circSETD3 Contributes to Acquired Resistance to Gefitinib in Non-Small-Cell Lung Cancer by Targeting the miR-520h/ABCG2 Pathway

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INTRODUCTION

Lung cancer is the most commonly diagnosed cancer and remains the leading cause of cancer death worldwide.1 About 80%–85% of lung cancer is non-small-cell lung cancer (NSCLC), with a 5-year survival rate of only 16%.2,3 In a vast majority of NSCLC tumors, the epidermal growth factor receptor (EGFR) has been found overexpressed, resulting in EGFR pathway overactivation, which leads to cellular proliferation, differentiation, and survival in the lung.4–6 In July 2015, the US Food and Drug Administration (FDA) approved gefitinib for the first-line treatment of advanced NSCLC, and it is currently used as first-line therapy for NSCLC patients with a sensitizing EGFR mutation.7 Unfortunately, despite initial and often dramatic responses of gefitinib treatment, nearly all patients will eventually develop acquired resistance after 10–14 months.8 Although a number of mechanisms have been elucidated, such as EGFR T790M mutation and MET amplification, in up to 30% of patients the underlying mechanisms still remain unknown.9,10 Moreover, a useful biomarker is highly and urgently needed to monitor the clinical response to gefitinib therapy.

Circular RNAs (circRNAs) are a class of non-coding RNAs that form covalently closed continuous loop structures.11 The unique circular structure makes circRNAs more stable and resistant to exonucleases (e.g., RNase R).11 In addition, circRNAs are often expressed in a tissue- and developmental stage-specific manner, and they are abundant in various tissues and body fluids such as blood, plasma, and serum.12 These characteristics of circRNAs make them an ideal biomarker for cancer and other diseases.12 Functional studies have strongly implicated that circRNAs are involved in gene expression regulation at the transcriptional, post-transcriptional, and translational levels and exert multiple physiological effects.13 Recently, accumulating evidence suggests that circRNAs are involved in acquired chemotherapy resistance. For example, circAKT3 and hsa_circ_0081143 contribute to the acquired resistance to cisplatin in gastric cancer, circPVT1 mediates doxorubicin and cisplatin resistance in osteosarcoma cells, and circ_100053 plays a key role in imatinib resistance in chronic myeloid leukemia.14–17 However, the function of circRNAs in acquired resistance to gefitinib in NSCLC has not been clearly elucidated so far.

In the present study, we found that circSETD3 (hsa_circ_0000567) was significantly upregulated in both gefitinib-resistant NSCLC cell lines and the plasma of gefitinib-resistant NSCLC patients. circSETD3 reduced gefitinib sensitivity by sponging miR-520h, thereby leading to increased expression of ATP-binding cassette subfamily G member 2 (ABCG2) and decreased intracellular gefitinib accumulation. Previous reports have shown that miR-520h is negatively related to the tumorigenesis of NSCLC, and, as a gefitinib efflux...
transporter, ABCG2 can decrease the intracellular concentration of gefitinib in NSCLC cells.\(^{18-20}\) Furthermore, we revealed that the serine/arginine splicing factor 1 (SRSF1) was responsible for the upregulation of circSETD3 in NSCLC cells with acquired resistance to gefitinib. Also, previous research has found that SRSF1 is closely related to the tumorigenesis and chemoresistance in NSCLC.\(^ {21}\)

**RESULTS**

**circRNA Expression Profiles in Gefitinib-Sensitive and -Resistant NSCLC Cell Lines**

We first established the acquired gefitinib-resistant NSCLC cell sublines PC9/GR and HCC827/GR, which were derived from the parental PC9 and HCC827 cell lines and were free from EGFR T790M mutation and MET amplification.\(^ {22}\) Results of a Cell Counting Kit-8 (CCK-8) assay showed that the 50% inhibitory concentration (IC\(_{50}\)) value for gefitinib in PC9/GR cells was 1.77 \(\mu\)M, with a 35.40-fold increase relative to that in PC9 cells (0.05 \(\mu\)M), and the IC\(_{50}\) value for gefitinib in HCC827/GR cells was 0.72 \(\mu\)M, with a 21.86-fold increase relative to that in HCC827 cells (0.03 \(\mu\)M) (Figure 1A). Additionally, after 1 \(\mu\)M gefitinib treatment, PC9 and HCC827 cells exhibited both apoptosis and G1 cell cycle arrest, but PC9/GR and HCC827/GR cells were not affected (Figures 1B and 1C). Moreover, unlike their parental cells, PC9/GR and HCC827/GR cells maintained high phosphorylation levels of EGFR and downstream signaling components even following treatment with 10 \(\mu\)M gefitinib (Figure 1D). These results confirmed that our gefitinib-resistant NSCLC cell lines were well established.

