (Addgene, #8454) using Lipofectamine 3000 Reagent (ThermoFisher, 10881775001) as guidelines suggested to produce lentivirus medium. Then, cells were incubated for 6 hr and changed to DMEM medium with only 10% FBS. Cells were then incubated for another 24 hr. The culture medium with lentivirus was collected and went through 0.45 µm filter to remove any floating cells or debris. At the same time, prepare target cells (MCF-7, A2780cis, and OVCAR4) in 6 cm dish. Used the collection lentivirus medium to infect target cells for 24hr with 8 µg/ml polybrene. Finally, select the target cells with puromycin (Fisher BioReagents, BP2956-100) to generate the stable cell line.

2.2 siRNA treatment and cloning

Cells in 6-well culture plates were transfected with 40 nM siRNA targeted *EPIC1*, Myc, and a control siRNA using Lipofectamine RNAiMAX reagent (ThermoFisher, 13778150) per the instructions by manufactures. After 48 hr, cells were collected, and western blot analysis and quantitative real-time PCR (qRT-PCR) were performed. siRNA sequences are listed as following: siControl (Santa Cruz, sc-37007), *siEPIC1*#1: CCUUCAGACUGUCUUUGAA; *siEPIC1*#2: GCUUUCUCUCGGAACGUG. Full length of *EPIC1* expression plasmid was followed the previous description.²⁴ Briefly, to construct *EPIC1* expression plasmids, full-length of *EPIC1* was cloned into pCDH-CMV-MCS-EF1-Puro (System Biosciences, #CD510B-1) with XbaI and EcoRI enzymes. Full-length of Flag-tagged MYC expression vectors were generated using a human MYC cDNA Clone (OriGene, #SC112715) as a DNA template.

2.3 SDS-PAGE and Western Blot

Cells were lysed in RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, and 1x protease inhibitor cocktail (Sigma-Aldrich, P8340)). Total protein concentrations were measured with BCA protein assay kit (ThermoFisher, 23225). 20 µg of protein with 5x SDS sample buffer were loaded into SDS-PAGE gel and then transferred to PVDF membranes (Bio-Rad, 162-0177). Membranes were blocked in 5% non-fat milk (Lab Scientific, M0841) for 1 hr and then incubated in primary antibodies diluted 1:1000 at 4 °C. β-Actin antibody was purchased from Sigma-Aldrich (A5441). The following antibodies were purchased from Santa Cruz: p-ERK (sc-7383), total-ERK (sc-514302), PTEN (sc-7974). The rest of antibodies are purchased from Cell Signaling Technology: p-AKT (4060) phosphorylation site, total-AKT (4691), p-S6K (9234), total-S6K (2788), p-S6 (4858), total-S6 (2217), p-4EBP1 (2855), total 4EBP1 (9644), Myc (13987), Flag (14793). On the second day, membranes were incubated in horseradish peroxidase-conjugated secondary antibodies for 1hr (Anit-mouse IgG: ThermoFisher 31430; anti-rabbit IgG: ThermoFisher, 31460). Bands were detected by ECL (electrogenenerated chemiluminescence) solution (ThermoFisher, 32106) on films.

2.4 Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using Trizol reagent. cDNAs were generated from 1 µg of RNA using cDNA Reverse Transcription Kit (Applied Biosystems, 4368813). qRT-PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems, 4367659) on 6 Flex Real-Time PCR System (Applied Biosystems). Relative mRNA expression levels were normalized to *GAPDH*. *GAPDH* primer sequences were used: forward: 5'-GGTGAAGGTCGGAGTCAACG-3'; and reverse: 5'-TGGGTGGAATCATATTGGAACA-3'. *EPIC1* primer sequences were used: forward: 5'-TATCCCTCAGAGCTCCTGCT-3'; and reverse: 5'-AGGCTGGCAAGTGTGAATCT-3'. *PTEN* primer sequences were used: forward: 5'-TGGATTCGACTTAGACTTGACCT-3'; and reverse: 5'-GGTGGGTTATGGTCTTCAAAAGG-3'.

