Hsa-miR-625 Upregulation Promotes Apoptosis in Acute Myeloid Leukemia Cell Line by Targeting Integrin-linked Kinase Pathway

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Abstract

Background: Growing evidence has demonstrated that microRNAs have a major effect on development of different types of cancer including AML. The overexpression of miR-625 could decrease tumorgenesis of acute myeloid leukemia cell lines through Integrin-linked kinase signaling pathway and reducing the associated oncopgenes. The aim of this study is to evaluate the effect of hsa-miR-625 upregulation on apoptosis and proliferation of KG1 cell line via ILK signaling pathway.

Methods: The KG-1 cell line was transfected with pLenti-III-premir625-GFP through viral method. Then, expression of miR-625 and genes were analyzed by quantitative PCR. Western blotting was used to evaluate for the protein level. Apoptosis was investigated by flow cytometry. Cell cycle analysis with PI and CCK-8 assay were performed to evaluate proliferation.

Results: KG-1 cells transfected with pLenti-III-pre mir625-GFP construct showed a significant increase in cell apoptosis. Gene expression of ILK and NF-κB were downregulated and AKT, c-fos, Caspase3, cyclin D1, KLF-4, OCT-4 and Nanog were upregulated but no alteration in GSK3 expression profile was observed. Downregulation of NF-κB and upregulation of Caspase 3 and p-β-catenin protein levels were indicated (p<0.05).

Conclusion: MiR-625 can be a promising approach to aid in the treatment of AML. However, further studies are required in this respect.

Keywords: miR-625- ILK- apoptosis- proliferation- KG-1- AML

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous malignancy characterized by a block in differentiation of hematopoiesis, resulting in clonal expansion of immature myeloid cells in bone marrow and peripheral blood through the acquisition of chromosomal rearrangements and multiple gene mutations (Rubnitz et al., 2008). AML accounts for more than 70% of acute leukemia and is commonly seen in adults with the median age of 67 years (Alasseiri et al., 2018; Wouters and Delwel 2016). Do to limitation of treatment options and drug resistance in advanced stage of AML, more clinically effective treatment approaches are required (de la Puente et al. 2015). The overexpression of Integrin-linked kinase (ILK) is often an important feature of human malignancies which correlates with poor patient outcome (McDonald et al., 2008).

ILK is a unique intracellular adaptor and serine/threonine kinase which is activated in a phosphoinositide 3-kinase (PI3K)-dependent manner. ILK links the cell-adhesion receptors, integrins and growth factors to the actin cytoskeleton and to a range of signaling pathways (Hannigan et al., 2005) such as growth, proliferation, survival, differentiation, migration, invasion and angiogenesis (McDonald et al., 2008; Zheng C-C et al., 2019). ILK overexpression was detected in Pancreatic cancer (Zhu et al., 2012), Bladder Transitional Cell Carcinoma (BTCC) (Wang et al., 2012a), Gastric cancer (Wang et al., 2012b), Colorectal Cancer (CRC) (Yan et al., 2014, Tsoumas et al., 2018), Lung cancer (Chen et al. 2013), Prostate cancer (Yuan et al., 2013), breast cancer (Hsu et al., 2016, Akrida et al., 2018) and hematological malignancies involving AML (Muranyi et al., 2006; Muranyi et al., 2009, 2010), CML, ALL (de la Puente et al., 2015), CLL (Krenn et al., 2016) and multiple myeloma (Steinbrunn et al., 2012; Wang et al., 2011).

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Asian Pacific Journal of Cancer Prevention, Vol 23 1159
Specific inhibitors of ILK kinase activity have resulted in growth arrest and apoptosis in cells in vitro and in vivo, providing support for ILK as a cancer therapeutic target in human (Yen et al., 2014).

