Circ_0001421 facilitates glycolysis and lung cancer development by regulating miR-4677-3p/CDCA3

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Abstract

Background: Lung cancer (LC) is a malignant tumor originating in the bronchial mucosa or gland of the lung. Circular RNAs (circRNAs) are proved to be key regulators of tumor progression. However, the regulatory effect of circ_0001421 on lung cancer tumorigenesis remains unclear.

Methods: The expression levels of circ_0001421, microRNA-4677-3p (miR-4677-3p) and cell division cycle associated 3 (CDCA3) were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Methyl thiazolyl tetrazolium (MTT), Transwell and Tumor formation assays were performed to explore the role of circ_0001421 in LC. Glucose consumption and lactate production were examined by a Glucose assay kit and a Lactic Acid assay kit. Western blot was utilized to examine the protein levels of Hexokinase 2 (HK2) and CDCA3. The interaction between miR-4677-3p and circ_0001421 or CDCA3 was confirmed by dual-luciferase reporter assay.

Results: Circ_0001421 was increased in LC tissues and cells, and knockdown of circ_0001421 repressed cell proliferation, migration, invasion and glycolysis in vitro. Meanwhile, circ_0001421 knockdown inhibited LC tumor growth in vivo. Mechanistically, circ_0001421 could bind to miR-4677-3p, and CDCA3 was a target of miR-4677-3p. Rescue assays manifested that silencing miR-4677-3p or CDCA3 overexpression reversed circ_0001421 knockdown-mediated suppression on cell proliferation, migration, invasion and glycolysis in LC cells.

Conclusion: Circ_0001421 promoted cell proliferation, migration, invasion and glycolysis in LC by regulating the miR-4677-3p/CDCA3 axis, which providing a new mechanism for LC tumor progression.

Keywords: Lung cancer, circ_0001421, miR-4677-3p, CDCA3
development. For example, circNRP1 boosted cell growth, migration and invasion through sponging miR-149-5p in gastric cancer [8]. Silencing circDENND4C repressed breast cancer cell glycolysis and metastasis by sponging miR-200b/c under hypoxia [9]. The effects of circRNAs on LC have also been reported. CircFGFR1 could elevate cell proliferation and invasion by targeting miR-381-3p in NSCLC [10]. CircRNA SMARCA5 played an inhibition role in the progression of LC by acting as miR-19b-3p sponge [11]. Circular RNA, circ_0001421 (circ-SEC31A) was upregulated in NSCLC and its high expression predicts unfavorable prognoses [12]. However, the role of circ_0001421 in lung cancer tumorigenesis and progression was needed to be elucidated further.

MiRNAs are also ncRNA molecules with 18 ~ 25 nucleotides in length, and they play a biological role by targeting the downstream mRNAs [13]. As reported, miR-196b-5p promoted tumor development in NSCLC by targeting TSPAN12 and GATA6 [14]. MiR-212-5p enhanced tumor growth by regulating Id3 in lung adenocarcinoma cells [15]. MiR-4677-3p was found to be decreased in lung adenocarcinoma tissue samples, and lncRNA TTN-AS1 elevated cell migration and invasion by acting as miR-4677-3p sponge [16], whereas the interaction between miR-4677-3p and circ_0001421 was not studied in LC.

Cell division cycle associated protein-3 (CDCA3) modulates cell cycle processes, also called as trigger of mitosis entry 1, and it was identified to play a procarcinogenic role in gastric cancer [17], colorectal cancer [18] and acute myeloid leukemia [19]. However, the function of CDCA3 in LC has been little studied, and its effect is not fully understood.

Here, we investigated the expression of circ_0001421 in LC and its effects on cell proliferation, migration, invasion and glycolysis. Additionally, we appraised the interaction between circ_0001421 and miR-4677-3p, as well as the downstream genes. In conclusion, the aim of this study was to explore the role of circ_0001421 in LC progression.

**Materials and methods**

**Clinical tissues and cell**

The 48 paired lung cancer tissues and adjacent normal tissues were collected from lung cancer patients, who were diagnosed in The Fourth Affiliated Hospital of Nantong University; The First People’s Hospital of Yancheng from June 2016 to December 2018. The samples were immediately transferred to liquid nitrogen for further research. Written informed consents were obtained from these LC patients. And this study was reviewed and approved by The Fourth Affiliated Hospital of Nantong University; The First People’s Hospital of Yancheng. Table 1 presents the clinical characteristics.