2.5 RNA immunoprecipitation (RIP)

RIP followed the description of our previous publication.²⁴ Briefly, cells were collected and washed once with pre-chilled 1x PBS solution, then cells were treated for 10 minutes with 0.3% formaldehyde at room temperature on a rotor and add glycine solution to a final concentration of 125 mM to treat for another 5 minutes. Cells were pelleted and lysed with RIP buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 0.5 mM DTT, 1 mM PMSF, and 1 x protease inhibitor cocktail (Sigma, P8340)) and incubate on rotor for 20 minutes at 4 °C. Cell lysates were incubated with Myc, MAX, and normal mouse or rabbit IgG used as a control overnight at 4 °C. On the second day, prewashed Dynabeads Protein G (Invitrogen, 10004D) were added and incubated for 2 hr at 4 °C. Finally, beads were washed six times with RIPA buffer. RNA was isolated with Trizol reagent, and qRT-PCR was performed.

2.6 Colony Formation Assay

5 x 10³ cells stably overexpressing *EPIC1* or a control vector were seeded into 12-well culture plate and cultured overnight, then cells were treated with a serial of dilution of rapamycin (Selleckchem, S1039) ranging from 1.25, 10, 30, 100, to 200 nM, and DMSO was used as a vehicle control. Medium and drug were changed every two days. MCF-7, A2780cis cells were cultured for 6 days, and OVCAR4 cells were cultured for 7 days. Then, cells were washed once with PBS solution and fixed with 2% formaldehyde for 10 minutes at room temperature, and stained for 20 minutes in 0.01% crystal violet-25% methanol solution. Representative images were scanned and analyzed. The colony quantification is done by using ImageJ software and relative cell survival for each concentration is normalized with the quantification at 0nM.

2.7 MTT Assay

5 x 10³ cells were seed into 96-well culture plates and incubated overnight. Cells were treated as indicated with a serial of dilution of rapamycin (Selleckchem, S1039) ranging from 0.05, 0.25, 1.25, 10, 30, 100, 200 nM, and DMSO as a vehicle control. MCF-7 and OVCAR4 cells were treated for 72 hrs, and A2780cis cells were treated for 96 hr. Finally, cells were incubated for 4 hr with MTT solution (Biosynth, 1329524) and solubilizing

solution (40% DMF, 16% SDS, 2% Acetic acid, PH4.7) were added to dissolve for overnight. The optical density of 570 nm and 630 nm were measured on the next day.

2.8 Chromatin Immunoprecipitation (ChIP)- sequencing

Use 1×10^7 cells per sample cross-link with 1.42 % formaldehyde on shaker for 10 minutes at RT. Then quenched by adding 1.25M glycine and shake for another 5 minutes at RT. Cells were rinsed by pre-chilled PBS for two times. Harvested cells into 50 ml tubes and add lysis buffer (50 mM pH 7.5 Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, and 1% Triton X-100) with proteasome inhibitor and sonicated to shear the chromatin to yield DNA fragment sizes 100-500bp. A small portion of DNA fragment lysis was used as input samples. Then add Dynabeads Protein G (Invitrogen, 10004D) that was pre-incubated with 30 μ g Myc antibodies for overnight at 4°C into DNA fragment lysis. Incubate beads and lysis for overnight at 4°C. On the next day, wash beads for six times using the lysis buffer and elute the DNA from beads. Reversed cross-link protein and DNA at 65°C for overnight. On the second day, digest protein and RNA used RNase A (Qiagen, 1007885) and Proteinase K (NEB, P8107S), respectively. Extracted DNA and run agarose gel to check the size of DNA fragments. Then performed library construction (BioLabs, E7645S) to enrich the DNA products. At last, send samples for sequencing.

2.9 Computational Analysis

RNA-seq data of MCF-7 and A2780cis cells after *EPIC1* knockdown were profiled using the STAR-RSEM pipeline as in our previous study.^{24,25} To interpret the function of regulated genes after *EPIC1* siRNA treatment, GSEA (version 2.2.0) was performed using the 50 cancer hallmark gene sets.^{26,27} The expression data for 4,962 patients for 20 cancer types were downloaded TCGA Pan-Cancer project (Data Freeze 1.3) processed as our previous publication.²⁴ To identify the pathways that are enriched with genes correlated with *EPIC1* expression in tumor samples, we performed a similar GSEA for each cancer type in the TCGA dataset. The False Discovery Rate (FDR) was used to show the significance of the enriched pathways. The Pearson and Spearman correlation was calculated between gene expressions, and significance was defined as $p < 0.05$.