The differentially expressed circRNAs in these cells were then examined using RNA sequencing (RNA-seq), which were shown in volcano map (Figure 2A). A total of 51 circRNAs were differentially expressed between PC9 and PC9/GR cells, consisting of 17 upregulated and 34 downregulated circRNAs. Also, 94 circRNAs were differentially expressed between HCC827 and HCC827/GR cells, consisting of 32 upregulated and 62 downregulated circRNAs (Tables S1 and S2). Among them, eight circRNAs were altered in both two paired cell lines with the same trend, and their expression levels were verified by quantitative real-time PCR (Figures 2B and 2C).

**Upregulated circSETD3 Reduces Sensitivity to Gefitinib in NSCLC Cells**

According to the results mentioned above, one of the most differentially expressed circRNAs, circSETD3 (hsa_circ_0000567), which is derived from exons 2–6 of the SET domain-containing 3 (SETD3) gene, was selected for further investigation. Its genomic structure is shown in Figure 3A. The distinct product of the expected size was amplified using outward-facing primers and confirmed by Sanger sequencing (Figure 3B). After RNase R treatment, the abundance of circSETD3 showed no significant decrease, while the linear SETD3 mRNA showed a marked reduction, which further confirmed that this RNA species exists in a circular form (Figure 3C; Figure S1). Moreover, we also investigated the stability and localization of circSETD3. After treatment with 2 \(\mu\)g/mL actinomycin D, an inhibitor of transcription, circSETD3 was highly stable, with a transcript half-life that exceeded 24 h, whereas the linear SETD3 mRNA exhibited a half-life of less than 8 h (Figure 3D). Quantitative real-time PCR analysis of nuclear and cytoplasmic expression demonstrated that the circSETD3 preferentially localized in the cytoplasm (Figure 3E).

We further determined the expression levels of circSETD3 in cultured gefitinib-sensitive and -resistant NSCLC cells, as well as in the plasma of gefitinib-sensitive and -resistant NSCLC patients. Compared to parental sensitive cells, significantly elevated expression of circSETD3 was observed not only in the whole-cell lysates, but also in the culture supernatants of PC9/GR and HCC827/GR cells (Figure 3F). Similarly, the level of circSETD3 in the plasma of NSCLC patients with acquired resistance to gefitinib was about 13.01-fold higher than that of gefitinib-sensitive patients (Figures 3G and 3H). These data provide evidence that circSETD3 is upregulated in NSCLC cells with acquired resistance to gefitinib both in vitro and in vivo, and is a potential biomarker for resistance to gefitinib therapy.

To study the influence of circSETD3 on gefitinib sensitivity in NSCLC cells, we modulated the expression level of circSETD3 either by overexpression in gefitinib-sensitive cells, or knockdown in gefitinib-resistant cells (Figures 4A and 4D). circSETD3 overexpression significantly upregulated the cell survival rate after various concentrations (0.01, 0.1, 1, 10, and 100 \(\mu\)M) of gefitinib exposure for 48 h, and the IC\(_{50}\) values showed a 44.32- and 9.79-fold increase in PC9 and HCC827 cells, respectively (Figure 4B). However, the IC\(_{50}\) values were reduced by 61.41% and 56.57% in PC9/GR and HCC827/GR cells after circSETD3 knockdown (Figure 4E). Additionally, after circSETD3 overexpression, gefitinib-induced apoptosis decreased from 40.73% to 16.25% in PC9 cells, and from 72.52% to 30.40% in HCC827 cells after 1 \(\mu\)M gefitinib exposure for 48 h (Figure 4C), whereas after circSETD3 knockdown, the gefitinib-induced apoptosis rate showed a 2.69-fold increase in PC9/GR cells and a 3.47-fold increase in HCC827/GR cells after 1 \(\mu\)M gefitinib exposure for 48 h (Figure 4F). Collectively, these data suggest that circSETD3 can significantly reduce the gefitinib sensitivity in NSCLC cells and is a potential target for overcoming gefitinib resistance.

