



Effect and Mechanism of Transthyretin Over-Expression on Proliferation and Cell Cycle of Lung Cancer A549 Cells

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Abstract

Background: The effects of transthyretin (TTR) over-expression on the proliferation and cell cycle of non-small cell lung cancer (NSCLC) A549 cells and its possible mechanism were verified.

Methods: A total of 196 LC patients and 20 healthy controls were enrolled at Tianjin Hospital, Tianjin, China between Apr 2017 and Oct 2017. The serum TTR content was detected by ELISA. Through lentiviral transfection method, NSCLC cells were divided into non-transfected group (group A), negative control group (group B) transfected with empty vector and experimental group (group C) transfected with TTR over-expression. Cell proliferation was detected by CCK-8 method, TTR mRNA expression was detected by real-time quantitative polymerase chain reaction (RT-qPCR), and TTR protein expression was tested by Western blot (WB). Cell cycle was detected by flow cytometry, Wnt3a/ β -catenin protein expression was detected by WB, and mRNA expression was detected by RT-qPCR.

Results: The serum TTR content in early, middle and late LC group was remarkably lower than that in healthy group ($P < 0.05$). Compared with late stage, TTR content in early and middle stages of LC group was higher, and the difference was statistically marked ($P < 0.05$). The absorbance value of group C was lower than that of groups A and B, indicating that the cell proliferation activity dramatically decreased, with statistically marked difference ($P < 0.05$). LC A549 cells in group C were obviously blocked in G2M, with statistical significance ($P < 0.05$).

Conclusion: TTR over-expression can inhibit the proliferation of NSCLC A549 cells, and the expression is related to Wnt3a/ β -catenin pathway. TTR in serum of patients was helpful for diagnosing LC and has certain clinical value.

Keywords: Non-small cell lung cancer; Transthyretin; Cell proliferation; Cell cycle; Serum marker

Introduction

Lung cancer (LC) is one of the most common malignancies in clinic. In recent years, the morbidity is increasing year by year, becoming more

youth-oriented. About 85% of clinical LC patients are non-small cell lung cancer (NSCLC), and the main pathological types are squamous



cell carcinoma and adenocarcinoma (1, 2). Clinical comprehensive treatment based on surgery has greatly improved the efficacy of early LC. Studies have shown that the 5-yr survival rate can be over 80% for early LC patients after surgery combined with neoadjuvant chemotherapy (3-8). Although targeted therapy and immunotherapy for LC are developing rapidly at present, the prognosis of advanced LC patients is still poor, with tumor metastasis, recurrence and drug resistance. Therefore, it is urgent to improve the early diagnosis rate of LC patients.

Transthyretin (TTR), also known as prealbumin (PA), is a tetramer composed of four identical subunits, each of which contains 127 amino acids. Recently, a large number of studies have shown that the change of serum expression level of TTR is relevant to the progression of various tumors, such as LC, colon cancer (CC), ovarian cancer (OC), gastric cancer (GC), pancreatic ductal cancer (PDC), etc. (9-12). But there are still very few reports in LC-related studies. Liu L (13) et al screened and identified TTR by SELDI technology, and confirmed that this protein could be used as a serum marker candidate for LC diagnosis. In the early stage, the research group found that TTR was a differentially expressed protein of LC through MALDI-TOF-MS, which was significantly lower in the serum of LC patients than that of healthy people and those with benign lung diseases (14).

Therefore, in order to further explore TTR's mechanism in LC, this study constructed TTR over-expression cell line, and then transfected LC A549 cell line. The influence on cell proliferation and cycle and the possible molecular mechanism was analyzed, and the serum TTR content was detected by ELISA to explore its value as a tumor marker. We would like to provide a reference for clinical diagnosis and treatment.