MicroRNAs (miRNAs) are small (18-25 nucleotides) non-coding RNA sequences which negatively regulate gene expression by binding to 3’ UTR of target mRNAs (Schotte et al., 2012). Evidence has shown that miRNAs could regulate tumor cell proliferation, apoptosis, and metastasis. In recent years, miRNAs were indicated to be involved in the pathogenesis of different types of leukemia, including breast cancer (Zhou et al., 2016), glioma (Zhang et al., 2017), colorectal carcinoma (Lou et al., 2013), non-small cell lung cancer (Li et al., 2015), malignant melanoma (Fang et al., 2017), hepatocellular carcinoma (Zhou et al., 2015), esophageal cancer (Li et al., 2015; Wang et al., 2014) and gastric cancer (Wang et al., 2012b). Previously, Wang et al., (2012b) detected that Down-regulated miR-625 suppresses invasion and metastasis of gastric cancer by targeting ILK. Furthermore, they identified that ILK is a direct target gene for miR-625.

However, the mechanism and function of miR-625 in AML have not been elucidated. Therefore, the aim of this study was to investigate the effect of hsa-miR-625 upregulation on apoptosis and proliferation of KG1 cell line through ILK signaling pathway.

Materials and Methods

Cell lines and culture

The KG-1 cell line (Pasteur Institute, Tehran, Iran) was characterized through flow cytometry by the cell bank. This cell line was cultured in RPMI-1640 (Gibco-BRL, Eggenstein, Germany) supplemented with 20% fetal bovine serum (FBS), 1% L-glutamine, 1% Non-Essential amino acid and cultured at 37°C in a humidified incubator with 5% CO₂.

Plasmids construct and extraction

The pLenti-III-pre mir625-GFP expression vector construct and pLenti-III-backbone-GFP were purchased from Bonyakhteh research center (Bonyakhteh, Tehran, Iran). DH5α strain harboring the vectors was cultured in LB broth medium with 100 mg/mL ampicillin. The plasmid was extracted with Macherey-Nagel NucleoBond® Xtra Maxi plasmid extraction kit (MACHEREY-NAGEL, Germany).

Transient transfection

Lentivirus production: Each of the pLenti-III-pre mir625-GFP and pLenti-III-backbone-GFP vectors with 2 packaging plasmids (psPAX2, and pMD2.G) were transfected in HEK-293T (human embryonic kidney, ATCC CRL-3216) cells using PI. At first, 5×10⁶ HEK-293T cells were seeded in a 10 cm plate in DMEM (Gibco, USA) with 15% FBS (Gibco, USA). On the next day, 5 μg of pLenti-III-pre mir625-GFP or pLenti-III-backbone-GFP vector, 5 μg of psPAX2 vector, 5 μg of pMD2.G vector and 22 μl PI were mixed in 800 μl DMEM no glucose and added drop wise to the cells after 15 minutes. 14 hours after the transfection, the medium was replaced with the fresh medium. The packaged recombinant lentiviruses were harvested from the supernatant of cell cultures 24 and 48 hours after transfection, filtered through a 0.4 μm filter and centrifuged at 37,565 rounds per minute (rpm) in 4°C for 2 hours. The recombinant viruses were stored at -70°C for subsequent experiments.

Transduction: the recombinant viruses were added to KG-1 cell line, after 48 hours, GFP expression was evaluated by fluorescence microscope and flow cytometry.

Fluorescence activated cell sorting

In order to separation of GFP+ KG-1 cells transfected with pLentiIII-pre mir625-GFP and backbone vector, the cells were washed with PBS-. Then for each one million of KG-1 cells, 500μl PBS- containing 1% BSA (5%) and 1% penicillin-streptomycin antibiotic were added to KG-1 cell pellet. The solution was filtered and then sorted with FACSArAII (Becton Dickinson, USA).

Apoptosis assay

KG-1 cells were transfected with pLentiIII-pre mir625-GFP expression construct and backbone vector and cultured for 96 hours in 12 well plates. Annexin V assay was performed by Annexin V-PE/7-AAD eBioscience kit (eBioscience, Inc., CA, USA). The cells were harvested and washed with PBS. Then, 2.5 μL Annexin V-PE was added to 100 μL of the cells suspended in binding buffer and incubated for 15 minutes in dark at room temperature. Next, the cells were washed in binding buffer and 2.5 μL of the 7-AAD solution was added to the cell suspension. Finally, the treated cells were analyzed versus untreated fresh cells by flow cytometry (Partec, Münster, Germany) and Flomax software (Partec) (n=3).

