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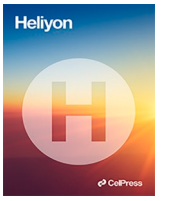

Research article

Impacts of TP53TG1 in cancer-associated fibroblasts-derived exosomes on epithelial-mesenchymal transition capacity of colorectal carcinoma cells by targeting miR-330-3p

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A R T I C L E I N F O

Keywords:
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A B S T R A C T

Objective: This research aims at clarifying the action and mechanisms of action of TP53TG1 in cancer-associated fibroblasts (CAF)-derived exosomes (EXs) on colorectal carcinoma (CRC) cells.
Methods: CAF and CAF-EXs isolated from CRC tissues were incubated with CRC SW480 cells to determine alterations in biological behavior, epithelial-mesenchymal transition (EMT) capacity, and TP53TG1 and miR-330-3p expression. In addition, a dual luciferase reporter (DLR) assay was conducted to verify the connection between TP53TG1 and miR-330-3p, and the impacts of the two genes on CRC cells were analyzed.
Results: CRC-CAF-EXs extracted from CRC tissues were successfully identified and were able to promote SW480 multiplication, invasiveness, migration, and EMT ability while inhibiting apoptosis ($P < 0.05$). In addition, TP53TG1 increased and miR-330-3p decreased in SW480 when cultured with CRC-CAF-EXs ($P < 0.05$). The DLR assay identified notably reduced fluorescence activity of TP53TG1-WT after transfection with miR-330-3p-mimics ($P < 0.05$). Furthermore, SW480 cell multiplication, invasiveness and migration were found to be enhanced and the apoptosis decreased after up-regulating TP53TG1, while suppressing TP53TG1 and up-regulating miR-330-3p contributed to quite the opposite effect ($P < 0.05$). Moreover, by elevating TP53TG1 and miR-330-3p simultaneously, we found a cell activity similar to the NC group ($P > 0.05$).
Conclusion: By targeting miR-330-3p, TP53TG1 in CRC-CAF-EXs can enhance CRC cell activity and EMT capacity and inhibit apoptosis.

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1. Introduction

Being a commonly seen malignancy of the gastrointestinal tract, colorectal carcinoma (CRC) ranks third in prevalence worldwide, second only to gastric and lung carcinomas [1]. According to statistics, CRC afflicts an average number of 300,000 every year, with a growing incidence in some developed countries [2]. Studies indicate that the global burden of CRC is expected to increase by approximately 5–6 times by 2030 [3]. Moreover, as one of the neoplastic diseases with extremely high malignancy, CRC has a very poor prognosis, with the prognostic survival and the 5-year mortality rate of advanced CRC patients being 8–20 months and 60–80 %, respectively [4]. The reason for the unsatisfactory prognosis of CRC also has a great relationship with its early concealment, as the disease may only be manifested as abdominal pain, diarrhea, etc., in the early stage. At present, the early diagnosis of neoplastic diseases is still mainly based on imaging, which has great limitations, resulting in a low diagnosis rate of early CRC [5]. Because of this, clinical efforts are being devoted to finding and exploring new diagnostic approaches and treatment options for CRC [6].

In recent years, the research on tumor diseases has focused on long non-coding Ribonucleic Acids (lncRNAs), which, as one of the human genetic materials, are considered as important potential regulatory genes in tumor biology, canceration, and metastasis [7]. A large number of studies have confirmed that lncRNAs are relatively stable and can be detected in blood, body fluids, tissues, cells, and other samples, suggesting their potential to become new tumor biomarkers [8]. In addition, exosomes (EXs) are a class of nanoscale extracellular vesicles that function as natural carriers of proteins and various non-coding RNAs, and are widely found in body fluids including plasma, serum, and urine. Some lncRNAs have been indicated to be selectively packaged in EXs to participate in cancer occurrence and development [9]. Cancer-associated fibroblasts (CAF) are the most active component of the tumor microenvironment, which can promote tumor angiogenesis, cell multiplication, migration, and metastasis by secreting various chemokines and cytokines interacting with tumor cells [10]. At present, it has been found that exosomal lncRNAs play an important role in CRC cell multiplication, migration, invasiveness, metastasis, stemness and cell drug resistance via targeting and inhibiting target genes. Therefore, the role played by CAF-derived exosomal lncRNAs in this process has attracted increasing attention [11].