**Transfection**

Small interfering RNA targeting circ_0001421 (si-circ_0001421, 5′-CCGGCTCTGGAGTTCTGATTGCATTCAGTGCAATCAGAACTCCAGAGTTTTTTTG-3′) and negative control (si-NC, 5′-UUCUCCGAACGUGUACUTT-3′), miR-4677-3p mimic (miR-4677-3p, 5′-UCAUCAAGAAACCAGAGUGUCU-3′), mimic NC (miR-NC, 5′-GAAAUGUACUUGAGCGUGGAGAC-3′), miR-4677-3p inhibitor (anti-miR-4677-3p, 5′-GUCUCCACGCUACAUUUUC-3′), inhibitor NC (anti-miR-NC, 5′-CUAAAACCGGCCGUACGCGGUU-3′), short-hairpin against circ_0001421 (sh-circ_0001421, 5′-CCGGGCTAAGATTCTCTCCAATCTCGAGATTGGAGTCTGAGTGCAATCAGAACTCCAGAGTTTTTTTG-3′).

| Clinical parameter | Hsa_circ_0001421 High (n = 24) | Hsa_circ_0001421 Low (n = 24) | P |
|--------------------|-----------------------------|-----------------------------|---|
| Age (years)        | ≤ 60                        | 9                          | 11 | 0.107 |
|                    | > 60                        | 15                         | 13 |       |
| Gender             | Male                        | 14                         | 12 | 0.116 |
|                    | Female                      | 10                         | 12 |       |
| Histological grade | Low or undiffer             | 10                         | 12 | 0.155 |
|                    | Middle or high              | 14                         | 12 |       |
| TNM stages         | I and II                    | 15                         | 16 | 0.018* |
|                    | III and IV                  | 9                          | 8  |       |
| Size               | ≤ 5 cm                      | 8                          | 7  | 0.015* |
|                    | > 5 cm                      | 16                         | 17 |       |
| Invasion depth     | T1 and T2                   | 14                         | 19 | 0.033* |
|                    | T3 and T4                   | 25                         | 12 |       |
| Lymphatic metastasis| Yes                        | 11                         | 10 | 0.153 |
|                    | No                          | 13                         | 14 |       |
| Distant metastasis | Yes                        | 12                         | 11 | 0.188 |
|                    | No                          | 12                         | 13 |       |
CAGAGATCTTAGCTTTTTTG-3′) and control (sh-NC, 5′-UUCUCCGAUGUGUCGUGUTT-3′) were acquired from GenePharma (Shanghai, China). Overexpression vector Vector-CDCA3 (CDCA3) and control (Vector) were acquired from GenePharma. Lipofectamine 3000 (Invitrogen) was employed to conduct cell transfection in this study.

Quantitative real-time polymerase chain reaction (qRT-PCR)
The RNA was extracted from LC tissues and cells via TRizol (Invitrogen), and then reversely transcribed to complementary DNA (cDNA) through the PrimeScript™ strand cDNA synthesis kit (Takara, Dalian, China). The reaction of qRT-PCR was carried out by a Power SYBR TM Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) on an ABI 7900 system (Thermo Fisher Scientific). U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as the internal controls for miRNA and circRNA or mRNA, respectively. Primer sequences were listed as follows: circ_0001421, F: 5′-TCTCTGGAGTTCTGAGACGAGG TGG-3′ and R: 5′-TGCTAGTAAATGGGGTGATTTC TGG-3′; miR-4677-3p, F: 5′-CTGTGAGACCAAAGAA CTACTGC-3′ and R: 5′-CTCTACAGCTATATTGCC AGCCAC-3′; CDCA3, F: 5′-GGACCCCTGAGACCTC CAGAT-3′ and R: 5′-GCGCTTACCCCTGCTAG-3′; GAPDH, F: 5′-CCATTTCAGTGGAAGAGATG TCTG-3′ and R: 5′-CACCACATTTGTAGTGG-3′; and U6, F: 5′-GCTTGCGGCAGCATACTAAAAAT-3′ and R: 5′-TACGTGCGTTAAGCATACTGC-3′.

