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Research Article

lncRNA MANCR Inhibits NK Cell Killing Effect on Lung Adenocarcinoma by Targeting miRNA-30d-5p

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Background. NK cells are imperative in spontaneous antitumor response of various cancers. Currently, lncRNAs are considered important modulators of the tumor microenvironment. This study investigated the molecular mechanism by which mitotically associated long noncoding RNA (MANCR) controls killing effect of NK cells on lung adenocarcinoma (LUAD) in the tumor microenvironment. *Methods.* The interplay between MANCR and miRNA-30d-5p was analyzed by bioinformatics. Expression of MANCR mRNA and miRNA-30d-5p was examined using qRT-PCR. Dual-luciferase reporter and RIP assays were utilized to verify the targeted relationship between MANCR and miRNA-30d-5p. To investigate regulation of MANCR/miRNA-30d-5p axis in NK cell killing effect on LUAD cells, western blot tested the protein level of perforin and granzyme B. ELISA determined the level of IFN-γ. CytoTox 96 Non-Radioactive Cytotoxicity Assay kit was applied for cytotoxicity detection of NK cells. Perforin and granzyme B fluorescence intensity was measured via immunofluorescence, and cell apoptosis levels were also revealed via flow cytometry. *Results.* MANCR was found to be upregulated, while miRNA-30d-5p expression was downregulated in LUAD tissues. Overexpression of MANCR in LUAD cells significantly reduced NK cell IFN-γ secretion, expression of granzyme B and perforin, and NK cell killing effect. In addition, MANCR could target and downregulate miRNA-30d-5p expression, and miRNA-30d-5p overexpression reversed the inhibition of NK cell killing effect caused by MANCR overexpression. *Conclusion.* MANCR inhibited the killing effect of NK cells on LUAD via targeting and downregulating miRNA-30d-5p and provided new ideas for antitumor therapy based on tumor microenvironment.

1. Introduction

Comprising about 40% of all lung cancer cases, LUAD is the most frequent type of lung cancer [1]. Despite great advances in diagnostic and therapeutic approaches over the last decades, the 5-year survival rate for LUAD is only 15% [2]. Hence, it is important to develop new treatments for LUAD to improve life quality of patients. Studies have indicated that tumor cells can perform various interactions in tumor microenvironment through cytokines, chemokines, and immune cells, thereby regulating tumor progression [3]. NK cells are important for tumor microenvironment and are considered the first line of

defense against tumors and viral infections because of their cytotoxic and regulatory functions [4, 5]. In recent years, the key role of NK cells in cancer immune monitoring has been recognized. NK cells are able to promote antitumor immunity by direct elimination of malignant cells [6], and activated NK cells can secrete inflammatory cytokines like TNF-α and IFN-γ and stimulate antigen-presenting cells, ultimately forming tumor-specific adaptive immune responses [7, 8]. NK cells are involved in spontaneous antitumor responses in cancers, such as gastric cancer, liver cancer, and breast cancer [9–11]. Additionally, tumor infiltration of NK cells is linked with long-term survival of cancer patients, and high NK cell toxicity

is associated with a reduction in cancer risk [12]. Therefore, exploring the mechanism of NK cells in patients with LUAD may be a new direction to seek novel strategies for LUAD treatment.

Long noncoding RNAs (lncRNAs) participate in biological processes like cell growth, apoptosis, differentiation, angiogenesis, migration, and invasion as tumor suppressor genes or oncogenes in varying cancers [13–15]. Previous reports have implied the crucial role of lncRNAs in NK cell responses. For example, lncRNA GAS5 enhances NK cell killing effect on liver cancer by modulating miRNA-544/RUNX3 [16]. Further studies by Wei et al. found that GAS5 could also promote killing effect of NK cells on gastric cancer by regulating miRNA-18a [17]. It can be seen that lncRNAs are able to regulate NK cell-mediated tumor cell killing process. As a novel lncRNA, MANCR plays a cancer-promoting role in progression of tumors, including mantle cell lymphoma, breast cancer, and gastric cancer [18–20]. As reported by Liu et al. [21], lncRNA MANCR facilitates the malignant progression of LUAD cells. Nevertheless, the modulatory role of MANCR on NK cell function in LUAD remains unclear so far.

Aberrant expression of miRNAs is involved in pathogenesis of multiple diseases including cancer. Available studies have shown that miRNA-30d-5p is generally downregulated in lung tumors and exerts cancer suppression effect [22]. For example, Chen et al. reported that miRNA-30d-5p repressed malignant progression of lung cancer by targeting CCNE2 [23]. miRNA-30d-5p can target and downregulate DBF4 to suppress migration, proliferation, invasion, and epithelial-mesenchymal transition of lung squamous cell carcinoma cells [24]. In addition, the functions of miRNAs are regulated by a variety of competitive endogenous RNAs including lncRNAs [25]. In thyroid cancer, LINC00284 binds miRNA-30d-5p to activate ADAM12-dependent Notch signaling pathway, thus accelerating tumor development [26]. Similarly, Zeng et al. discovered that miRNA-30d-5p was negatively mediated by lncRNA POU3F3 in LUAD cells, leading to increased proliferation, migration, and invasion of cells [27]. Notably, studies have found that miRNAs can be involved in regulating tumor immunosuppression through NK cells. For example, Tang's study demonstrated that miRNA-20a knockout increased the sensitivity of colorectal cancer cells to NK cells [28]. Another report indicated that miRNA-140-3p inhibited NK cell killing effect on ovarian cancer cells by mediating MAPK1 [29]. The previous results of this study revealed a targeted relationship between MANCR and miRNA-30d-5p, but how the MANCR/miRNA-30d-5p axis affects the cytotoxicity of NK cells is not clear.

By summarizing the results of previous studies and bioinformatics analysis, we performed a set of cell function assays and confirmed that MANCR inhibited NK cell killing effect on LUAD cells by targeting and downregulating miRNA-30d-5p. The MANCR/miRNA-30d-5p axis may serve as a promising target in NK cell-based therapy for LUAD.

2. Material and Methods

2.1. Bioinformatics Analysis.
MANCR expression level analysis was performed using TCGA-LUAD database. Subse-quently, the patients were grouped based on the median value of MANCR expression, and KEGG pathway enrichment analysis was performed for MANCR using GSEA software. For upstream miRNA prediction, miRNA expression profiling data were acquired from TCGA-LUAD database. Differentially expressed miRNAs were obtained by differential analysis using the edgeR package [30]. miRNAs with interactions with MANCR were predicted using the lncBase database, and the prediction results of lncBase and differential miRNAs were intersected to select miRNAs with low expression in LUAD. The correlation between miRNA expression and MANCR was subsequently calculated to confirm miRNAs that may have a regulatory relationship with MANCR.

