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*Full Length Research Paper*

# Expression of E2F3, miR-17-5p, miR-20a and their interplay in bladder cancer

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**In order to explore the expression levels of E2F3, miR-17-5p, miR-20a and find out if they interact with one another in bladder cancer, 50 transitional cell carcinoma tissues, 10 normal mucosa tissues and 5637 cell line were used in this study. miR-17-5p, miR-20a, E2F3 gene and E2F3 proteinum were significantly amplifed and overexpressed in cancer tissues, and with advancing of the tumor grade, E2F3 gene and E2F3 proteinum increased. The 5637 cell showed significantly high expression of E2F3 proteinum and the proteinum could be down-regulated by miR-17-5p and miR-20a, whereas enforced overexpression of E2F3 was shown to activate transcription of miR-17-5p and miR-20a. The miR-17-5p, miR-20a, E2F3 gene and E2F3 proteinum were significantly amplified and overexpressed, and they interacted with one another in bladder cancer.**

**Key words:** E2F3, miR-17-5p, miR-20a, bladder cancer, gene regulation.

## INTRODUCTION

The essential role of E2F1-3 family of transcription factors in progression of the cell cycle is well known, especially, the E2F3, as an activator of cell cycle progression which promotes the entry of quiescent cells into S phase (Wu et al., 2001). It closely correlates with the cell cycle, differentiation, apoptosis and proliferation (Giangrande et al., 2003). Thereby, E2F is implicated in human diseases including cancer. Amplification of E2F3 gene and overexpression of E2F3 proteinum in bladder cancer have been reported, especially, E2F3 which is an oncogene of bladder cancer (Feber et al., 2004). MicroRNAs (miRs) are an abundant class of small non-coding RNA that function as negative gene regulators by binding to the sites within the 3'-untranslated regions (3'-UTRs) of targets mRNA. They can control cell growth, differentiation and apoptosis, consequently, miRs expression have been implicated in tumorigenesis. Many miRs are present within the genome as clusters of multiple miRs. Recently, a polycistronic cluster of miR-17-92 was a tumor research area of intense interest. The entire mir-17–92 cluster is located within the third intron of an open reading frame termed the C13orf25 (chromosome 13 open reading frame 25) gene located at 13q31–q32. It includes seven miRNAs: miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1. Researchers demonstrated that E2F induce expression of miR-17–92 cluster, two of the miRs within the cluster, miR-17-5p and miR-20a, inhibit E2F mRNA translation and downregulate the E2F proteinum (O'Donnell et al., 2005; Sylvestre et al., 2007; Woods et al., 2007). The promoter of the cluster contains E2F transcription factor binding sites and chromatin immunoprecipitation demonstrates that E2F3 is the primary E2F family member that occupies the promoter (Sylvestre et al., 2007; Woods et al., 2007). In several studies, which involved lung, breast, colon, pancreas and prostate cancer, miRs from this cluster were overexpressed (Hayashita et al., 2005; Calin et al., 2004). The miRs from the cluster can function as tumour suppressors or oncogenes depending on which cell types these miRs are expressed in and what tissue-specific target mRNAs are present to be regulated. Here, we

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explored the expression levels of E2F3, miR-17-5p, miR-20a and whether these interact with one another in bladder cancer.

## MATERIALS AND METHODS

### Tumor samples and cell lines

Freshly frozen (-80℃) clinical tissues from 50 bladder cancer (transitional cell carcinoma, TCC) patients who underwent radical or partial cystectomy were obtained from Tianjin Institute of Urological Surgery (Tianjin, China). 20 of the specimens were classified as high-grade TCC (grade 1), 14 were moderate- grade (grade 2) and 16 were low-grade (grade 3). The 10 controls were normal bladder mucosas that were obtained from the patients who underwent prostatectomy and were ruled out of canceration strictly by pathologist. All patients gave their informed consent, and the Ethical and Scientific Committees of the participating institutions approved the study. Tumor histological types and differentiated grade were determined according to WHO 1973 classification. The bladder cancer cell line 5637 was obtained from the cell bank of Chinese Academy of Science. The cell line were cultured under the recommended conditions (RPMI-1640 , 5% $CO_2$, 37℃, 10% FBS). TRIzol reagent was used to isolate total RNA from tissue samples and cell line. The entire assay was under a condition of RNase protection.