Cell proliferation detection
The proliferation of A549 and H1299 cells was determined through the Methyl thiazolyl tetrazolium (MTT, Sigma-Aldrich, St. Louis, MO, USA) reagent. Cells were inoculated into the 96-well plates. 10 μL MTT was added into the cells. Then, 100 μL Dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added into the cells. The absorbance at 490 nm was detected at 0 h, 24 h, 48 h and 72 h by a microplate reader (Bio-Rad, Hercules, CA, USA).

Transwell assay
Transwell assay with the chamber was performed to assess cell migration (uncoated Matrigel) and invasion (coated Matrigel, Thermo Fisher Scientific). Transfected LC cells suspended in serum-free medium were seeded into the top chambers, and 600 μL medium with 10% FBS was added into the lower chamber. 24 h later, cells on the lower surface of chambers were fixed by methanol and then stained with 0.1% crystal violet for 20 min. Finally, a microscope was used to quantify the number of migrated or invaded cells.

Glucose consumption and lactate production analysis
The transfected A549 and H1299 cells were harvested at 48 h post-transfection, and the cell culture medium were collected. Then, the concentration of glucose in cell culture medium was examined by a Glucose assay kit (Sigma-Aldrich, St. Louis, MO, USA), and lactate concentration was determined by a Lactic Acid assay kit (Seebio, Shanghai, China).

Western blot
Proteins were extracted from cell and tissue lysates using RIPA buffer (Invitrogen), and then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, samples were transferred onto polyvinylidene fluoride (PVDF, Millipore, Billerica, MA, USA) membranes. 5% skim milk powder was used to block these membranes for 2 h. Then, the bands were incubated with the primary antibodies against Hexokinase 2 (HK2, 1:1000, Abcam, Cambridge, MA, USA), CDCA3 (1:1000, Abcam) or GAPDH (1:2000, Abcam) at 4 °C overnight. The next day, the membranes were mixed with the secondary antibodies (1:4000, Abcam) for 1 h. The immunoblots were detected with the enhanced chemiluminescence reagent (Millipore).

Dual-luciferase reporter assay
To affirm the interaction between miR-4677-3p and circ_0001421 or CDCA3, the wild-type (WT) sequences of circ_0001421 contained miR-4677-3p binding sites or its mutant (MUT) were cloned into the pmirGLO vector (Promega, Madison, WI, USA) to form circ_0001421-WT orcirc_0001421-MUT reporter vector. CDCA3-3′UTR-WT and CDCA3-3′UTR-MUT were constructed in the same way. These reporter vectors were co-transfected into cells with miR-4677-3p or miR-NC by using Lipofectamine 3000. 48 h later, the luciferase activity was determined by a Dual-Luciferase Reporter assay system (Promega).

Xenograft tumors in nude mice
Twelve male nude BALB/c mice (20–22 g, 4 weeks) were purchased from Shanghai Laboratory Animal Center (SLAC, Shanghai, China). A549 cells transfected with sh-circ_0001421 or sh-NC were injected into the nude mice. Tumor volume was measured once a week. Five weeks later, all the mice were euthanatized with an overdose of ether, and tumor weight was measured. Western blot and qRT-PCR were performed to examine the expression of CDCA3 and circ_0001421 or miR-4677-3p, respectively.

Statistical analysis
Data were displayed as the mean ± standard deviation (SD). The difference of two groups was evaluated by Wilcoxon test, and one-way analysis of variance
(ANOVA) was applied for more than two groups. \( P < 0.05 \) was considered as statistically distinct. Each test was repeated at least three times.

Result

Circ_0001421 was overexpressed in LC tissues and cells

Firstly, circ_0001421 expression in tumor and normal tissues of 48 LC patients was measured by qRT-PCR. The results showed that circ_0001421 expression in tumor tissues was significantly higher than that in normal tissues (Fig. 1a). Additionally, circ_0001421 was overexpressed in LC cell lines, including A549 and H1299 cells, compared to 16HBE cells (Fig. 1b).

