MIR-182 AFFECTS NERVE CELL DAMAGE AFTER EPILEPSY BY REGULATING THE EXPRESSION OF TARGET GENE APLN

Peng Lin[1,#], Zhenyu Zhang[1,#], Jing Chu[1,#], Yingjie Zhou[2], Meifeng Li[1], Wenbo Yu[1,*]
[1]Department of Intensive Care Unit, The Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai 264000, PR China -
[2]Department of Rehabilitation, Yantai Haigang Hospital, Yantai 264000, PR China
[#]*These authors contributed equally to this work as co-first author*

ABSTRACT

Objective*: To analyze the mechanism of miR-182 and APLN genes in neuronal injury after epilepsy.*

Methods: *The over-expression vector and silencing vector of miR-182 were constructed, and the neurons were transfected with miR-shNC. The negative control group was established. The expression of APLN mRNA was detected using real-time quantitative PCR, and the expression of caspase, Bax, and Bcl-2 apoptosis proteins were detected by Western blot. In addition, pentylenetetrazol was used to establish the epileptic model in rats. Three kinds of plasmids, including the over-expression of APLN, silencing of APLN, and over-expression of miR-182, were injected into rats respectively, and a negative control group was set up. An epileptic cell model of hippocampal neurons was established. The expression of APLN mRNA in the hippocampal tissue of rats in each group was detected by real-time quantitative PCR, and the Western blot method was used to detect the expression of caspase, Bax, and Bcl-2 apoptotic proteins in the neuronal epilepsy cells and hippocampus of each group of rats.*

Results: *The expression of APLN mRNA in the over-expressed miR-182 group was significantly lower than that in the silenced miR-182 group (P<0.05). The expression of caspase and Bax protein in the over-expression miR-182 group was significantly higher than that in the negative control group (P<0.05), and the expression of Bcl-2 protein was significantly lower than that in the negative control group (P<0.05). The expression of caspase and Bax in the neurons of the miR-182 group was significantly lower than that of the negative control group (P<0.05), and the expression of Bcl-2 was significantly higher than that of the negative control group (P<0.05). The expression of APLN mRNA in the over-expression APLN group was significantly higher than that in the negative control group (P<0.05), and the expression of APLN mRNA in the silenced APLN group and the over-expression miR-182 group was significantly lower than that in the negative control group (P<0.05). The expression of caspase and Bax protein in epileptic cells and hippocampal tissues of rat hippocampal neurons increased gradually in the over-expression APLN group, negative control group, over-expression miR-182 group, and silenced APLN group, and the Bcl-2 protein decreased gradually.*

Conclusion: *The APLN gene can reduce apoptosis by regulating apoptosis proteins, inhibit miR-182, up-regulate APLN gene expression, and protect neurons from injury after epileptic seizure.*

Keywords: *MiR-182, APLN gene, epilepsy, nerve cell injury, mechanism of action.*

DOI: 10.19193/0393-6384_2021_3_237

Received *March 15, 2020;* ***Accepted*** *October 20, 2020*

Introduction

Epilepsy refers to temporary brain dysfunction caused by the sudden abnormal discharge of brain neurons. The World Health Organization reports that more than 50 million people suffer from epilepsy worldwide. The overall prevalence rate of epilepsy in China is 7.0%, and it has become the second most common disease after headache in neurology[(1)]. Because of the differences in the initial position and transmission mode of the abnormal discharge, the clinical manifestations of epilepsy are various, and it can manifest as delirium, muscle rigidity, myoclonus, and other symptoms. With the continuous deepening of research on epilepsy, breakthroughs have been made in the theory of its pathogenesis. It is generally believed that the abnormal discharge of brain neurons during seizure is closely related to glial cells, synaptic connections, neurotransmitters, genetics, etc.[(2-3)]. Neural cell injury is an important

cause of epilepsy during development, and this process is mainly achieved through the regulation of apoptosis-related genes. miRNA is a kind of endogenous non-coding small molecular RNA, which negatively regulates the expression of target genes and protein synthesis at the transcription level. In recent years, it has been confirmed that human temporal lobe epilepsy can cause a change in specific miRNA in brain tissue, revealing that miRNA may participate in the pathogenesis and mutation process of epilepsy[4]. Apelin is encoded by the APLN gene, which is expressed in many tissues and can regulate the inflammatory response in the process of myocardial ischemia-reperfusion injury and oxidative stress[5].