### Plasmids, mimics of miR and the 2`-O-methyl anti-miRNA oligonucleotides (AMO)

The plasmids of pcDNA3.1-HA-E2F3 were kind gifts from Dr. J. Nevins (Duke University Medical Center, Durham, North Carolina of USA). The Adeno-associated virus vectors plasmids pAAV-siRNA-E2F3 was kindly provided by Dr HaiLong HU (Tianjin Institute of Urological Surgery). The mimics of miR-17-5p, miR-20a and the AMO of miR-17-5p, miR-20a, endogenous control U6 (house-keeping) were all purchased from ABI (Ambion, Inc).

### Real-time fluorescent quantitative PCR (qRT-PCR)

qRT-PCR was performed by using 2×PCR TaqMan® Universal PCR and standard TaqMan® MicroRNA Reverse Transcription kit protocol on an Applied Biosystems 7900HT Sequence Detection System (ABI Prism®) to detect the level of miRs and E2F3 in tissues and 5637 cells. All RNA and miR samples were normalized based on the TaqMan® Gene Expression Assays for human or mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 endogenous (Ambion, Inc) controls, following the relative quantitative $2^{-\Delta\Delta Ct}$ method. 5×Reverse Transcription (RT) Buffer was used as primer for reverse transcription of miRs, 10× and 20×TaqMan® Gene Expression Assay Mix as fluorescent probe of miRs and E2F3 PCR. All reactions were run according to the manufacturer's instructions (TaqMan® Gene Expression Assays). qRT-PCR was also used to detect the miRs and E2F3 changes, and they respectively transfected the mimics of miR-17-5p, miR-20a and their AMO with HiPerFect Reagent into 5637 cell line; pcDNA3.1-E2F3 with Attractene transfection reagent were transfected, according to the manufacturer's instructions (HiPerFect transfection Reagent handbook and Attractene transfection Reagent handbook, QIAGEN®). The cell was infected with pAAV-siRNA-E2F3 at an optimized multiplicity of infection (MOI), based on pilot dose response studies conducted which was $1 \times 10^5$ (v.g/cell).

### Western blot

Western blot analysis was performed to detect the E2F3 proteinum in tissues and cells, the changes of transfecting the mimics of miR-17-5p, miR-20a and their AMO into the cell line, respectively. Proteinum samples were prepared with sodium dodecyl sulfate (SDS) and run non-reduced and reduced on an appropriate SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The proteinum were transferred to a polyvinylidene fluoride (PVDF) membrane using a semi-dry transfer apparatus. The antibodies against E2F3, anti-GAPDH (Sigma) as endogenous controls, were used for Western blot. Analysis of grayscale was performed by using BandScan5.0 software.

### Statistical analysis

All the assays were conducted at least three times in triplicate. The statistical significance of differences between the expression level of miR-17-5p, miR-20a and E2F3 in cancerous tissues, 5637 cell line and controls tissues was determined by one-way ANOVA. The cell transfecting date was determined by paired-samples T test. $P$ value of <0.05 was considered statistically significant. All data were analyzed using SPSS software ver. 11.5 (SPSS, Inc).

## RESULTS

E2F3, miR-17-5p and miR-20a gene were found to be significantly amplified in the tissue samples from bladder cancer and 5637 cell line when compared with the control (normal bladder tissues) ($P$<0.05) (Figure 1.). Total RNA were collected 48 h after transfection, the results are shown in Figure 2A and B. All the proteinum were collected 72 h after transfection. The results of Western blot analysis are shown in Figure 3A and B.