Considering that the epithelial-mesenchymal transition (EMT) and minor subpopulations of cancer stem cells (CSCs) have been reported to be associated with acquired resistance of gefitinib in NSCLC,\(^ {23,24}\) we used qRT-PCR to study the effect of circSETD3 on the expression
of EMT markers (vimentin, N-cadherin, E-cadherin, and keratin 18) and stemness-related markers (CD133, OCT4, NANOG, and SOX2). Our results showed that there were no significant differences in these markers after circSETD3-overexpression plasmid (OE-circSETD3) and circSETD3-short hairpin RNA (shRNA) (sh-circSETD3) (Figure S2), which indicates that the function of circSETD3 in gefitinib resistance was irrelevant with EMT or stem cell properties in NSCLC.

circSETD3 Acts as a Sponge for miR-520h

Given that exonic circRNAs localized in the cytoplasm can act as a microRNA (miRNA) sponge to regulate gene expression,25 we next explored the miRNAs that could potentially interact with circSETD3, as well as their targeting genes. Using online bioinformatics databases (TargetScan, DIANA Tools, miRanda, and Circinteractome), we found that circSETD3 was potentially targeted by many miRNAs, and the five miRNAs with the highest interaction scores were miR-520h, miR-1236, miR-191, miR-149, and miR-421. Their potentially targeting genes were also predicted. Then, we used Cytoscape software to delineate the interaction network of circSETD3-miRNAs-target genes (Figure 5A). Among these miRNAs, we have significant interest in miR-520h, which has been reported to be associated with cancer progression, metastasis, as well as an anticancer drug response.26–28

We then observed the interaction between circSETD3 and miR-520h using a luciferase reporter system. Our results showed that miR-520h could interact with circSETD3 via a complementary seed region (Figures 5B and 5C). Moreover, a significantly reduced level of miR-520h was found in gefitinib-resistant NSCLC cells compared with their parental sensitive cells (Figure 5D). Overexpression of circSETD3 in PC9 and HCC827 cells markedly decreased the miR-520h level, whereas circSETD3 knockdown in PC9/GR and HCC827/GR cells exhibited an opposite effect (Figures 5E and 5F). However, miR-520h failed to influence the expression of circSETD3 (Figure 5G). These results imply that circSETD3 directly binds to miR-520h and acts as a miR-520h sponge.

Among the target genes in Figure 5A, we have significant interest in ABCG2, a member of the ATP-binding cassette family. Previous studies have reported that ABCG2 can decrease the intracellular accumulation of gefitinib in NSCLC cells and plays an important role in acquired resistance to gefitinib in NSCLC.19,20 Also, previous studies have reported that miR-520h can suppress the expression of ABCG2 by binding to its 3′ UTR region.29 Consistent with previous observations, we also found that miR-520h significantly inhibited the expression of ABCG2 in NSCLC cells, which could be rescued by a miR-520h inhibitor (Figures 5H–5J).

circSETD3 Upregulates the Expression and Function of ABCG2

In order to further explore the functional mechanisms of circSETD3 in gefitinib resistance, we observed its influence on ABCG2 expression and efflux activity. Our results showed that circSETD3 could upregulate the luciferase activity of the reporter containing the wild-type ABCG2 3′ UTR sequence in HEK293 cells, which was remarkably suppressed by the miR-520h mimic. However, these effects were not shown in the mutant group (Figure 6A). Accordingly, circSETD3 overexpression significantly upregulated the ABCG2 level in PC9 and HCC827 cells, which could be abolished by miR-520h mimic (Figures 6B and 6D). However, circSETD3 knockdown remarkably downregulated the ABCG2 levels in PC9/GR and HCC827/GR cells, which could be rescued by the miR-520h inhibitor (Figures 6C and 6E). These data indicate that circSETD3 can abolish the inhibitory effect of miR-520h on ABCG2 expression.

