RESEARCH ARTICLE

Exosome-derived microRNA-29c Induces Apoptosis of BIU-87 Cells by Down Regulating BCL-2 and MCL-1

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Abstract

Background: Aberrant expression of the microRNA-29 family is associated with tumorigenesis and cancer progression. As transport carriers, tumor-derived exosomes are released into the extracellular space and regulate multiple functions of target cells. Thus, we assessed the possibility that exosomes could transport microRNA-29c as a carrier and correlations between microRNA-29c and apoptosis of bladder cancer cells. Materials and Methods: A total of 28 cancer and adjacent tissues were examined by immunohistochemistry to detect BCL-2 and MCL-1 expression. Disease was Ta-T1 in 12 patients, T2-T4 in 16, grade 1 in 8, 2 in 8 and 3 in 12. The expression of microRNA-29c in cancer tissues was detected by quantitative reverse transcriptase PCR (QRT-PCR). An adenovirus containing microRNA-29c was used to infect the BIU-87 human bladder cancer cell line. MicroRNA-29c in exosomes was measured by QRT-PCR. After BIU-87 cells were induced by exosomes-derived microRNA-29c, QRT-PCR was used to detect the level of microRNA-29c. Apoptosis was examined by flow cytometry and BCL-2 and MCL-1 mRNA expressions were assessed by reverse transcription-polymerase chain reaction. Western blotting was used to determine the protein expression of BCL-2 and MCL-1. Results: The expressions of BCL-2 and MCL-1 protein were remarkably increased in bladder carcinoma ($p<0.05$), but was found mainly in the basal and suprabasal layers in adjacent tissues. The expression of microRNA-29c in cancer tissues was negatively correlated with the BCL-2 and MCL-1. The expression level of microRNA-29c in exosomes and BIU-87 cells from the experiment group was higher than that in control groups ($p<0.05$). Exosome-derived microRNA-29c induced apoptosis ($p<0.01$). Although only BCL-2 was reduced at the mRNA level, both BCL-2 and MCL-1 were reduced at the protein level. Conclusions: Human bladder cancer cells infected by microRNA-29c adenovirus can transport microRNA-29c via exosomes. Moreover, exosome-derived microRNA29c induces apoptosis in bladder cancer cells by down-regulating BCL-2 and MCL-1.

Keywords: Exosomes - microRNA-29c - bladder cancer cells - apoptosis

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Introduction

The incidence of bladder carcinoma is in the first in Chinese urological tumor, which includes the 70% non invasive cancer and 30% invasive cancer. The patients accompanied with systemic metastasis have poor prognosis and a lifetime risk of recurrence.

Tumor-derived exosomes had been regarded as a good carrier of anti-tumor therapy for its advantages of tumor specific antigen recognition, easy to obtain. For example, exosomes from tumor cells treated by heat shock or genetic modification were associated with ODN-CpG adjuvants to deal with the tumor bearing mice, showing that it enhanced the antitumor activity of the secretion of exosomes (Chaput et al., 2004). Researches on exosomes-derived miRNA indicated that it could be used as a new disease biomarker and possesses therapeutic potential of tumor, inflammation and other diseases (Kosaka et al., 2010; Mittelbrunn et al., 2011).

MicroRNA (miRNA) is a kind of non-coding single strand RNA molecules coded by endogenous gene of 20-22 nucleotides, bound with target gene 3 'non-coding region so as to promote the degradation of target gene or inhibit its translation. At present it has been found more than 800 human miRNAs (Kozomara et al., 2011), and confirmed that the abnormal expression of miRNA associated with a particular tumor can play the role of oncogenes or tumor suppressor genes. MicroRNA-29 (miR-29) family including miR-29a, miR-29b and miR29c in human is an important regulator of the tumor (Wang et al., 2013). It can modulate the complex process of tumor through acting on multiple targets. The present study