Materials and Methods

Experimental materials

NSCLC cell A549 (Shanghai Tiancheng Medical Technology Co., Ltd.), fetal bovine serum (FBS)

and PRIM 1640 (HyClone), cell transfection reagent (QIAGEN), pancreatin (Beyotime), lentiviral vector (it was synthesized by Beijing Hesheng Gene Technology Co., Ltd., the vector was HS-LV001, and the element sequence was EF1 α -TTR (human)-3 \times FLAG-IRES-EGFP-Puro), Trizol (Invitrogen, USA), qRT-PCR kit (TaKaRa), and Human TTR PicoKine ELISA Kit (Boster Biological Technology Co., Ltd.), PCR primer (synthesized by Sangon Biotech, Shanghai), goat anti-mouse IgG (GeneText), protein quantitative kit (Thermo, USA), GADPH monoclonal antibody and goat anti-rabbit IgG combined with horseradish peroxidase HRP (Zhongshan, Beijing), rabbit anti-human TTR monoclonal antibody (Abcam, USA), microplate reader (Thermo, USA), PCR instrument (Agilent).

Research objects

Experimental group: From Apr 2017 to Oct 2017, 196 hospitalized LC patients were enrolled. There were 84 with stage I (early stage), 46 with stage II-IIIa (middle stage) and 66 with stage IIIb-IV (late stage) according to TNM staging standard. The inclusion criteria of the experimental group: 1) Tumor cells were found in bronchoscope biopsy tissue and tissue brush, postoperative pathological tissue and pleural effusion culture, and finally diagnosed as LC by immunohistochemical staining and imaging examination. 2) No anti-tumor treatment such as radiotherapy and chemotherapy was given before blood collection.

Overall 20 healthy people were enrolled as control group. They were medically examined in the physical examination center of Tianjin Chest Hospital at the same time. All of them did not have respiratory diseases and other malignancies. All participants signed an informed consent form. This study was approved by the Ethics Committee of Tianjin Hospital, China.

Cell culture

NSCLC cells were cultured in RPMI1640 medium (containing 10% FBS) at saturated humidity, 5% CO₂, 37 °C, and were passaged when they fused to 80%.

Construction of A549 cells with up-regulated TTR expression

Lentivirus infection of A549 cells: A549 cells in logarithmic growth phase were selected and digested with pancreatin. The cell density was adjusted to $(3-5) \times 10^4$ /ml, and cells were inoculated into a 6-well plate and cultivated all night until the cell confluence reached 30%-50%. Before transfection, they were replaced with fresh culture medium, and divided into three groups. Group A was non-transfected (blank) group, group B was the group added with lentivirus empty vector virus solution (negative control group), and group C was the group added with over-expression of lentivirus vector virus solution (experimental group). After cells were cultured for 12-16 h, cell morphology was observed and the medium was replaced.

Screening of stable expression cell lines: A549 cells transfected with lentivirus were cultured 48 h in 5% CO₂ incubator at 37 °C, and then replaced with complete culture medium containing puromycin with appropriate concentration. Finally, those with stable expression of TTR were screened.

Identification of A549 cells with up-regulated TTR expression

1) RT-qPCR: Total RNA extracted from A549 cells in logarithmic growth phase was digested by Trizol, and then reverse transcribed into cDNA: A549-TTR upstream primer 5'-TGGGAGCCATTTCCTCTG-3', downstream primer 5'-AGCCGTGGTGGGAATAGGAGTA-3'. GAPDH was used as internal reference, primer was synthesized by Sangon Biotech, Shanghai. PCR reaction conditions were as follows: 94 °C for 45 s, 55 °C for 50 s, 72 °C for 75 s, 40 cycles. The relative expression of target gene was calculated by $2^{-\Delta\Delta C_t}$ method.

