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Research Article

Depression Accelerates Tumor Cell Proliferation Via Regulating Serotonin/miR-144 Axis in NSCLC Mice

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Abstract

Non-small cell lung cancer is known as a malignant tumor with low survival rate and poor prognosis. Depression affects various diseases. However, the effect of depression on the progression of NSCLC remains unclear. In our current study, chronic mild stress (CMS) mice was used as depression animal model. Depression prompted the tumor progression *in vivo* analysis, including increasing tumor indexes and reducing survival rate. Serotonin secretion was observed to be remarkable elevation in both serum and tumor tissue, which was positively related with tumor progression. *In vitro* assays, serotonin promoted the proliferation of A549 cells. Additionally, we observed that miR-144 expression was significantly downregulated in serotonin stimulated group. Further loss-of- and gain-of-function assays verified that miR-144 was the downstream factor of serotonin underlying the condition of CMS. Taken together, our research indicated that CMS-induced serotonin secretion accelerates NSCLC proliferation via inhibiting miR-144 expression, suggesting the potential therapeutic direction in NSCLC patients.

Keywords: Depression; Chronic Mild Stress; miR-144; NSCLC

Introduction

Non-small cell lung cancer (NSCLC) threatens patients with high morbidity and mortality with low 5-year survival [1]. It has been reported that around 85% patients with lung cancer were diagnosed as NSCLC [2], suggesting its urgent need in promising therapeutic treatment. Various risk factors accelerates the progression of NSCLC, promoting its tumor proliferation, including inflammation [3], oxidation [4], and also, the psychiatric factors, such as depression [5,6].

Depression is generally induced by continuous chronic stress. Long-term exposure to chronic stress causally leads to various physio- and pathological changes from the central nervous system to peripheral organs [7,8]. Ample researches have documented positive relationship between depression and the risk of ischemic or coronary heart disease, causally increasing their morbidity and mortality [9]. Depression is also considered as a chronic, inflammation-related disorder, abnormally altering immune system, oxidative reaction and nitrosative stress [10]. In addition, the link between depression and chronic obstructive pulmonary disease has been well addressed [11], emphasizing the critical role of depression in chronic disease. Currently, depression has been gradually considered as the pro-carcinoma factor, which is the most malignant chronic diseases nowadays. Increasing studies have provided an overall view of depression-related carcinoma progression [12-15]. However, there still little research on NSCLC with depression

exist. More efforts are in requirements with this direction. The mechanisms underlying depression-induced disease progression step in the developing level.

Serotonin has been recognized as an important emotion-related factor, participating in various regulation of diseases. Researches have described serotonin as a biomarker and potential target against heart failure [16,17]. Additionally, its regulatory role beyond neurotransmitter has also been discovered. Serotonin attenuates LPS-induced systemic inflammation [18], as well as intestinal inflammation [19]. For carcinoma, serotonin caused a dose-dependent increase in the proliferation of bladder [20] and prostate cancer cells [21]. However, the effect of serotonin on NSCLC remains unclear. Based on these theories, our study was designed to investigate the relationship between depression, serotonin and NSCLC, providing a novel therapeutic direction and strategy against NSCLC.

Materials and Methods

Animals

Animal experimental protocols were consented by the Ethic Committees of Harbin Medical University, and were in accordance with the Guide for the Use and Care of Laboratory Animals published by US National Institutes of Health (NIH Publication No. 85-23, revised 1996). NSCLC tumor-bared mice were established as previous study [22]. In brief, six-week old BALB/c nude mice, which were purchased from the Animal Center of the 2nd Affiliated Hospital of Harbin Medical University (Harbin, China). All mice were housed in a dedicated room (12h dark/light cycle, controlled temperature at 22 ± 1°C, constant humidity at 55 ± 5%) for 1-week acclimatization. According to the experimental design, nude mice bearing A549 tumor xenografts were divided into following groups randomly: controls, CMS, CMS+apocynin, CMS+SSRI, serotonin, serotonin+miR-144, and serotonin+negative control (NC). The volumes of the tumors were measured every week for 3 month.