Some scholars have found that the expression of APLN in the hippocampus and hypothalamus of the nervous system increased significantly[6]. Therefore, the purpose of this study is to analyze the mechanism of miR-182 and the APLN gene in neuron injury after epilepsy.

Materials and methods

Experimental reagents and instruments

Rat hippocampal neurons were purchased from Shanghai Kanglang Biotechnology Co., Ltd.; cell culture and pancreatin cell cryopreservation solution were purchased from Zhongqiao Xinzhou Biological Company; TRIzol® reagent was purchased from Invitrogen Corporation of the United States; RNase-free water was purchased from Beijing Tiangen Biotechnology Company; APLN goat anti-rabbit antibody was purchased from American Abcam; rabbit anti-mouse caspase, Bax, Bcl-2 polyclonal antibodies were purchased from Beijing Bosen Biotech Co.; the ECL chemiluminescence development kit was purchased from Thermo Fisher Scientific Co., Ltd. ; RIPA protein lysate was purchased from Wuhan Boshide Biological Co., Ltd.

Cell incubators were purchased from Thermo Fisher Technology Co., Ltd.; low-speed centrifuges were purchased from Heraeus company in Germany; protein electrophoresis instrument was purchased from Shanghai Junyi Co., Ltd.; an inverted microscope was purchased from OLYMPUS company; a vertical electrophoresis tank and a membrane transfer instrument were purchased from BIO-RAD company in the United States; a micro vortex mixer was purchased from Beijing Tongzheng biotechnology development company; a micro pipette was purchased from Eppendorf company in Germany -80 °C and -20 °C refrigerators were purchased from Qingdao Haier company.

Neuron cell culture, transfection, and detection methods

Neurons in good condition were cultured in a medium containing penicillin streptomycin and neurotrophic factors. When the fusion degree reached 90%, the cells were sub-cultured. During trypsin digestion, we waited for more than 60% of cells to digest the trypsin, and then stopped the digestion. The cells were evenly divided into six well plates. To each well was added with 1 ml of basic medium for the culture, and the growth of cells was observed. A mature sequence of miR-182 was obtained from the miRBase database, and the over-expression vector and silencing vector for miR-182 were designed, respectively. The neurons were transfected with miR-182, and the negative control group was established by using an empty miR-shNC transfection.

• Real-time quantitative PCR method to detect the effect of miR-182 transfection on the expression of APLN mRNA in rat neurons: The TRIzol® method was used to extract the total RNA of the neurons. According to the instructions of the reverse transcription kit, the designed miR-182 reverse transcription primer and the primer mix of the APLN target gene were used to reverse-synthesize cDNA with the total RNA as template. The expression of the APLN gene in neurons was detected using SYBR green mix fluorescent dye in a real-time fluorescent quantitative PCT amplification instrument. Using cDNA as template and β-actin as internal control, the APLN gene was pre-denatured for 5 min at 95 °C, 5 s at 95 °C, and 30 s at 60 °C for 40 cycles. The data were analyzed using the $^{2-\triangle\triangle}$CT formula.

• Western blot method for detecting the effect of miR-182 transfection on neuronal apoptosis protein: we collected neurons transfected with the miR-182 vector and silencing vector, and extracted protein using the BCA kit for protein quantification. We added 10 μL of loading buffer to the sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to PVDF membrane after 1 h. We blocked with 5% skim milk powder for 1 h and added the rabbit anti-mouse caspase diluted 1:500. The Bax and Bcl-2 polyclonal antibodies were shaken in a refrigerator at 4 °C overnight, and the corresponding secondary antibodies were added and incubated at room temperature for 2 h. The electro-chemiluminescent reagent was developed and exposed, and then scanned with a scanner.

Preparation, transfection, and detection of epileptic cells in rat hippocampus and neurons

The rats, 24 healthy females, 8-10 weeks old, were purchased from Beijing Weitonglihua Experimental Animal Technology Co., Ltd. and raised at a room temperature of 22-24 °C and a humidity of 50-60%. They were fed for 12 h alternately day and night and were permitted to drink and eat freely.