Knockdown of circ_0001421 repressed LC cell proliferation, migration, invasion and glycolysis in vitro

Subsequently, several experiments were performed to explore the function of circ_0001421 in LC. As shown in
Fig. 2a, the lower expression of circ_0001421 was found in A549 and H1299 cells by transfection of si-circ_0001421 relative to that in si-NC-transfected cells. MTT assay demonstrated that circ_0001421 inhibition retarded proliferation in A549 and H1299 cells comparison with the control group (Fig. 2b). Transwell assay results manifested that circ_0001421 knockdown notably curbed cell migration and invasion of A549 and H1299 cells (Fig. 2c-d). Glucose consumption and lactate production assays were performed to assess the effect of circ_0001421 on glycolysis. Circ_0001421 knockdown significantly reduced glucose consumption and lactate production in A549 and H1299 cells (Fig. 2e-f). Meanwhile, HK2 protein expression in si-circ_0001421 transfected A549 and H1299 cells was declined (Fig. 2g). Collectively, these results supported that circ_0001421 promoted in LC cell proliferation, migration, invasion, glycolysis, and functioned as an oncogenic circRNA.

**Circ_0001421 could act as a sponge for miR-4677-3p**

To investigate the molecular mechanism of circ_0001421 regulating the progression of LC cells, Starbase 3.0 was employed to screen the target miRNAs sponged by circ_0001421. The results showed that there were binding sites between circ_0001421 and miR-4677-3p (Fig. 3a), and the relationship between them was then confirmed by dual-luciferase reporter assay. The expression of miR-4677-3p was increased in A549 and H1299 cells by transfection of miR-4677-3p (Fig. 3b). Overexpression of miR-4677-3p drastically degraded the luciferase activity of circ_0001421-WT, but did not distinctly reduce the luciferase activity of circ_0001421-MUT in A549 and H1299 cells (Fig. 3c). Then, we examined miR-4677-3p expression in LC tissues by qRT-PCR. As displayed in Fig. 3d, miR-4677-3p expression was down-regulated in tumor tissues compared to normal tissues. In addition, miR-4677-3p expression was exceptionally decreased in A549 and H1299 cells (Fig. 3e). The correlation between circ_0001421 and miR-4677-3p expression in 48 LC patients was then analyzed and a significant negative correlation between them was observed (Fig. 3f). We also found that knockdown of circ_0001421 up-regulated miR-4677-3p expression in A549 and H1299 cells (Fig. 3g). We concluded that circ_0001421 could target miR-4677-3p to negatively regulate miR-4677-3p expression in LC cells.

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**Fig. 3** Circ_0001421 could act as a sponge for miR-4677-3p. **a** The predicted binding sites between circ_0001421 and miR-4677-3p were shown. **b** The expression of miR-4677-3p in H1299 and A549 cells transfected with miR-NC or miR-4677-3p was detected by qRT-PCR. **c** Dual-luciferase reporter assay was performed to verify the relationship between circ_0001421 and miR-4677-3p in H1299 and A549 cells. **d** miR-4677-3p expression in tumor and adjacent normal tissues was examined by qRT-PCR. **e** qRT-PCR was used to determine miR-4677-3p expression in A549, H1299 and 16HBE cells. **f** The correlation between circ_0001421 and miR-4677-3p expression was analyzed in LC tissues by Pearson coefficient analysis. **g** miR-4677-3p expression in H1299 and A549 cells transfected with si-NC or si-circ_0001421 was measured by qRT-PCR. *P < 0.05
**Circ_0001421 regulated LC cell progression by targeting miR-4677-3p in LC cells**

To understand whether miR-4677-3p was involved in circ_0001421 regulation of cell biological behaviors, si-circ_0001421 and anti-miR-4677-3p were co-transfected into A549 and H1299 cells. The results showed that miR-4677-3p was inhibited by transfection of anti-miR-4677-3p in A549 and H1299 cells than cells transfected anti-miR-NC (Fig. 4a). MTT and Transwell assay indicated that miR-4677-3p inhibition reversed the inhibition effects of si-circ_0001421 on the proliferation (Fig. 4b), migration (Fig. 4c) and invasion (Fig. 4d) of A549 and H1299 cells. Moreover, glucose consumption and lactate production were decreased by circ_0001421 knockdown and then recovered by miR-4677-3p inhibition knockdown in A549 and H1299 cells (Fig. 4e-f), and the inhibition effect of si-circ_0001421 on HK2 expression could be reversed by silencing miR-4677-3p (Fig. 4g). These findings suggested that circ_0001421 executed its carcinogenic role in the progression of LC cells by targeting miR-4677-3p.