LncRNA TP53TG1, a newly discovered lncRNA, is abnormally expressed in gliomas and lung cancer [12], but its role in CRC remains to be defined. Current studies have found that TP53TG1 lipid nanosystem has certain regulatory ability on colorectal tumor behavior [13]. Therefore, it is preliminarily suspected that TP53TG1, an exosome derived from CAF, also has a certain influence on CRC cells. Previous studies have indicated that miR-330-3p has a close relationship with TP53TG1, and that miR-330-3p is essential in regulating CRC growth [14]. Therefore, we speculate that the action pathway of CRC-CAF may be related to miR-330-3p and TP53TG1. In this study, we will analyze this and provide a new direction for the future diagnosis and treatment of CRC, as well as laying the foundation for subsequent research.

2. Data and methods

2.1. Cell source

CRC-CAF and normal fibroblasts (NF) were extracted from fresh CRC tissues and adjacent counterparts. Human CRC SW480 cells supplied by the American Type Culture Collection (ATCC) were cultivated in 10 % fetal bovine serum (FBS)-added Dulbecco's Modified Eagle Medium (DMEM) in a 5 % CO_2 and 37 °C incubator, and digested with trypsin. Passage was carried out when the cells fused to 75%–80 %.

2.2. CRC-CAF culture

After rinsing the CRC tissues and adjacent counterparts with phosphate buffer saline (PBS) for 3 times, they were cut into pieces, digested with a mixed solution composed of collagenase, neutral protease and hyaluronidase for 50min, and centrifuged (352×*g*, 4 °C, 5min) to obtain precipitates, which were then suspended with Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F-12) complete medium mixed with 12 % FBS for inoculation in Petri dishes. The medium was replaced after 3 days, and the tissue fragments and non-adherent cells were washed away. Finally, the epithelial cells were digested with trypsin to obtain CRC-CAF and NF.

2.3. Fibroblast (Fb) identification

After being isolation from CRC-CAF and NF, the total proteins were moved to a polyvinylidene fluoride (PVDF) membrane to be sealed with 5 % skim milk for 2 h, and added with α-smooth muscle actin (α-SMA), Human Fibroblast Specific Protein 1 (FSP-1), Fibroblast activating protein (FAP), and Vimentin (Abcam, USA) for overnight incubation (4 °C). At ambient temperature, the membrane was washed the next day and cultivated with Horseradish Peroxidase (HRP)-labeled secondary antibody for 2 h. The protein expression was analyzed after enhanced chemiluminescence (ECL) development.

2.4. Extraction and identification of EXs

The cell culture medium was replaced with a 10 % FBS-added EXs-free complete culture medium. After 48h of culture, the supernatant was collected for centrifugation (3000×*g*, 15min) to remove dead cells, followed by another centrifugation (6000×*g*, 40min) to remove cell fragments, the third centrifugation (10000×*g*, 1h) to obtain supernatant, and the fourth centrifugation (100000×*g*, 1h) to collect precipitate to obtain EXs, which were refrigerated at −80 °C. The exosomal standard proteins CD9, CD63, and TSG101

(Abcam, USA) were measured by Western blot (WB).

2.5. Polymerase chain reaction (PCR) detection of TP53TG1

TRIzol-isolated total RNA from the cells were reverse transcribed to cDNA, which was then amplified with a PCR kit. Reaction conditions (40 cycles): 95 °C/30s; 95 °C/5s, and; 60 °C/34s. TP53TG1 expression was normalized with GAPDH and obtained via $2^{-\triangle\triangle CT}$ (Applied Biosystems Veriti PCR instrument, Thermo Fisher, USA). The primer sequences are shown in Table 1.

2.6. Cell multiplication testing

SW480 was diluted to 1×10^5/ml in EXs-free DMEM comprising FBS, inoculated into the wells of a 96-well plate (100 μL/well), and added with EXs extracted from CRC-CAF, which was set up CAF-SW480 group. Besides, the cells added with the same amount of PBS were set as PBS-SW480 group. The final concentration was 10 μg/ml. At intervals of 24 h, 10 μL Cell Counting Kit-8 (CCK-8) (MedChemExpress, USA) solution was added into a well and measured by a microplate reader (Agilent BioTek Multifunction Enzyme Labeler Synergy H1, USA) for absorbance (OD 450 nm) after incubation for 4 h. Besides, cells (200 cells/ml) were inoculated into the wells of 6-well plates, and added with FBS (500 μL/well) on the 5th day after plating. The supernatant was discarded after colony formation, and the cells were subjected to 4 % paraformaldehyde immobilization and 0.1 % crystal violet staining for cloning rate calculation.

2.7. Cell migration testing

The 6-well plates where the treated cells (4×10^5/mL) were inoculated, were scratched to make vertical lines with a 10 μL pipette tip when cell growth reached 80–90 %. After 24 h, the scratch area was observed, and the cell mobility was calculated = (scratch distance0h - scratch distance24h)/scratch distance0h × 100 %.