Cell cycle analysis

The cell cycle distribution was analyzed by flow cytometry. We harvested 106 KG-1 cells transfected with pLentiIII-pre mir625-GFP and backbone vector. These cells were washed twice with cold PBS- and fixed with 1 ml of 70% cold ethanol for 2 hours at 4°C. After fixation, the cells were washed twice with PBS-, and resuspended in 100 μl of RNase A (100 μg/ml) for 10 minutes at 37°C. Prior to analysis, the cells were incubated with 200 μl of PI (50 μg/ml) for 5 minutes at 37°C. Cell cycle analysis was performed on a BD FACS-Calibur flow cytometer and the Cell Quest program (Becton-Dickinson, San Jose, CA).

Proliferation assay

Cell Counting Kit-8 (CCK-8) analysis was performed to determine the proliferation of KG-1 cells transfected with miR-625 and backbone vector. Briefly, treated KG-1 cell line was seeded in 96-well plates at a density of 5×10⁴ cells/per well, and incubated at 37°C for 24 h. Cell proliferation was determined by CCK-8 detection kit (Sigma-Aldrich, USA) at every 24 h for four consecutive days, according to the protocols of manufac–turer. The absorbance of each group was assessed at 450 nm using
an ELISA reader (Elx800; Bio-Rad Laboratories, Inc.).

RNA extraction and cDNA synthesis

48 hours after transfection, total RNA was extracted from GFP+ KG-1 cells by Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Then the quality of RNA was determined by electrophoresis. cDNA of total RNA was synthesized by Fermentaze cDNA Synthesis kit (Fermentaze, Massachusetts, USA).

cDNA synthesis and stem-loop qPCR of miR-625

Stem-loop primer as well as forward and reverse primers were designed to synthesize cDNA of miR-625. Snord47 was selected as reference gene (Table 1). Relative expression was evaluated by the ΔΔCT method (n=3).

qPCR for ILK, AKT, GSK3, c-fos, NF-kb, Caspase 3, Cyclin D1, KLF-4, OCT-4 and Nanog genes expression

ILK was determined as direct target of miR-625 via miRNAs target prediction site (miRTarbase.mbc.nctu.edu.tw) and according to the study of Wang et al and Hannigan G et al. AKT, NF-kb, Caspase 3 are involved in survival signalling pathway and GSK3, c-fos, Cyclin D1 participating in proliferation signaling pathway based on Hannigan G et al study . KLF-4, OCT-4 and Nanog are stemness genes. Primers of these genes were designed by PerlPrimer and Gen Runner software and GAPDH was used as reference gene (Table 1). Quantitative PCR (qPCR) was performed for genes using Takara SYBR green PCR kit (Takara Bio Inc., Shiga, Japan). Relative expression was calculated for target genes using the ΔΔCT method (n=3).

Western blotting analysis

After 48 hours, Total cellular protein of GFP+ KG-1 cells transfected by pLentiIII-pre mir155-GFP expression vector and backbone vector were obtained by lysing cells with Trizol (Invitrogen 15596-026) lysis reagent and quantified for total protein by BCA assay with a standard curve generated using a BSA.

Total protein extract (10 µg) from each sample was separated on 12% SDS-polyacrylamide gels for 120 min at 100 V and transferred to PVDF membrane (Bio-Rad, USA) by a wet transfer system (Bio-Rad, USA) at 20 V overnight. Transferred blots were blocked with 5% BSA and 0.1% Tween-20 (Sigma-Aldrich). Afterward, blots were incubated with primary antibodies including 1:700 dilution of NF-κb rabbit primary antibody (Abcam, Inc., Cambridge, MA, USA), 1:400 dilution of Caspase 3 rabbit primary antibody (Abcam, Inc., Cambridge, MA, USA) and 1:200 dilution of p-β-catenin rabbit primary antibody (Santa Cruz) at 4°C. Blots were then washed three times, 20 min each, with TBST and incubated with 1:3,000 dilution of secondary antibody, HRP-conjugated anti-rabbit antibody (Abcam ab16284) for 1h at room temperature. After three washes, 15 min each, with TBST blots were incubated with chemiluminescent peroxidase substrate (Sigma-Aldrich, PQ0201, Germany) and analyzed with Gel doc. Densitometry of bands was analyzed by ImageJ software (http://rsb.info.nih.gov/ij) (n=3).