The epilepsy model was established by pentylenetetrazol. After the model was established successfully, three different plasmids, i.e., the over-expression APLN, silencing APLN, and over-expression miR-182, were injected into the rats in a mixed solvent, and the empty carrier miR-shNC was injected to establish the negative control group. Establishment of epileptic cell model of hippocampal neurons: the hippocampal neurons of rats were cultured in normal medium for 14 days. The original medium was discarded, washed with low magnesium extracellular solution, and then cultured in low magnesium extracellular solution (2 ml per hole).

After it had cultured in normal cell solution for 3 h in an incubator, the extracellular solution was washed twice, and then cultured in normal maintenance solution (2 ml per hole) for 14 days. Finally, the medium was used for a follow-up test.

• Real-time quantitative PCR was used to detect the effect of APLN on the expression of APLN mRNA in the hippocampus of epileptic rats.

• Western blot was used to detect the effect of APLN on caspase, Bax, and Bcl-2 apoptotic proteins in the epileptic cells and hippocampus of rats. The procedure was the same as 1.2[(2)].

Statistical methods

All the measurement data for this study are expressed as ($\bar{x}\pm s$).

The mean comparison between the two groups was tested by independent sample t, and the mean comparison between multiple groups was analyzed by ANOVA; $P<0.05$ was regarded as statistically significant. The data for this study were analyzed using the SPSS 20.0 software package.

Results

Effect of miR-182 on APLN mRNA expression in rat neurons

The expression of APLN mRNA in the over-expression miR-182 group was significantly lower than that in the silenced miR-182 group ($P<0.05$). See Figure 1.

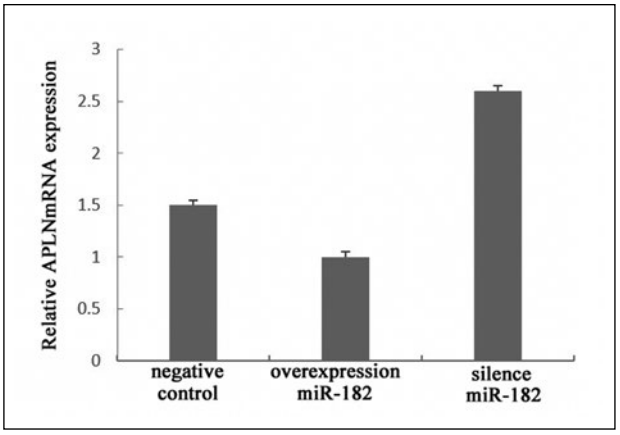

Figure 1: Effect of miR-182 transfection on APLN mRNA of rat neurons.

The effect of miR-182 transfection on neuronal caspase, Bax, Bcl-2 apoptosis protein

Western blot analysis showed that the expression of caspase and Bax protein in neurons of the over-expression miR-182 group was significantly higher than that in the negative control group ($P<0.05$), and the expression of Bcl-2 protein was significantly lower than that in the negative control group ($P<0.05$).

The expressions of caspase and Bax protein in neurons in the silenced miR-182 group were significantly lower than that in the negative control group ($P<0.05$), and the expression of Bcl-2 protein was significantly higher than that in the negative control group ($P<0.05$). See Figure 2 and Table 1.

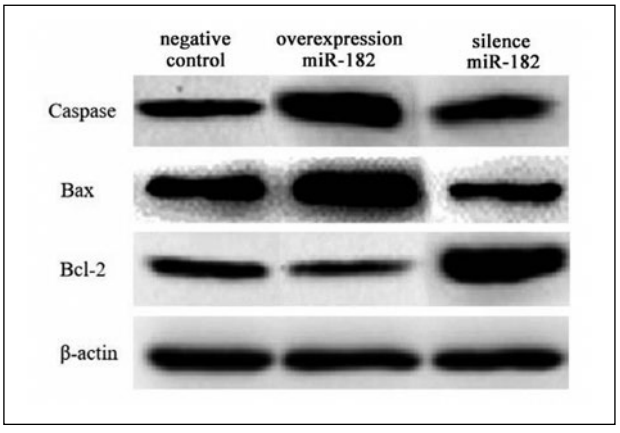

Figure 2: Effect of miR-182 transfection on neuronal caspase, Bax, Bcl-2 apoptosis protein.