## DISCUSSION

miR-17-5p and miR-20a can inhibit apoptosis in lung cancer cells (Matsubara et al., 2007), breast cancer cells; miR-17-5p has antiproliferative effects (Hossain et al., 2006), in prostate cancer cell lines, and miR-20a can inhibit apoptosis (Sylvestre et al., 2007). These findings implicated that the miRs within the mir-17–92 cluster can act as oncogene or anti-oncogene depending on the tissular and cellular contexts. In this study, we found that the E2F3, miR-17-5p and miR-20a gene were all amplified in bladder transitional cell carcinoma tissues, these tissues and cell line also contained high levels of E2F3 proteinum, and with advancing of the tumor grade, E2F3 proteinum increased. E2F3 has been an oncogene of bladder cancer (Feber et al., 2004). What role do miR-17-5p and miR-20a play in human bladder cancer? To answer this question, in *in vitro* studies, we demonstrated a regulatory feedback loop of E2F3, miR-17-5p and miR-20a in human bladder cancer, and E2F3 mRNA and proteinum level could be down-regulated by miR-17-5p and miR-20a, whereas enforced overexpression of E2F3 has been shown to activate transcription of miR-17-5p

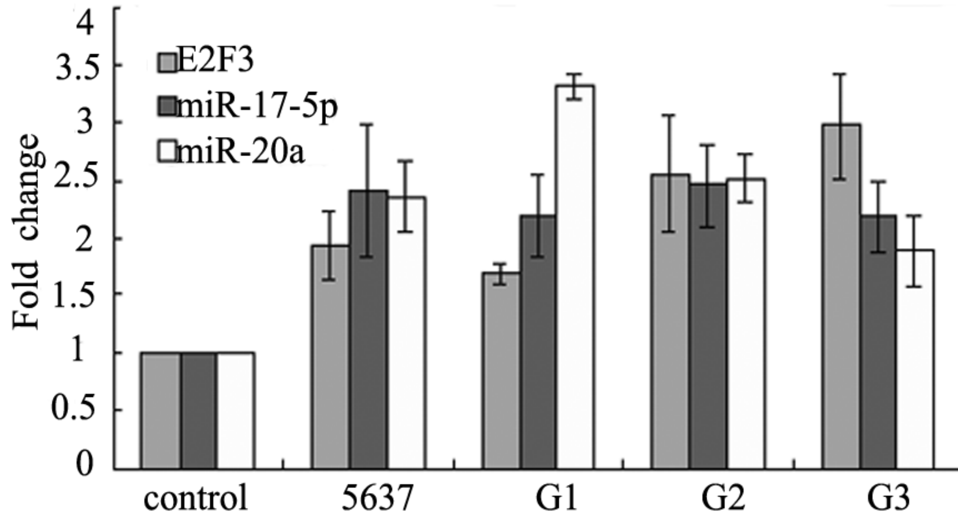


**Figure 1.** E2F3 amplified in the cancer tissues when compared with the control (normal tissues) ($P$<0.05). With advancing of the tumor grade, E2F3 showed increasing trend. miR-17-5p and miR-20a was significantly amplified in the tissues ($P$<0.05). With advancement of the tumor grade, the miR-20a showed decreasing trend ($P$>0.05) using one-way ANOVA. Bars, SD.