2.8. Cell invasiveness testing

The treated cells (5×10^4/mL) were inoculated into a Transwell upper chamber (Corning, USA), and serum-free medium was added to 300 ml. Cell culture medium comprising 10 % FBS was added to the lower chamber, and Matrigel gel was added to the Transwell chamber. After 24 h of culture, the surface-suspended cells were swabbed with Q-tips, fixed and stained, and the transmembrane cells were counted in 5 target fields randomly.

2.9. Apoptosis testing

In the same way, 5×10^5/mL cells in CAF-SW480 and PBS-SW480 groups after 24h of cultivation were placed in the centrifuge tube and mixed with 500 μL of Binding Buffer for re-suspension, followed by dyeing with Annexin V-FLTC and PI solution (5 μL each) and light tight incubation (15min). Fluorescence value was detected by flow cytometry (Merck Millipore), and apoptosis number was analyzed.

2.10. Cell transfection

After constructing TP53TG1-si (targeted silencing of TP53TG1 expression), TP53TG1-sh (targeted elevation of TP53TG1 expression), negative control NC and miR-330-3p-mimics (miR-330-3p mimic sequence) by GenePharma, they were transfected into SW480 according to Lipofectamine 2000 (Thermo Fisher, USA) instructions.

2.11. Double luciferase reporter (DLR) gene assay

Prediction of TP53TG1's downstream target genes was made by online target gene database starbase (https://rnasysu.com/encori/). The wild-type (TP53TG1-WT) and mutant (TP53TG1-MUT) luciferase reporter vectors of TP53TG1 were constructed, which were then co-transfected into SW480 with either miR-330-3p-mimics or negative control for the determination of luciferase activity with the use of DLR assay kit (Thermo Fisher, USA).

Table 1
Primer series.

	Forward primer (3′-5′)	Reverse primer (3′-5′)
TP53TG1	TC-CCCTCAGATTTTGGTGGCA	GCGG-GAGATATTGGCTGGTA
GAPDH	GCTGAGTACGTCGTGGAGTC	CCCATTCCCCAGCTCTCATA
miR-330-3p	GCCAACAATATCCTGGTGCTG	GAGGTATTCGCACTGGATACGACTCTCTG
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT

2.12. Statistical processing

The experimental results, recorded as ($\bar{\chi}\pm s$), were repeatedly determined three times. SPSS23.0 (IBM, USA) was used for statistical analysis, and the significance level was $P < 0.05$ in this study. Independent sample *t*-test, one-way Analysis of Variance (ANOVA) and Least-Significant Difference (LSD) post-hoc test were used for the data conforming to normal distribution, and nonparametric Mann-Whitney *U* test and Kruskal-Wallis H test were used otherwise.

3. Results

3.1. Fb extraction and EXs identification results

As indicated by WB, α-SMA, FSP-1, FAP, and Vimentin were markedly higher in CRC-CAF versus NF ($P < 0.05$, Fig. 1A and B), which accorded with the characteristics of CAF and NF, indicating successfully Fb extraction. EXs were between 30 and 100 nm in size under transmission electron microscopy, with an average of (74.63 ± 21.42) nm. and had a double-layer membrane structure and a "cup holder" shape, which accorded with the structural characteristics of EXs (Fig. 1C). WB analysis revealed elevated CD9, CD63, and TSG101 protein levels in CRC-CAF and NF-derived EXs, which was in line with the structure and biological characteristics of EXs, indicating successful extraction (Fig. 1D and E).

3.2. Impacts of CRC-CAF-EXs on CRC cells

As indicated by biological behavior test results, the cell multiplication (Fig. 2A–C), invasiveness (Fig. 2D and E), and migration (Fig. 2F and G) capacities were enhanced in CAF-SW480 group versus PBS-SW480 group ($P < 0.05$), while the apoptosis rate was reduced ($P < 0.05$, Fig. 2H and I). These results suggest that CRC-CAF-EXs could promote SW480 activity and accelerate CRC growth. WB revealed lower E-cadherin protein and higher N-cadherin and Vimentin protein levels in CAF-SW480 group versus PBS-SW480 group ($P < 0.05$, Fig. 2J and K). Subsequently, PCR analysis determined higher TGF-β and VEGF mRNA levels in CAF-SW480 group compared with PBS-SW480 group ($P < 0.05$, Fig. 3L).