For CMS animals were obtained as a gift from substance-dependent laboratory of Qiqihar Medical University. As shown in previous study [23], CMS includes limited room restraint, forced warm water bath, water/food deprivation, housing in wet saw dust, and reversed day/night cycle, etc.

Serotonin was treated as previously documented [24]. In brief, serotonin (Sigma-Aldrich Co., Ltd, Louis, MO, USA) was administrated via oral gavage (7.5 mg/kg/day). Apocynin (5 mg/kg/day, Calbiochem, Gibbstown, NJ) was administrated orally, dissolved in drink water [25]. SSRI (10 mL/kg fluoxetine dissolved) was injected intraperitoneally, dissolved in 0.9% saline (0.9% NaCl, pH 7.4) accordingly [26]. Controls received the same volume of dissolved solution.

Cell culture and treatment

NSCLC cell line, A549 cells, was purchased from Shanghai Institutes for Biological Sciences (SIBS, China). Standard culture medium includes the RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA), 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (100 μg/mL). Cells were incubated in 5% CO_2 at 37°C. Before experiments, cells were staffed overnight in FBS-free restriction/treatment medium.

In vitro assays, serotonin (5 μM) and SSRI (10 μM fluoxetine hydrochloride, Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO) according to previous study [27,28].

Cell transfection

A549 cells were seeded into 6-well plate at a density of 2×10^6 cells/well. At the confluence of 80%, cells were starved in serum-free medium overnight before transfection. miR-144, mutated miR-144 (negative control, NC), anti-miR-144 antisense oligoribonucleotide (AMO-144), and AMO-NC (RiboBio Co., Ltd, Guangzhou, China) were transfected into A549 cells as experimental design. For the protocol of transfection, A549 cells were cultured with serum-free medium for 12 h. miRNAs and lipofectamine 2000 (Invitrogen, Carlsbad, USA) were mixed for 5 minutes prior to transfection. Then, the two mixtures were combined and incubated at room temperature for 15 minutes and finally, the mixture was added to A549 cells. The transfection medium was replaced by regular growth medium after 6h transfection. as previous study showed.

Enzyme-linked immunosorbent assay

Serum contents of serotonin (serotonin; BOSTER, Wuhan, China) were determined using an ELISA kit following the manufacturer's instructions.

Western blot

Protein samples (NSCLC tumor tissues) were extracted and dissolved with RIPA buffer (Solarbio, Beijing, China) with protease inhibitors (Sigma, Louis, MO, USA). BCA (Beyotime, Shanghai, China) method was used to quantify the concentration. SDS-PAGE (10% polyacrylamide gels) was used to separate proteins with different molecular weight, and then transferred to nitrocellulose

membrane. Non-fat milk (5%) was used for blocking subsequently, and the membranes were incubated with the primary antibodies for serotonin (ab66047, Abcam, USA) and GAPDH (TA-08, Zhongshan Golden Bridge Biotechnology, Beijing, China), which was used as internal control. After overnight incubation, florescence-labeled secondary antibody was incubated in dark. Odyssey Infrared Imaging System (LI-COR, Lincoln, NB, USA) was used to calculate the relative expression level of serotonin compared with the internal control. which GAPDH was used as internal control.

Real-time PCR

Total RNA was harvested from A549 cells via TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA; Cat. no.4368814). The levels of serotonin and miR-144were determined by SYBR Green I incorporation method and ABI 7500 fast real-time PCR system (Applied Biosystems, USA). GAPDH and U6 were used as internal control, respectively.

Proliferation assay

Proliferation detection of A549 cells was conducted by CCK-8 assay. 100 μL medium containing A549 cells were seeded in a 96-well plate before detection. CCK-8 reagent was added to each well (10 μL/well). Cells were incubated for 4 h in cell-culture circumstance. Then, the optical density (OD) value (450 nm) was determined by an enzyme-linked immunosorbent assay plate reader (Bioreader).