2.2. Cell Culture and Cell Transfection.
Human normal bronchial epithelial cells 16HBE (BNCC338044), human LUAD cell lines A549 (BNCC337696), H1975 (BNCC340345), H1299 (BNCC100859), and HCC827 (BNCC342007) were all purchased from the BeNa Culture Collection (China), and cells were maintained in DMEM with 10% fetal bovine serum (FBS). All cell cultures were performed under standard conditions.

Human NK cell line NK92 (PTA-6967) was offered by ATCC (USA) and cultured with α-MEM containing 1.5 g/L sodium bicarbonate, 12.5% horse serum, 12.5% FBS, and 2 mM L-glutamine at 37°C with 5% CO_2. 100 U/mL of IL-2 (BD Biosciences, USA) was utilized to stimulate NK92 cells for 24 h [16].

The oe-MANCR, si-MANCR, miRNA-30d-5p mimic (miR-mimic), and their negative controls (oe-NC, si-NC, and mimic-NC) were all purchased from Invitrogen (USA). Lipofectamine 2000 kit (Thermo Fisher, USA) was applied to transfect the above-mentioned plasmids into H1299 or HCC827 cells. After 24 h, the transfected cells were utilized for subsequent experiment.

2.3. Cell Coculture.
H1299 and HCC827 cells were diluted to 1.5×10^5 cells/mL in DMEM with 10% FBS. NK92 cells activated by IL-2 were cocultured with H1299 or HCC827 cells treated with different transfections for 4 h at 37°C at a 10:1 effector/target (E:T) ratio. Culture supernatants were then collected for subsequent assays.

2.4. Cytotoxicity Assay.
CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, USA) was applied for cytotoxicity detection of NK cells. Briefly, IL-2-activated NK92 cells were cocultured with differently transfected H1299 or HCC827 cells at a 10:1 E:T ratio in 96-well plates for 4 h at 37°C, supplemented with 10 μL 10× lysate 45 min prior to addition of CytoTox 96 reagent. Later, 50 μL of CytoTox 96 reagent was supplemented to each well. Next, 50 μL of stop buffer was added after 30 min of incubation at room temperature in the dark, and absorbance at 490 nm was measured within 1 h. The cytotoxicity percentage was calculated with the formula: Percent cytotoxicity = 100 × Experimental LDH Release (OD490)/Maximum LDH Release (OD490).

2.5. ELISA. Supernatants were collected from IL-2-activated NK92 cells after coculture with H1299 or HCC827 cells treated for 4 h with different transfections, and the level of IFN-γ was measured using an IFN-γ human ELISA kit (Invitrogen, USA). Absorbance at 450 nm was measured using an iMark™ Microplate Absorbance Reader (Bio-Rad, USA).

2.6. Immunofluorescence Detection. Cells were fixed with 4% formalin for 8 h at 4°C, embedded in paraffin blocks, and cut into sections (3 μm), which were mounted on glass slides. The slides were then permeabilized in 0.2-0.5% Triton X-100 and blocked in 5% normal donkey serum for 1 h at room temperature. Then, they were cultured overnight with anti-perforin (sc-373943, Santa Cruz, USA) and antigranzyme B (ab225473, Abcam, UK) antibodies, followed by culture with DAPI and fluorescently conjugated goat antimouse/rabbit IgG HampL. Finally, the slides were fixed and photographed for observation [31].

2.7. Apoptosis Detection. The treated cells were gathered and washed with prechilled PBS. 1×10^6 cells were dyed with 500 μL binding buffer, 5 μL FITC-labeled Annexin V, and 5 μL PI solution and cultured for 10 min in the dark. Cell apoptosis was evaluated using a FACSCalibur flow cytometer system (BD Biosciences, USA) [32].

2.8. qRT-PCR. RNA extraction and PCR analysis were performed according to the steps described by Fang et al. [16]. GAPDH was an internal reference for relative quantification of total RNA in cells, and PowerUp™ SYBR™ Green Master Mix (Invitrogen, USA) was introduced for qRT-PCR analysis. GAPDH and U6 were internal reference genes for detecting lncRNA MANCR and miRNA-30d-5p expression, respectively. The relative gene expression was calculated using $2^{-\Delta\Delta Ct}$ method (primers refer to Table 1).

2.9. Western Blot. Total proteins were extracted using RIPA Lysis Buffer (Thermo Fisher, USA), and equal amounts of proteins were separated by 10% SDS-AGE, which were then transferred to PVDF membranes, blocked with 5% skimmed milk, and cultured with primary antibodies overnight at 4°C. Subsequently, membranes were probed with secondary antibody for 1 h at room temperature. Finally, the immunoblot results were visualized using Bio-Rad ChemiDoc XRS system. The antibodies were antigranzyme B (ab243879, Abcam, UK), antiperforin (ab256453, Abcam, UK), anti-GAPDH (ab181602, Abcam, UK), and goat antirabbit IgG H&L (HRP) (ab6721, Abcam, UK).

2.10. Dual-Luciferase Assay. PmirGLO-MANCR-3′-UTR-WT and pmirGLO-MANCR-3′-UTR-MUT luciferase reporter vectors (Promega, USA) were first constructed. Then miRNA-30d-5p mimic/mimic-NC and MANCR-WT/MANCR-MUT plasmids were, respectively, cotransfected into H1299 cells for 48-h culture. The luciferase activity was detected using the luciferase activity assay kit (Promega, USA).

TABLE 1: Primers of qRT-PCR.

Gene	Primer sequence (5′→3′)
MANCR	F: CAATACCACAATTGCAATC
	R: CATGTTCTTCCTCATATGGA
miR-30d-5p	F: CCTGTTGGTGCACTTCCTAC
	R: TGCAGTAGTTCTCCAGCTGC
U6	F: ATGACGTCTGCCTTGGAGAAC
	R: TCAGTGTGCTACGGAGTTCAG
GAPDH	F: GAGTCAACGGATTTGGTCGT
	R: TTGATTTTGGAGGGATCTCG

2.11. RIP Assay. Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) was introduced for RIP assay. After washing H1299 cells with prechilled PBS, the supernatant was discarded, and 100 μL of RIP lysis buffer containing 0.25 μL RNase inhibitor and 0.5 μL protease inhibitor was added to lyse the cells on ice. The supernatant was discarded after centrifugation. Cells were incubated with RIP buffer containing magnetic bead-protein antibody complex and anti-AGO2 antibody (Invitrogen, USA) or negative control IgG (Invitrogen, USA) for coprecipitation. After obtaining RNA-binding protein complexes, the relative enrichment of MANCR in the precipitate was examined by qRT-PCR.

