Atorvastatin suppressed proliferation and facilitated apoptosis of A549 cells through mediating recruitment of Fas and CD59 in lipid raft

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INTRODUCTION
Lung cancer is one of the most malignant cancers worldwide with the 5-year overall survival rate low to 16.6% [1,2]. Lung cancer contains small cell lung cancer (SCLC) and non-
small cell lung cancer (NSCLC). The former on
takes about 15% of all kinds of lung cancer and
NSCLC occupies approximately 85% [3].

Although diagnosis of lung cancer has made
huge progress, it is still unfortunate that most
patients are diagnosed at advanced stage[4].

Based on analysis of NSCLC, its poor prognosis
has correlation with lacking diagnostic
biomarkers in early stage. Therefore, it is
urgently needed to Figure out potential
mechanism of occurrence and progression of
NSCLC.

Lipid raft is a dynamically formed by lipid and
protein with many kinds of regulatory molecules
and receptors, which is regarded as a platform
for signaling pathways [5]. Lipid raft was also
found to play important role in cell activation,
polarization and signal transduction [6].

Moreover, cancers can be occurred and
processed according to those signaling pathways
[7,8]. Previous studies have proven that lipid raft
plays important role in many kinds of cancers.
Merkel cell polyomavirus (MCPyV) entered cells
through endocytosis of lipid raft, resulting in
Merkel cell carcinoma (MCC), a skin cancer [9].

Besides that, lipid raft was gathered with
migrated gastric cancer cells by RANKL [10].

Under ultraviolet light, cholesterol in lipid raft
was increased and Fas receptor was congregated,
leading to apoptosis of tumor cells in skin cancer
[11]. Flotillin (Flot) is a protein family in lipid raft
that can mediate cell survival, proliferation and
apoptosis [12,13]. FLOT2 is one of the
homologous types of Flot family, which was
proved as a target of miR-133 in
adenocarcinoma of lung [14]. Stomatin-like
Protein 2 (SLP-2) was discovered to be highly
expressed and accelerated proliferation in
NSCLC through mediating surviving expression
[15]. In addition
to that, SLP-2 suppression blocked response of
macrophage to TLR ligands through reducing
total protein mass in rafts [16]. Hence, we
selected these two biomarkers for measuring
functions of lipid raft in NSCLC. Moreover, Lipid
raft can also gather Fas to transmit apoptosis
[17]. Besides that, CD59 can promote cancer cell
progression in binding proteins in lipid raft[18].

Based on those detections, Fas and CD59 was
also chosen to measure mechanism of lipid raft
in progression of NSCLC.

**EXPERIMENTAL**

**Cell culture and treatment**

Human non-small cell lung cancer cell line, A549
was acquired from American Type Culture
Collection (ATCC, USA). Thereafter, A549 cells
were cultured in Dulbecco’s Modified Eagle
Medium (DMEM, Gibco, USA) supplemented
with 10% FBS (Gibco, USA) at 37°C, 5%CO2.

Then, A549 cells were grouped as NC group,
MβCD (lipid raft inhibitor, 5mM and 10mM)
group[19], atorvastatin (0, 5mM, 10mM and
15mM) group, MβCD (10 mM)+atorvastatin
(15mM) group, 3BDO (60μM)+ MβCD (10mM)
group and 3BDO (60μM)+atorvastatin (15mM)+
MβCD (10mM) group. Cells in log phase were
used for following experiments.

**RT-qPCR**

In accordance with manufacturer’s instruction of
TRizol Reagent (Invitrogen, USA), total RNA was
separated and extracted from cells. Later, cDNA
was compounded through reverse transcription
of RNA by using High-Capacity cDNA Reverse
Transcription Kit from Applied Biosystems (USA).

Thereafter, 7500 Fast Food Safety Real-Time
PCR System (Applied Biosystems, USA) was
applied for amplification. Sequences of primers in
this study were listed as follows: FLOT2: forward,
5’-CCCATGTCCTGCACAAA-3’ and reverse,
5’—TCCACTGAGACCACAATCTCTA-3’[20];
SLP-2: forward, 5’-CTGGAGCCGTTTGTGAA
CAT-3’ and reverse, 5’-AGGATCTGGCGCTG
TTCTT-3’ [21], mTOR: forward, and GAPDH:
forward, 5’-GAAGATGGTGATGGGATTTC-3’ and
reverse, 5’-GAAGATGGTGATGGGATTTC-3’
[21]. GAPDH was considered as internal
reference and expressions of FLOT and SLP-2
were calculated by 2 -△△Ct method [22]. As for
PCR condition, pre-denaturation was first
performed at 95°C for 15min. Following,
denaturation was at 95°C for 45s and annealing
was at 60°C for 30s. Finally, extension was
applied for 1min at 72°C. The program contained
a total of 30 cycles.