Statistical analysis

All values were presented as mean ± S: E: M: Statistical comparisons were performed by Student's t-test between two groups or one-way ANOVA for multiple comparisons. $p < 0:05$ was considered to indicate a significant difference. Data were analyzed using the GraphPad Prism 7.0 software. Correlations between miR-144 and serotonin were assessed by using Pearson, Spearman, and Kendall's rank correlation coefficient analyses [22].

Results

Depression positively related with malignant ending in NSCLC mice

Depression plays vital roles on the progression of various diseases, promoting the deterioration, and finally leads to a bad end. To detect the influence of depression on NSCLC, we established NSCLC with CMS, and further investigate the criteria of tumor progression, including tumor size, volume, weight, and survival rate. Apocynin is commonly recognized as an anti-depression drug, which we used as the positive control group. The result of Figure 1 shows that CMS remarkably increased the values of tumor size (Figure 1A), volume (Figure 1B), and weight (Figure 1C), reducing the survival rate (Figure 1D) compared non-CMS stimulated controls and Apocynin treated CMS group. These data indicated that depression is positively related with the progression of NSCLC, emphasizing the importance tote further detect the underlying mechanism.

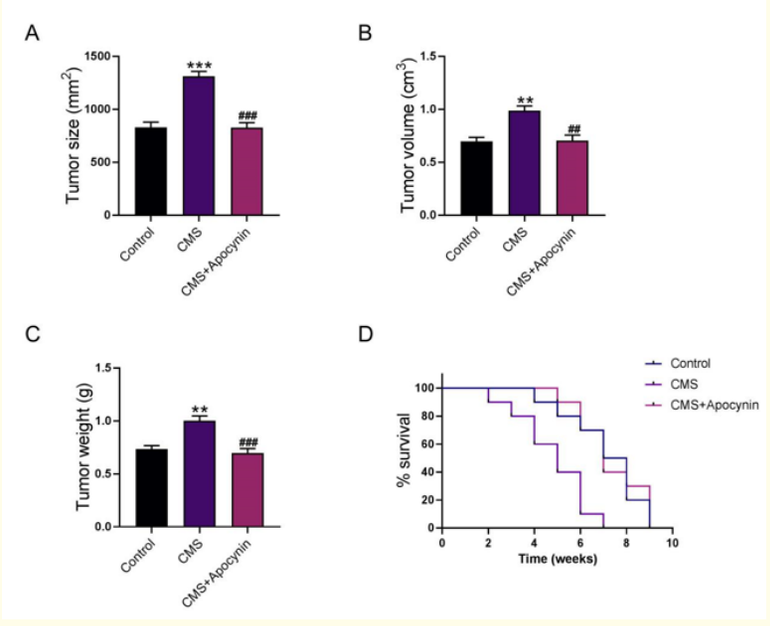

Figure 1: Depression induced by CMS promotes the progression of NSCLC. (A-D) Tumor size, volume, weight and survival rate were continuously recorded and calculated 3 months after animal model establishment. N = 10 in each batches. **p < 0.01, ***p < 0.001. Compared with controls, ##p < 0.01, ###p < 0.001 compared with CMS group.

Serotonin involved in depression-related NSCLC tumor progression

Serotonin is a key regulatory factor on emotion, especially on depression. To gain the insight of the mechanism, we examined the levels of serotonin in serum and tumor tissue homogenate. The results show that serotonin was significantly elevated in CMS group, whereas repressed after Apocynin treatment (Figure 2A-2C). To detect the relationship between serotonin and NSCLC tumor progression, we treated CMS mice with selective serotonin reuptake inhibitor (SSRI). The results show that serotonin inhibition by SSRI ameliorated the condition of NSCLC, including the tumor size (Figure 2D), tumor volume (Figure 2E), tumor weight (Figure 2F), as well as survival rate (Figure 2G). Relationship analysis results show that serotonin is positively related with the progression of NSCLC (Figure 2H-2J), suggesting its regulatory role of NSCLC.