2.12. Statistical Analysis. All quantification experiments were carried out in triplicate. Data were expressed as mean ± SD and statistically analyzed by GraphPad Prism 6 software (GraphPad Software, USA). Differences were compared by t-test or one-way analysis of variance, *, # representing $P < 0.05$, indicating statistical significance.

3. Results

3.1. MANCR Expression Is Upregulated in LUAD. Previous analysis based on TCGA-LUAD database in this study revealed that MANCR expression was significantly upregulated in LUAD tissues (Figure 1(a)). qRT-PCR result showed that MANCR was notably highly expressed in different LUAD cell lines compared to human normal bronchial epithelial cells 16HBE (Figure 1(b)). Among LUAD cell lines, MANCR expression was relatively low in H1299 and HCC827 cells. These two cells were therefore chosen for subsequent experiments. MANCR was enriched in NK cell-mediated cytotoxic pathways as revealed by GSEA pathway enrichment analysis (Figure 1(c)). Thus, we hypothesized that MANCR may promote LUAD development by inhibiting killing effect of NK cells. For subsequent analyses, we cultured NK92 cells and activated them using IL2. ELISA result showed that IFN-γ secretion was significantly increased in the supernatant of NK92 cells from the IL-2-treated group versus the control group (Figure 1(d)). In summary, MANCR was remarkably upregulated in LUAD and may affect the cytotoxicity of NK cells.
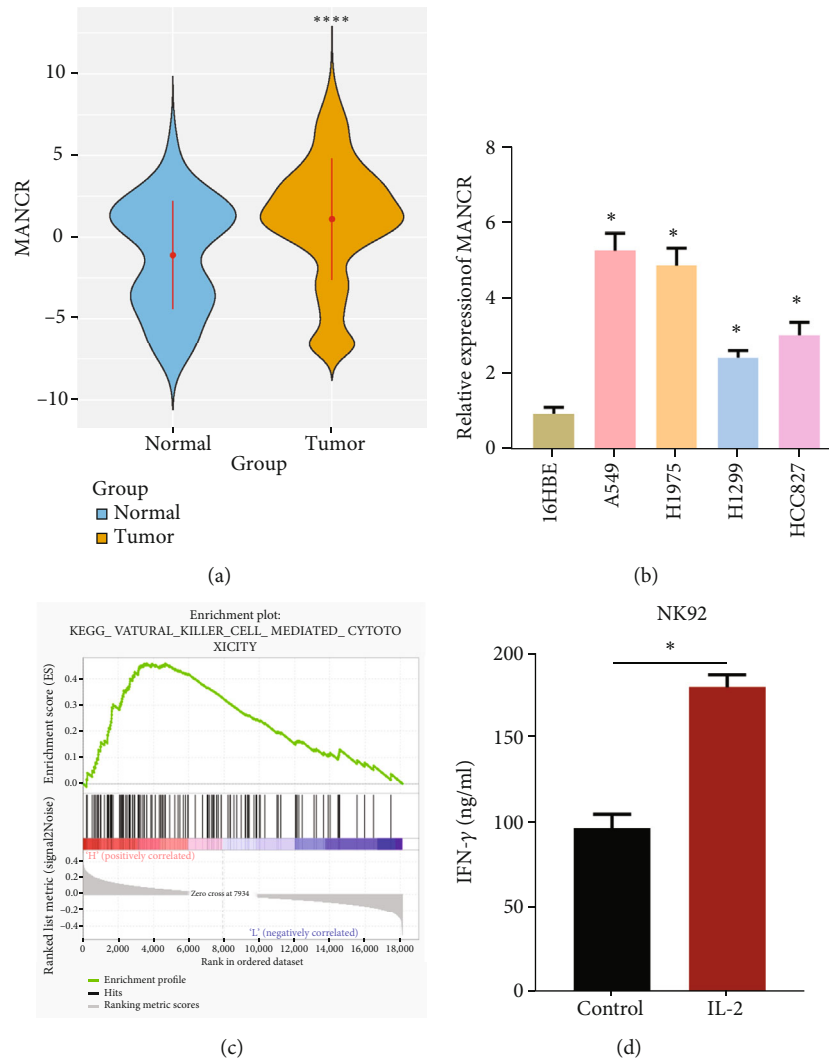
(a)

(b)

(c)

(d)

FIGURE 1: MANCR is upregulated in LUAD. (a) Analysis of MANCR expression using TCGA-LUAD database, blue indicating normal tissue samples and yellow indicating tumor tissue samples; (b) the expression of MANCR in different cell lines; (c) the GSEA pathway enrichment analysis result of MANCR; (d) the concentration of IFN-γ in NK92 cells before and after IL-2 stimulation; $*$ represents $P < 0.05$.

3.2. MANCR Inhibits the Killing Effect of NK Cells on LUAD Cells. H1299 and HCC827 cells were first transfected with oe-MANCR and oe-NC, and the transfection efficiency was examined via qRT-PCR (Figure 2(a)), followed by coculture of treated cells with IL-2-activated NK92 cells. ELISA result showed that IFN-γ content in cell supernatant was reduced in oe-MANCR group, which suggested that MANCR reduced killing effect of NK cells on tumor cells (Figure 2(b)). Further examination displayed that cytotoxicity of NK92 cells against LUAD cell lines HCC827 and H1299 was significantly reduced after MANCR overexpression, compared with the control group (Figure 2(c)). Granzyme B and perforin are cytotoxic molecules manipulated by NK cells to induce cell apoptosis [33]. Western blot result exhibited that granzyme B and perforin expression levels were prominently reduced in NK cells of oe-MANCR group (Figure 2(d)). Immunofluorescence experiment yielded the reduced fluorescence intensities of perforin and granzyme B in NK cells of oe-MANCR group in comparison with the

control group (Figure 2(e)). Tumor cell apoptosis level was subsequently evaluated by flow cytometry. It was observed that the apoptotic rates of LUAD cell lines HCC827 and H1299 were reduced in the coculture system after MANCR overexpression (Figure 2(f)). The above results showed that MANCR significantly weakened the killing effect of NK cells on LUAD cells.