**CCK-8**

A549 cells treated with NC, MβCD (5mM and
10mM); NC, atorvastatin (0, 5mM, 10mM and
15mM); NC, atorvastatin (15mM), MβCD (10mM)
+ atorvastatin (15mM); NC, 3BDO (60μM) and
3BDO (60 μM)+atorvastatin (15mM) were
seeded into 95-well plate with 5×10^3 cells per
well and incubated at 37°C, 5% CO₂. For cell
proliferation detection, 10μl CCK-8 (Beyotime,
Shanghai, China) was added into wells at 24h,
48h and 72h. After CCK-8 was mixed, cells were
incubated for another 3h. Finally, Varioskan™
LUX Microplate Reader (Thermo Scientific, USA)
was used to measure optical density (OD) values
at 450nm wavelength. In cytotoxicity test, 10μl
different concentrations of atorvastatin (0, 5mM,
10mM and 15mM) was added into A549 cells in
96-well plate and cultured for 72h. Later, 10μl

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CCK-8 was added and incubated for 2h. OD values of cells were measured at 450 nm wavelength.

**Western blot method**

Cells were collected and protein was extracted using RIPA. The BCA kit was applied to determine the protein concentrations (Bioss, Beijing, China). Furthermore, proteins were separated by SDS-PAGE method and then transferred to PVDF membranes (Millipore, Shanghai, China). The primary antibody against mTOR (bs-1992R, 1:500, Bioss) was added to incubate the membranes at 4°C overnight, with β-actin (bs-0061R, 1:500, Bioss) as the internal reference. On the second day, the membranes were incubated in secondary antibody after washing using TBST for thrice. ECL kit (Chuanshi Biotech, Tianjin, China) was added onto the membranes. The blotting bands were imaged and the protein expression was analyzed using ImagePro (MD, USA).

**Flow cytometry**

Flow cytometry was applied to measure apoptosis rate of A549 cells, NC, MβCD (5mM and 10mM); NC, atorvastatin (15 mM), MβCD (10mM)+atorvastatin (15mM); NC, 3BDO (60μM), 3BDO (60μM)+atorvastatin (15mM) were used to treat A549 cells. After treatment, cells were rinsed by PBS. Then, Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China) was used for apoptosis detection. A549 cells were resuspended by Annexin V-FITC binding buffer and following, 5μl Annexin V-FITC (20μg/ml) and 10μl PI (50μg/ml) were added and incubated at room temperature for 15min without light. Finally, Agilent NovoCyte Advanteon Flow Cytometer (Agilent, USA) was performed to measure apoptosis rate of A549 cells.

**Statistical analysis**

All data were shown as mean±SD and analyzed by Graph Pad Prism 7.0 (USA) and SPSS19.0 (USA). Experiments were all run in a triplicate. Student’s t-test and one way ANOVA were used to measure comparisons of groups. P<0.05 was considered to have meanings.

**RESULTS**

MβCD treatment suppressed FLOT-2 and SLP-2 expressions and mediated cell viability, apoptosis and expressions of Fas and CD59

In order to analyze function of lipid raft in progression of NSCLC, MβCD was performed to suppress lipid raft. After different concentrations of MβCD (5mM and 10mM) were added, both FLOT-2 and SLP-2 RNA expressions were significantly downregulated in a dose dependent manner (Figure 1A). Meanwhile, viability of A549 cells was detected revealing that cell viability was markedly reduced as MβCD concentration increased (Figure 1B). Apoptosis of A549 cells was analyzed later, indicating that MβCD noticeably promoted apoptosis of A549 cells (Figure 1C). Then, Fas and CD59 protein levels were evaluated, revealing that Fas protein expression was remarkably increased with up-regulated concentrations of MβCD while CD59 was significantly declined (Figure 1D).
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Figure 1 MβCD treatment suppressed FLOT-2 and SLP-2 expressions and mediated cell viability, apoptosis and recruitments of Fas and CD59: A: FLOT-2 and SLP-2 RNA expressions were assessed by RT-qPCR in A549 cells treated with MβCD (5mM and 10mM), \( P < 0.05 \). B: Cell viability of A549 cells with different concentrations of MβCD (5mM and 10mM) were measured by CCK-8, \( P < 0.05 \). C: Flow cytometry was applied to analyze apoptosis rate of A549 cells after treated by MβCD (5mM and 10mM), \( P < 0.05 \). D: Protein levels of Fas and CD59 after MβCD treatment (5mM and 10mM) in lipid raft of A549 cells were measured by ELISA, \( P < 0.05 \). Experiments were all repeated three times.