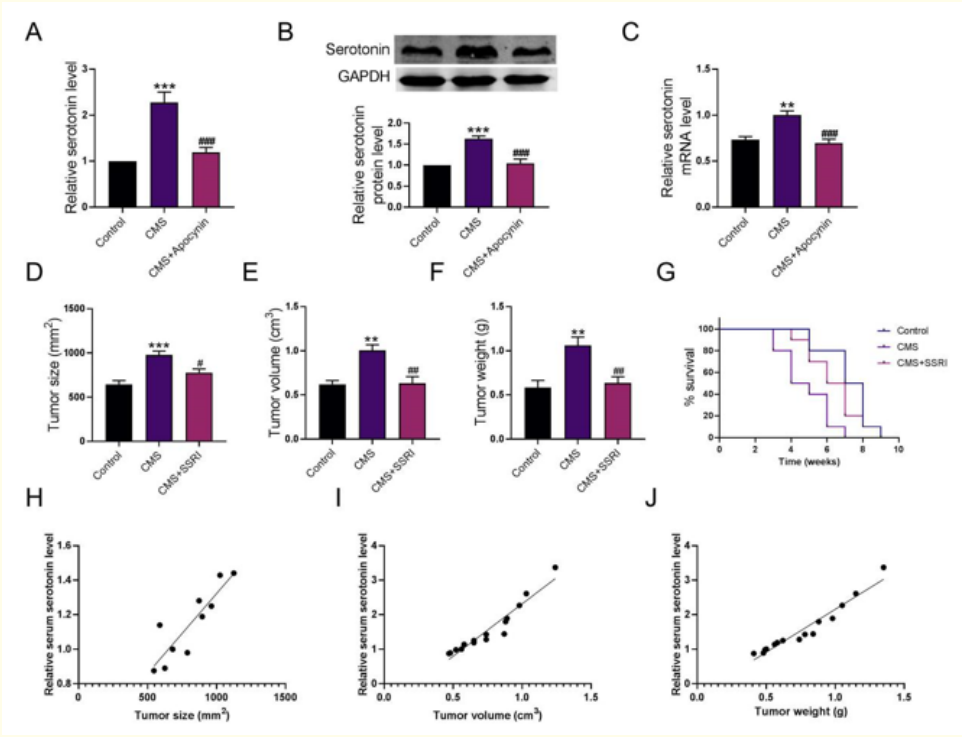

Figure 2: Serotonin is positively related with depression-affricated tumor progression of NSCLC. (A) Serotonin level was examined using ELISA kit in mice serum. n = 5 in each group, ***p < 0.001 compared with control group, ###p < 0.001 compared with CMS group. (B-C) Serotonin levels were detected by real-time PCR and western blot in mice tissue homogenate, respectively. n = 5, **p < 0.01, ***p < 0.001 compared with control group, ###p < 0.001 compared with CMS group. (D-G) Tumor size, volume, weight and survival rate were recorded and calculated in control, CMS and CMS+SSRI groups. n = 5 in each batches, **p < 0.01, ***p < 0.001 compared with control group, #p < 0.05, ##p < 0.01 compared with CMS group. (H-J) Relationship between serum serotonin levels and tumor index (tumor size, volume and weight), X axis represents relative tumor index, and Y axis represents relative serum serotonin levels, n = 10 in each batches.

Serotonin promotes the progression of NSCLC via downregulating miR-144

Previous studies have showed that miR-144 is not only participates in the proliferation of NSCLC cells [29,30], but also regulates depression-related physiological processes [31-33]. Based on these researches, we further verified the underlying mechanism with the involvement of miR-144. A549 cells were used as *in vitro* materials. The proliferation of A549 cells was measured as figure 3A and

3B show. Compared with controls, serotonin stimulates A549 cell proliferation, increasing the cell number (Figure 3A and 3B). Additionally, miR-144 was reduced in serotonin stimulated group, as well as CMS group (Figure 3C and 3D), suggesting the regulatory role of serotonin on miR-144. To further investigating the effect of serotonin/miR-144 on cell proliferation of A549, we used gain- and loss-of-function approaches. The results reveal that miR-144 is the downstream factor of serotonin, participating the regulation of serotonin on cell proliferation of A549 (Figure 3E and 3F).