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showed that miR-29 was involved the regulation of cell proliferation, cell cycle, cell apoptosis and function of immune by acting on the pathways of p53, c-myc, NF-kB and others (Wang et al., 2013). In terms of cell apoptosis, Park et al had shown that miR-29 could induce the cell apoptosis by targeting on the suppressor gene p85 and CDC42 of p53 (Park et al., 2008). Additionally, both miR-29a and miR-29b did not only result in the degradation of antiapoptotic genes directly, but also up-regulated the apoptosis gene BIM (BCL2L11) and the tumor suppressor programmed cell death 4 (PDCD4). Therefore, by targeting antiapoptotic gene (Cdc42, p85a, Mcl1 and Tcl1) and enhancing the inhibiting of tumor transcription, miR-29 chould inhibits the tumor growth effectively (Garzon et al., 2009). In several researches, the genetic screening showed that the expression of miR-29 a/c in bladder cancer was down-regulated (Dyrskjot et al., 2009; Friedman et al., 2009; Wang et al., 2010). Baffa R et al showed that the expression of miR-29a/b was up-regulated in paired primary and metastatic cancers. It might associate with the classification of bladder cancer (Baffa et al., 2009). However, the specific function of miR-29 in bladder cancer is still lack of research.

Our prophase researches have also confirmed that miR-29c in bladder cancer cells is low expression. However, it has not been reported whether miR-29c is transported by exosomes from bladder cancer cells, and the machanism of miR-29c action in bladder cancer. So we suppose miR-29c may be a tumor suppressor and design the experiment to provide a idea of tumor treatment. In this study, we will transfect the exogenous miR-29c into bladder cancer cells, identify whether it was secreted through exosomes, detect the apoptosis of bladder cancer cells after dealing it with exosomes-derived miR-29c, and then examine the expression of BCL-2 and MCL-1 in bladder cancer cells, to initially investigate the effect of miR-29c in bladder cancer and provide a new idea of treatment for bladder cancer.

Materials and Methods

Patients and Specimens

28 pairs of human TCCB (bladder transitional cell carcinoma) and corresponding adjacent tissue samples were obtained from patients during 2011 and 2013, who were underwent total cystectomy at the Department of Urology, First Affiliated Hospital of Chongqing Medical University. All tissue samples were examined to be TCCB or normal tissue in Department of Pathology. According to UICC guidelines, the cancer samples included stage Ta-T1 in 12 and T2-T4 in 16, and grade 1 in 8, 2 in 8 and 3 in 12. All specimens were stored at-80°C. The socioeconomic status of all patients was similar. Informed consent was obtained from all patients and the study protocol was approved by the ethical committee.

Cell culture and virus infection

Bladder cancer cell lines BIU-87 was cultured in RPMI 1640 supplemented with 10% newborn calf serum (GIBCO®) in 5% carbon dioxide in a humid incubator at 37°C, Cells were cultured at 37°C in 5% CO_2. when BIU-87 growing to 70%~80% confluency in plate, medium without serum was used, then adenovirus was added, incubated in 37°C for 1h, then equal amounts of RPMI1640 containing 10% fetal bovine serum culture medium was added. The cells were divided into 3 groups: Ad-miR group (infected by Ad-RFP-miR-29c), Ad group (infected by Ad-RFP), and control group (not infected).

Separation and identification of exosomes

Culture supernatants (100ml) from 3 groups of BIU-87 were collected, the cell debris were cleaned by serial centrifugation. Then the clarified supernatant was concentrated by centrifugation at 1000×g for 30 min in a prerinsed 100 kDa MWCO Centrifugal Filter Device and gain about 20ml concentrated exosomes. The ultracentrifuge supernatant was removed to the centrifuge tube which was spread with 30% sucrose/D_2O density cushion, and concentrated by ultracentrifuging at 100, 000×g for 60 min. At the bottom, the cushion was collected and diluted in 10 ml of PBS. The exosomes were further concentrated by centrifuging for 30 min at 1000×g in prerinsed 100 kDa MWCO Amicon ultra-15 to a volume of about 3 ml. Membrane filter (0. 22μm) was used to sterilize exosomes. Bladder cancer cells derived exosomes was configured as 100 ng /mL with RPMI1640 and stored at -80°C. Bradford method was used for the quantification of the total protein. (EXO: exosomes derived from cells of control group. EXO/Ad: exosomes derived from cells of Ad group. EXO/Ad-miR: exosomes derived from cells of Ad-miR group). Morphology of exosomes was identified with transmission electron microscopy.

Immunohistochemical procedures

Paraffin sections were dewaxed and rehydrated. Antigen was repaired by 10mM sodium citrate (pH6.0). 3%H_2O_2 blocked endogenous peroxidase for 10min, polyclonal rabbit antibody against BCL-2 or MCL-1 (Immunoway USA) was added to incubate 4°C overnight. Goat anti-rabbit Ig conjugated with horseradish peroxidase was used to incubate at 37°C for 45min. Slides were stained with the chromogen diaminobenzidine (Zhongshan, Beijing, China) until a brown color appeared. Hematoxylin was used as counterstain, ethanol dehydrate. The calculation methods of positive expression quote from the paper (Jamiyandorj et al., 2013). All images were analyzed by IPP 6.0 (Intel®).