3. Results

3.1. The high expression level of EPIC1 is correlated with activated mTORC1 signaling pathway

To determine the function of lncRNA *EPIC1*, we first used Gene Set Enrichment Analysis (GSEA) to identify *EPIC1* related signaling pathways in 20 different cancer types. This analysis revealed that the mTORC1 signaling pathway was positively correlated with *EPIC1* expression in 12 out of 20 cancer types (Figure 1A), including breast (Figure 1B **left**) and ovarian cancer (Figure 1B **right**). *EPIC1* has been shown to significantly associate with breast cancer poor prognosis in our previous publication.²⁴ Moreover, we have further performed survival analysis between *EPIC1* expression and ovarian cancer patient prognosis. The analysis indicates that *EPIC1* overexpressed ovarian cancer tumors show a nonsignificant trend toward poor survival (Supplemental Figure 1C). In addition, RNA-sequencing analysis indicated that genes involved in the mTORC1 signaling pathway were

significantly downregulated in the *EPIC1*-knockdown MCF-7 and A2780cis cell lines (Figure 1A). In the breast (Figure 1C) and ovarian (Supplemental Figure 1A) cancer patient samples, mTORC1 signaling core genes were positively correlated with *EPIC1* expression. For example, the expression of AURKA (serine/threonine-protein kinase 6), GSK3B (glycogen synthase kinase 3 beta), and PLK1 (polo-like kinase 1), which were all reported as major regulators of mTORC1 signaling pathway,²⁸⁻³⁰ were positively associated with *EPIC1* expression in breast (Figure 1D) and ovarian (Supplemental Figure 1B) cancer tumors. These analyses suggested *EPIC1* might play an important role in promoting the mTORC1 signaling pathway in cancer.

3.2. *EPIC1* promotes the AKT-mTORC1 pathway through upregulating the phosphorylation level of 4EBP1 and S6K

In order to determine if *EPIC1* regulates the AKT-mTORC1 signaling pathway and its downstream targets, we examined the relationship between *EPIC1* and p-AKT. Meanwhile, we also studied two downstream mTORC1 targets, p-S6K and p-4EBP1. Out of ten breast and ovarian cancer cell lines, we chose three that had relatively high expression levels of *EPIC1* identified by qRT-PCR (Figure 2A) for further studies. We further generated *EPIC1* overexpression stable cell lines in MCF-7, OVCAR-4, and A2780cis cells (Figure 2E). Western Blot demonstrated that three *EPIC1* overexpressed cells all showed an increased signal of p-AKT, p-S6K, and p-4EBP1 compared with control cells without affecting the total expression level of these proteins (Figure 2B, 2C, and 2D). In addition, we investigated the protein expression level of p-ERK, which was known to be another upstream regulator of mTORC1 besides AKT.⁴⁻⁷ We observed that p-ERK was also highly upregulated in *EPIC1* overexpressed cells (Figure 2B, 2C, and 2D). To verify *EPIC1*'s regulation of mTORC1 signaling, we knockdown *EPIC1* by treating these three cell lines with *EPIC1* siRNAs (Figure 2F, 2G, and 2H **right**). Knockdown *EPIC1* downregulated the expression of p-ERK and two mTORC1 downstream targets p-S6K and p-4EBP1 without changing the total S6K or 4EBP1 protein level. These data suggested that *EPIC1* promoted the AKT-mTORC1 pathway by upregulating the phosphorylation levels of AKT and mTORC1 downstream targets.

3.3 Activation of AKT-mTORC1 signaling pathway by *EPIC1* is mediated by its interaction with Myc

There are several critical proteins that have been established as direct regulators of the AKT-mTORC1 pathway. PTEN is a well-known tumor suppressor gene, which can suppress the AKT-mTORC1 pathway through inhibiting AKT phosphorylation and activation.³¹ We first examined if *EPIC1* regulated PTEN expression level. Western blot data demonstrated that there were no significant differences of PTEN at the protein level between *EPIC1* overexpressed cells and control MCF-7, A2780cis, and OVCAR4 cells (Figure 3A, 3B, and 3C **left**). Furthermore, qRT-PCR indicated comparable PTEN mRNA expression level between control and *EPIC1* overexpression cells (Figure 3A, 3B, and 3C **right**). These observations suggested that *EPIC1*'s regulation of the AKT-mTORC1 pathway is not mediated by PTEN. Our previous study found that *EPIC1* is located in the nucleus and binds with transcription factor Myc²⁴, which has demonstrated as a regulator of AKT-mTORC1 pathway by previous studies. We thus sought to determine if *EPIC1* activates the AKT-