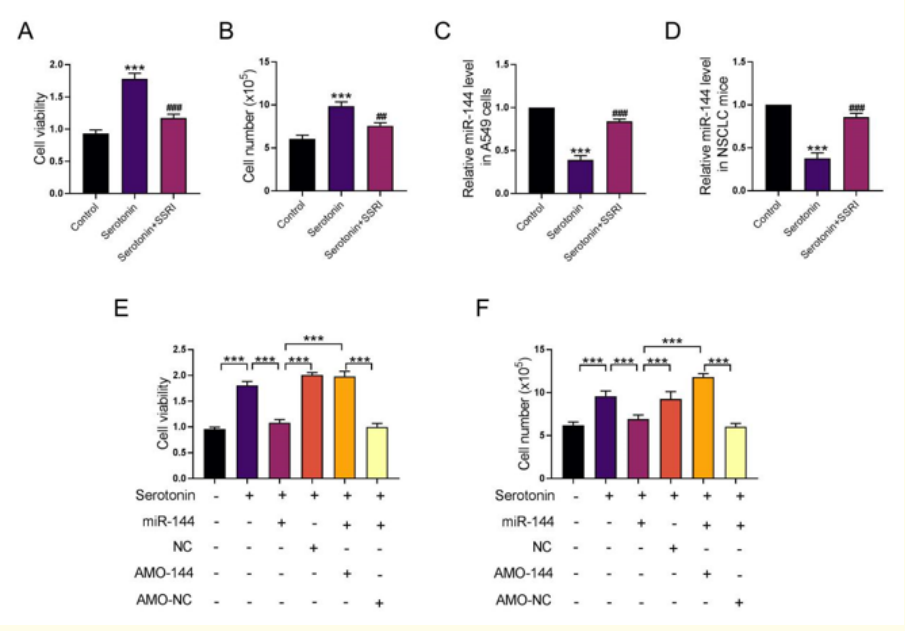

Figure 3: miR-144 inhibits A549 cell proliferation as the downstream factor of serotonin. (A, B) A549 cell viability and number were detected by CCK-8 and trypan blue assay, respectively. N = 5 in each group, **p < 0.01, ***p < 0.001 compared with controls, ##p < 0.01, ###p < 0.001 compared with serotonin group. (C, D) The levels of miR-144 in A549 cells and tumor tissue homogenate were detected by real-time PCR, calibrated by U6 expression. n = 5, ***p < 0.001 compared with controls, ###p < 0.001 compared with serotonin group. (E-F) Cell viability and number were measured by CCK-8 and trypan blue assays in different groups, n = 5 in each batches, ***p < 0.001.

Serotonin/miR-144 axis regulates depression-related tumor progression in NSCLC mice

According to the *in vitro* results, we then verified the effect of serotonin/miR-144 on depression-induced NSCLC aggravation by *in vivo* analysis. A549 cells were transfected with miR-144 mimics, as well as its mutated vectors (NC) before transferred into mice. After serotonin administration, tumor indexes were measured as figure 4A-4D show. Overexpression of miR-144 in A549 mice significantly reduced the tumor size, volume and weight, ameliorating the survival rate (Figure 4A-4D). Similarly, miR-144 shows the beneficial effects on tumor progression in CMS stimulated mice (Figure 4E-4H). These data indicate that serotonin/miR-144 regulates depression-promoted tumor progression of NSCLC, suggesting a potential therapeutic target against NSCLC.