Quantitative reverse transcriptase PCR (QPCR)

Total RNA was extracted from 28 pairs of human bladder cancer tissues and three groups of exosomes derived from bladder cancer by Trizol, (Invitrogen), and reversely transcribed into cDNA. MiR-29c reverse transcriptionprimer:5'-GTCGTATCCAGTGCAGGGT CCGAGGTATTCGCACTGGATACGACTAACCG-3', QPCR detected the expression of miR-29c. MiR-29c forward primer: 5 '- GCCTAGCACCATTTGAAATCG-3'; reverse primer: 5 '- GTGCAGGGTCCGAGGT-3', U6 was used as the internal standard. QPCR parameters for cycling: predegeneration at 95°C for 30s, degeneration at 95°C for 10s, annealing at 53°C for 20s, extension at 72°C for 30s, repeating for 40 cycles, the dissolution curve: 65°C -95°C, increasing 0.5°C per 5s.

Reverse transcription (RT) PCR

The total RNAs were extracted from the BIU-87 after co-cultured with exosomes (EXO, EXO/Ad, EXO /Ad-miR) for 48h, and was reversely transcribed into cDNA. The parameters for cycling: predegeneration at 94°C for 5min, degeneration at 94°C for, 30sannealing at 56°C for 30s, extension at 72°C for 1min, repeating for 35 cycles. BCL-2 forward primer: 5'-CGACGACTTCTCCCGCCGCTACCGC-3', reverse primer 5'-CCGCATGCTGGGGCCGTACAGTTCC-3', MCL-1 forward primer: 5'-TAAGGACAAAACGGGACT GG-3', reverse primer 5'-ACCAGCTCCTACTCCAGC AA-3'.

Western blot

BIU-87 were co-culture with exosomes (EXO, EXO/Ad, EXO/Ad-miR) for 48h, cells were collected, then lysed in RIPA lysis buffer with Protease Inhibitor (PMSF) and phosphatase inhibitor (NaF and Na_3VO_4) (Roche, Switzerland) on ice for 30 min. The solution was centrifuged 12000×g to obtain supernatant (to identify exosomes protein, 40 μL exosomes were collected, ultrasonic crushed, and boiled for 5 min after adding 5×SDS sample buffer). The protein sample was electrophoresed for 2h, and transferred to PVDF membranes (Amersham Biosciences, Beijing, People's Republic of China). Membranes were blocked in 5% skim milk in TBS-T for 1h at room temperature. The monoclonal rabbit antibody against BCL-2, MCL-1 diluted 1:500 was added in and incubated at 4°C overnight. After washed 3 times in 0.1% TBST, membranes were incubated with horseradish peroxidase conjugated secondary antibody (Zhongshan) diluted 1:1000 for 1 hour at 37°C. The blots were then detected by using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) and analyzed with Adobe Photoshop 7.0 software.

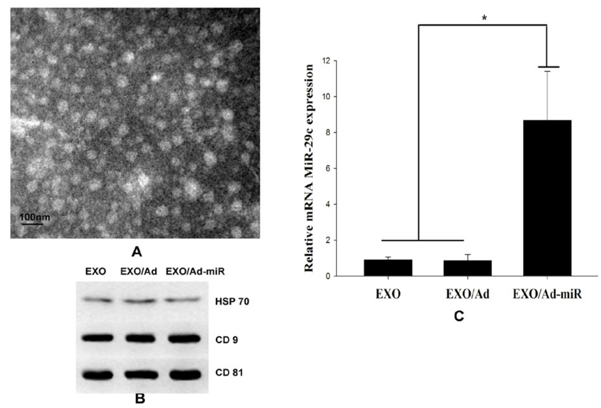

Figure 1. Exosomes Morphology and the Expressions of Mark Protein and miR-29c in Exosomes Showed we Isolated the Exosomes and Obtained Exosomes with Over-expression miR-29c. A) Exosomes morphology was observed by transmission electron microscope, **B)** the expressions of HSP70,CD9 and CD81 were detected by western blot, **C)** QRT-PCR revealed miR-29c expression in exosomes from the group of EXO, EXO/Ad and EXO/Ad-miR (*p<0.05)

Statistical analysis

The software of SPSS version 13.0 for Windows (SPSS Inc, IL, USA) was used for statistical analysis. Unpaired two-tailed t tests were used to compare significant differences between 2 groups. χ^2 and Fisher's exact tests were applied to compare the levels of rates. Data shown as $p<0.05$ was considered statistically significant.