Figure 2A). Then, FLOT-2 and SLP-2 RNA expressions with atorvastatin were detected, revealing that atorvastatin significantly reduced RNA levels of FLOT-2 and SLP-2. Moreover, combination of MβCD and atorvastatin resulted in the lowest levels of FLOT-2 and SLP-2 (Figure 2B). Cell viability was then analyzed, indicating that atorvastatin caused much lower viability of A549 cells and compounds of MβCD and atorvastatin obviously reduced cell viability of A549 compared to atorvastatin only (Figure 2C). Meanwhile, detection of apoptosis indicated that atorvastatin resulted in markedly higher apoptosis rate of A549 cells and added MβCD sharply up-regulated apoptosis of A549 cells on the basis of atorvastatin treatment (Figure 2D). Furthermore, Fas and CD59 protein levels were analyzed, showing that atorvastatin treatment significantly up-regulated Fas protein expression and declined CD59 protein levels. Moreover, combination of MβCD and atorvastatin remarkably magnified up-regulation of Fas and inhibition of CD59 in A549 cells (Figure 2E).

Atorvastatin repressed FLOT-2 and SLP-2 in lipid raft and mediated viability and apoptosis of A549 cells via mTOR signaling pathway

After correlation between MβCD and atorvastatin was Figure out, deep mechanism of atorvastatin mediating lipid raft in A549 cells was measured. In normal A549 cells and MβCD treated A549 cells, mTOR protein was significantly downregulated in A549 cells after MβCD treatment compared to normal A549 cells (Figure 3A). Therefore, A549 cell with MβCD (10mM) treatment was chosen or getting connections among MβCD, mTOR and atorvastatin. As 3BDO (mTOR agonist) added, mTOR was remarkably promoted while atorvastatin treatment restored up-regulation of mTOR caused by 3BDO (Figure 3B). Besides that, cell viability was analyzed, indicating that 3BDO treatment obviously up-regulated viability of A549 cells but added atorvastatin resumed up-regulation of 3BDO resulting in low cell viability (Figure 3C). Meanwhile, 3BDO suppressed apoptosis rate was also reversed to be up-regulated with...
Figure 2: Combination of atorvastatin and MβCD downregulated FLOT-2 and SLP-2 expressions and mediated viability, apoptosis and aggregation of Fas and CD59 A: Cell toxicity of A549 with atorvastatin (0, 5mM, 10mM and 15mM) were evaluated by CCK-8. B: FLOT2 and SLP-2 RNA levels in A549 cells with atorvastatin (15mM) and MβCD (10 mM)+atorvastatin (15mM) were examined by RT-qPCR, P<0.05. C: CCK-8 was applied to measure cell viability of A549 cells after treated by atorvastatin (15mM) and MβCD (10 mM)+atorvastatin (15mM), P<0.05. D: Apoptosis rate of A549 cells were detected through flow cytometry in A549 cells with atorvastatin (15mM) and MβCD (10 mM)+atorvastatin (15mM) treatment, P<0.05. E: Fas and CD59 protein levels were evaluated after atorvastatin (15mM) and MβCD (10 mM)+atorvastatin (15mM) treatment in A549 cells through ELISA, P<0.05. Each experiment was run in a triplicate

DISCUSSION

Statins are the most common drugs for preventing cardiovascular diseases and reducing mortality and morbidity through downregulating plasma cholesterol levels[25]. According to previous studies, statins could also anti-tumors via inducing apoptosis and inhibiting cancer cell growth and angiogenesis[26, 27]. Atorvastatin, an antilipemic agent, was found to suppress progression of NSCLC through promoting expressions of cleaved caspase-3 and PARP and repressing autophagy[28]. Besides that, atorvastatin was discovered to be a lipid raft inhibitor by breaking cholesterol[29]. Moreover, atorvastatin treatment in A549 cells (Figure 3D). Fas and CD59 protein expression were analyzed as well, revealing that Fas protein level was elevated after 3BDO treatment and decreased with atorvastatin usage while CD59 was inhibited by 3BDO and then rescued by atorvastatin (Figure 3E).