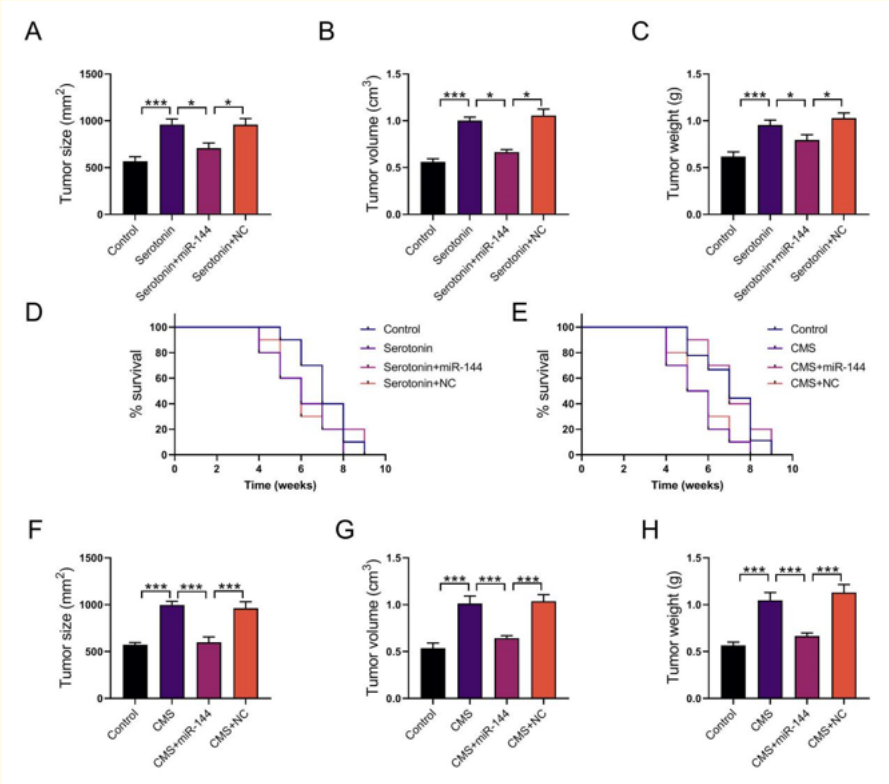

Figure 4: Serotonin/miR-144 ameliorates tumor progression of NSCLC induced by depression. Tumor indexes, including tumor size, volume, weight and survival rate were recorded and calculated in different groups. n = 10 in each group, *p < 0.05, ***p < 0.001.

Discussion

Our research has observed that 1) Depression promotes the progression of NSCLC, and 2) serotonin, at least in part, participates in depression-induced NSCLC aggravation. 3) miR-144 is the downstream effector of serotonin and 4) serotonin/miR-144 axis is the key regulatory pathway, mediating depression accelerated NSCLC cell proliferation. We have analyzed the multiple pathogenic factor of NSCLC, and uncovered the influence of psychiatric factor, as well as the underlying mechanism in this carcinoma. In this case, this study provides not only the discovery in therapeutic strategy and potential targets of NSCLC, but also initiates the cross-disciplinary direction for NSCLC treatment.

Nowadays, lung cancer remains the leading cause of mortality worldwide, especially NSCLC, possessing the most common proportion of cases. Although multiple treatments in development, including such as thoracic surgery, chemotherapy, radiotherapy and targeted drug treatments, the survival rate of 5 year, as well as the prognostic result is still under-satisfied. Tumor cell proliferation contributes to the main malignancy in NSCLC, requiring the effective approach to block. Signalling pathways involved in the proliferation of NSCLC tumor cells includes Wnt pathway [34], NF-κB pathway [35], and E2F2 pathway [36], *et al*. Researches have also highlighted the importance of miRNAs in this process, and discovered the regulatory effect of Notch-1/miR-137, as well as PDCD4/miR-421 axis on NSCLC proliferation [37,38]. Additionally, miR-124 has been reported to exert its beneficial effect on NSCLC [39,40], recognized as a tumor suppressor and prognostic marker. Based on these theories, researches of NSCLC focused on miR-124 are of great value of clinical treatments.