It is well known that gefitinib is a substrate of ABCG2 at clinically relevant concentrations.30 We therefore speculated that the contribution of circSETD3 to gefitinib resistance might be, at least partially, owing to the enhancement of ABCG2-mediated gefitinib efflux. We first evaluated the impact of circSETD3 on intracellular accumulation
of Hoechst 33342, a typical substrate of ABCG2. We found that, in PC9 cells, circSETD3 overexpression caused a 36.8% reduction in the intracellular Hoechst 33342 concentration, which was completely reversed by Ko143, an ABCG2 inhibitor. However, in PC9/GR cells, circSETD3 knockdown led to about a 2.6-fold increase in Hoechst 33342 accumulation, which was completely rescued by ABCG2 over-expression (Figure 6F). Consistent with these results, the level of circSETD3 was also inversely related to the intracellular concentration of gefitinib (Figure 6G). These results suggest that circSETD3 enhances the expression and function of ABCG2 by sponging miR-520h, resulting in decreased intracellular accumulation of gefitinib and reduced gefitinib sensitivity in NSCLC cells.

**circSETD3 Is Responsible for the Gefitinib Resistance in NSCLC Cells In Vivo**

To determine whether circSETD3 influences gefitinib sensitivity in NSCLC cells in vivo, PC9 xenograft models were established. Although the average body weight of mice showed no significant difference among groups (Figure 7A), circSETD3 was shown to significantly increase the tumor volume and tumor weight (Figures 7B–7D). H&E staining in xenograft models showed that circSETD3 increased the malignant degree of tumors (Figure 7E). Immunohistochemical staining of Ki-67 and ABCG2 in xenograft models showed that circSETD3 enhanced the expression of Ki-67, a well-known proliferation marker, as well as ABCG2 (Figures 7F and 7G). According with our results in vitro, circSETD3 increased the phosphorylation levels of EGFR and downstream signaling components in NSCLC xenograft mice models (Figure S3). These results suggest that circSETD3 can suppress the sensitivity of NSCLC cells to gefitinib in vivo, and inhibition of circSETD3 expression can partially restore the gefitinib sensitivity.

**circSETD3 Expression Is Regulated by SRSF1 in NSCLC Cells**

Finally, we explored the potential mechanism of circSETD3 upregulation in gefitinib-resistant NSCLC cells. Previous research has shown that for most host genes, there is a balance between circular and linear RNA biogenesis. Functional dysregulation of some splicing factors may be involved in the perturbation of this balance and can induce a change in circRNAs expression. Therefore, we evaluated the levels
of SETD3 precursor mRNA (pre-mRNA) and linear SETD3 mRNA in all cell lines. The SETD3 pre-mRNA level showed no difference, but the linear SETD3 mRNA was markedly decreased in gefitinib-resistant cells (Figure 8A). Then, we selected 20 differentially expressed splicing factors between gefitinib-sensitive and -resistant cells based on RNA-seq results and verified their expression by quantitative real-time PCR (Figures 8B and 8C). We chose three common significantly downregulated splicing factors, i.e., HNRNPK, HNRNPH1, and SRSF1, for further study. After knocking down these splicing factors, we found that only SRSF1 showed significant influence on the circSETD3/SETD3 balance (Figure S4). Then, SRSF1 was chosen for further study. In NSCLC cells, knockdown of SRSF1 resulted in an increase in circSETD3 levels, as well as a decrease in linear SETD3 mRNA expression. However, overexpression of SRSF1 led to a decrease in circSETD3 levels and an increase in linear SETD3 mRNA expression (Figures 8D and 8E). Taken together, these results suggest that SRSF1 is responsible for the upregulation of circSETD3 in gefitinib-resistant NSCLC cells.

**DISCUSSION**

In this study, we present the first study investigating the function of circRNAs in acquired resistance to gefitinib in NSCLC. First, we identified that circSETD3 was significantly upregulated in NSCLC cell lines as well as in the plasma of patients with acquired resistance to gefitinib. Recent studies of colorectal cancer (CRC) and hepatocellular carcinoma (HCC) have shown that circSETD3 was downregulated in these tumor tissues, and its expression was negatively related to tumor size and differentiation. Thus, circSETD3 may play an unignorable role in the regulation of proliferation, differentiation, and chemosensitivity of tumor cells. Considering that circSETD3 is localized in the cytoplasm, we suppose it may act as a scaffold to modulate the function of multiple molecules. It is not surprising that it plays a distinct role in different types of tumors and under different circumstances. According to our *in vitro* and mouse xenograft studies, circSETD3 could significantly decrease the sensitivity of NSCLC cells to gefitinib. Downregulation of circSETD3 expression could markedly increase the gefitinib sensitivity in gefitinib-resistant NSCLC cells, but it could not bring back these resistant cell lines to the sensitive level of their parental sensitive cell lines. This happens because the acquired gefitinib resistance is a multi-factorial phenomenon, and some of the mechanisms are still unclear. Therefore, the influence of other mechanisms cannot be ruled out in this study.