atorvastatin attenuated TLR4 recruitment into lipid raft to affect anti-inflammatory response[30]. Though atorvastatin has been detected to be an inhibitor in NSCLC, its correlation with lipid raft in NSCLC was never mentioned. Therefore, in this study, we have analyzed correlation between atorvastatin and lipid raft and mechanisms. At first, we have proven that lipid raft facilitated FLOT-2 and SLP-2 expression in NSCLC while suppression of lipid raft through MβCD retarded FLOT-2 and SLP-2 RNA expressions, viability of A549 cells and promoted apoptosis with up-regulating aggregation of Fas and reducing CD59 in lipid raft. Based on these detections, we primarily clarified that lipid raft accelerated progression of NSCLC through up-regulating recruitments of FLOT-2, SLP-2 and CD59 and MβCD blocked functions of lipid raft.

Moreover, functions of atorvastatin were examined later. With concentrations of atorvastatin up-regulating, cell toxicity was increased. Meanwhile, atorvastatin treatment
significantly decreased recruitments of FLOT-2 and SLP-2 in lipid raft and combination of MβCD with atorvastatin caused lower levels of FLOT-2 and SLP-2 than atorvastatin only. As for cell viability detection, added atorvastatin obviously declined viability of A549 cells and mixed MβCDenhanced suppression of atorvastatin while apoptosis was significantly up-regulated. Recruitments of Fas and CD59 were checked later, revealing that atorvastatin treatment markedly increased protein level of Fas and repressed CD59 and added MβCD magnified regulation of atorvastatin in promoting Fas and reducing CD59. After functions of atorvastatin were measured, deeper mechanism of atorvastatin in mediating lipid raft was examined. Atorvastatin was proved to retard inflammation in RAW264.7 with suppressing phosphorylation of mTOR[31, 32]. Lipid raft was discovered to mediate activation of mTOR signaling pathway in cancers[33]. Based on these studies, we measured role of mTOR in progression of NSCLC with lipid raft and atorvastatin. With MβCD treatment, mTOR protein expression was obviously reduced. After 3BDO treatment, mTOR was promoted while atorvastatin treatment reversed function of 3BDO and downregulated mTOR. Moreover, increased cell viability of 3BDO was also restrained with atorvastatin but apoptosis suppressed by 3BDO was promoted after atorvastatin added. Furthermore, Fas was promoted by 3BDO in lipid raft and then reversed by atorvastatin while CD59 inhibited by 3BDO was recovered by atorvastatin. Results reminded that atorvastatin retard functions of lipid raft in promoting progression of NSCLC through blocking mTOR signaling pathway.

CONCLUSION

Atorvastatin inhibited FLOT-2 and SLP-2 RNA expressions and cell viability but promoted apoptosis of NSCLC cells and mediated recruitments of CD59 and Fas in lipid raft via mTOR signaling pathway, suggesting

**Figure 3:** Atorvastatin repressed FLOT-2 and SLP-2 in lipid raft and mediated viability and apoptosis of A549 cells via mTOR signaling pathway. A: Protein expression of mTOR in A549 cells and MβCD (10mM) treated A549 cells were assessed by western blot, P<0.05. B: Protein levels of mTOR in MβCD (10mM) treated A549 cells were measured by western blot after 3BDO (60μM) and 3BDO (60μM)+atorvastatin (15mM) treatment, P<0.05. C: CCK-8 was performed to measure cell viability of MβCD (10mM) treated A549 cells under 3BDO (60μM) and 3BDO (60μM)+atorvastatin (15mM) treatment, P<0.05. D: Apoptosis rate of MβCD (10mM) treated A549 cells was detected by flow cytometry after treated by 3BDO (60μM) and 3BDO (60μM)+atorvastatin (15mM), P<0.05. E: Protein levels of Fas and CD59 were measured by ELISA in MβCD (10mM) treated A549 cells after 3BDO (60μM) and 3BDO (60μM)+atorvastatin (15mM) treatment, P<0.05. All experiments were repeated three times.
atorvastatin could be used for blocking functions of lipid raft in NSCLC. Nevertheless, further detections in vivo and in clinical stage are needed for getting more knowledge.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Jiahuan Ludesigned the work and carried out the literature search, data analysis, manuscript preparation and review. Huiping Qiang conceptualized the work, and was involved in the design, data acquisition and manuscript editing. Tianqing Chu was also involved in literature search and definition of intellectual content.

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