3.3. Relationship between TP53TG1 and miR-330-3p

The detection of TP53TG1 expression in EXs showed markedly elevated TP53TG1 in CRC-CAF-EXs than in NF-derived EXs ($P < 0.05$, Fig. 3A). While miR-330-3p was found to be lower in CRC-CAF-EXs than in NF-derived EXs ($P < 0.05$, Fig. 3B). Through online target gene prediction website screening, we found miR-330-3p when searching the downstream target genes of TP53TG1. The binding complementary sites between them are shown in Fig. 3C. Subsequently, the DLR assay showed notably decreased fluorescence activity of TP53TG1-WT after miR-330-3p-mimics transfection in CRC cells ($P < 0.05$), confirming the presence of a targeted relationship between them (Fig. 3D). Then, PCR detection results determined lower miR-330-3p in TP53TG1-sh group versus TP53TG1-si and TP53TG1-NC groups, and higher miR-330-3p in TP53TG1-si group compared with TP53TG1-NC group ($P < 0.05$, Fig. 3E).

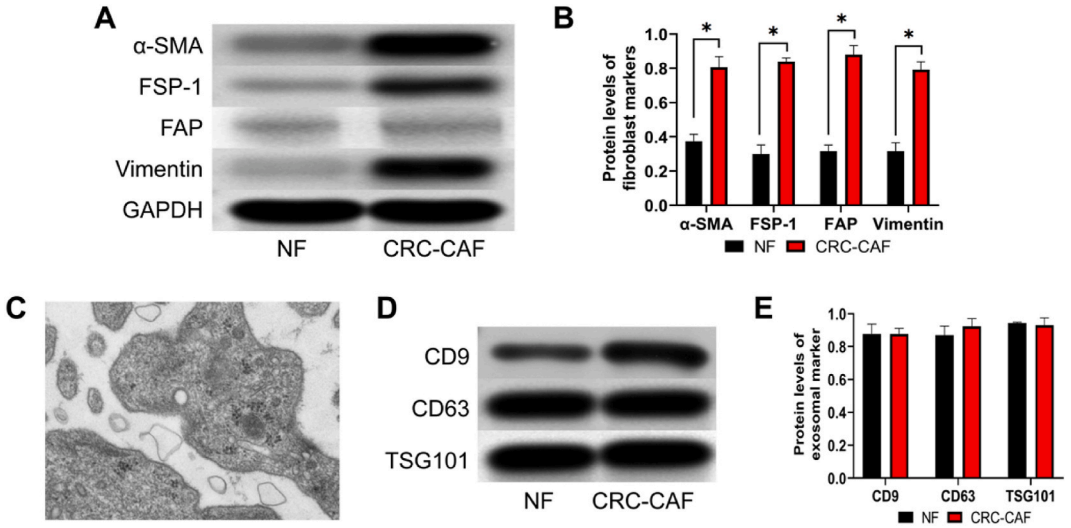

Fig. 1. Fb extraction and EXs identification results. (A) Western blot diagram. (B) Protein levels of fibroblast markers. (C) Transmission electron microscopy of exosomes. (D) Western blot diagram. (E) Protein levels of exosomal marker. *$P < 0.05$. The uncut of Fig. 1A–D is shown in Supplementary Figs. S1 and S2.