Results

The identification of exosomes showed that we obtained exosomes from BIU-87successfully

Culture Supernatant contains a variety of substances, such as protein fragments, exosomes and vesicles. In order to successfully isolate exosomes from the supernatant fluid, we use the ultrafiltration of sucrose gradient centrifugation to isolate exosomes, and both morphological method and marker protein was used to identify exosomes. Transmission electron microscopy showed that the exosomes were spherical vesicles with different sizes and surrounded by lipid membrane, whose diameter was about 30~100 nm (Figure 1A). The expression of HSP70, CD9 and CD81 which are marker proteins of exosomes were positive by western blot (Figure 1B). There was not significant difference among the marker proteins expression in exosomes derived from cells of three groups.

The expression of BCL-2 and MCL-1 in bladder cancer was higher than adjacent tissues

Antiapoptotic gene BCL-2 and MCL-1 plays an important role in the occurrence and development of cancer. To deeply understand their expression in bladder cancer, we tested the expression of BCL-2 and MCL-1 in 28 cases of bladder cancer tissue. Among 28 cases of bladder cancer and adjacent tissues, BCL-2 protein

Table 1. BCL-2 and MCL-1 Protein Expression and Clinicopathological Parameters

	No. Specimens	BCL-2 expression			MCL-1expression		
		+ (%)	- (%)	*p* value	+ (%)	- (%)	*p* value
Tissue:				0.01			<0.01
Adjacent	28	5 (17.9)	23 (82.1)		3 (10.7)	25 (89.3)	
Bladder Ca	28	17 (60.7)	11 (39.3)		23 (82.1)	5 (17.9)	
Histological grade				0.01			0.393
Ta–T1	12	4 (33.3)	8 (66.7)		9 (75)	3 (25)	
T2–T4	16	13 (81.3)	3 (18.8)		14 (87.5)	2 (12.5)	
Histological stage				0.032			0.223
G1	8	2 (25)	6 (75)		5 (62.5)	3 (37.5)	
G2	8	5 (62.5)	3 (37.5)		7 (87.5)	1 (12.5)	
G3	12	10 (83.3)	2 (16.7)		11 (91.7)	1 (8.3)	

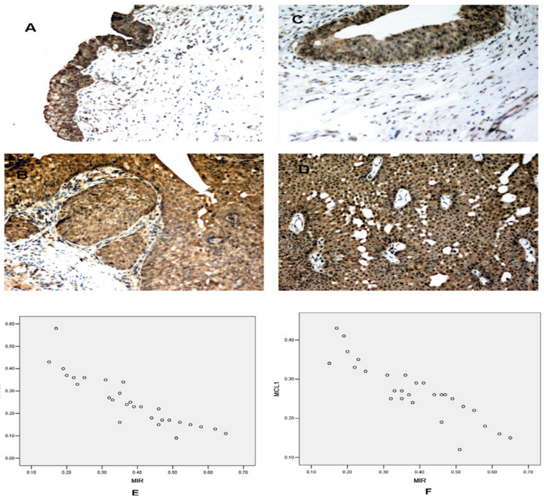

Figure 2. The Expressions of BCL-2 and MCL-1 in Bladder Cancer were Lower than in Adjacent Tissues, the Expression of microRNA-29c in Cancer Tissues was Negatively Correlated with the BCL-2 and MCL-1. The expression of Bcl-2 was shown in **A)** non-tumoral bladder tissue (DAB; 200×), **B)** urothelial carcinoma (DAB; 200×), and the expression of mcl-1 was shown in **C)** non-tumoral bladder tissue (DAB; 200×), **D)** urothelial carcinoma (DAB; 200×).The linear correlation analysis showed that the expression of microRNA-29c in cancer tissues was negatively correlated with the BCL-2 **E)** and MCL-1 **F)** ($p<0.01$)