3.3. MANCR Targets and Regulates the Expression of miRNA-30d-5p. To further determine how MANCR regulates NK cell killing, we screened the miRNAs with the highest correlation with MANCR expression through bioinformatics databases. The result demonstrated that miRNA-30d-5p expression was significantly negatively correlated with MANCR expression (Figure 3(a)), and miRNA-30d-5p was remarkably downregulated in LUAD tissues (Figure 3(b)). In addition, MANCR was predicted to have potential binding sites targeting miRNA-30d-5p based on bioinformatics methods (Figure 3(c)). H1299 cells
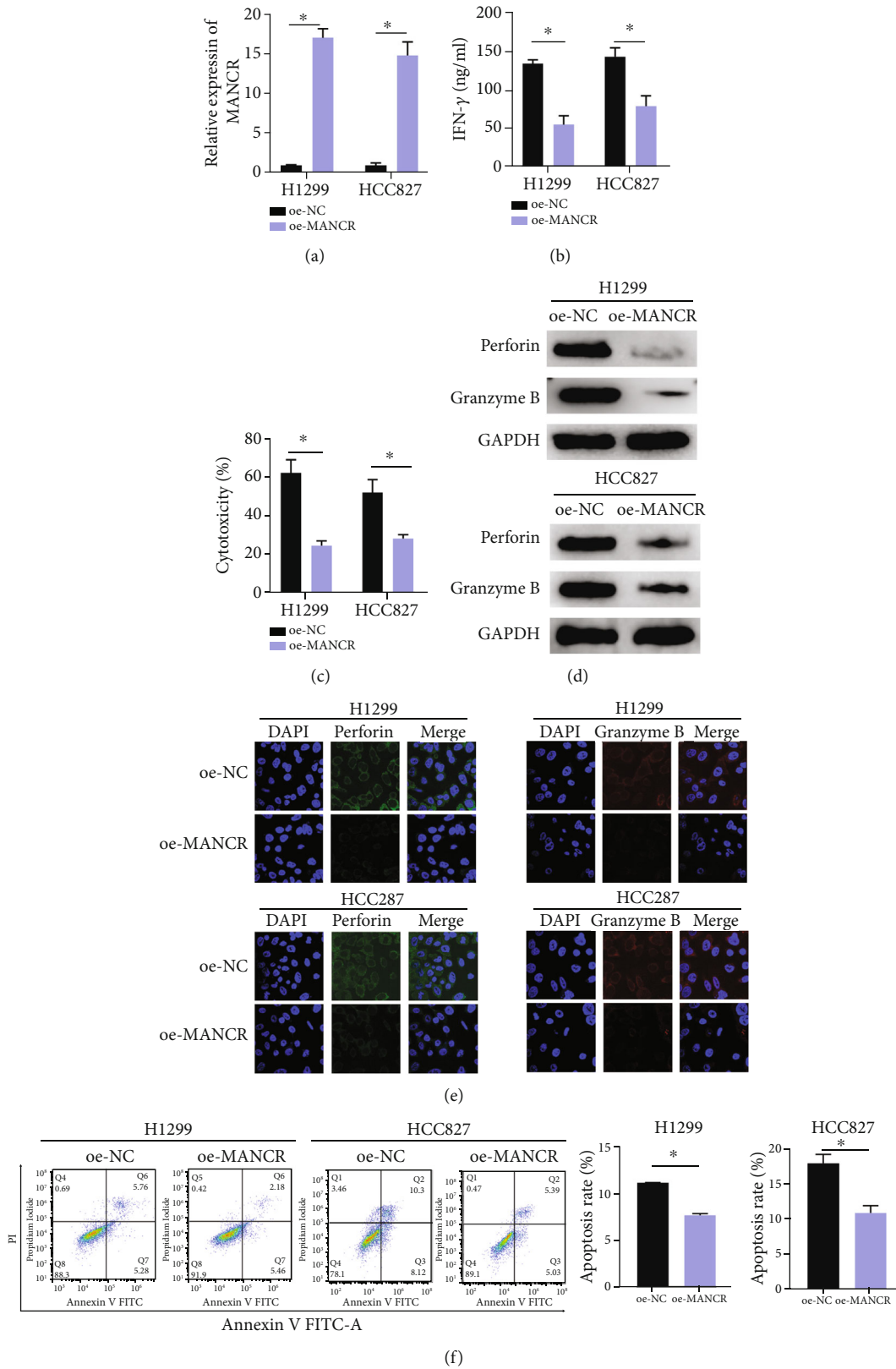
(a)

(b)

(c)

(d)

(e)

(f)

FIGURE 2: MANCR suppresses the killing effect of NK cells on LUAD cells. (a) Detection of transfection efficiency of oe-MANCR in LUAD cells; (b) the IFN-γ secretion level in supernatant after coculture of IL-2-activated NK92 cells with LUAD cells; (c) NK cell cytotoxicity; (d) the protein level of perforin and granzyme B after coculture of NK92 cells with LUAD cells of different treatments; (e) the fluorescence intensity of perforin and granzyme B after coculture of NK92 cells with LUAD cells of different treatments; (f) the apoptosis level of LUAD cells; ∗ represents $P < 0.05$.

Cor=0.29 (pvalue=4.92e-11)

(a)

(b)

MANCR -WT   3' - CATTTTTAGGGGGGCTGAAGGGT -5'

hsa-miR-30d-5p   3' - TGTAAACATCCCCGACTGGAAG - 5'

MANCR -MUT   3' - CATAAATAGCCCCGACTAGGGT - 5'
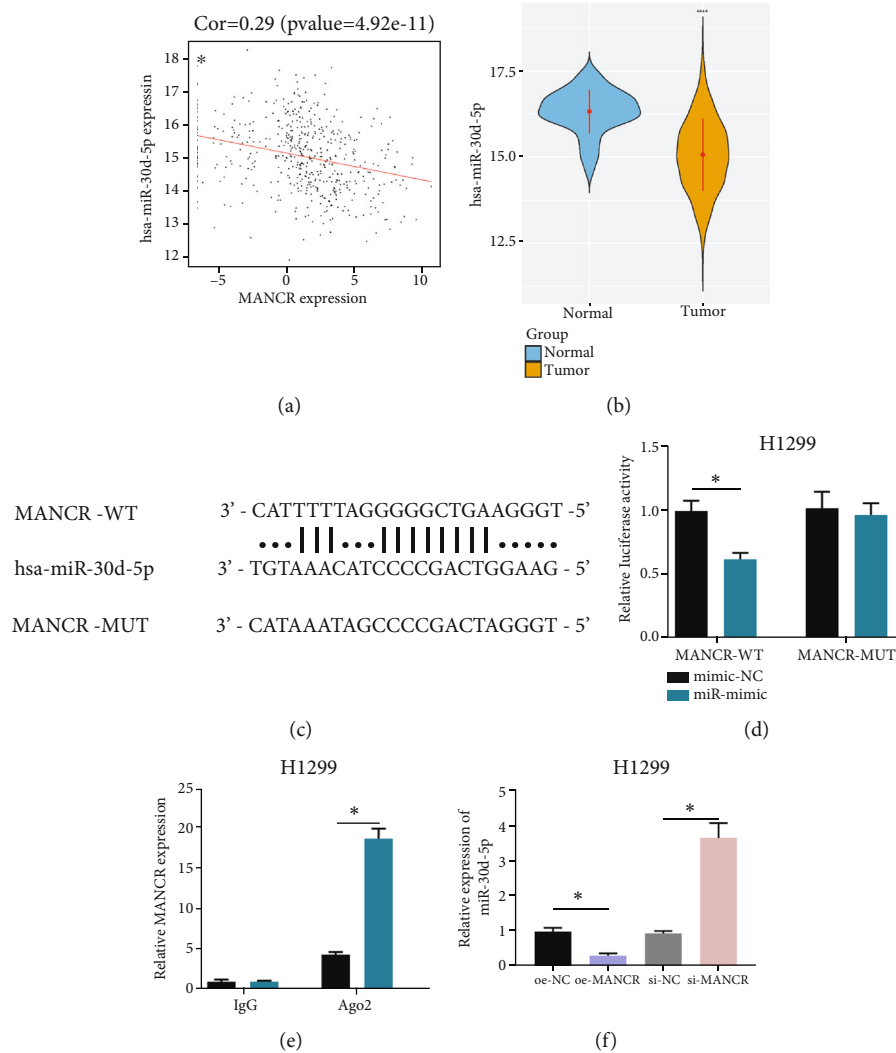
(c)