2) WB detection: The total proteins of groups A, B and C in logarithmic growth phase were extracted routinely. Then the cells were fully lysed and the total proteins were extracted by RIPA lysate, and the proteins were quantified by BCA

method. Altogether 15 µg protein was applied to each well. The initial voltage was 80V, and it increased to 100V after the leading edge of bromophenol blue dye entered the upper edge of the separation gel, and it ended after the electrophoresis of bromophenol blue out of the lower edge of the separation gel. Protein electrotransfer in PVDF membrane was performed by semi-dry electrotransfer instrument with 30 mA constant current for 90 min. The PVDF membrane was taken out and sealed with 5% TBST skimmed milk powder and shaken for 60 minutes. Afterwards, the membrane was washed with TNS-T rinse solution for 10 min, three times, and then it was transferred to hybridization bag. The antibody was diluted by appropriate rinse solution and it was incubated at 4 °C all night. Next, it was washed with TBST rinse solution for 10 min, three times, the secondary antibody labeled with peroxidase was diluted with rinse solution, and shaken for 60 min. The PVDF membrane was placed in ECL chromogenic solution and incubated for 5 min. Then it was exposed, developed and fixed in a dark room. After that, it was rinsed with branch water, dried and scanned. Gray value on the target strip of scanned image was analyzed by IPP.

Detection of cell growth curve by CCK-8 method

The lentivirus infected cells in each group were collected and inoculated into a 96-well plate with 3×10^3 cells/well, 6 multiple wells in each group. After being cultured in an incubator for 24, 48 and 72 h respectively, the culture medium was discarded from the culture plate, washed twice with PBS, and 10 µL CCK-8 solution was added to each well. After cells were incubated 4 h under dark conditions, the absorbance of cells in each group at the 450 nm wavelength was recorded by microplate reader. Each experiment was repeated three times, and the cell growth curve was drawn by GraphPad Prism8.

Detection of cell cycle by flow cytometry

Transfected cells in each group were washed twice with ice-cold PBS, and fixed 30 min with 75% ethanol. The fixed cells were washed twice with ice-cold PBS, and stained 30 min with 300 μ L PI. Cells in G0/G1, S and G2/M phases were counted respectively, and the cell cycle was analyzed by Modfit.

Expression of Wnt3a/ β -catenin protein is detected by RT-qPCR and WB

A549 cells after lentivirus TTR over-expression were collected. Total protein was extracted from cell lysate, and protein was quantified by BCA method. After loading, the protein was transferred to PVDF membrane by polyacrylamide gel electrophoresis, sealed 1 h with 5% skimmed milk powder at indoor temperature, incubated with primary antibody at 4 °C all night, and cleaned 3 times with TBST, each time for 10 min. Soon afterwards, it was then incubated 1 h with secondary antibody at indoor temperature. The PVDF membrane was placed in ECL chromogenic solution for 5 min, exposed, developed and fixed in dark room. It was rinsed with branch water, dried and scanned. Gray value on the target strip of scanned image was analyzed by IPP.

Serological ELISA verification of LC patients

1) Specimen collection and treatment: About 4 mL elbow venous blood was collected on an empty stomach in the early morning in all LC patients and healthy controls, placed in a non-anticoagulation common tube, and centrifuged 10 min at 3,000 r/min, 4 °C. The serum was absorbed, packed and stored in a refrigerator at -80 °C. All specimens were thawed once at normal temperature before laboratory test.

2) Main reagent: Human TTR PicoKine ELISA Kit was purchased from Boster Biological Technology Co., Ltd.

3) Detection methods: TTR content in patients' serum was detected by enzyme linked immunosorbent assay (ELISA) based on the instructions.

Statistical analysis

SPSS 23.0 (Chicago, IL, USA) was employed for data analysis. The experimental data were tested first to verify if they were in line with normal distribution. The measurement data were represented by ($x \pm s$). The comparison between the two groups was analyzed via independent-samples *t* test, comparison among multiple groups was assessed via one-way analysis of variance, and pairwise comparison within groups was analyzed via LSD-t. $P < 0.05$ indicated that the difference was statistically remarkable. If ELISA serum TTR experimental data did not conform to normal distribution, then they were detected by SPSS normal test. Non-parametric test [Kruskal-Wallis (K-S) test] was fully utilized. The measurement data were described statistically by median and interquartile distance, and the difference was statistically significant if $P < 0.05$.