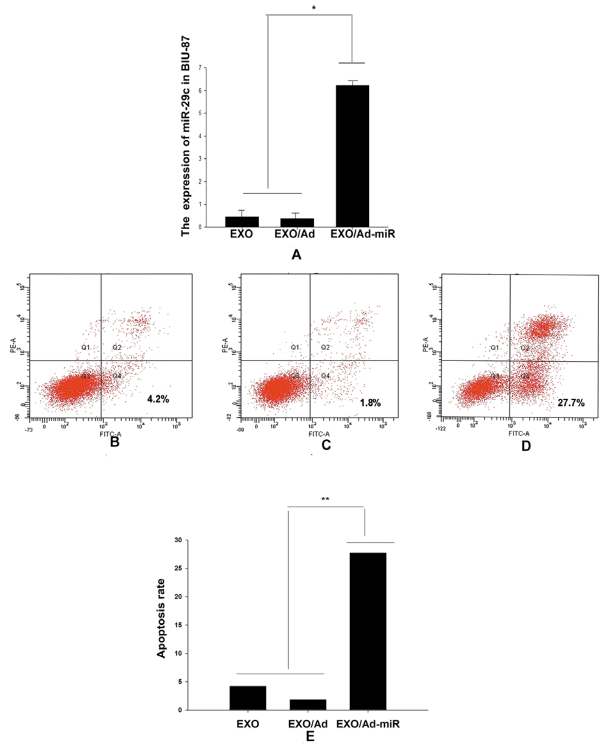

Figure 3. Over-Expression miR-29c in Exosomes was Transfected into BIU-87 and Induced Apoptosis in Bladder Cancer Cells A) The results of the microRNA-29C in BIU-87 by QRT-PCR; B: EXO group; C: EXO/Ad group; D: EXO/Ad-miR group) (*$p<0.05$; **$p<0.01$)

mainly expressed in the basal and suprabasal layers in adjacent tissues and appeared everywhere in TCCB. The positive expression rate in TCCB was obviously higher ($p=0.01$). Interestingly, the expression of MCL-1 protein was extremely similar with BCL-2. Dyeing results (Figure 2) showed that BCL-2 protein expression in cancer tissue was closely interrelated with staging and clinical grading of TCCB ($p<0.05$), but MCL-1 protein was not interrelated ($P>0.05$) (Table 1).

The expression of BCL-2 and MCL-1 was negatively correlated with the microRNA-29c in bladder caner tissues

Our research group have found that the expression of microRNA-29c in bladder cancer tissues were lower than the adjacent tissues (data are not shown), but it is not known that the relationship between the expression of BCL-2, MCL-1 and microRNA-29c. So the expression of microRNA-29c and BCL-2 and MCL-1 in bladder cancer tissues was measured. The linear correlation analysis showed that the expression of microRNA-29c in cancer tissues was negatively correlated with the BCL-2 and MCL-1 ($p<0.01$) (Figure 2 E-F).

Quantitative reverse transcriptase PCR showed that we obtained over-expression microRNA-29c in exosomes and BIU-87 successfully

For confirming that the exosomes from EXO/Ad-miR group contained the over-expressed microRNA-29c, BIU-87 was infected by Ad-RFP-miR-29c or Ad-RFP for 48h separately. Then the expression of miR-29c in exosomes from the BIU-87 of 3 groups was examined. The EXO/Ad-miR group represented higher expression of miR-29c (EXO/Ad-miR *vs* EXO/Ad *vs* EXO) (Figure 1 C), with a significant difference among 3 groups ($p<0.05$).

BIU-87 was treated with three groups of exosomes (EXO, EXO/Ad, EXO/Ad-miR) for 48h, then the QRT-PCR was used to detected the level of miR-29c in BIU-87, The EXO/Ad-miR group represented higher expression of miR-29c (EXO/Ad-miR *vs* EXO/Ad *vs* EXO) (Figure 3 A), with a significant difference among 3 groups ($p<0.05$).

Over-expression miR-29c in exosomes induced apoptosis in bladder cancer cells

The function of exosomes-derived over-expression miR-29c in BIU-87 is unknown, so BIU-87 was treated with three groups of exosomes (EXO, EXO/Ad, EXO/Ad-miR), and the apoptosis rate was measured by flow cytometry (Figure 3 A B). The rate of apoptosis in EXO group (3.47±0.81) % and EXO/Ad group (1.53±0.25) % was lower than the EXO/Ad-miR group (27.77±1.30) %, and it was statistically significant ($p<0.01$). From these we concluded that over-expression miR-29c in exosomes could induce apoptosis in bladder cancer cells.