(d)

(e)

(f)

Figure 3: MANCR targets and regulates the expression of miRNA-30d-5p. (a) Pearson correlation analysis diagram of MANCR and miRNA-30d-5p; (b) analysis of miRNA-30d-5p expression using TCGA-LUAD database. Blue indicates normal tissue samples, and yellow indicates tumor tissue samples; (c) schematic diagram of binding sites between MANCR and miRNA-30d-5p predicted by bioinformatics; (d and e) verification of the targeted relationship between MANCR and miRNA-30d-5p; (f) miRNA-30d-5p expression in different treatment groups; ∗ represents $P < 0.05$.

were selected for subsequent molecular experiments to verify correlation between MANCR and miRNA-30d-5p. As suggested by dual-luciferase reporter assay, overexpression of miRNA-30d-5p reduced wild-type MANCR 3′-UTR luciferase activity, but had no prominent effect on mutant MANCR 3′-UTR luciferase activity (Figure 3(d)). The binding relationship between MANCR and miRNA-30d-5p was then further verified using RIP assay, and the result showed remarkable MANCR enrichment in miRNA-30d-5p overexpressed cells (Figure 3(e)). Additionally, qRT-PCR result showed that overexpression of MANCR evidently reduced miRNA-30d-5p expression, while silencing MANCR could increase miRNA-30d-5p expression (Figure 3(f)), which illustrated that MANCR targeted and negatively regulated the expression of miRNA-30d-5p.

3.4. MANCR Inhibits Killing Effect of NK Cells through miRNA-30d-5p. To confirm that MANCR can inhibit killing

effect of NK cells through miRNA-30d-5p, we set up 4 different treatment groups and performed subsequent experiments: oe-NC + mimic-NC, oe-MANCR+mimic-NC, oe-NC + miR-mimic, and oe-MANCR+miR-mimic. qRT-PCR suggested that miRNA-30d-5p was prominently upregulated after miRNA-30d-5p overexpression, indicating a good transfection efficiency. Meanwhile, miRNA-30d-5p expression in LUAD cells returned to the level of control group after overexpression of MANCR (Figure 4(a)). The result of ELISA showed that miRNA-30d-5p overexpression in LUAD cells restored the inhibitory effect of MANCR overexpression on IFN-γ secretion from NK cells (Figure 4(b)). Later, NK cell cytotoxicity was examined after coculture of IL-2-activated NK92 cells and LUAD cells treated with different transfections. Compared with the control group, NK92 cells presented remarkably increased cytotoxicity against miRNA-30d-5p overexpressed H1299 and HCC827 cells, while overexpression of MANCR reversed the
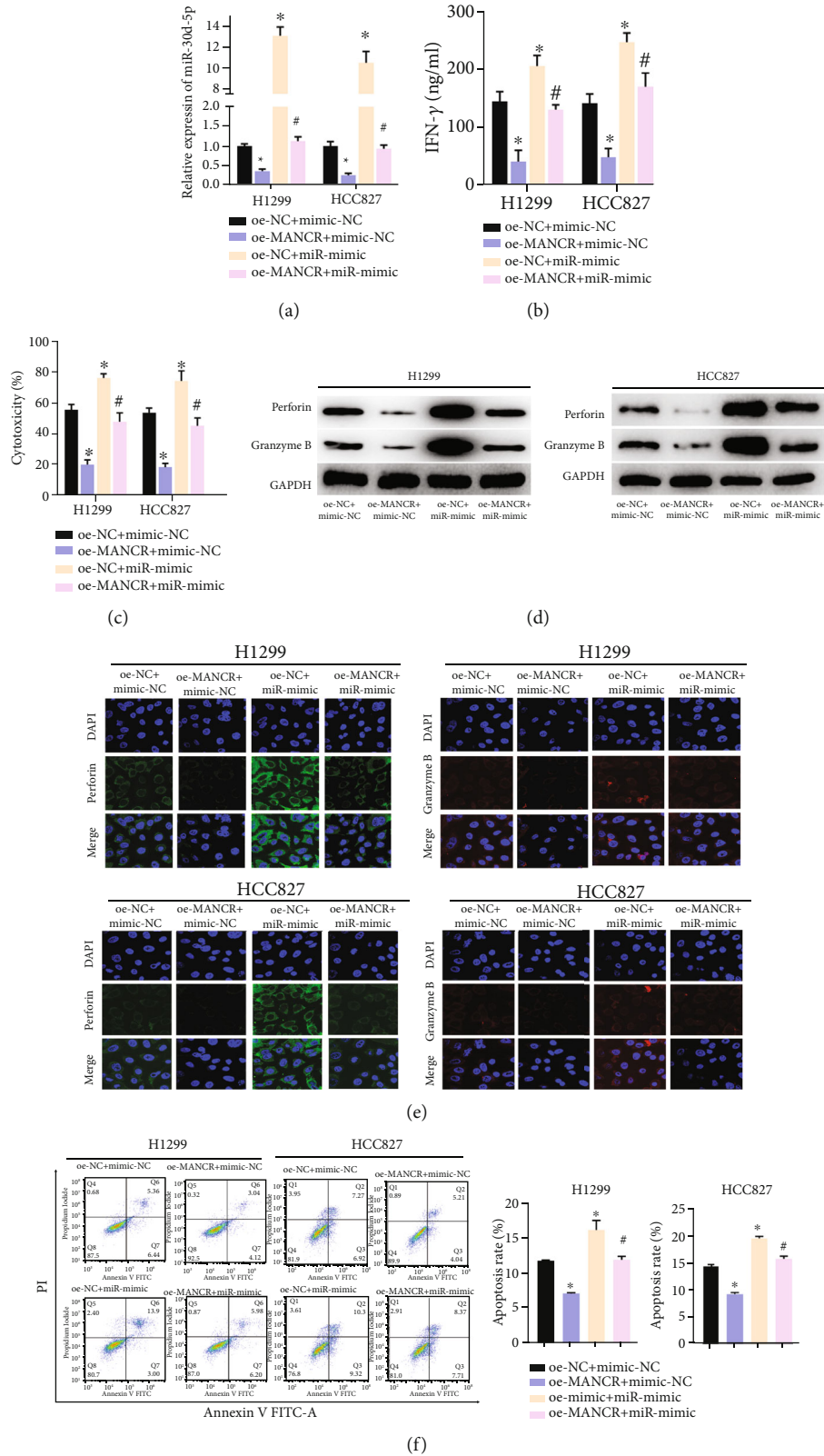
FIGURE 4: MANCR inhibits the killing effect of NK cells by miRNA-30d-5p. (a) Detection of the transfection efficiency of miRNA-30d-5p in LUAD cells; (b) the IFN-γ secretion level in supernatant after coculture of IL-2-activated NK92 cells and LUAD cells; (c) NK cytotoxicity; (d) the protein expression of perforin and granzyme B after coculture of NK92 cells and LUAD cells with different treatments; (e) the fluorescence intensities of perforin and granzyme B after coculture of NK92 cells and LUAD cells with different treatments; (f) the level of apoptosis of LUAD cells; $^*P < 0.05$ (vs. oe-NC + mimic-NC) and $^\#P < 0.05$ (vs. oe-MANCR+mimic-NC) indicate significant statistical differences.