mTORC1 signaling pathway through interacting with Myc.^{8,9,11,12} We first verified the binding between *EPIC1* and Myc by performing RNA immunoprecipitation assay (RIP) in 293T cells. We also included Flag-tagged MAX (Myc-associated protein X) as a negative control.³² The RIP assay indicated that compared with control and Flag-MAX, only Flag-Myc could successfully pull down *EPIC1* after normalized with input (Figure 3D **right**). We further used siRNA to knockdown Myc in MCF-7 control and *EPIC1* overexpressed cells. Western Blot demonstrated that compared with siControl treatment, knockdown of Myc abolished the *EPIC1*'s activation effect on p-AKT, p-ERK, and p-4EBP1 in *EPIC1* overexpression cells (Figure 3E). We have also performed the siMyc treatment in two ovarian cancer cell lines A2780cis (Figure 3F) and OVCAR4 (Figure 3G) and observed similar results. These data suggested that *EPIC1* promoted the AKT-mTORC1 signaling pathway through its interaction with Myc.

3.4. Activation of AKT-mTORC1 by EPIC1 leads to rapamycin resistance

mTORC1 signaling pathway has been established to be an important drug target for cancer treatment.¹² Rapamycin, also named Sirolimus is a class I mTOR inhibitor approved to treat breast cancer.¹⁸ However, drug resistance of rapamycin and other types of mTOR inhibitors remain a current challenge in treating cancer patients.^{33,34} Based on our previous studies, we hypothesized that overexpression of *EPIC1* could lead to the resistance of mTOR inhibitors. After treated with rapamycin in MCF-7 control and *EPIC1* overexpression cells, Indeed, *EPIC1* overexpression dampened rapamycin's inhibition of S6K and S6 phosphorylation (Figure 4A). This result suggested that *EPIC1* may play an important role in rapamycin resistance. There was no significant difference in the protein level of another mTORC1 downstream target p4EBP1. This observation was concordant with previous studies, which showed that rapamycin has a strong effect on p-S6K, but not p-4EBP1.³⁵ Consistently, siRNA knockdown of *EPIC1* (Figure 4B **right**) enhanced rapamycin's inhibition of S6K and S6 phosphorylation (Figure 4B **left**). MTT experiment was performed by seeding 5,000 MCF-7 control and *EPIC1* overexpressed cells in 96-well plate and treated with different concentrations of rapamycin for 72 hr. The cell viability curve indicated that *EPIC1* overexpressed cells had a significantly higher IC₅₀ for treating rapamycin (Figure 4C). Further colony formation assay confirmed that *EPIC1* overexpressed cells exhibited an increased resistance to rapamycin and higher rate of relative cell survival (Figure 4D). In ovarian cancer cell lines A2780cis and OVCAR4, we also observed that *EPIC1* overexpression lead to rapamycin resistance; as indicated by p-S6K and p-S6 (Figure 5A and 5B) and MTT assay (Figure 5C, 5D). These observations suggested that the promotion of the AKT-mTORC1 pathway by *EPIC1* could lead to mTORC1 inhibitor-rapamycin resistance in breast and ovarian cancer.

3.5. Knockdown of EPIC1 results in cells sensitive to rapamycin treatment

In order to further determine the role of *EPIC1* in rapamycin resistance of breast and ovarian cancers, we performed cell viability assays in MCF-7, A2780cis and OVCAR4 cell lines treated with rapamycin after knockdown *EPIC1* by using siRNA. The cell viability curves of MTT experiments indicated that MCF-7 (Figure 6A), A2780cis (Figure 6B), and OVCAR4 (Figure 6C) parental cells with *EPIC1* knockdown showed a significantly lower IC₅₀ while treating rapamycin compared with control cells. Further colony formation assays

demonstrated that MCF-7 (Figure 6D), A2780cis (Figure 6E), and OVCAR4 (Figure 6F) cells with *EPIC1* knockdown showed a significantly lower number of colonies and lower relative cell survival rate after treated with rapamycin. These results suggested that inhibition of *EPIC1* could lead to rapamycin sensitivity in breast and ovarian cancer cells.