Exosomes-derived miR-29c could down-regulate the protein level of BCL-2 and MCL-1

We have demonstrated that exosomes-derived miR-29c could induce apoptosis in bladder cancer cells, but the machanism of miR-29c action in bladder cancer has not been reported. So after BIU-87 were treated by exosomes from 3 groups (EXO, EXO/Ad, EXO/Ad-miR) for 48h, we detected the protein expression of BCL-2 and MCL-1 in BIU-87. The result showed the expression of BCL-2 and MCL-1 in the EXO/Ad-miR group significantly decreased compared with control groups (Figure 4 A, B, C).

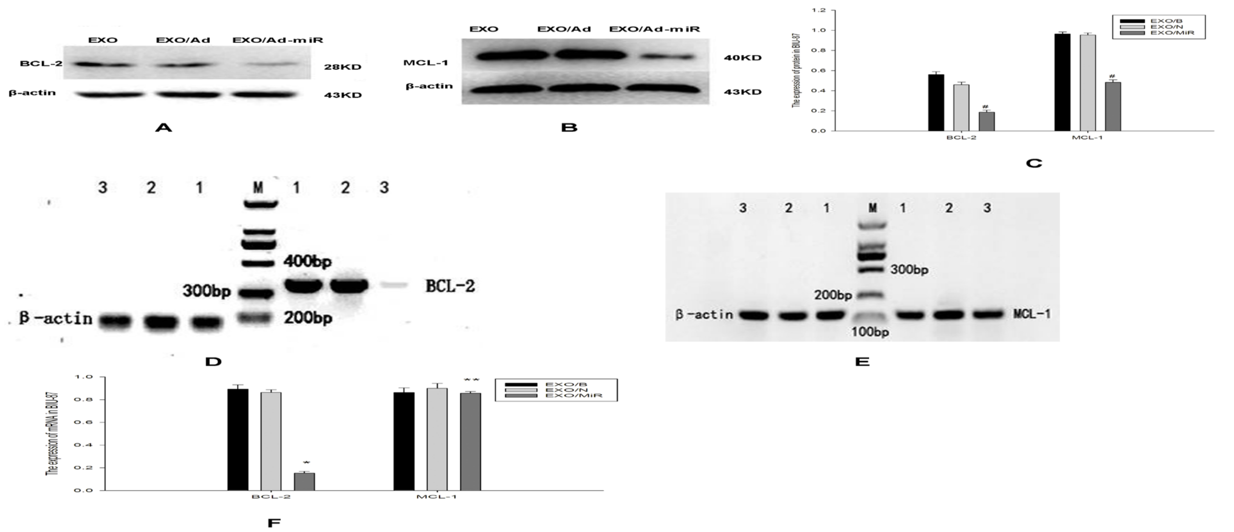

Figure 4. Over-Expression miR-29c in Exosomes Down-regulated the Protein Levels of BCL-2 and MCL-1, and the mRNA Level of BCL-2. After the BIU-87 was Treated by Exosomes from 3 groups (EXO, EXO/Ad, EXO/Ad-miR)for 48h, the mRNA Expressions of BCL-2 (D) and MCL-1 (E) in BIU-87 were Detected by Semi-quantitative RT-PCR. The protein levels of BCL-2 (A) and MCL-1 (B) in BIU-87 were detected by western blot. ($^{\#}p$<0.05) (EXO/Ad-miR group vs EXO/Ad group *vs* EXO group)

Exosomes with over-expression miR-29c could decrease the mRNA of BCL-2 but not MCL-1

MiR-29c can promote the degradation of target gene or inhibit its translation. It is unknown that miR-29c degrade mRNA of BCL-2 and MCL-1 or only inhabit their translation in BIU-87. So after BIU-87 were treated by exosomes from 3 groups (EXO, EXO/Ad, EXO/Ad-miR) for 48h, we detected the mRNA expression of BCL-2 and MCL-1 in BIU-87. The result showed the mRNA expression of BCL-2 in the EXO/Ad-miR group decreased compared with control groups, and MCL-1 mRNA expression did not change (Figure 4 D, E, F).