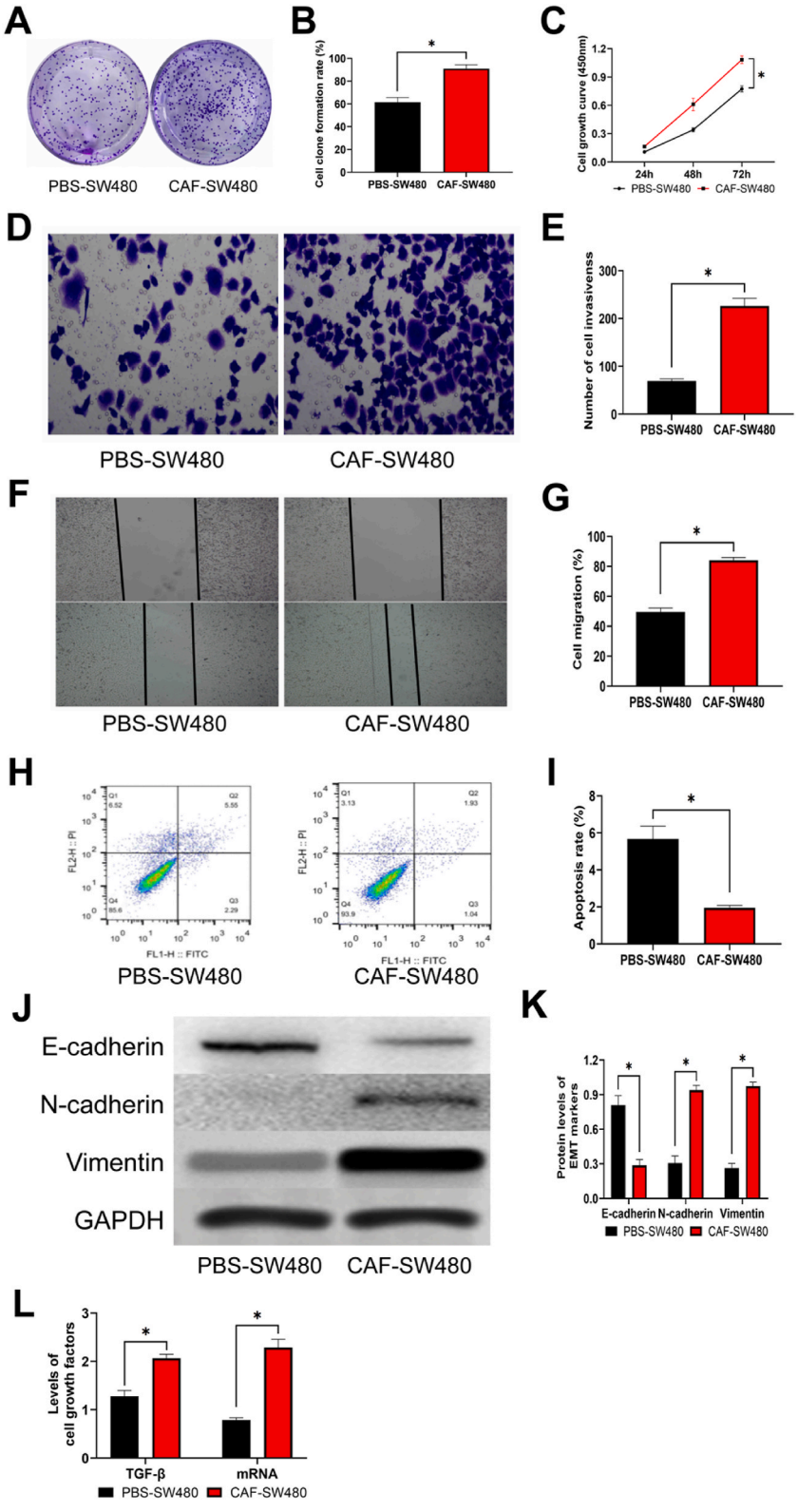

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Fig. 2. Impacts of CRC-CAF-EXs on CRC cells. (A) Results of cell cloning experiments. (B) Cell clone formation rate. (C) Cell growth curve. (D) Invasive cell staining. (E) Number of cell invasiveness. (F) Cell wound-scratch assay results. (G) Cell migration. (H) Flow cytometry results. (I) Apoptosis rate. (J) Western blot diagram. (K) Protein levels of EMT markers. (L) Levels of cell growth factors. *P < 0.05. The uncut of Fig. 2J is shown in Supplementary Fig. S3.

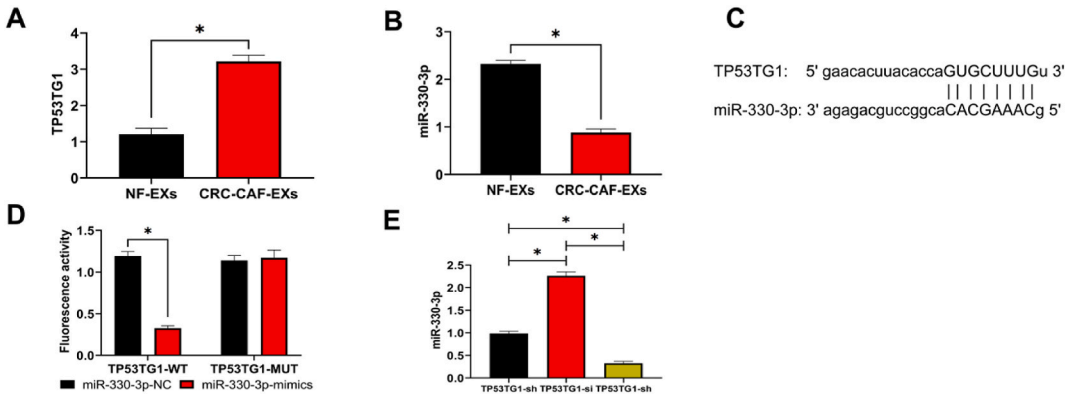

Fig. 3. Relationship between TP53TG1 and miR-330-3p. (A) Comparison of TP53TG1 expression levels. (B) Comparison of miR-330-3p expression levels. (C) Binding complementary sites of TP53TG1 and miR-330-3p. (D) DLR assay. (E) Impacts of TP53TG1 on miR-330-3p in CRC. *P < 0.05.