enhancement of NK cell cytotoxicity induced by miRNA-30d-5p (Figure 4(c)). Western blot result exhibited that the protein level of granzyme B and perforin in NK cells of the oe-NC + miR-mimic group was increased over the oe-NC + mimic-NC control group, and their expression in NK cells of oe-MANCR+miR-mimic group returned to the level of the control group, indicating that MANCR overexpression reversed the promoting effect of miRNA-30d-5p (Figure 4(d)). Immunofluorescence experiment revealed that the fluorescence intensities of granzyme B and perforin in NK cells in the oe-NC + miR-mimic group were increased compared with the oe-NC + mimic-NC control group, which in the oe-MANCR+miR-mimic group returned to the level of the control group (Figure 4(e)). Further, the apoptosis of LUAD cells with different treatments was detected by flow cytometry. The apoptosis rates of H1299 and HCC827 cells in the oe-NC + miR-mimic group were significantly increased, which were remarkably decreased in the oe-MANCR+miR-mimic group compared with those in the oe-NC + miR-mimic group and returned to the level of the control group, indicating that MANCR overexpression reversed the apoptosis-promoting effect of miRNA-30d-5p on LUAD cells (Figure 4(f)). The above data indicated that MANCR restrained the killing effect of NK cells on LUAD cells by modulating miRNA-30d-5p.

4. Discussion

Numerous studies implied that improving immune function and inhibiting immune escape can inhibit malignant progression of LUAD [34–36]. NK cells take a crucial part in the prognosis of tumors because they can induce immune responses after cancer treatment [37]. In addition, NK cells dissolve target cells by releasing cytotoxic granules containing granzymes and perforin, which mediate contact-dependent killing of target cells [38]. In recent years, researchers have begun to focus on the application of NK cell immunotherapy in LUAD treatment. Song et al. revealed that overexpression of PTPRN promoted LUAD metastasis and inhibited NK cell cytotoxicity [39]. In the current study, we found that MANCR gene upregulation in LUAD cells could repress the killing ability of NK cells, which provided support for developing novel immune therapeutic strategies for LUAD patients.

In LUAD, aberrant expression of lncRNAs is involved in regulating proliferation, invasion, migration, and tumor immunity of LUAD cells [40, 41]. MANCR is an lncRNA associated with mitosis [19]. Recent studies have suggested that MANCR is highly expressed in LUAD and can enhance proliferation, migration, and invasion of LUAD cells and reduce apoptosis [21]. In this work, MANCR was significantly upregulated in LUAD, which may be a potential molecular target for LUAD treatment. As reported, NK cell-mediated tumor cell killing effect is strengthened in the context of molecularly targeted therapies [42]. However, the effect of MANCR on NK cell tumor killing has not been elucidated. Here, we discovered that overexpression of MANCR significantly reduced secretion level of cytotoxic factor IFN-γ and protein expression of granzyme B and per-

forin in NK cells, and upregulation of MANCR prominently repressed killing effect of NK cells on LUAD cells, confirming that MANCR can be used as a potential target for NK cell-based immunotherapy of LUAD.

Several studies have stated that lncRNAs can be widely involved in the modulation of NK cytotoxicity in a variety of cancers by specifically binding to the corresponding miRNAs [43, 44]. In this study, we analyzed and predicted by bioinformatics means that there were potential binding sites between MANCR and miRNA-30d-5p. miRNA-30d-5p has been elucidated as a potential biomarker for the treatment of various cancers, including LUAD, prostate cancer, and gallbladder cancer [45–47]. It has been shown that silencing miRNA-30d-5p expression can reduce the percentage of CD8$^+$ T cells, which leads to immune escape of prostate cancer [48]. However, the mechanism by which miRNA-30d-5p regulates NK cell function remains unclear. Therefore, we hypothesized that the MANCR/miRNA-30d-5p axis could affect NK cell killing effect on LUAD. Based on the hypothesis, this study found that MANCR negatively regulated the expression of miRNA-30d-5p. More importantly, upregulation of miRNA-30d-5p expression reversed the inhibitory effect induced by MANCR overexpression on cytotoxicity of activated NK cells. These conclusions provide a molecular mechanism by which the MANCR/miRNA-30d-5p axis acts on NK cell killing effect.