Discussion

As a non-coding RNA, miRNA could target specific genes and promote target gene's degradation or inhibit its translation. Recent studies have shown that miRNA plays an important role in the tumorigenesis and cancer progression by regulating the expression of multiple genes. The miR-29 family has been found having obviously abnormal expression in multiple tumors, but its functions are still poorly understood. Now, more and more reports revealed the potential biological function of miR-29 family. They participate in a number of pathological and physiological processes and become a promising therapy target with clinical value (Calin et al., 2005; Garzon et al., 2009; Castilla et al., 2011). MiR-29a was up-regulated in esophageal Cancer with well prognosis (Zhao et al., 2013) , it may affect the prognosis of the tumor, but it was lack of in-depth research, In bile duct carcinoma cell lines, human hepatocellular carcinoma cell lines and acute myeloid leukemia cell lines, miR-29 could regulate apoptosis by targeting MCL-1. Over-expression of miR-29 could down-regulate the expression of MCL-1 (Mott et al., 2007; Garzon et al., 2009; Xiong et al., 2010). According to nasopharyngeal darcinoma, miR-29c could promote the apoptosis of cancer cells and increase the sensitivity of cancer cells to radiotherapy and chemotherapy (Zhang et al., 2013). In human lung cancer cells, matrine could reduce proliferation of cancer cells by inducing apoptosis and down-regulating the expression of BCL-2, author detected the expression of miR and found that the mechanism may be associated with the up-regulating of miR-29 c (Liu et al., 2014).

Exosomes are membrane vesicles of endocytic origin released from cells by exocytosis way, which were discovered in electron microscopic and range from 30~100 nM in diameter. By transporting the protein, lipid, RNA, and other molecules to the specific organization, exosomes regulate the biological function of target in vivo. Research has shown that miRNA transported by exosomes could have an important impact on the target cells. Exosomes from class II transactivator (CIITA) gene transfected CT26 cells could induce anti-tumour responses by enhancing splenocyte proliferation and IFN-γ production of CD4+T cells (Fang et al., 2013). Hu G in vitro confirmed that the release of miR-29 through exosomes secreted from astrocytes was increased under astrocytes were exposed to morphine and human immunodeficiency virus Tat protein, over-expression miR-29 would lead to neuronal cell damage (Hu et al., 2012).

Bladder cancer cells were infected by adenovirus of miR-29c, our study proved that the exosomes infected by adenovirus represented higher expression of miR-29c and infer that miR-29c can be transported by exosomes. The detection of BCL-2 and MCL-1 protein expression in bladder cancer and adjacent tissue by immunohistochemistry also showed that the positive rate of cancer tissues was significantly higher than that in adjacent tissue. The expression of BCL-2 and MCL-1 in adjacent tissue focused more on the basal and suprabasal layers, and rarely did express in the muscular layer. Moreover, the association of the expression of BCL-2 with the grading and staging of cancer indicate that there is a correlation between BCL-2 and cancer progression. The bladder cancer cell apoptosis rate increased significantly after bladder cancer cells were treated by exosomes-derived miR-29c, and the BCL-2 and MCL-1 protein were down regulated. We infer that the miR-29c carried by exosomes induced apoptosis in BIU-87 cells by down regulating the expression of BCL-2 and MCL-1. However, the mRNA of MCL-1 was not down regulated evidently by miR-29c. MiR-29 can target on 3 'non-coding region of MCL-1, and degrade mRNA of MCL-1 in acute leukemia (Castilla et al., 2011), through repeated experience for many times, the mRNA of MCL-1 was not degrade, but the protein expression was significantly down-regulated. Because microRNA can combine with gene 3 'non-coding region so as to promote the degradation of target gene or inhibit its translation, we hypothesized that miR-29c just inhibited the translation of MCL-1 but not degraded the mRNA in bladder cancer.

Exosomes is an important carrier of cellular cargo to extracellular microenvironment. Comparing to the intracellular RNA, miRNA in exosomes is more stable, and has advantages in storage for the resistance to degradation in frozen environment (Reid et al., 2011). Nowadays, the utilize of exosomes for targeted therapy has become the major topic of debate. The Wood laboratory intravenously injected RVG-targeted exosomes which were loaded with exogenous siRNA to the mice, and delivered siRNA specifically to neurons, microglia, oligo-dendrocytes in the brain, resulting in a specific gene knockdown (Alvarez-Erviti et al., 2011). We induced apoptosis of bladder cancer cells by exosomes-derived miRNA-29c successfully, and provided a valuable reference for the study of the role of miR-29c in bladder cancer and the treatment of bladder cancer.

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