Results

Up-regulation of TTR expression in A549 cells

qRT-PCR results showed that the TTR mRNA expression level in group C was markedly higher than that in groups A and B (Table 1, Fig. 1).

Table 1: Comparison of mRNA expression in each group

| Group | TTR mRNA |
|---------|------------------|
| Group A | 0.98 \pm 0.05 |
| Group B | 1.02 \pm 0.08 |
| Group C | 6.78 \pm 0.22# |

Note: compared with groups A and B, # $P < 0.05$

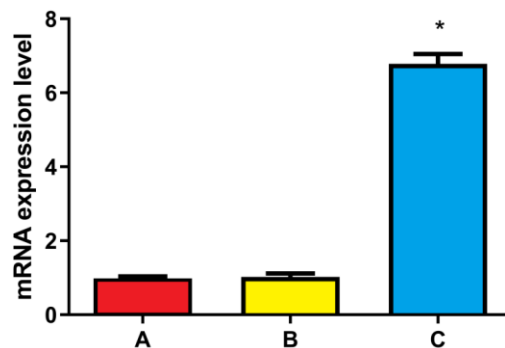


Fig. 1: *TTR* mRNA expression of cells in each group is detected by RT-qPCR

WB results manifested that the *TTR* protein expression in group C was markedly higher than that in groups A and B (Fig. 2).

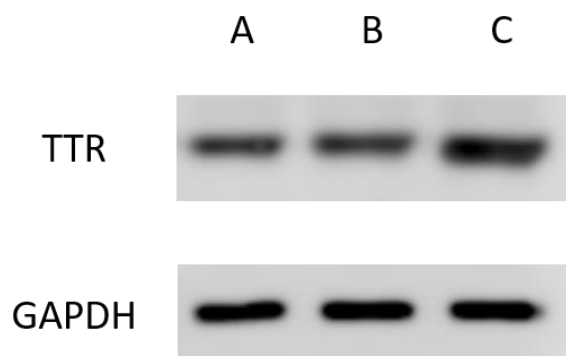


Fig. 2: WB protein expression bands of cells in each group

TTR over-expression inhibits A549 cell proliferation

CCK-8 cell proliferation experiment results showed that *TTR* over-expression in group C could inhibit A549 cell proliferation, and the difference was statistically remarkable ($P < 0.05$), as shown in Fig. 3.

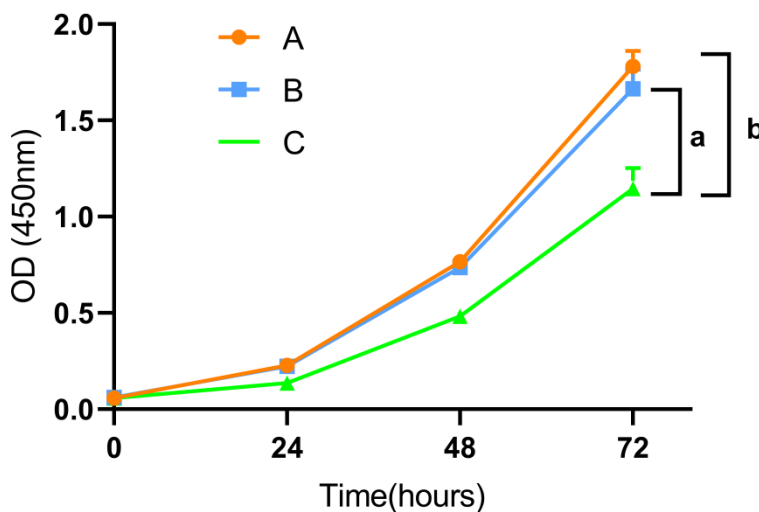


Fig. 3: Effect of *TTR* over-expression on A549 cell proliferation

(The horizontal axis is time, the vertical axis is absorbance value, a: The difference between group C and group B was statistically marked ($P < 0.05$). b: The difference between group C and group A was statistically marked ($P < 0.05$))

Effect of TTR over-expression on A549 cell cycle

Flow cytometry identified that *TTR* over-expression could affect the cell cycle of LC A549

cells, and G2/M phase block appeared in group C, indicating that A549 over-expression could inhibit cell proliferation, as shown in Fig. 4 and Fig. 5.