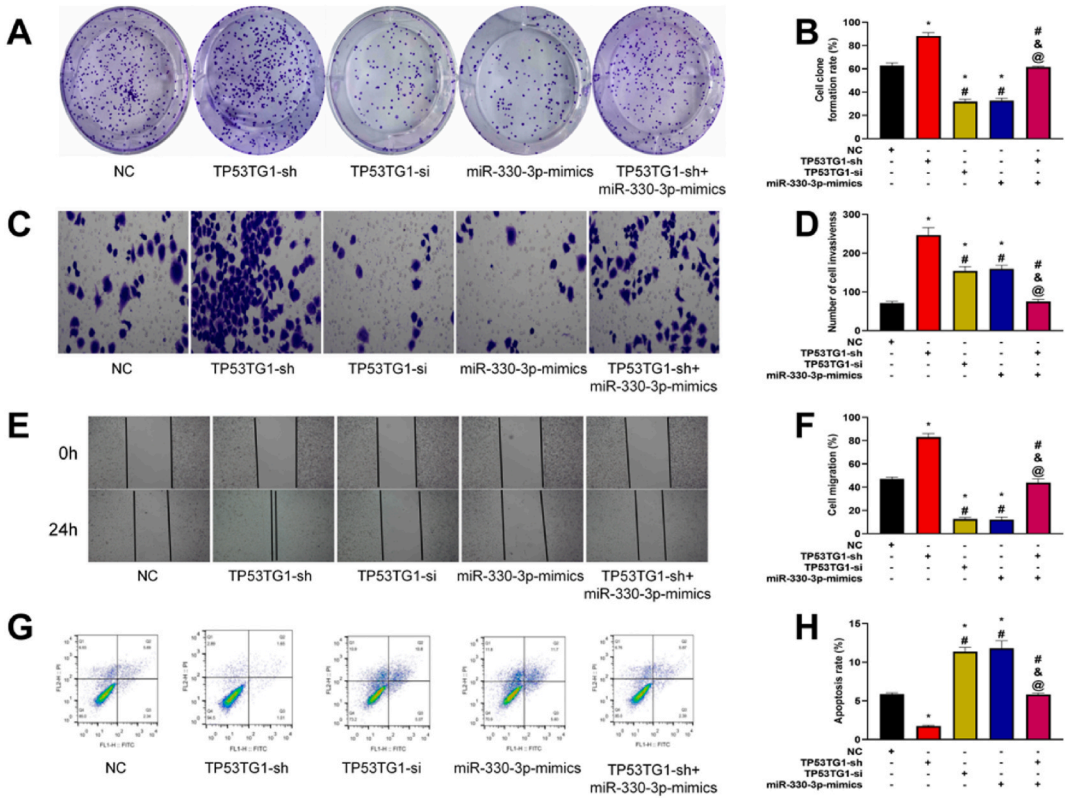

Fig. 4. TP53TG1 affects CRC via sponge adsorption of miR-330-3p. (A) Results of cell cloning experiments. (B) Cell clone formation rate. (C) Invasive cell staining. (D) Number of cell invasiveness. (E) Cell wound-scratch assay results. (F) Cell migration. (G) Flow cytometry results. (H) Apoptosis rate. Compared with the NC group *P < 0.05. Compared with the TP53TG1-sh group #P < 0.05. Compared with the TP53TG1-si group &P < 0.05. Compared with the miR-330-3p-mimics group @P < 0.05.

3.4. Highly expressed TP53TG1 affects CRC cell biological behavior via targeting miR-330-3p

Biological behavior test results showed similar cell multiplication (Fig. 4A and B), migration (Fig. 4C and D), invasiveness (Fig. 4E and F), and apoptosis rate (Fig. 4G and H) between TP53TG1-sh + miR-330-3p-mimics and NC groups ($P > 0.05$). Nor was there a significant difference between TP53TG1-si and miR-330-3p-mimics groups ($P > 0.05$), which showed decreased multiplication, migration and invasiveness capacities while enhanced apoptosis compared with TP53TG1-sh + miR-330-3p-mimics group ($P < 0.05$). Of the five groups, the highest multiplication, invasiveness and migration capacities and the lowest apoptosis rate were determined in TP53TG1-sh group ($P < 0.05$).

3.5. Highly expressed TP53TG1 promotes CRC cell epithelial-mesenchymal transition (EMT) via targeting mir-330-3p

Similarly, TP53TG1-sh + miR-330-3p-mimics and NC groups showed no evident difference in EMT capacity ($P > 0.05$), whose E-cadherin protein was lower than that of TP53TG1-si and miR-330-3p-mimics groups but higher than TP53TG1-sh group, while N-cadherin and Vimentin were higher than those of TP53TG1-si and miR-330-3p-mimics and lower than TP53TG1-sh group ($P < 0.05$, Fig. 5A–D). And the detection results showed that TGF-β and VEGF were the highest in TP53TG1-sh group among the five groups, while were the lowest in TP53TG1-si and miR-330-3p-mimics groups ($P < 0.05$, Fig. 5E and F).