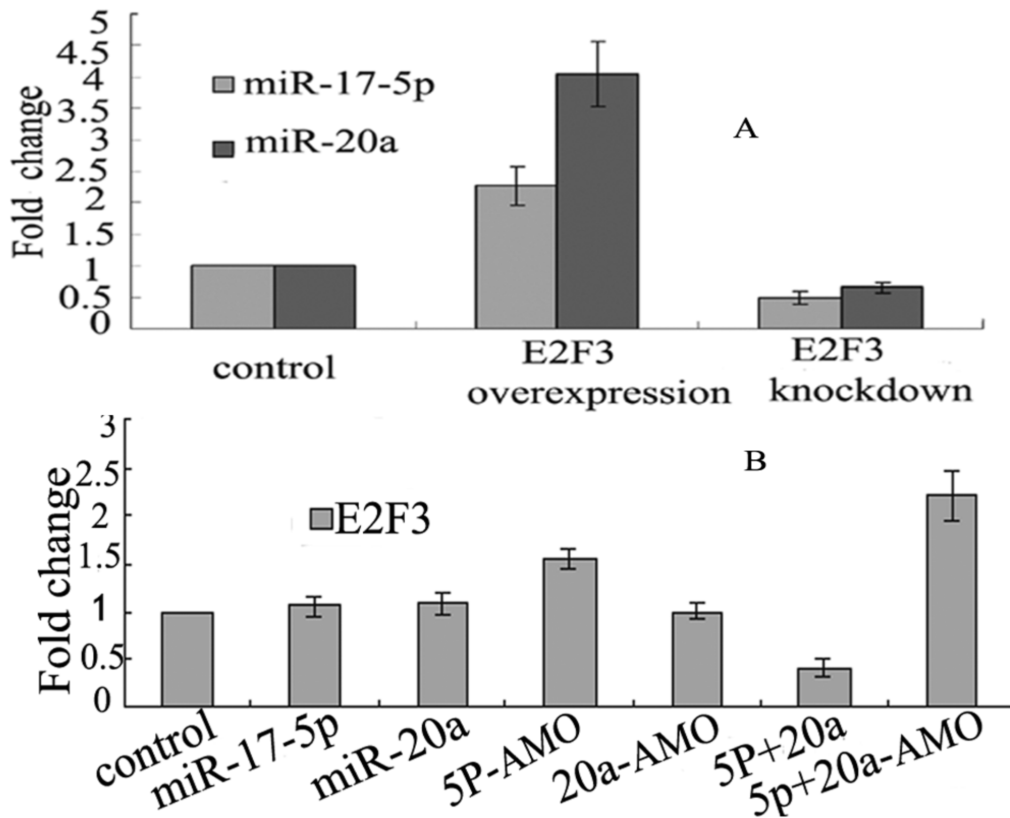


**Figure 2.** A, The expression of miR-17-5p and miR-20a were increased in 5637 cells by transfecting pcDNA3.1-HA-E2F3, while they were decreased by transfecting pAAV-siRNA-E2F3 when compared with the control (P<0.05) (control were 5637 cells without treatment) using paired-samples T test. B, The E2F3 mRNA was increased by pooled transfection of the AMO, and it was decreased by pooled transfection of mimics when compared with the control (P<0.05). The effect of individual mimics or its AMO was faint ($P$>0.05), the control was 5637 cell without treatment using paired-samples T test. Bars, SD.