Group	Caspase	Bax	Bcl-2
Negative control group	0.73±0.04	1.19±0.08	0.76±0.09
Over-expression miR-182 group	0.92±0.09*	2.52±1.01*	0.50±0.04*
Silenced miR-182 group	0.40±0.08*	0.52±0.07*	1.38±0.12*

Table 1: Effect of miR-182 transfection on neuronal caspase, Bax, Bcl-2 apoptosis proteins ($\bar{x}\pm s$).
*Note: Compared with the negative control group *P<0.05.*

Effect of APLN transfection on the expression of APLN mRNA in hippocampus of epileptic rats

The expression of APLN mRNA in the over-expression APLN group was significantly higher than that in the negative control group (P<0.05), and the expression of APLN mRNA in the silenced APLN group and the over-expression miR-182 group was significantly lower than that in the negative control group (P<0.05). See Figure 3.

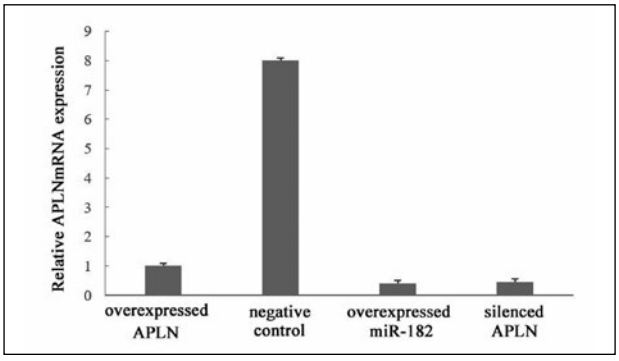

Figure 3: Effect of APLN transfection on the expression of APLN mRNA in the hippocampus of epileptic rats.

Effect of APLN transfection on caspase, Bax, and Bcl-2 apoptosis proteins in rat hippocampal neuronal epilepsy cells

The expression of caspase and Bax protein in rat hippocampal epilepsy neurons increased gradually in the over-expressed APLN group, negative control group, over-expressed miR-182 group, and silenced APLN group, and the Bcl-2 protein decreased gradually. See Figure 4 and Table 2.

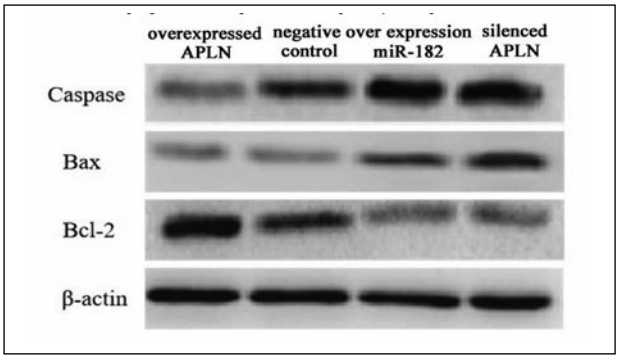

Figure 4: Effect of APLN transfection on the expression of APLN mRNA in the hippocampus of epileptic rats.

Group	Caspase	Bax	Bcl-2
Over-expression APLN group	0.50±0.01	0.48±0.02	1.00±0.02
Negative control group	0.75±0.05	0.51±0.06	0.80±0.07
Over-expression miR-182 group	1.12±0.16	0.76±0.08	0.53±0.05
Silenced APLN group	1.13±0.07	1.02±0.09	0.49±0.07

Table 2: Effects of APLN transfection on apoptosis protein in rat hippocampal epilepsy neurons ($\bar{x}$±s).

Effect of APLN transfection on caspase, Bax, and Bcl-2 apoptosis proteins in hippocampus of epileptic rats

The expression of caspase and Bax protein in the hippocampus of epileptic rats increased gradually in the over-expressed APLN group, negative control group, over-expressed miR-182 group, and silenced APLN group, and the Bcl-2 protein decreased gradually. See Figure 5 and Table 3.

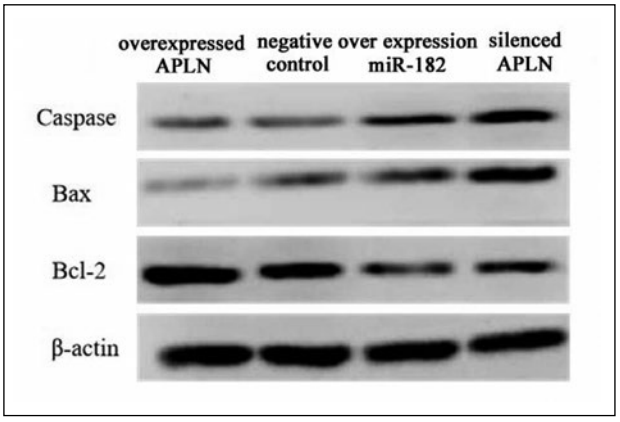

Figure 5: Effect of APLN transfection on caspase, Bax, and Bcl-2 apoptosis proteins in the hippocampus of epileptic rats.