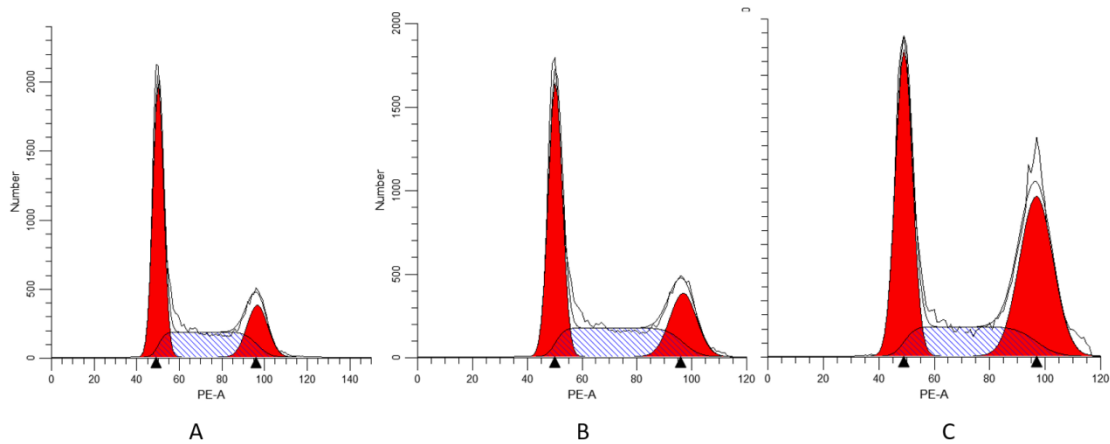


Fig. 4: Flow cytometry results show that *TTR* over-expression can affect A549 cell cycle

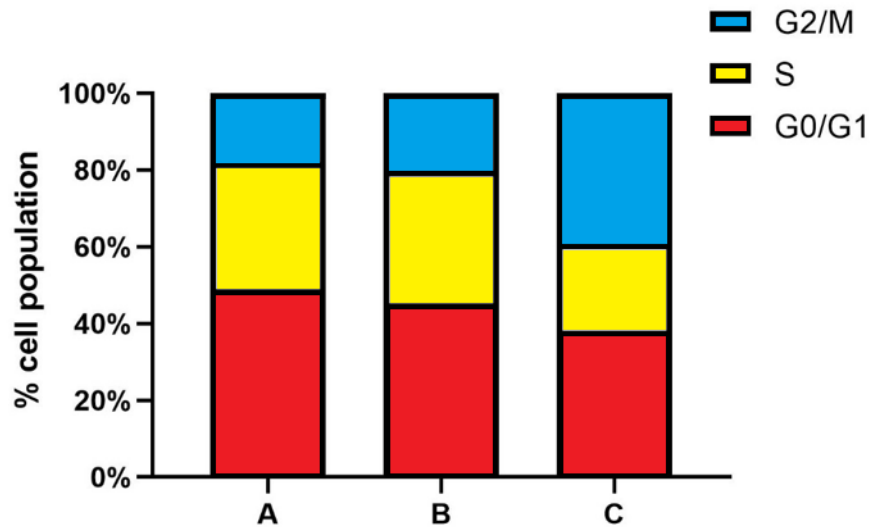


Fig. 5: Flow cytometry results show that G2/M phase of cells in group C is obviously blocked

***TTR* over-expression can participate in regulating *Wnt3a*/ β -catenin pathway**

The *Wnt3a*/ β -catenin mRNA expression was detected by qRT-PCR (Fig. 6). The results manifested that the *Wnt3a* and β -catenin mRNA expression levels in *Wnt* signaling pathway in group

C were down-regulated, and the expression of *wnt3a*/ β -catenin protein was detected by WB (Fig. 7). It turned out that the *Wnt3a* and β -catenin expression levels in *Wnt* signaling pathway were down-regulated in group C.