4. Discussion

In this study, we found that TP53TG1 in CRC-CAF exosomes could promote the activity of CRC cells with EMT by targeting miR-330-3p, and these findings could provide new research directions for the future treatment of CRC.

As a high-incidence malignancy worldwide, CRC has a great potential threat [15]. Fully understanding its pathogenesis is the key to finding a new diagnosis and treatment scheme, but clinical breakthroughs have not yet been achieved [16]. Fbs are the most common component of connective tissue and one of the main sources of CAF in tumors. During quiescence, Fbs are mitotically inactive until their phenotype transitions to an activated state when they respond to tissue damage or different types of stress [17]. CAF activation is a key event in ECM protein synthesis and secretion, leading to extracellular matrix remodeling and increased tumor cell invasiveness [18]. The relationship between CAFs and tumor cells is mediated through a complex signaling network, with each cell type interacting through synergistic or antagonistic signaling axes. Many studies have revealed that CAF affects CRC progression through the release of

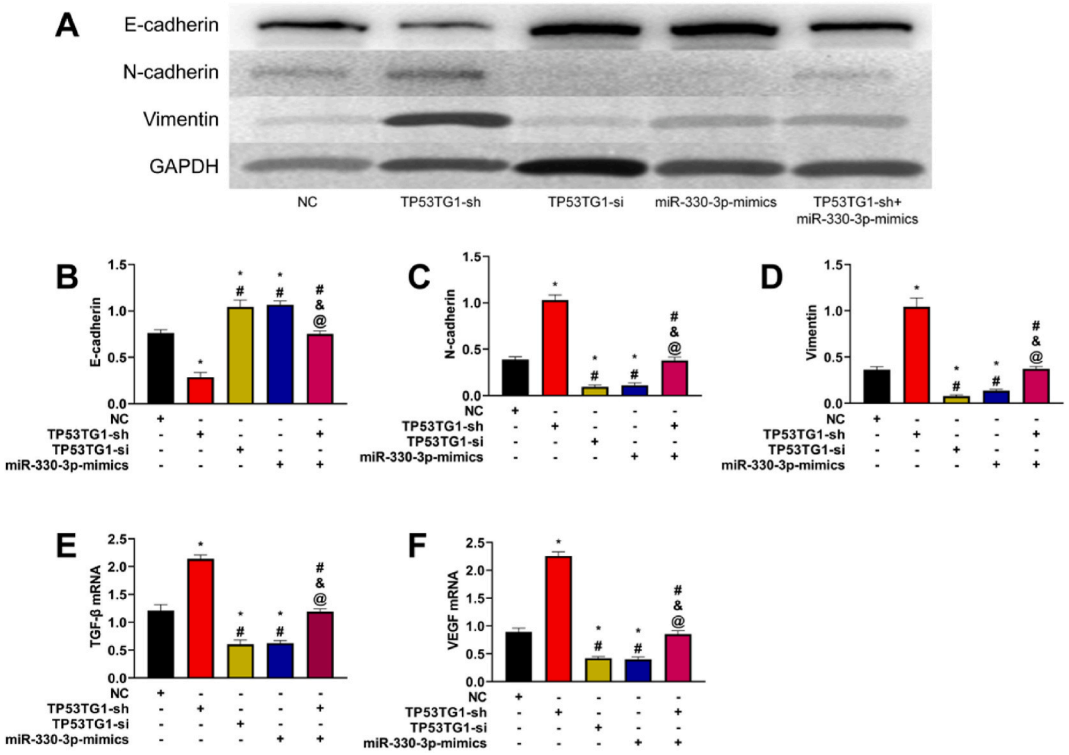

Fig. 5. Highly expressed TP53TG1 promotes CRC cell EMT via targeting miR-330-3p. (A) Western blot diagram. (B) Expression of E-cadherin protein. (C) Expression of N-cadherin protein. (D) Expression of Vimentin protein. (E) Expression of TGF-β mRNA. (F) Expression of VEGF mRNA. Compared with the NC group $^{*}P < 0.05$. Compared with the TP53TG1-sh group $^{\#}P < 0.05$. Compared with the TP53TG1-si group $^{\&}P < 0.05$. Compared with the miR-330-3p-mimics group $^{@}P < 0.05$. The uncut of Fig. 5A is shown in Supplementary Fig. S4.

tumor-related factors such as growth factors, chemokines, and stromal cell-derived factor-1 [19,20]. EXs are extracellular vesicles formed by lipid bilayers that facilitate intercellular communication by carrying various types of molecules and enhance chemoresistance and radioresistance by modifying gene expression in recipient tumor cells [21]. Exosomal lncRNAs have been shown to exert a significant impact on CRC cell activity [22,23]. Thus, a thorough understanding of the release of CAF-EXs may be the key to inhibiting tumor progression and metastasis and altering chemotherapy resistance in the future. Therefore, this study is of great significance for future CRC research.