In conclusion, this study elucidated the effect of the MANCR/miRNA-30d-5p axis on NK cell cytotoxicity against LUAD. Upregulation of MANCR expression in LUAD cells decreased IFN-γ secretion, granzyme B, and perforin expression in NK cells and cytotoxicity of NK cells by negatively regulating miRNA-30d-5p expression. However, there are also some limitations, such as the failure to validate the suppressive effect of MANCR on NK cell killing in LUAD at the animal level. The results of this study indicate that the MANCR/miRNA-30d-5p axis may be a potential target for immunotherapy of LUAD, providing new ideas for developing NK cell-based anticancer drugs.

Data Availability

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

Ethical Approval

No animal or human cell was used.

Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, or publication of this article.

Authors' Contributions

(I) Conception and design were contributed by Yunping Lu.
(II) Administrative support was contributed by Xiao Rao

References

[1] T. V. Denisenko, I. N. Budkevich, and B. Zhivotovsky, "Cell death-based treatment of lung adenocarcinoma," *Cell Death & Disease*, vol. 9, no. 2, p. 117, 2018.

[2] J. Minna and J. Schiller, "Lung Cancer," in *Harrison's Principles of Internal Medicine*, pp. 551–562, McGraw-Hill Professional, 2008.

[3] S. D. Soysal, A. Tzankov, and S. E. Muenst, "Role of the tumor microenvironment in breast cancer," *Pathobiology*, vol. 82, pp. 142–152, 2015.

[4] A. Jewett, J. Kos, Y. Fong et al., "NK cells shape pancreatic and oral tumor microenvironments; role in inhibition of tumor growth and metastasis," *Seminars in Cancer Biology*, vol. 53, pp. 178–188, 2018.

[5] C. Sun, H. Y. Sun, W. H. Xiao, C. Zhang, and Z. G. Tian, "Natural killer cell dysfunction in hepatocellular carcinoma and NK cell-based immunotherapy," *Acta Pharmacologica Sinica*, vol. 36, no. 10, pp. 1191–1199, 2015.

[6] C. Zhang, P. Oberoi, S. Oelsner et al., "Chimeric antigen receptor-engineered NK-92 cells: an off-the-shelf cellular therapeutic for targeted elimination of cancer cells and induction of protective antitumor immunity," *Frontiers in Immunology*, vol. 8, p. 533, 2017.

[7] C. Yang, H. Cheng, Y. Zhang et al., "Anergic natural killer cells educated by tumor cells are associated with a poor prognosis in patients with advanced pancreatic ductal adenocarcinoma," *Cancer Immunology, Immunotherapy*, vol. 67, no. 12, pp. 1815–1823, 2018.

[8] M. A. Degli-Esposti and M. J. Smyth, "Close encounters of different kinds: dendritic cells and NK cells take centre stage," *Nature Reviews. Immunology*, vol. 5, no. 2, pp. 112–124, 2005.

[9] Y. Du and Y. Wei, "Therapeutic potential of natural killer cells in gastric cancer," *Frontiers in Immunology*, vol. 9, p. 3095, 2018.

[10] P. Liu, L. Chen, and H. Zhang, "Natural killer cells in liver disease and hepatocellular carcinoma and the NK cell-based immunotherapy," *Journal of Immunology Research*, vol. 2018, Article ID 1206737, 8 pages, 2018.

[11] R. Kim, A. Kawai, M. Wakisaka et al., "A potential role for peripheral natural killer cell activity induced by preoperative chemotherapy in breast cancer patients," *Cancer Immunology, Immunotherapy*, vol. 68, no. 4, pp. 577–585, 2019.

[12] A. B. Bigley and R. J. Simpson, "NK cells and exercise: implications for cancer immunotherapy and survivorship," *Discovery Medicine*, vol. 19, no. 107, pp. 433–445, 2015.

[13] J. Yu, Y. Liu, Z. Gong et al., "Overexpression long non-coding RNA LINC00673 is associated with poor prognosis and promotes invasion and metastasis in tongue squamous cell carcinoma," *Oncotarget*, vol. 8, no. 10, pp. 16621–16632, 2017.

[14] F. Fei, Y. He, S. He et al., "LncRNA SNHG3 enhances the malignant progress of glioma through silencing KLF2 and p21," *Bioscience Reports*, vol. 38, no. 5, 2018.

[15] X. Dong, X. He, A. Guan et al., "Long non-coding RNA Hotair promotes gastric cancer progression *via* miR-217-GPC5 axis," *Life Sciences*, vol. 217, pp. 271–282, 2019.

[16] P. Fang, L. Xiang, W. Chen et al., "LncRNA GAS5 enhanced the killing effect of NK cell on liver cancer through regulating miR-544/RUNX3," *Innate Immunity*, vol. 25, no. 2, pp. 99–109, 2019.

[17] M. F. Wei, Z. S. Gu, L. L. Zheng, M. X. Zhao, and X. J. Wang, "Long non-coding RNA GAS5 promotes natural killer cell cytotoxicity against gastric cancer by regulating miR-18a," *Neoplasma*, vol. 67, no. 5, pp. 1085–1093, 2020.

[18] S. Wen, M. Zeng, Y. Li et al., "Downregulation of MANCR inhibits cancer cell proliferation in mantle cell lymphoma possibly by interacting with RUNX2," *Acta Biochim Biophys Sin (Shanghai)*, vol. 51, no. 11, pp. 1142–1147, 2019.

[19] K. M. Tracy, C. E. Tye, P. N. Ghule et al., "Mitotically-associated lncRNA (MANCR) affects genomic stability and cell division in aggressive breast cancer," *Molecular Cancer Research : MCR*, vol. 16, no. 4, pp. 587–598, 2018.

[20] L. Yao, J. Yan, L. Gan, S. Huang, F. Cheng, and N. Fang, "Upregulation of MANCR predicts poor survival in patients with gastric cancer," *Oncology Letters*, vol. 18, no. 6, pp. 6801–6806, 2019.

[21] C. Liu, H. Li, X. Li, X. Zhao, and X. Zhang, "LncRNA MANCR positively affects the malignant progression of lung adenocarcinoma," *BMC Pulmonary Medicine*, vol. 21, no. 1, p. 272, 2021.

[22] S. M. Hosseini, B. M. Soltani, M. Tavallaei et al., "Clinically significant dysregulation of *hsa-miR-30d-5p* and *hsa-let-7b* expression in patients with surgically resected non-small cell lung cancer," *Avicenna Journal of Medical Biotechnology*, vol. 10, no. 2, pp. 98–104, 2018.

[23] D. Chen, W. Guo, Z. Qiu et al., "MicroRNA-30d-5p inhibits tumour cell proliferation and motility by directly targeting CCNE2 in non-small cell lung cancer," *Cancer Letters*, vol. 362, no. 2, pp. 208–217, 2015.