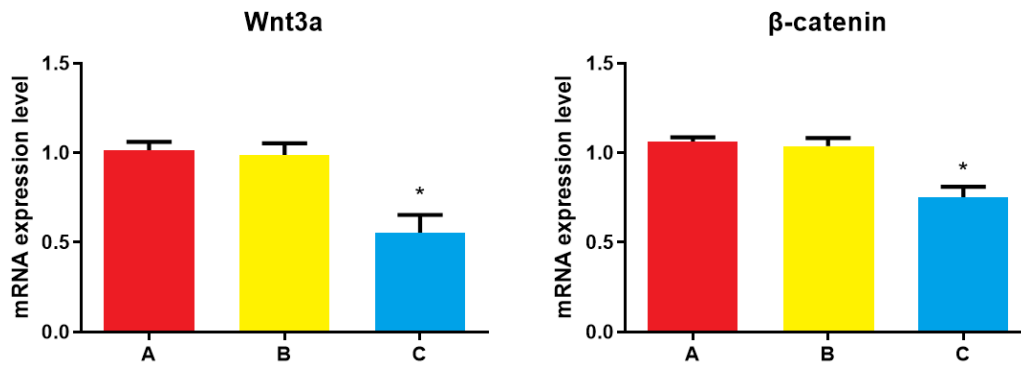


Fig. 6: Wnt3a/β-catenin pathway related mRNA expression is detected by qRT-PCR

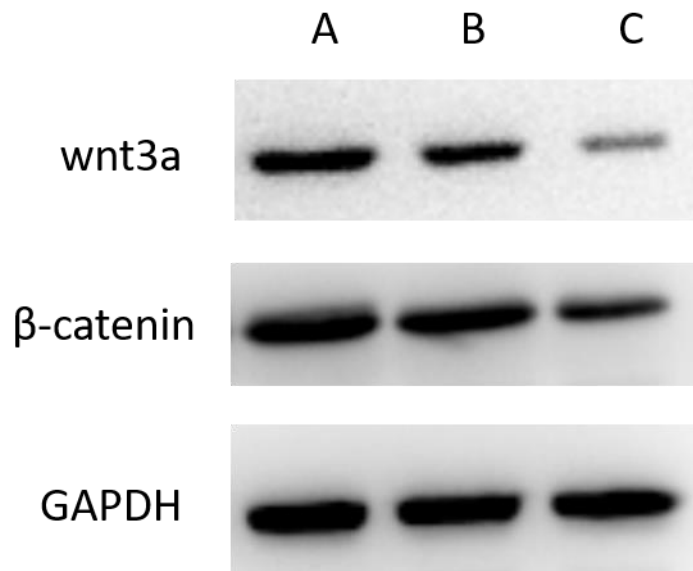


Fig. 7: Wnt3a/β-catenin protein expression is detected by WB

Serum TTR content in LC patients is detected by ELISA

ELISA serum results showed that there were 84 patients with stage I (early stage), 46 with stage II-IIIa (middle stage), 66 with stage IIIb -IV (late stage). The data of 20 healthy controls were imported into SPSS 23.0, and there was no marked difference in age and gender among the groups ($P>0.05$). The data were assessed via normality test, and the results showed that each group did not conform to the normal distribution ($P<0.05$). Thus, the measurement data were expressed by the median and quartile distance Md (Q), the

comparison between groups was performed by non-parametric rank sum test, and the pairwise comparison was performed by K-W test. It revealed that the serum TTR content in the healthy group was 319.37 (238.03-400.40) ug/ml, that in early stage LC was 162.77 (116.68-226.81) ug/ml, that in middle stage LC was 119.00 (138.17-260.37) ug/ml, and that in advanced LC was 94.57 (138.17-260.37) ug/ml. Except that there was no marked difference in serum between early and middle stage patients ($P>0.05$), there was remarkable difference between the other groups ($P<0.01$) (Fig. 8 and Fig. 9)