Statistical analysis

Statistical data analysis was done using student t test by GraphPad Prism software version 6.07 (La Jolla, California, USA). P < 0.05 was considered statistically significant. All the experiments of this study were carried out in triplicate (n = 3).

Results

Overexpression of miR-625 in KG-1 cells

Fluorescent microscopy confirmed transfection efficacy after 48 hours (Figure 1). Approximately 67% of cells were transfected and from these cells, GFP+ KG-1 cells were sorted (data not shown). qPCR shows significantly increased expression of miR-625 in the cells after 48 hours of transfection. The expression of miR-625 was approximately 27.11-fold higher relative to backbone vector transfected cells (P < 0.001).

miR-625 induces apoptosis in KG-1 cells

Next, programmed cell death in the KG-1 cells was evaluated by Annexin V-PE / 7-AAD kit 96 hours post-transfection. As shown in Figure 2A-2C, the number of apoptotic cells in KG-1 cell line transfected with pLentiIII-pre mir625-GFP construct was higher than backbone transfected cells. The number of apoptotic cells transfected with pLentiIII-pre mir625-GFP construct was about 7.6% higher than those transfected with backbone vector (Figure 2D).

Figure 1. Transfection Efficacy (GFP expression) was Confirmed 48 hours after Transfection of pLenti-III-premiR625-GFP and Backbone Vectors in KG-1 Cells by Fluorescent Microscopy.
The effects of miR-625-5p on cell cycle and cell proliferation in KG-1 cell line

We examined the effects of hsa-miR-625-5p on KG-1 cells proliferation by performing CCK-8 assay. Our results demonstrated that hsa-miR-625-5p overexpression doesn’t affect proliferation from 1 day to 4 days (Figure 3).

Figure 3. Effect of miR625 Overexpression on KG-1 Cells Proliferation from 1 day to 4 days. Proliferation of KG-1 cells transfected with pLentIII-pre mir625-GFP construct and backbone vector were evaluated by performing CCK-8 assay. Overexpression of miR-625 showed no significant change on proliferation of KG-1 cells compared to backbone (Mean ±SD, P > 0.05).
Cell cycle analysis was detected by Flowcytometry. Our results showed that 48 hours after transfection with hsa-miR-625-5p, there was no significant change in the cell cycle compared to the backbone group (Figure 4).

The effects of miR-625-5p on the mRNA expression levels
Forty-eight hours after transfection, the mRNA expression levels of ILK, AKT, NF-κB, Caspase3, GSK3, c-fos, Cyclin D1 and stemness genes including KLF-4, OCT-4 and Nanog genes expression were assessed by qPCR. The results indicated that the expression of ILK in KG-1 cells transfected with pLentiIII-pre mir625-GFP construct were about 0.53 and 0.58-fold higher than the cells transfected with backbone vector, respectively (P < 0.01). There was no significant change in mRNA expression level of GSK3 (p> 0.05). Compared with the backbone group the mRNA expression level of AKT, c-fos, Caspase3, Cyclin D1 and KLF-4, OCT-4 and Nanog displayed different levels of upregulation (p <0.05) (Figure 5).

The effects of miR-625-5p on the protein expression levels of NF-κB, Caspase 3 and p-β-catenin
Forty-eight hours after transfection, the protein levels of NF-κB, Caspase 3 and p-β-catenin were evaluated by western blot analysis. The data indicated that when KG-1 cells were transfected with pLentiIII-pre mir625-GFP construct, the levels of NF-κB protein decreased and the levels of Caspase 3 and p-β-catenin protein increased compared with backbone group (P <0.05) (Figures 6-8).

Discussion
High proliferation and reduced apoptosis are prominent features of AML cells. The apoptotic defect is a major challenge, and limits the efficacy of cancer chemotherapy. On the other hand, side effects of chemotherapeutic agents are other concerns (Fathabad et al., 2017). The miRNAs like miR-625-5p play a crucial role in cell fate as small potent molecules. Expression of miR-625 has decreased in acute myeloid leukemia cell lines (Xiong et al., 2014). miR-625 has different targets including NTRK3, ILK, FHIT and etc. ILK is one of the direct targets of miR-625-5p (mirtarbase.mbc.nctu.edu.tw) which is confirmed with validation methods such as Reporter assay, qPCR and NGS. Also, Wang et al., (2012b) identified that ILK is a direct target gene for miR-625. Through ILK downstream targets AKT, GSK3, NF-κB, Caspase 3, c-fos, β-catenin and Cyclin D1, ILK can be linked to two tumorgenesis-related events including cell proliferation.