Group	Caspase	Bax	Bcl-2
Over-expression APLN group	0.48±0.04	0.20±0.03	1.26±0.09
Negative control group	0.49±0.02	0.42±0.05	0.74±0.02
Over-expression miR-182 group	0.71±0.05	0.62±0.08	0.51±0.05
Silenced APLN group	0.92±0.11	0.90±0.12	0.50±0.06

Table 3: Effect of APLN transfection on caspase, Bax, and Bcl-2 apoptosis proteins in the hippocampus of epileptic rats ($\bar{x}$±s).

Discussion

Epilepsy is one of the common diseases of the nervous system. Its long-term and recurrent chronic attacks seriously affect the life, study, and work of patients. It is a syndrome with complicated etiology, pathophysiology, and clinical manifestations. Its pathogenesis is not yet clear, and most of the clinical control is based, so it is important for clinical diagnosis, prevention, and treatment to clarify the pathogenesis[7]. MiRNAs are a class of endogenous non-coding RNA, which can regulate gene expression by pairing with a target gene mRNA to form a silencing complex, inhibiting the translation or degradation of target mRNA, and thus playing an important role in regulating an organism[8]. In recent years, it has been reported that a variety of

miRNA populations are involved in nervous system diseases. The abnormal expression of an miRNA biotransformation pathway is related to epilepsy, Alzheimer's disease, Parkinson's disease, and other nervous system diseases[9]. miR-182 is a member of miR-183/96/182 family. Its content is abundant in the brain of adults and embryos. It is believed that miR-182 may participate in the transformation of neuro-hepatocytes to mature neurons. Some scholars have found that miR-182 can aggravate brain injury by inhibiting the expression of the iASPP gene, and it can also promote the expression of the caspase-3 protein to further aggravate brain injury[10].

Apelin is an endogenous ligand of the G-protein-coupled receptor APJ, which is encoded by the APLN gene. It is widely distributed in the central nervous system in the hippocampus, amygdala, striatum, and lateral hypothalamus, suggesting that it may play an important physiological role in the central nervous system[11]. It has been confirmed that APLN may play an important role in the process of neuronal excitotoxic injury and apoptosis[12]. The APLN gene is widely distributed in the nervous system, but its mechanism of action in nerve injury is not clear. Some scholars have found through the establishment of an epilepsy model in rats that APLN pretreatment can significantly increase the threshold of epileptic onset, inhibit the application of inflammation and apoptosis, and play a protective role in cortical neurons[13]. The results of real-time quantitative PCR have showed that the expression of APLN mRNA in the over-expression miR-182 group was significantly lower than that in the silenced miR-182 group ($P<0.05$), which confirmed that over-expression miR-182 could significantly inhibit the expression of APLN mRNA.

Studies have found that miR-182 also plays an important role in the apoptosis of neurons[14]. Repeated seizures are closely related to patients' cognitive dysfunction and neuron damage caused by frequent epileptic activity, including neuronal apoptosis. The caspase family plays a key role in the process of cell apoptosis. After it is activated, caspase can play an important role in many kinds of functional proteins that maintain cell activity and cell apoptosis[15]. BCL2 is an anti-apoptotic gene, and BAX is an apoptotic gene. Both of them regulate the function of apoptosis by inducing the release of apoptotic factors and regulating the mitochondrial cytochrome[16]. The Western blot showed that caspase and Bax protein expression in the over-expression miR-182 group was significantly higher than in the negative control group ($P<0.05$), and Bcl-2 protein expression was significantly lower than in the negative control group ($P<0.05$), which suggested that over-expression of miR-182 might promote neuron apoptosis through caspase, Bax, Bcl-2 and other apoptotic proteins. In this study, we found that the expression of caspase and Bax protein in the epileptic cells of hippocampal neurons and the hippocampal tissues of rats increased gradually in the over-expression APLN group, negative control group, over-expression miR-182 group, and silenced APLN group, and the expression of Bcl-2 protein decreased gradually. This suggests that APLN can regulate the expression of apoptotic genes to reduce the apoptosis of neurons, and has a protective effect on cell damage after epilepsy.