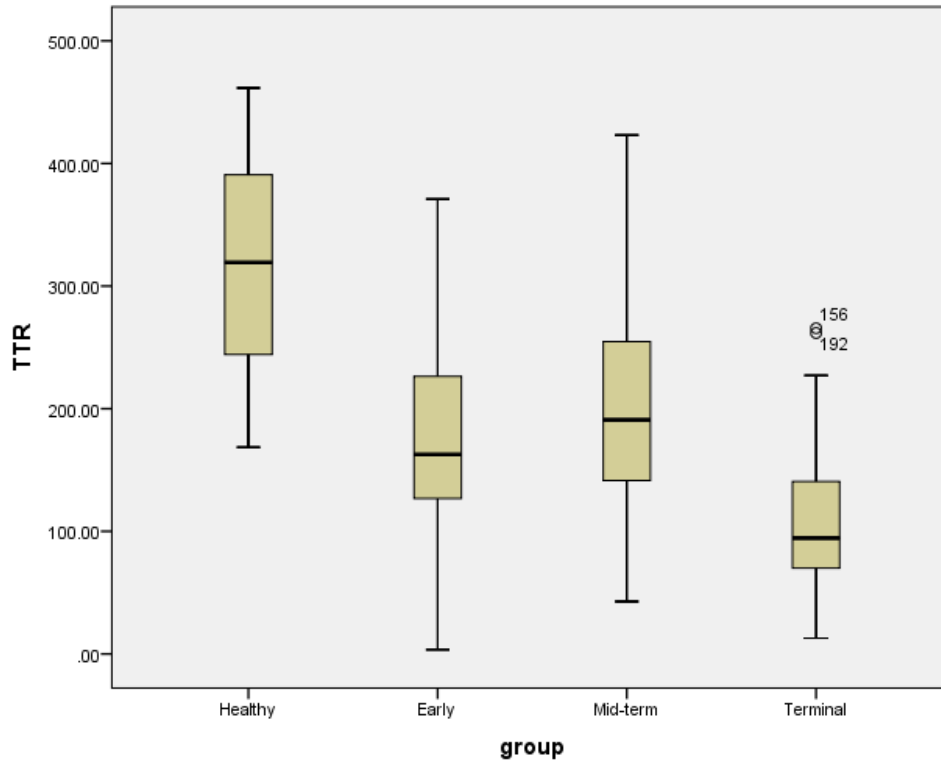
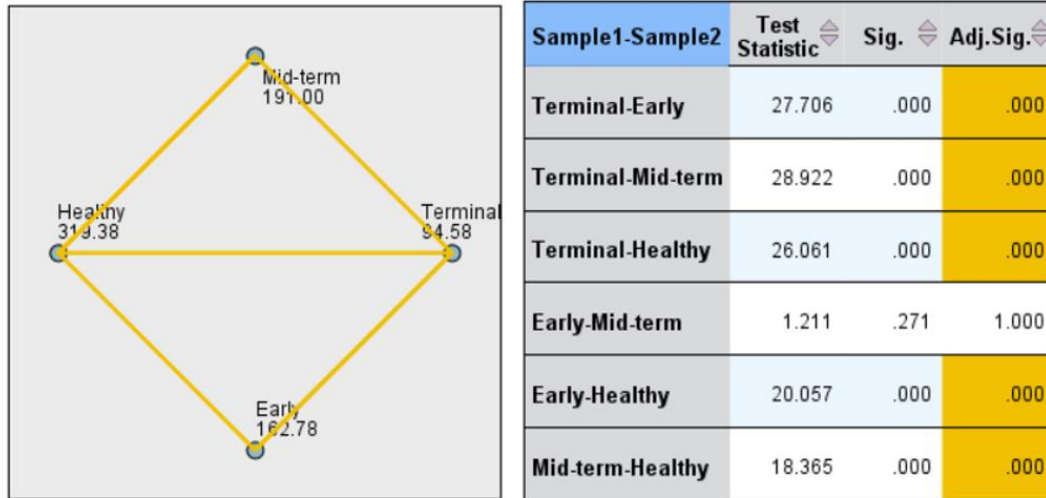


Fig. 8: Comparison of TTR content in serum between experimental group and control group



Each node shows the sample median of group.