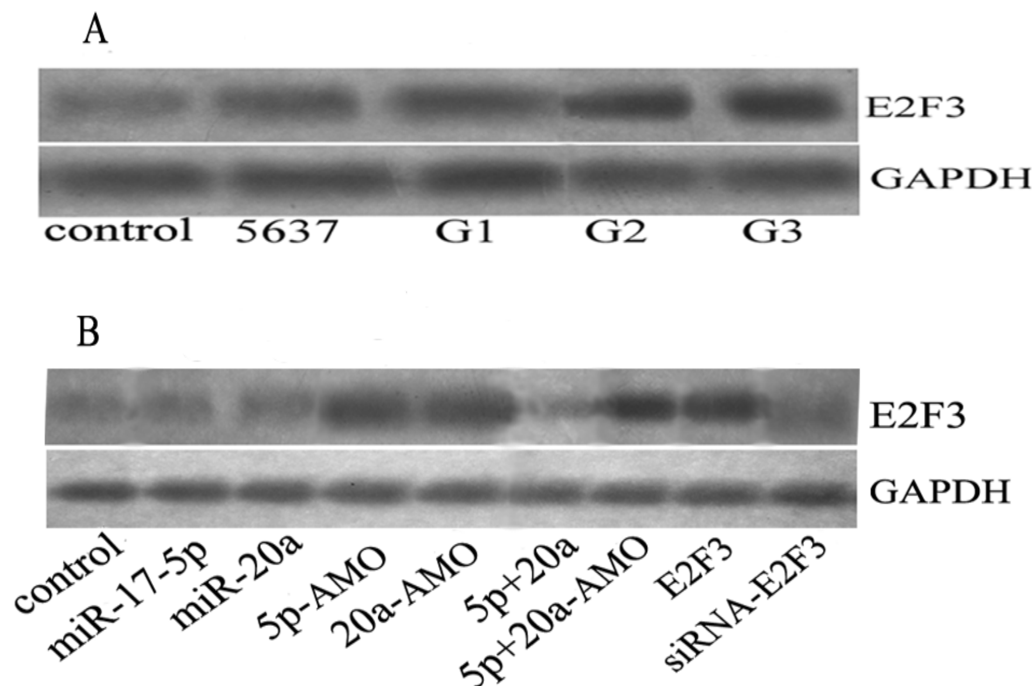


**Figure 3.** A, E2F3 proteinum were amplified in the cancer tissues when compared with normal tissues ($P$<0.05). With advancement of the tumor grade, E2F3 proteinum showed increasing trend, the 5637 cells showed significantly high expression of E2F3 proteinum when compared with normal tissues ($P$<0.05). B, Transfecting individual or pooled mimics, E2F3 proteinum were significantly decreased ($P$<0.05), and E2F3 proteinum were increased by transfecting pooled or individual AMO ($P$<0.05). To overexpress E2F3 by pcDNA3.1-HA-E2F3, E2F3 proteinum were significantly increased ($P$<0.05), knockdown E2F3 gene by pAAV-siRNA-E2F3 and E2F3 proteinum were significantly decreased ($P$<0.05). The control was 5637 cell without treatment using Paired-samples T test. E2F3 = pcDNA3.1-HA-E2F3, siRNA-E2F3 = pAAV-siRNA-E2F3.

and miR-20a. Altogether, these results suggest that miR-17-5p and miR-20a have an important role in preventing an abnormal accumulation of E2F3, miR-17-5p and miR-20a which might act as anti-oncogene in bladder cancer overexpressing the E2F3.

A miR will directly destruct the target mRNA if it has perfect or near-perfect complementarity to the sites within the 3'-UTRs (Hutvagner and Zamore, 2002), the partially complementary will downregulate the proteinum without affecting mRNA abundance (Bartel, 2004). New findings have revealed, however, that mammalian miRs can affect the mRNA abundance under the partially complementary (Wu et al., 2006). In our study, the individual miR-17-5p, miR-20a or its AMO did not affect the E2F3 mRNA abundance, but pooled transfection could affect the mRNA abundance. The finding suggest that the more the miRs, the more perfect or near-perfect complementarity to the sites within the 3'-UTRs. Our data showed that in different tissular and cellular contexts, the raised or descended rate of miR-17-5p and miR-20a was different, and with increasing tumor grade, the miR-20a showed decreasing trend (Figures 1 and 2A). We postulated that the miRs within the mir-17-92 cluster might form different assembly and have different effect, and determine the cell response to the microenvironment or cellular contexts. Study showed that the entire mir-17–92 cluster could accelerate tumor development and inhibit apoptosis in B-cell lymphomas (He et al., 2005), but the individual miR-18, miR-19a or miR-20a within the cluster has no effect on the entire cluster (Hayashita et al., 2005). Aguda and colleagues had attempted to find a mathematical model to predict the change of regulatory loop of mir-17–92 cluster and E2F (Aguda et al., 2008). We also suggest that the miRs within the mir-17–92 cluster can act as oncogene or anti-oncogene not only depending on their different assembly, but also their levels of expression, and their levels were regulated by the target mRNAs. miR-17-5p and miR-20a have an important role in preventing an abnormal accumulation of E2F3. The regulatory feedback loop may play a key role in bladder cancer. Future studies might contribute to understanding the complex regulatory feedback loop.

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