In conclusion, the APLN gene can reduce apoptosis by regulating apoptotic protein, protect neurons from damage after seizure, inhibit miR-182, and up-regulate the expression of APLN gene.

References

1) Sawchuk T, Buchhalter J, Senft B. Psychogenic nonepileptic seizures in children-Prospective validation of a clinical care pathway & risk factors for treatment outcome. Epilepsy Behav 2020; 105: 106971.
2) Moavero R, Benvenuto A, Emberti Gialloreti L, Siracusano M, Kotulska K, et al. Early Clinical Predictors of Autism Spectrum Disorder in Infants with Tuberous Sclerosis Complex: Results from the EPISTOP Study. J Clin Med 2019; 8: 788.
3) Dreier JW, Pedersen CB, Gasse C, Christensen J. Antiepileptic Drugs and Suicide: Role of Prior Suicidal Behavior and Parental Psychiatric Disorder. Ann Neurol 2019; 86: 951-961.
4) Liu L, Pan J, Wang H, Ma Z, Yin J, et al. von Willebrand factor rescued by miR-24 inhibition facilitates the proliferation and migration of osteosarcoma cells in vitro. Biosci Rep 2018; 38: 6.
5) Yan J, Wang A, Cao J, Chen L. Apelin/APJ system: an emerging therapeutic target for respiratory diseases. Cell Mol Life Sci 2020; 10: 107.
6) Kartal H, Comu FM, Kucuk A, Polat Y, Dursun AD, et al. Effect of apelin-13 on erythrocyte deformability during ischaemia-reperfusion injury of heart in diabetic rats. Bratisl Lek Listy 2017; 118: 133-136.
7) Tisser L, Palmini A, Paglioli E, Portuguez M, Azambuja N, et al. Pre- and post-operative Wisconsin card sorting test performance in patients with temporal lobe epilepsy due to hippocampal sclerosis. Dement Neuropsychol 2018; 1: 173-180.

8) Zhang J, Zhang H, Qin Y, Chen C, Yang J, et al. MicroRNA-200c-3p/ZEB2 loop plays a crucial role in the tumor progression of prostate carcinoma. Ann Transl Med 2019; 7: 141.
9) Segura MF, Jubierre L, Li S, Soriano A, Koetz L, et al. Krüppel-like factor 4 (KLF4) regulates the miR-183~96~182 cluster under physiologic and pathologic conditions. Oncotarget 2017; 8: 26298-26311.
10) Sharifi M, Fasihi-Ramandi M, Sheikhi A, Moridnia A, Saneipour M. Apoptosis induction in acute promyelocytic leukemia cells through upregulation of CEBPα by miR-182 blockage. Mol Biol Res Commun 2018; 7: 25-33.
11) Wang Y, Song J, Bian H, Bo J, Lv S, et al. Apelin promotes hepatic fibrosis through ERK signaling in LX-2 cells. Mol Cell Biochem 2019; 460: 205-215.
12) Shin K, Kenward C, Rainey JK. Apelinergic System Structure and Function. Compr Physiol 2017; 8: 407-450.
13) Koval S, Iushko K, Starchenko T. Relations of Apelin with Cardiac Remodeling in Patients with Hypertension and Type 2 Diabetes. Folia Med 2018; 60: 117-123.
14) Sadeg N, François G, Petit B, Dutertre-Catella H, Dumontet M. Automated liquid-chromatographic analyzer used for toxicology screening in a general hospital: 12 months' experience. Clin Chem 2020; 43: 498-504.
15) Ancuceanu R, Dinu M, Neaga I, Laszlo FG, Boda D. Development of QSAR machine learning-based models to forecast the effect of substances on malignant melanoma cells. Oncol Lett 2019; 17: 4188-4196.
16) Azimian H, Dayyani M, Toossi MTB, Mahmoudi M. Bax/Bcl-2expression ratio in prediction of response to breast cancer radiotherapy. Iran J Basic Med Sci 2018; 21: 325-332.

Corresponding Author:
Wenbo Yu
Email: dwi5pg@163.com
(China)