While plenty of the pathogenesis of NSCLC, the psychiatry factor of depression remains an obscure step. Physical uncomfortableness from illness and treatments, as well as organ functional

impairment adversely promote the formation of depression. In turn, depression adversely accelerates the progression of chronic illnesses. The mortality rate in cardiac infarction patients with depression is remarkably elevated compared with normal mental condition patients (26% vs 7%) [41]. Researches have also revealed depression as an independent risk factor of heart failure, threatening the life-span of patients with cardiovascular diseases [42]. Long-term pressure and anxiety from illness, family, and medical cost in NSCLC patients frequently contribute to depression. NSCLC has been identified to be the highest rates of co-morbid depression among all cancer types. Studies focused on NSCLC and depression has showed that NSCLC patients with mutant EGFR is negatively associated with depression [5]. Also, depression is related with worse survival in patients with newly diagnosed NSCLC [43]. Although several researches exist, the underlying reason for this association, together with its mechanism is not entirely clear, raising the requirement for further detection.

Serotonin is a biogenic monoamine, characterized as a neuromodulatory factor regulating neoplastic capabilities. Additionally, it also acts as the local mediator in the gut and vasoactive agent in the blood, exerting its biological effects via interacting with receptors, as well as multiple pathways [44]. The biological underpinning of serotonin on depression is becoming increasingly understood. Serotonin participates in the development of neuronal networks, and its dysfunction thereby contributes to brain disorders. The relationship between serotonin and the pathophysiology of depression has been well reviewed [45], however, the biological relevance, triggers and molecular mechanisms are only beginning to be understood. Previous study has revealed the growth inhibition of prostatic carcinoma by serotonin antagonists [46], and serotonin activates MAP kinase and PI3K/Akt signaling pathways in the progression of prostate cancer [47]. In addition, serotonin has been observed to be involved in of small cell lung carcinoma cells, colonic adenocarcinoma, breast carcinoma, and Bladder carcinoma., *et al*. [44], whereas the relationship between serotonin and NSCLC remains incomprehensive. Thus, our study has initially uncovered this relationship, providing the novel therapeutic direction against NSCLC.

Importance of miRNAs has been recognized in various physiopathological processes. Researches of miRNAs focused on carcinoma have been gradually emphasized. The development of gastric cancer has been reported to be regulated by miR-183 via LncRNA MALAT1/miR-183/SIRT1 axis and PI3K/AKT/mTOR signals [48]. MiR-135a-5p promotes lung cancer progression via targeting LOXL4 [49]. MiR-155/miR-143 axis participates in TGF-β1 promoted colorectal cancer immune escape [50]. The involvement of miR-150/β-catenin axis in colorectal cancer progression has also been identified recently [51]. Among these miRNAs, miR-144 has been considered as a key regulator on cervical cancer [52], gastric cancer [53], colorectal cancer [54], as well as NSCLC. Studies have addressed the inhibitory effects of miR-144 on radiosensitivity of NSCLC via regulating ATF2 [30] and cancer proliferation by targeting CDKL1 [55]. According to previous studies, miR-144 is also tightly related with depression. Expression level of miR-144-5p is significantly downregulated in depressive patients [31], suggesting its potential peripheral biomarker for pathologic processes related to depression. Our current study has verified that miR-144 mediates depression-promoted NSCLC progression via inhibiting cell proliferation, indicating the clinical potential of miR-144 in psychiatric-combined carcinoma treatment.

Conclusion

In this study, we found that depression is positively related with malignancy of NSCLC via elevating serotonin expression. Serotonin/miR-144 axis regulates depression-promoted NSCLC progression via inhibiting cancer cell proliferation property. Future investigations are needed to verify the downstream targets of miR-144 and define the specific serotonin receptor involved in the process. Also, the resource of serotonin and the secretion mechanisms are still in requirement, raising the need of joint contribution cross disciplines.

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Conflict of Interests

The authors declare no conflict of interests.

Data Available

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

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