Discussion:

¹³ Rapamycin, along with its derivatives and other mTOR inhibitors, has been demonstrated to have a therapeutic effect for cancer.³⁶ However, emerging evidence indicated that drug resistance has become a serious problem in patients who have been treated with rapamycin derivatives and other classes of mTOR inhibitors^{37,38}. In the current study, we have demonstrated that Myc binding lncRNA *EPIC1* is a potential regulator of the AKT-mTORC1 signaling pathway. In 927 breast and 264 ovarian cancer patients, *EPIC1* expression level was significantly correlated with genes involved in ribosome biogenesis and protein synthesis that were affected by the AKT-mTORC1 signaling pathway. These observations suggested that lncRNA *EPIC1* played an essential role in mTORC1 mediated tumorigenesis. Indeed, *EPIC1* overexpression increased the protein phosphorylation level of crucial kinases involved in the AKT-mTORC1 signaling pathway. We have further shown that knockdown of Myc abolished *EPIC1*'s regulation of the AKT-mTORC1 signaling pathway. Finally, our study demonstrated that *EPIC1* overexpression could lead to rapamycin resistance in breast and ovarian cancer cells.

Recent studies have shown that lncRNAs are promising in serve as potential biomarkers for cancer diagnosis, prognosis, and treatment. For example, one clinical study demonstrated that colorectal cancer patients who had higher expression of lncRNA *MALAT1* appeared worse disease-free survival and overall survival compared with patients who had lower *MALAT1* expression. This result suggested that the expression level of *MALAT1* might serve as a prognostic biomarker in colorectal cancer patients.³⁹ There was another preclinical study revealed that the expression of ten lncRNAs were associated with stage progression in ovarian cancer patients, which suggested that these lncRNAs were able to serve as prognostic biomarkers treating ovarian cancer patients.⁴⁰ In a recent study that integrated cancer cell line pharmacogenomics data and cancer patient drug response data, our group have also shown that lncRNAs can be used to predict cancer drug response.²⁵ In this study, our computational and experimental result suggested that overexpression of lncRNA *EPIC1* might serve as a clinically actionable biomarker in breast and ovarian cancer patients.

From our study, we concluded that *EPIC1* regulated the AKT-mTORC1 signaling pathway through its interaction with Myc. However, the specific mechanism of how *EPIC1*-Myc axis regulates mTORC1 signaling pathway still needs to be answered. In the future, we aim to pinpoint out the targets in the *EPIC1*-Myc axis that mediates their regulation of mTORC1 signaling pathway. This goal can be achieved by performing Myc ChIP-seq analysis in *EPIC1* control and knockdown MCF-7 cells. Our preliminary ChIP-seq results showed that after *EPIC1* knockdown, there was a significant downregulation of the binding between Myc transcription factor and *IGF1R* (Supplemental Figure 2A), *PI3KR2* (Supplemental Figure 2B), and *AKTIP* (Supplemental Figure 2C) at the promoter region. *IGF1R* encodes insulin

growth factor receptor protein, which is a receptor tyrosine kinase binding with growth factor and activating AKT-mTORC1 signaling pathway.⁴¹ *PI3KR2* encodes p85 protein, which is a regulatory subunit of PI3K. p85 facilitates the function of PI3K enzyme to activate and phosphorylate AKT.⁴² Decreasing the expression of p85 partially inhibits the function of PI3K enzyme.⁴² *AKTIP* gene encodes AKT interacting protein, which directly interacts with AKT and modulates AKT activity by enhancing the phosphorylation of AKT's regulatory sites.⁴³ Whether *EPIC1*-MYC axis regulates mTORC1 signaling pathway through transcriptional regulation of *IGF1R*, *PI3KR2*, and *AKTIP* warrant future study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

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Data Availability Statement

The data that support the findings of this study are openly available in GEO with reference number [GSE98538].