[24] Y. Qi, Y. Hou, and L. Qi, "miR-30d-5p represses the proliferation, migration, and invasion of lung squamous cell carcinoma via targetingDBF4," *Journal of Environmental Science and Health, Part C: Toxicology and Carcinogenesis*, vol. 39, no. 3, pp. 1–18, 2021.

[25] Z. Zhao, W. Sun, Z. Guo, J. Zhang, H. Yu, and B. Liu, "Mechanisms of lncRNA/microRNA interactions in angiogenesis," *Life Sciences*, vol. 254, p. 116900, 2020.

[26] C. Hu, Z. Kang, L. Guo, F. Qu, and R. Qu, "The role of LINC00284 in the development of thyroid cancer via its regulation of the MicroRNA-30d-5p-mediated ADAM12/notch axis," *Frontiers in Oncology*, vol. 11, p. 643039, 2021.

[27] Q. Zeng, Y. Dai, C. Duan, R. Zeng, Q. Zeng, and C. Wei, "Long noncoding RNA POU3F3 enhances cancer cell proliferation, migration and invasion in non-small cell lung cancer (adenocarcinoma) by downregulating microRNA-30d-5p," *BMC Pulmonary Medicine*, vol. 20, no. 1, p. 185, 2020.

[28] S. Tang, H. Fu, Q. Xu, and Y. Zhou, "miR-20a regulates sensitivity of colorectal cancer cells to NK cells by targeting MICA," *Bioscience Reports*, vol. 39, no. 7, 2019.

[29] J. Wang, M. Zhu, X. Zhou et al., "MiR-140-3p inhibits natural killer cytotoxicity to human ovarian cancer via targeting MAPK1," *Journal of Biosciences*, vol. 45, no. 1, 2020.

[30] M. D. Robinson, D. J. McCarthy, and G. K. Smyth, "edgeR: a Bioconductor package for differential expression analysis of digital gene expression data," *Bioinformatics*, vol. 26, no. 1, pp. 139-140, 2010.

[31] Y. Huang, Y. Luo, W. Ou et al., "Exosomal lncRNA SNHG10 derived from colorectal cancer cells suppresses natural killer cell cytotoxicity by upregulating INHBC," *Cancer Cell International*, vol. 21, no. 1, p. 528, 2021.

[32] L. Liang, Z. Yang, Q. Deng et al., "miR-30d-5p suppresses proliferation and autophagy by targeting ATG5 in renal cell carcinoma," *FEBS Open Bio*, vol. 11, no. 2, pp. 529–540, 2021.

[33] M. C. Morissette, J. Parent, and J. Milot, "Perforin, granzyme B, and FasL expression by peripheral blood T lymphocytes in emphysema," *Respiratory Research*, vol. 8, no. 1, p. 62, 2007.

[34] L. Li, L. Yang, S. Cheng et al., "Lung adenocarcinoma-intrinsic GBE1 signaling inhibits anti-tumor immunity," *Molecular Cancer*, vol. 18, no. 1, p. 108, 2019.

[35] L. Schmidt, B. Eskiocak, R. Kohn et al., "Enhanced adaptive immune responses in lung adenocarcinoma through natural killer cell stimulation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 116, no. 35, pp. 17460–17469, 2019.

[36] L. Shao, Q. He, J. Wang et al., "MicroRNA-326 attenuates immune escape and prevents metastasis in lung adenocarcinoma by targeting PD-L1 and B7-H3," *Cell Death Discovery*, vol. 7, no. 1, p. 145, 2021.

[37] C. Dendle, P. Y. Gan, K. R. Polkinghorne et al., "Natural killer cell function predicts severe infection in kidney transplant recipients," *American Journal of Transplantation*, vol. 19, no. 1, pp. 166–177, 2019.

[38] M. J. Smyth, E. Cretney, J. M. Kelly et al., "Activation of NK cell cytotoxicity," *Molecular Immunology*, vol. 42, no. 4, pp. 501–510, 2005.

[39] X. Song, X. Jiao, H. Yan et al., "Overexpression of PTPRN promotes metastasis of lung adenocarcinoma and suppresses NK cell cytotoxicity," *Frontiers in Cell and Development Biology*, vol. 9, p. 622018, 2021.

[40] X. Deng, W. Xiong, X. Jiang et al., "LncRNA LINC00472 regulates cell stiffness and inhibits the migration and invasion of lung adenocarcinoma by binding to YBX1," *Cell Death & Disease*, vol. 11, no. 11, p. 945, 2020.

[41] Y. Chen, X. Zhang, J. Li, and M. Zhou, "Immune-related eight-lncRNA signature for improving prognosis prediction of lung adenocarcinoma," *Journal of Clinical Laboratory Analysis*, vol. 35, no. 11, article e24018, 2021.

[42] N. Shimasaki, A. Jain, and D. Campana, "NK cells for cancer immunotherapy," *Nature Reviews. Drug Discovery*, vol. 19, no. 3, pp. 200–218, 2020.

[43] X. Qin, M. Zhou, H. Lv et al., "Long noncoding RNA LINC00657 inhibits cervical cancer development by sponging miR-20a-5p and targeting RUNX3," *Cancer Letters*, vol. 498, pp. 130–141, 2021.

[44] Y. Zhang, X. Li, J. Zhang, and H. Liang, "Natural killer T cell cytotoxic activity in cervical cancer is facilitated by the LINC00240/microRNA-124-3p/STAT3/MICA axis," *Cancer Letters*, vol. 474, pp. 63–73, 2020.

[45] X. Hua, H. Chu, C. Wang, X. Shi, A. Wang, and Z. Zhang, "Targeting USP22 with miR-30-5p to inhibit the hypoxia-induced expression of PD-L1 in lung adenocarcinoma cells," *Oncology Reports*, vol. 46, no. 4, 2021.

[46] Y. Song, C. Song, and S. Yang, "Tumor-suppressive function of miR-30d-5p in prostate cancer cell proliferation and migration by targeting NT5E," *Cancer Biotherapy & Radiopharmaceuticals*, vol. 33, no. 5, pp. 203–211, 2018.

[47] Y. He, X. Chen, Y. Yu et al., "LDHA is a direct target of miR-30d-5p and contributes to aggressive progression of gallbladder carcinoma," *Molecular Carcinogenesis*, vol. 57, no. 6, pp. 772–783, 2018.

[48] Z. Chen, Z. Chen, S. Xu, and Q. Zhang, "LncRNA SOX2-OT/miR-30d-5p/PDK1 regulates PD-L1 checkpoint through the mTOR signaling pathway to promote non-small cell lung cancer progression and immune escape," *Frontiers in Genetics*, vol. 12, p. 674856, 2021.