Second, we found that the influence of circSETD3 on gefitinib sensitivity was mediated at least partially by targeting the miR-520h/ABCG2 pathway. As a member of the miR-520 family, miR-520h has been reported to play an essential role in the growth, invasion, migration, and chemoresistance of various cancer cells through targeting multiple molecules. For example, miR-520h enhances cell proliferation, migration, and invasion in epithelial ovarian cancer cells by targeting Smad7. It also influences cell sensitivity to doxorubicin and paclitaxel by targeting histone deacetylase 1 (HDAC1) and death-associated protein kinase 2 (DAPK2) in gastric and breast cancer cells, respectively. ABCG2 is also a direct target of miR-520h. It can actively pump out a variety of substrates from cells, including some anti-cancer drugs such as doxorubicin, mitoxantrone, as well as gefitinib. Numerous studies have indicated that ABCG2 is involved in acquired resistance to gefitinib. The level of ABCG2 is upregulated in gefitinib-resistant NSCLC tissues and cell lines, resulting in decreased intracellular gefitinib accumulation. However, the mechanisms controlling ABCG2 upregulation in gefitinib-resistant NSCLC are not clear. Our present study suggests for the first time that the expression and function of ABCG2 can be modulated by circSETD3 through the miR-520h sponge.

Finally, we reported that the splicing factor SRSF1 contributed to the upregulation of circSETD3 in gefitinib-resistant NSCLC. In general, circRNAs are produced by spliceosome-mediated pre-mRNA backsplicing. Although the efficiency of back-splicing is much lower than that of linear splicing in human cells, functional alteration of spliceosomal components has been shown to cause preferred circRNA expression. SRSF1, as the archetype member of the serine/arginine-rich (SR) protein family of splicing regulators, is a multifunctional protein involved in the regulation of alternative splicing and other processes related to RNA metabolism. In addition, SRSF1 is also a proto-oncogene and plays a central role in tumorigenesis and cancer therapy. Therefore, dysregulation of SRSF1 may lead to significant changes in alternative splicing patterns, including back-splicing. Previous studies have indicated that depletion of *Drosophila* splicing factor SF2 (homolog of human SRSF1) facilitates back-splicing from the endogenous Laccase2 gene, resulting in an increased Laccase2 circular RNA level and a decreased linear Laccase2 mRNA level. Consistent with these results, we found that the downregulation of SRSF1 in gefitinib-resistant NSCLC cells resulted in an increased circSETD3 level and a corresponding decreased linear SETD3 mRNA level, but the underlying mechanisms need further investigation.

SETD3 is a member of the protein lysine methyltransferase (PKMT) family, which catalyzes the addition of methyl group to lysine residues. Recent findings have implicated a role of SETD3 in tumorigenesis and cancer therapy, but its role may vary depending on the cancer type. For instance, SETD3 is upregulated in HCC tissues and positively correlated with proliferation of HCC cells, but it is
Supplemental Information

circSETD3 Contributes to Acquired Resistance to Gefitinib in Non-Small-Cell Lung Cancer by Targeting the miR-520h/ABCG2 Pathway

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Table S1. Differentially expressed circRNAs in PC9 and PC9/GR cells