Abbreviations:

LncRNA	Long noncoding RNA
AKT	Protein kinase B
mTORC1	Mammalian-target of rapamycin complex 1
EPIC1	Epigenetically-induced long noncoding RNA 1
AKT	Protein kinase B
ERK	Extracellular-signal-regulated kinase
S6K	p70S6 kinase
S6	Ribosomal protein S6
4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
p-AKT	Phosphorylated protein kinase B
p-ERK	Phosphorylated extracellular-signal-regulated kinase
p-S6K	Phosphorylated p70S6 kinase
p-S6	Phosphorylated ribosomal protein S6

p-4EBP1	Phosphorylated eukaryotic translation initiation factor 4E-binding protein 1
PTEN	Phosphatase and tensin homolog
Rap	Rapamycin
<i>EPIC1</i> O/E	Epigenetically-induced long noncoding RNA 1 overexpression
FDR	False discovery rate
BRCA	Breast cancer
OV	Ovarian cancer cell
IP	Immunoprecipitation
RIP	RNA immunoprecipitation assay
qRT-PCR	Quantitative real-time Polymerase chain reaction
PCR	Polymerase chain reaction
MAX	Myc-associated protein X
GS	Glutamine synthetase
GSEA	Gene set enrichment analysis
AURKA	Serine/threonine-protein kinase 6
GSK3B	Glycogen synthase kinase 3 beta
PLK1	polo-like kinase 1
IGF1R	Insulin-like growth factor 1 receptor
PI3KR2	Phosphatidylinositol 3-kinase regulatory subunit beta
AKTIP	AKT-interacting protein
ChIP-Seq	Chromatin immunoprecipitation sequencing
MALAT1	Metastasis associated lung adenocarcinoma transcript 1
siRNA	Small interfering RNA

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Figure 1: High expression level of *EPIC1* is correlated with activated mTORC1 signaling pathway.

A. Correlation between *EPIC1* expression and mTORC1 signaling pathway by Gene set enrichment analysis (GSEA) in 20 cancer types and *EPIC1* knockdown MCF-7 (m1, m2, m12), A2780cis cells (ac1, ac2, ac12). The heatmap indicates the GSEA scores. B. Association between the enrichment of mTORC1 signaling and *EPIC1* expression in breast cancer patients (left) and ovarian cancer patients (right) by GSEA analysis (A). FDR, False Discovery Rate. C. Expression analysis showing that *EPIC1* expression is associated with hallmark mTORC1 genes (B) in 927 breast cancer patients. Each column represents one patient. D. Correlation analysis between specific genes that involved in the mTORC1 signaling pathway and *EPIC1* expression in breast cancer patients.

Figure 2: *EPIC1* promotes AKT-mTORC1 pathway through upregulating the phosphorylation level of 4EBP1 and S6K.

A. qRT-PCR analysis of *EPIC1* RNA level in breast and ovarian cancer cell lines. *EPIC1* RNA level was normalized to *GAPDH*. B-D. Western blot of AKT-mTORC1 signaling pathway proteins in MCF-7 (B), A2780cis (C) and OVCAR4 (D) cells stably overexpressing *EPIC1* or a control vector. E. PCR validation for *GAPDH* and *EPIC1* overexpression in MCF-7, A2780cis and OVCAR4 cell lines. F-H. Western blot of AKT-mTORC1 signaling pathway proteins in MCF-7 (F), A2780cis (G) and OVCAR4 (H) cells treated with *EPIC1* siRNA or a control siRNA. *EPIC1* knockdown efficiency was also shown by qRT-PCR (right). Error bars indicate mean $\pm$ SD, n = 3. *p < 0.05, **p < 0.01, and ***p < 0.001. The quantification of indicated protein phosphorylation level of western blots were shown above each signal band. All western blots were repeated three times independently with similar results, and representative images are shown.

Figure 3: Activation of AKT-mTORC1 signaling pathway by *EPIC1* is through the interaction with Myc.