In this study, we extracted CRC-CAF-EXs from CRC tissues and adjacent counterparts for identification. The results showed that both CRC-CAF and EXs obtained were consistent with their biological characteristics, which laid a foundation for the accuracy of the research. After culturing CRA-CAF-EXs with SW480, it was observed that CRC cell multiplication, invasiveness, and migration were obviously increased, while the apoptosis rate was decreased, suggesting that CRC-CAF-EXs can activate cell activity and accelerate CRC progression. Similarly, Zhou et al. reported that CAF-derived EXs can promote CRC growth [24]. Then, we detected TP53TG1 levels in CRC-CAF-EXs and found highly expressed TP53TG1. TP53TG1, with a length of 751 nt, is a newly discovered lncRNA closely linked to many tumor diseases, with aberrant expression in CRC [25–29]. However, the role played by TP53TG1 in CAF and CAF-derived EXs is still unclear. Therefore, we conducted relevant detection, and results consistent with previous studies were obtained [30], preliminarily confirming that CRC-CAF-EXs can activate TP53TG1 expression. Therefore, we speculate that the action pathway of CRC-CAF may be related to miR-330-3p and TP53TG1.

Subsequently, in order to confirm the action mechanism of TP53TG1 and miR-330-3p on CRC, we verified the relationship between them. The results showed that TP53TG1 and miR-330-3p had complementary binding sites, and the fluorescence activity of TP53TG1 was inhibited by miR-330-3p, confirming the targeting relationship between the two. Besides, after transfection of the two into SW480, the multiplication, invasiveness, and migration of SW480 were enhanced and the apoptosis was decreased after increasing TP53TG1, while the results were completely opposite after the inhibition of TP53TG1 and the increase of miR-330-3p, indicating that high expression of TP53TG1 can promote the malignant growth of CRC, while increasing miR-330-3p can inhibit this process, consistent with the previous research results [31,32]. Moreover, by simultaneously increasing TP53TG1 and miR-330-3p, we found no difference in cell activity between TP53TG1 and NC groups, indicating that the effect of increasing TP53TG1 on CRC can be completely reversed by increasing miR-330-3p, which confirms the presence of a targeted competition between TP53TG1 and miR-330-3p.

EMT, a process known to be crucial to tumor growth, can modify the adhesion molecules expressed by cells to enable them to migrate and become aggressive [33]. In previous studies, both CAF and TP53TG1 have been found to have important effects on EMT of cells [34,35]. Therefore, in this study, we also detected the EMT of cells. First of all, the EMT capacity of cells was found to be significantly activated under the action of CRC-CAF or after the elevation of TP53TG1, confirming the role of oncogene of high TP53TG1 expression in CRC and supporting the accuracy of the above experimental results. Similarly, the effect of TP53TG1 on the EMT capacity of CRC cells was reversed after miR-330-3p was simultaneously increased, which once again verified the targeted regulation relationship between them.

This study preliminarily explored the influence mechanism of CRC-CAF-EXs on CRC cells, but there are still many limitations to be addressed. In the follow-up study, we need to purchase more types of CRC cell lines to further confirm the mechanism of action of CRC-CAF, TP53TG1, and miR-330-3p. In addition, tumorigenesis experiments in nude mice are needed to verify the effects of the three factors on living tumors. Finally, we need to carry out clinical trials as soon as possible to analyze the clinical expression of TP53TG1 and miR-330-3p, so as to provide a more reliable reference for clinical practice.

5. Conclusion

TP53TG1 in CRC-CAF-EXs can promote the activity and EMT of CRC cells and inhibit their apoptosis via targeting miR-330-3p, which may become a breakthrough in future CRC diagnosis and treatment, with great clinical implications.

Data availability statement

The data in this article can be obtained from the corresponding author under reasonable circumstances.

Consent for publication

All authors consent to the publication of this study.

CRediT authorship contribution statement

Yawei Liu: Writing – review & editing, Writing – original draft, Methodology, Investigation. **Youwei Wang:** Formal analysis, Data curation. **Zhijuan Yu:** Visualization, Validation, Supervision. **Ziheng Wang:** Project administration, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ziheng Wang is the assocaited editor of Heliyon If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

All authors have no acknowledgements to disclose.
The following is the Supplementary data to this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30301.

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