Fig. 9: Comparison of serum TTR levels between groups

Discussion

LC is one of the malignancies with high incidence all over the world. Its clinical mortality has been high for many years, which poses a serious threat

to patients' life safety. The ideal treatment for LC patients is early radical operation, while the survival rate of advanced patients is not ideal and their quality of life is very poor. Therefore, improving the diagnosis rate of LC is one of the hot

spots for current scholars (15-17). However, there are very few sensitive tumor markers that can accurately predict LC early incidence. At present, there are many LC markers used clinically, such as soluble fragment of cytokeratin 19 (cyfra21-1), neuron-specific enolase (NSE) and CEA, etc. But their specificity are low.

TTR is a highly stable tetrameric structural protein, which is composed of four identical subunits bonded by non-covalent bonds, and each subunit contains 127 amino acids. It is mainly secreted by hepatocytes in human body, and its main role is to transport thyroxine (mainly T₄) and retinol (vitamin A). However, TTR tetramer can be decomposed into monomer under pathological or abnormal physiological conditions (such as stress and inflammatory reaction). TTR can form a wide variety of amyloid fibers, which leads to abnormal physiological aggregation of amyloid fibers in cells. Abnormal deposition of amyloid in cells can lead to abnormal metabolism of cells and even disorder and change of all tissue functions, thus bringing about diseases.

As early as 2005, Schweigert and Sehouli found that TTR levels in serum of patients with early OC markedly changed (18). The levels in serum and ascites of OC patients were dramatically different (19). The differential expression of TTR had important biological significance in the development of cutaneous T-cell lymphoma (20). Katare et al found four mutations of TTR gene in LC rats, which changed three amino acids at positions 61, 100 and 115 (21). The mutation at position 115 interfered with the formation of TTR tetramer, leading to TTR accumulation (21). With the deepening of TTR research, TTR could be used as a potential biomarker for pancreatic ductal adenocarcinoma (12). Serum TTR level was related to the prognosis of GC patients, and patients with lower serum TTR level had poor prognosis (9). With more and more researches on the correlation between TTR and disease development and progression (22), it can be seen that TTR has high clinical value for diagnosing and detecting various cancers (23).

In a few studies on LC and TTR, we could see that TTR may be a new tumor marker. The TTR expression level in different subtypes of LC was remarkably different, among which adenocarcinoma was the highest, reaching 84.4% (10). Previously, the research group found that the serum TTR content in early LC patients showed a decreasing trend compared with healthy people (14). In view of the relationship between TTR expression and LC, this paper focused on the effects of TTR over-expression on proliferation and cell cycle of NSCLC A549 cells. And the results showed that the proliferation of A549 cells decreased after over-expression. Pathways related to tumor signal transduction, such as Wnt, PI3K/AKT or NF- κ B, may play an important role in tumor genesis and development. In addition, more and more evidences showed that the typical Wnt/ β -catenin signaling pathway is the key signaling pathway involved in the development of various cancers (24-32). In order to further study the mechanism of TTR on NSCLC tumor progression, we found that the WNT 3A and β -catenin expression levels in Wnt signaling pathway were down-regulated after TTR over-expression. It indicated that TTR inhibited the proliferation of LC A549 cells to some extent by inhibiting Wnt/ β -catenin signaling pathway. ELISA test confirmed that serum TTR of LC patients showed an obvious downward trend compared with healthy people, and the difference was statistically remarkable.

Conclusion

TTR in serum has certain value for LC diagnosis, and it plays a certain role in its proliferation, occurrence and development.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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