A-C. Western blot and qRT-PCR analysis of PTEN protein and RNA level in MCF-7 (A), A2780cis (B), and OVCAR4 (C) cells stably overexpressing *EPIC1* or a control vector. D. RNA immunoprecipitation (RIP) experiment showing by qRT-PCR analysis of *EPIC1* enrichment in Flag-Myc, Flag-MAX, and control samples of 293T cells (right). Western blot of IP efficiency and input was also shown (left). E-G. Western-Blot of AKT-mTORC1 signaling pathway proteins in MCF-7 (E), A2780cis (F), and OVCAR4 (G) stably overexpressing *EPIC1* or a control vector after silencing by Myc siRNA or a control siRNA for 48 hours. Error bars indicate mean $\pm$ SD, $n = 3$. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. NS, not significant. The quantification of the indicated protein phosphorylation level of western blots were shown above each signal band. All western blots were repeated three times independently with similar results, and representative images are shown.

Figure 4: Activation of AKT-mTORC1 by *EPIC1* leads to rapamycin resistance in MCF-7 cells.

A. Western-Blot of AKT-mTORC1 signaling pathway proteins in MCF-7 cells stably overexpressing *EPIC1* or a control vector after treatment with a serial dosage of rapamycin (Rap: 0, 0.05, 0.1, 0.2, 0.4, 1, 10 nM) for 2 hrs. B. Western-Blot of AKT-mTORC1 signaling pathway proteins in MCF-7 cells transfected for 48 hr with *EPIC1* siRNA or a control siRNA then treated with a serial dosage of rapamycin (Rap: 0, 0.05, 0.1, 0.2, 0.4, 1, 10 nM) for 2 hrs. *EPIC1* knockdown efficiency was analyzed by qRT-PCR (Right). C. Cell viability was detected by MTT assay after treatment with a serial dosage of rapamycin as indicated for 72 hr in MCF-7 cells stably overexpressing *EPIC1* or a control vector. D. Colony formation assay of MCF-7 cells stably overexpressing *EPIC1* or a control vector after treatment for 6 days with a serial dosage of rapamycin as indicated. The quantification of relative cell survival is on the right. Error bars indicate mean $\pm$ SD, $n = 3$. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. The quantification of indicated protein phosphorylation level of

western blots were shown above each signal band at significant concentrations as indicated. All western blots were repeated three times independently with similar results, and representative images are shown.

Figure 5: Activation of AKT-mTORC1 by *EPIC1* leads to rapamycin resistance in A2780cis and OVCAR4 cells.

A-B. Western-Blot of AKT-mTORC1 signaling pathway proteins in A2780cis (A) and OVCAR4 (B) cells stably overexpressing *EPIC1* or a control vector after treatment with a serial dosage of rapamycin (Rap: 0, 0.05, 0.1, 0.2, 0.4, 1, 10 nM) 2 hrs. C-D. Cell viability was detected by MTT assay after treat with rapamycin for 72 hr and 96 hr in OVCAR4 and A2780cis cells stably overexpressing *EPIC1* or a control vector, respectively. Error bars indicate mean $\pm$ SD, $n = 3$. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. The quantification of indicated protein phosphorylation level of western blots were shown above each signal band at significant concentrations as indicated. All western blots were repeated three times independently with similar results, and representative images are shown.

Figure 6: Knockdown of *EPIC1* results in cells sensitive to rapamycin treatment.

A. Cell viability was detected by MTT assay after treated with a serial dosage of rapamycin as indicated for 72 hr in MCF-7 cells silencing by control siRNA or *EPIC1* siRNA. B. Cell viability was detected by MTT assay after treated with a serial dosage of rapamycin as indicated for 96 hr in A2780cis cells silencing by control siRNA or *EPIC1* siRNA. C. Cell viability was detected by MTT assay after treated with a serial dosage of rapamycin as indicated for 72 hr in OVCAR4 cells silencing by control siRNA or *EPIC1* siRNA. D. Colony formation assay of MCF-7 cells treated with control siRNA or *EPIC1* siRNA then treated with a serial dosage of rapamycin as indicated for 6 days. E. Colony formation assay of A2780cis cells treated with control siRNA or *EPIC1* siRNA then treated with a serial dosage of rapamycin as indicated for 6 days. F. Colony formation assay of OVCAR4 cells treated with control siRNA or *EPIC1* siRNA then treated with a serial dosage of rapamycin

as indicated for 7 days. The quantification of relative cell survival is on the right (D-F). Error bars indicate mean $\pm$ SD, n = 3. *p < 0.05, **p < 0.01, and ***p < 0.001.