### Table 1. The List of Primers Used in qPCR Analysis

| Primers     | Product size (bp) | Primers     | Product size (bp) |
|-------------|------------------|-------------|------------------|
| miR-625-5p  | Stem-loop RT: AGGGUAGAGGGAUGAGGGGAAAGUUCUAUAGUUCGUGAAUUGAGAU- CUCAGGACUAAAGAUCUCCCCCUCUACCCUCUGCCCU  | 503 |
|             | Forward: 5'-CTCTGCTCTGACTGTGCTG-3' | ILK         | 209             |
|             | Reverse: 5'-TACCCAGCTCTCCTAACCACGTG-3' | Forward: 5'-AACGGATTGATGATCTGAG-3' | 204 |
|             | GSK3             | Forward: 5'-AGTGTGTAAGAAAGATGAGTT-3' | 207             |
|             | Reverse: 5'-GAGGTTCGCTGGTTATATAATCAGT-3' | c-fos       | 85              |
|             | NF-κB            | Forward: 5'-ACTGGCCAATTTAACAACCTG-3' | 220             |
|             | Reverse: 5'-CATCACTGGCTCTTAAGGAAAGG-3' | Caspase 3   | 175             |
|             | Forward: 5'-AAAGGAAATATGAGAAGCTG-3' | Reverse: 5'-CAAGTTTCTGATGATTTCTCTTGTGAG-3' | 179 |
|             | Reverse: 5'-CTCTGAGAGAGATGCTGAG-3' | Cyclin D1   | 150             |
|             | GSK3             | Forward: 5'-AGTGGTGAGAAAGAAAGATGAG-3' | 128             |
|             | Reverse: 5'-GAGGTTCGCTGGTTATATAATCAGT-3' | Oct-4       | 150             |
|             | Nanog            | Forward: 5'-AAAGAATCTTACCTATGAC-3' | 110             |
|             | Reverse: 5'-GAAGGAAAGAGGAGGAGAAGCTG-3' | GAPDH       | 224             |
|             | SNORD47          | Stem-loop RT: GTCTATGCTCGAGGGTGCTTCGAGGT TCGACTGCAAGGACAAAACCC  | 71              |
|             | Forward: 5'-ATCACTGTCAAACACCTACTGAGT-3' | Reverse: 5'-GAGCAGGGGTCAGGAGGT-3' |
and apoptosis. ILK is aberrantly activated in malignancies such as AML (Alasseiri et al., 2018).

In this study, we evaluated the potential anti-cancer effect of miR-625 to target ILK pathway for inhibiting tumorigenesis as a promising approach to aid in the treatment of AML. Viral transduction method, which is a versatile method in terms of efficiency of transfection, was used to transfect KG-1 cells. Our results revealed that about 67% of the transfected cells express GFP and thereby dramatically induce miR-625. However, one of

Figure 4. Effect of miR-625 Overexpression on Cell Cycle in KG-1 Cells 48 hours Post Transfection. Flow cytometric analysis of cell cycle phase in (A) KG-1 cells were used as a control. (B) KG-1 cells transfected with pLentiIII-backbone-GFP construct. (C) KG-1 cells transfected with pLentiIII-premir625-GFP. (D) Overexpression of miR-625 in KG-1 cells demonstrated no significant change compared to backbone (Mean ±SD, P > 0.05).