| ID          | Gene Symbol | circRNA_Length | PC-9  | PC-9/GR | FoldChange | Up/Down | P value    |
|-------------|-------------|----------------|-------|---------|------------|---------|------------|
| hsa_circ_0006276 | ANXA7       | 436            | 39.6097631 | 0       | 0          | Down    | 1.58E-05   |
| hsa_circ_0065299 | SMARCC1     | 233            | 14.4913767 | 0       | 0          | Down    | 0.00603411 |
| hsa_circ_0053535 | BIRC6       | 1395           | 12.5591932 | 0       | 0          | Down    | 0.01213801 |
| hsa_circ_0019170 | EXOC6       | 357            | 11.5931014 | 0       | 0          | Down    | 0.01755605 |
| hsa_circ_0012144 | ERI3        | 269            | 11.5931014 | 0       | 0          | Down    | 0.01755605 |
| hsa_circ_0072081 | MTMR12      | 502            | 10.6270096 | 0       | 0          | Down    | 0.02576458 |
| hsa_circ_0008148 | BTA1F       | 614            | 10.6270096 | 0       | 0          | Down    | 0.02576458 |
| hsa_circ_0005386 | NF2         | 333            | 10.6270096 | 0       | 0          | Down    | 0.02576458 |
| hsa_circ_0005023 | STAG1       | 720            | 10.6270096 | 0       | 0          | Down    | 0.02576458 |
| hsa_circ_0001172 | PPP4R1L     | 1513           | 10.6270096 | 0       | 0          | Down    | 0.02576458 |
| hsa_circ_0072386 | HMGS1       | 1483           | 9.66091783 | 0       | 0          | Down    | 0.03879450 |
| hsa_circ_0003720 | UBAC2       | 358            | 9.66091783  | 0       | 0          | Down    | 0.03879450 |
| hsa_circ_0008189 | C11orf30    | 831            | 9.66091783  | 0       | 0          | Down    | 0.03879450 |
| hsa_circ_0007979 | DYNC1H1     | 823            | 9.66091783  | 0       | 0          | Down    | 0.03879450 |
| hsa_circ_0071131 | LRBA        | 450            | 9.66091783  | 0       | 0          | Down    | 0.03879450 |
| hsa_circ_0006248 | TBC1D1      | 227            | 9.66091783  | 0       | 0          | Down    | 0.03879450 |
| hsa_circ_0003537 | FOCAD       | 11350          | 9.66091783  | 0       | 0          | Down    | 0.03879450 |
| hsa_circ_002402  | ECE1        | 442            | 9.66091783  | 0       | 0          | Down    | 0.03879450 |
| hsa_circ_0003933 | MROH1       | 579            | 9.66091783  | 0       | 0          | Down    | 0.03879450 |
| hsa_circ_0000419 | YLPM1       | 3411           | 9.66091783  | 0       | 0          | Down    | 0.03879450 |
| hsa_circ_0056078 | PBRM1       | 57305          | 9.66091783  | 0       | 0          | Down    | 0.03879450 |
| hsa_circ_0007797 | VCL         | 1962           | 9.66091783  | 0       | 0          | Down    | 0.03879450 |
| hsa_circ_0008274 | UGGT2       | 244            | 55.0672316   | 8.28078671 | 0.15037594 | Down    | 0.00417256 |
| hsa_circ_0001402 | TBC1D1      | 507            | 33.8132124   | 5.1754917  | 0.15306122 | Down    | 0.00959426 |
| hsa_circ_0000419 | RAB3IP      | 242            | 42.5080385   | 7.24568837 | 0.17045455 | Down    | 0.00903546 |
| hsa_circ_0032649 | MLH3        | 3343           | 38.6436713   | 7.24568837 | 0.1875    | Down    | 0.01412636 |
| hsa_circ_0003848 | PSEN1       | 222            | 21.2540192   | 4.14039336 | 0.19480519 | Down    | 0.01422712 |
| hsa_circ_0008282 | MARK3       | 6317           | 33.8132124   | 7.24568837 | 0.21428571 | Down    | 0.02571049 |
| hsa_circ_0016601 | DNAH14      | 1140           | 36.7144878   | 8.28078671 | 0.22556391 | Down    | 0.02698474 |
| hsa_circ_0006410 | TUSC3       | 490            | 35.7453960   | 8.28078671 | 0.23166023 | Down    | 0.03024911 |
Table S2. Differentially expressed circRNAs in HCC827 and HCC827/GR cells