![Fig. 4 Circ_0001421 regulated LC cell progression by adsorbing miR-4677-3p in LC cells. a]（a）The expression of miR-4677-3p in H1299 and A549 cells transfected with anti-miR-NC or anti-miR-4677-3p was detected by qRT-PCR. b-g H1299 and A549 cells were transfected with si-NC, si-circ_0001421, si-circ_0001421 + anti-miR-NC or si-circ_0001421 + anti-miR-4677-3p. b MTT assay was employed to analyze cell proliferation. c-d Cell migration and invasion were evaluated by Transwell assay. e-f Glucose consumption and lactate production were assessed by a Glucose assay kit and a Lactic Acid assay kit. g Western blot was used to check the protein expression of HK2. *P < 0.05
CDCA3 was a target gene of miR-4677-3p in LC

MiRNAs often play biological roles by binding to the 3’UTR of mRNAs [20]. To verify the target mRNAs of miR-4677-3p, Starbase 3.0 was employed. As shown in Fig. 5a, the 3’UTR of CDCA3 contained miR-4677-3p binding sites. To further confirm this prediction, dual-luciferase reporter assay was performed in A549 and H1299 cells. We observed that the luciferase activity of CDCA3–3’UTR-WT was reduced in A549 and H1299 cells transfected with miR-4677-3p, while the luciferase activity of CDCA3–3’UTR-MUT was no obvious variation (Fig. 5b). To explore the role of CDCA3 in LC, CDCA3 expression in LC tissues and cells was detected by western blot. As shown in Fig. 5c-d, CDCA3 protein expression in LC tissues and cells was drastically increased compared to normal tissues and cells. Unsurprisingly, CDCA3 expression was negatively correlated with miR-4677-3p expression and positively related to circ_0001421 level (Fig. 5e-f). Besides, miR-4677-3p overexpression reduced the protein expression of CDCA3 in A549 and H1299 cells (Fig. 5g). Western blot results also revealed that circ_0001421 knockdown degraded CDCA3 expression, while this effect could be weakened by miR-4677-3p deficiency in A549 and H1299 cells (Fig. 5h-i). Collectively, miR-4677-3p could bind to CDCA3 and regulate its expression in LC.

Fig. 5 CDCA3 was a target gene of miR-4677-3p in LC. a The predicted binding sites between miR-4677-3p and CDCA3 were shown. b The relationship between miR-4677-3p and CDCA3 in H1299 and A549 cells was analyzed by dual-luciferase reporter assay. c-d The protein of CDCA3 in LC tissues and cells versus that in normal tissues and cells was examined by western blot. e The correlation between miR-4677-3p and CDCA3 expression was appraised in LC tissues through Pearson coefficient analysis. f The correlation between miR-4677-3p and circ_0001421 level was appraised in LC tissues through Pearson coefficient analysis. g The protein of CDCA3 in H1299 and A549 cells transfected with miR-4677-3p or miR-NC was measured by western blot. h-i The protein of CDCA3 in H1299 and A549 cells transfected with si-NC, si-circ_0001421, si-circ_0001421 + anti-miR-NC or si-circ_0001421 + anti-miR-4677-3p was determined by western blot. *P < 0.05.
CDCA3 overexpression reversed the inhibitory effects of circ_0001421 knockdown on LC cell proliferation, migration, invasion and glycolysis

Considering that circ_0001421 could regulate the expression of CDCA3, we then investigated whether circ_0001421 played a role in LC cells by regulating CDCA3. As shown in Fig. 6a, CDCA3 protein expression in A549 and H1299 cells transfected with CDCA3 was significantly increased than the control group. In above data, we demonstrated that circ_0001421 knockdown impeded cell proliferation (Fig. 6b), migration (Fig. 6c) and invasion (Fig. 6d), all of which could be neutralized by overexpression of CDCA3 in A549 and H1299 cells. Meanwhile, CDCA3 overexpression offset the repression of circ_0001421 knockdown on glucose consumption (Fig. 6e), lactate production (Fig. 6f) and the protein of HK2 (Fig. 6g) in A549 and H1299 cells. These results suggested that circ_0001421 could regulate the development of LC cells by regulating CDCA3.