Figure 5. Expression of ILK, AKT, GSK3, c-fos, NF-κB, Caspase 3 and Cyclin D1 Genes by qPCR. KG-1 cells transfected either with the plentiIII-premir625-GFP construct or backbone vector followed by expression evaluation of ILK, AKT, GSK3, c-fos, NF-κB, Caspase 3 and Cyclin D1 genes 48 hours after transfection. Overexpressed miR-625 resulted in upregulation of AKT, c-fos, Caspase 3, Cyclin D1 and downregulation of ILK and NF-κB expression but caused no alteration in GSK3 expression (Mean ± SD).
Figure 6. (A) Western blot analysis of NF-κB protein expression in KG-1 cells transfected by either plentiIII-premir625-GFP construct or backbone vector 48 hours post-transfection. miR-625 downregulated NF-κB protein. (B) Densitometry analysis of bands by ImageJ software indicated downregulation of nearly 0.6-fold. β-actin was used as loading control (Mean ±SD, * P < 0.05).

Figure 7. (A) Western blot analysis of Caspase 3 protein expression in KG-1 cells transfected by either plentiIII-premir625-GFP construct or backbone vector 48 hours post-transfection. miR-625 upregulated Caspase 3 protein. (B) Densitometry analysis of bands by ImageJ software indicated upregulation of nearly 2.87-fold. β-actin was used as loading control (Mean ±SD, * P < 0.05).

Figure 8. (A) Western blot analysis of p-β-catenin protein expression in KG-1 cells transfected by either plentiIII-premir625-GFP construct or backbone vector 48 hours post-transfection. miR-625 upregulated p-β-catenin protein. (B) Densitometry analysis of bands by ImageJ software indicated upregulation of nearly 10.15-fold. β-actin was used as loading control ( Mean ±SD, *P < 0.05).
the disadvantages of viral transduction method could be specific biosafety condition which is needed (Schlimmen et al., 2016).

Our findings demonstrated that upregulation of miR-625 in AML cells leads to downregulation of ILK. Wang et al., (2012b) showed that miR-625 is significantly down-regulated in gastric cancer compared with adjacent non-tumor tissues. They detected that up-regulation of miR-625 suppresses invasion and metastasis of gastric cancer by targeting ILK. miR-625 inhibits the protein expression of ILK by directly targeting 3' - UTR of ILK. Our results showed that downregulation of ILK is followed by downregulation of NF-κB and upregulation of Caspase 3 and consequently apoptosis occurs. Our results revealed that apoptosis was significantly induced in KG-1 cells following overexpression of miR-625. However, the number of apoptotic cells in the KG-1 cell line transfected with backbone vector was also high, which may be due to viral transduction method. Liu et al., (2013) identified that using OSU-T315, as an ILK inhibitor (OSU-T315 docks into AKT-binding site of ILK), induces apoptosis by suppressing both AKT and ERK pathway in CLL cells. Muranyi et al., (2006) identified that down-regulation of PI-3K pathway by inhibition of ILK kinase activity (QLT0267) is cytotoxic to leukemic blasts and progenitors in many patient samples. Increased miR-625 expression upregulated Cyclin D1 mRNA level and p-β-catenin protein level while ultimately it didn’t result in significant alteration on proliferation and cell cycle analysis. In our experiment, miR-625 upregulated the mRNA expression level of KLF-4, OCT-4 and Nanog. It shows that miR-625 cannot induce differentiation in KG-1 cells. De la Puente et al., (2015) reported that using the small molecule ILK inhibitor, Cpd22, and ILK knockdown, inhibits the proliferation of AML, CML and ph+ ALL. Treatment of CML cells with Cpd22 lead to induction of apoptosis and autophagy. Knockdown of ILK with lentiviral shRNA vector particles against ILK in human CML and AML cell lines resulted in inhibition of cell proliferation. In our study, contradicting De la Puente findings, inhibition of ILK did not effect on proliferation of KG-1 cells.

In this study, we propose a new potential anticancer agent in AML cell line, i.e. miR-625. We showed that overexpression of miR-625 can induce apoptosis in KG-1 cell line via downregulation of ILK but it has no effect on proliferation. Signaling for apoptosis occurs through down regulation of NF-xB and upregulation of Caspase 3. However, further and complementary studies are needed to address some other aspects, including in vivo studies and safety issues.

Author Contribution Statement

BA, SSD and MM performed the experimental work. SHM and ME participated in data analysis. SHM, ME, ShA and AAH conceived and participated in the design of the study. BA wrote the manuscript. All authors read and approved the final manuscript.

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Data availability statement

Not applicable.

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Asian Pacific Journal of Cancer Prevention, Vol 23

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