|          | Symbol     | NAA15 | 52.8873994 | 12.4211801 | 0.23486086 | P value          | Down | 0.03091876 |
|----------|------------|-------|-------------|-------------|-------------|-----------------|-------|-------------|
| hsa_circ_0006867 | NAA15     | 263   | 51.2028645  | 14.4913767  | 0.28301887  | Down | 0.04294951 |
| hsa_circ_0009061 | KDM1A     | 360   | 154.574685  | 46.5794253  | 0.3013392   | Down | 0.03674825 |
| hsa_circ_0031584 | ARHGAP5   | 4033  | 22.220111   | 74.5270804  | 3.35403727  | Up   | 0.04162171 |
| hsa_circ_0088030 | PT3R1     | 551   | 23.1862028  | 80.7376704  | 3.48214286  | Up   | 0.03486354 |
| hsa_circ_0083709 | DOCK5     | 1311  | 12.5591932  | 44.5092286  | 3.54395604  | Up   | 0.04499706 |
| hsa_circ_0005676 | SETD3     | 683   | 6.76264248  | 27.9476552  | 4.13265306  | Up   | 0.04522852 |
| hsa_circ_002191 | C9orf3    | 567   | 8.6942605   | 46.5794253  | 5.35714286  | Up   | 0.01037715 |
| hsa_circ_0006837 | RERE      | 582   | 2.89827535  | 17.5966718  | 6.07142857  | Up   | 0.04119238 |
| hsa_circ_0009144 | FKBP8     | 394   | 1.93218357  | 14.4913767  | 7.50000000  | Up   | 0.04557846 |
| hsa_circ_0003511 | LMBR1     | 578   | 2.89827535  | 21.7370651  | 7.50000000  | Up   | 0.01672487 |
| hsa_circ_0007528 | PIGN      | 595   | 1.93218357  | 15.5264751  | 8.03571429  | Up   | 0.03455423 |
| hsa_circ_0001147 | RBM39     | 475   | 1.00858384  | 10.3509834  | 10.2628883  | Up   | 0.0253606 |
| hsa_circ_0000994 | SLC8A1    | 1832  | 3.86436713  | 42.4390319  | 10.9821429  | Up   | 0.00112344 |
| hsa_circ_0005695 | SLC30A6   | 314   | 1.00858384  | 11.3860817  | 11.2891771  | Up   | 0.01647701 |
| hsa_circ_0001610 | TNFRSF21  | 1147  | 1.00858384  | 13.4562784  | 13.3417548  | Up   | 0.00735154 |
| hsa_circ_0000655 | CHD2      | 413   | 1.00858384  | 22.7721635  | 22.5783543  | Up   | 0.00038722 |
| hsa_circ_0007222 | GSE1      | 219   | 1.00858384  | 22.7721635  | 22.5783543  | Up   | 0.00038722 |
| hsa_circ_0008901 | PTPN12    | 393   | 3.86436713  | 87.9833588  | 22.7678571  | Up   | 1.67E-05  |
| hsa_circ_0006200 | AAGAB     | 462   | 1.00858384  | 33.1231468  | 32.8412426  | Up   | 3.68E-05  |

Table S3. Clinicopathological factors of plasma samples of patients with non-small lung cell cancer and expression of circSETD3

| Characteristic | N (%) | P value          | Down | 0.03091876 |
|---------------|-------|-----------------|-------|-------------|
| Characteristic | Total | Sensitive | Resistant | >0.999 |
| Age (yr)      |       |            |          |       |
| ≥ 60          | 17(56.7) | 9(30) | 8(27) |       |
| < 60          | 13(43.3) | 6(20) | 7(23) |       |
| Gender        |       |            |          | 0.7152 |
| Male          | 14(46.7) | 6(20) | 8(27) |       |
| Female        | 16(53.3) | 9(30) | 7(23) |       |
| Smoking status|       |            |          | 0.1862 |
| Never         | 13(43.3) | 6(20) | 7(23) |       |
| Former        | 3(10)   | 1(3)   | 2(6)   |       |
| Current       | 10(33.3) | 4(13) | 6(20) |       |
| Unknown       | 4(13.3) | 4(13) | 0(0)   |       |
Expression of circ-SETD3

| Expression     |     |     |     |
|----------------|-----|-----|-----|
| Low expression | 18(60) | 14(47) | 4(13) |
| High expression| 12(40) | 1(3)  | 11(37) |

*the cutoff value of circ-SETD3 was calculated using Youden index20,21.*

Table S4. Primer pairs used in this study
Fig S1. The abundance of circSETD3 and linear SETD3 mRNA in PC9 and HCC827 cells treated with RNase R.

Fig S2. qRT-PCR analysis of EMT markers (Vimentin, N-cadherin, E-cadherin and keratin 18) and stemness-related markers (CD133, OCT4, NANOG and SOX2). *P < 0.05

Fig S3. Western Blot analysis of EGFR signaling pathway in xenograft tumor models.
Fig S4. qRT-PCR analysis of relative expression of circSETD3 and SETD3 after si-SRSF1, si-HNRNPK and si-HNRNPH1. *P < 0.05