Knockdown of circ_0001421 inhibited cell growth of LC cells in vivo

To further certify the promotion of circ_0001421 in LC development in vivo, the xenograft experiments were...
carried out in nude mice. Our data showed that circ_0001421 was downregulated in A549 cells (Fig. 7a). As demonstrated in Fig. 7b-c, the volume and weight of the tumors in sh-circ_0001421 group were smaller than those tumors in sh-NC group. Meanwhile, qRT-PCR indicated that the expression of circ_0001421 was reduced, while miR-4677-3p expression was increased in tumors of sh-circ_0001421 group (Fig. 7d-e). The protein expression of CDCA3 in sh-circ_0001421 group was markedly reduced (Fig. 7f). In summary, these results identified that inhibition of circ_0001421 restrained LC cell growth in vivo.

Discussion

Despite major breakthroughs in the treatment for LC in recent years, LC still poses a great threat to human health. Many reports demonstrated that abnormal circRNA expression was related to the development of a variety of human cancers, including LC [21, 22]. A previous study claimed that circ_0001421 was up-regulated in NSCLC tissues [12]. In this research, we manifested the high expression of circ_0001421 in LC tissues and cell lines. Furthermore, we explored the functional effects of circ_0001421 and revealed that circ_0001421 deficiency restrained cell proliferation, migration and invasion, and reduced glucose consumption, lactate production and the protein of HK2, suggesting silencing circ_0001421 also inhibited cell glycolysis. The lactate produced during glycolysis could to disrupt the extracellular matrix, leading to the rapid cell proliferation, inhibiting apoptosis, and facilitating cell metastasis [23]. These results elucidated that circ_0001421 might play a role in promoting the development of LC.

Mechanistically, it has been determined that circRNAs functioned as competing endogenous RNAs (ceRNAs) of miRNAs to regulate mRNA expression [24]. For instance, circRNA-002178 served as a ceRNA of miR-34 to promote PDL1 expression in lung adenocarcinoma [25]. Circ-ZKSCAN1 modulated FAM83A expression via sponging miR-330-5p in NSCLC cells [26]. In this paper, miR-4677-3p was a downstream target of circ_0001421. Furthermore, miR-4677-3p expression and role have been mentioned in the study of Han et al., lncRNA LINC02418 elevated proliferation and invasion of NSCLC cells via sponging miR-330-5p in NSCLC cells [27]. As expected, circ_0001421 could negatively modulate miR-4677-3p expression, while interference with miR-4677-3p abrogated the suppressive effects of circ_0001421 knockdown on cell progression, suggesting that miR-4677-3p has an inhibition effect on the development of LC. Taken together, our results confirmed that circ_0001421 contributed to the development of LC by acting as a miR-4677-3p sponge.

The downstream gene of the circ_0001421/miR-4677-3p axis was further explored. CDCA3 was identified as the target of miR-4677-3p by Starbase 3.0 prediction and dual-luciferase reporter assay. A previous report using a microarray data set from 163 tumor types for gene network analysis, demonstrated that CDCA3 was
prominently up-regulated in breast, lung, and ovarian cancers relative to that normal tissues [28]. In line with these results, we revealed that CDCA3 was enhanced in LC tissues and cells. Additionally, we observed that CDCA3 overexpression inverted the inhibition role of circ_0001421 knockdown in LC cell progression. Furthermore, Adams et al. revealed that high expression of circ_0001421 knockdown in LC cell progression. Furthermore, Adams et al. revealed that high expression of CDCA3 was associated with poor prognosis in patients with NSCLC, and interference with CDCA3 inhibited cell proliferation [29]. Consistent with the results, we revealed that CDCA3 was enhanced in circ_0001421 decreased group.

Conclusion
The present study revealed that circ_0001421 participated in cell proliferation, migration, invasion and glycolysis in LC cells via modulating the miR-4677-3p/CDCA3 axis. The results of this study might provide the meaningful inspiration for exploring LC progression.

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