

# MicroRNA profiling of tacrolimus-stimulated Jurkat human T lymphocytes

Ho Kyun Lee, Sang Young Chung, Soo Jin Na Choi

Department of Surgery, Chonnam National University Medical School, Gwangju, Korea

**Purpose:** This study investigated the Jurkat T cell line expresses cytotoxicity when treated with different concentrations of FK506, and analyzed the expression pattern of microRNA when stimulated by FK506 using the microRNAs microarray, as well as the expression pattern of a gene that is related to the differentiation, activation and proliferation of T cells after being affected by the change of microRNAs.

**Methods:** To investigate the effects of FK506 on microRNA expression, we purified total RNA of Jurkat cells treated with 20  $\mu$ M FK506 for 72 hours and used to analyze microRNA profiling by using Agilent's chip.

**Results:** These results demonstrated that treatment with FK506 markedly induced the down-regulation of 20 microRNAs as well as the up-regulation of 20 microRNAs in a time-dependent manner. The genes that down-regulated by FK506 include let-7a\*, miR-20a\*, and miR-487a. Otherwise miR-202, miR-485-5p, and miR-518c\* are gradually up-regulated in expression. Sanger Institute and DAVIDs bioinformatics indicated that microRNAs regulated the several transcriptomes including nuclear factor of activated T cell-related, T cell receptor/interleukin-2 signaling, and Ca<sup>2+</sup>-calmodulin-dependent phosphatase calcineurin pathways.

**Conclusion:** As a result of treating FK506 to a Jurkat cell line and running the microRNA microarray, it was found that FK506 not only took part in the suppression of T cell proliferation/activation by inhibiting calcineurin in Jurkat apoptosis, but also affected the microRNAs that are involved in the regulation of various signal transduction pathways.

## INTRODUCTION

Tacrolimus (FK506) is an immunosuppressive drug, discovered in 1984 from *Streptomyces tsukubaensis*, that is used to prevent rejection of transplanted organs following the organ transplantation [1-4]. FK506 suppresses T cell activation by inhibiting calcineurin and the calcineurin-dependent transcription factors, the nuclear factor of activated T cells (NFATc), which are the central regulators of the T cell function. However, the molecular mechanisms underlying how FK506 regulates NFATc in T cell activation are obscure. Previous studies have indicated that FK506 efficiently blocks proliferation of T cells by inducing a G<sub>0</sub>/G<sub>1</sub> cell cycle arrest.

MicroRNAs (miRNAs) are small RNAs that regulate the expression of complementary messenger RNAs. Hundreds of microRNA genes have been found in diverse animals, and many of these are phylogenetically conserved [5-7]. Recently, microRNAs have been shown to target particular sets of mRNAs, thereby preventing

### Corresponding Author

Soo Jin Na Choi  
Department of Surgery, Chonnam National University Medical School, 160 Baekseo-ro, Dong-gu, Gwangju 501-746, Korea  
Tel: +82-62-220-6456  
Fax: +82-62-227-1635  
E-mail: choisjn@chonnam.ac.kr

### Key Words

Tacrolimus, MicroRNAs, Jurkat T lymphocyte

Received May 29, 2013

Revised July 18, 2013

Accepted July 24, 2013

J Korean Surg Soc 2013;85:161-168  
<http://dx.doi.org/10.4174/jkss.2013.85.4.161>

Copyright © 2013, the Korean Surgical Society

© Journal of the Korean Surgical Society is an Open Access Journal. All articles are distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

translation or accelerating mRNA turnover [5,6,8,9]. Therefore miRNAs play important roles in normal regulation of gene expression for developmental timing, cell proliferation, and apoptosis.

This study investigated the Jurkat T cell line expresses cytotoxicity when treated with different concentrations of FK506, and analyzed the expression pattern of miRNA when stimulated by FK506 using the miRNAs microarray, as well as the expression pattern of a gene that is related to the differentiation, activation and proliferation of T cells after being affected by the change of miRNAs. Furthermore, by analyzing the relationship between the expression pattern of the genes related to apoptosis and the biological pathway, this study was expected to provide the new approach to the biological effect of FK506.

## METHODS

Jurkat human T-lymphocyte lines obtained from the Korean type Culture collection (Seoul National University, Seoul, Korea) were maintained in RPMI-1640 (Gibco BRL, Grand Island, NY, USA) tissue culture medium supplemented with 10% fetal calf serum at 37°C with 5% CO<sub>2</sub>. FK506 was solubilized at a concentration of 5 mg/mL in dimethylsulfoxide, maintained at 20°C, and diluted in the RPMI-1640 to the final concentrations of 0.1 to 100 mol/L. All cell cultures were incubated at 37°C with 5% CO<sub>2</sub>. Cell viability was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromid assay at 24 and 48 hours after FK506 treatment.

### RNA extraction

RNA was extracted from cell lines using a standard Trizol (Invitrogen, Carlsbad, CA, USA) protocol, and modified by washing the final RNA pellet with 70% EtOH. The cell lines were sectioned on a cryostat prior to homogenization in Trizol. RNA quantity and quality were assessed by Nanodrop (Genomic Tree, Daejeon, Korea) and Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively.

MicroRNA labeling and hybridization were done by using Cy3-dyed total RNA. Our miRNA microarray (Agilent's Human genome 4 × 1.5 K glass slide) contained a probe for 534 mature human microRNAs and 64 mature viral microRNAs (Sanger miRBase release 9.1).

Unbound sample was removed from the beads by washing with 1× TE and resuspending in 1× tetramethylammonium chloride buffer after the hybridization. Streptavidin-phycoerythrin, premium grade (Invitrogen) was added to the beads (1:100 dilution) and incubated for 10 minutes at 50°C to bind to biotin moieties on the cDNA. Samples were scanned by the

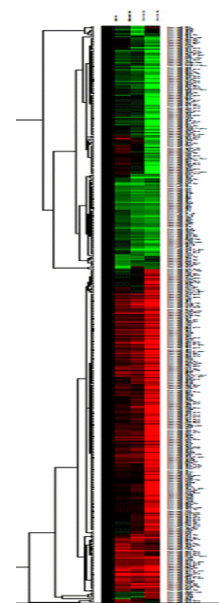
laser confocal scanner (G2565BA, Agilent Technologies) and median fluorescence intensity values were acquired using the Feature extraction software (Agilent Technologies).

- Amplification and labeling: performed using the Agilent's Low RNA Input Linear Amplification kit PLUS.
- Microarray hybridization: performed using the Agilent's Gene Expression Hybridization Kit.
- Microarray wash: used the Agilent's Gene Expression Wash Buffer Kit.
- Scan and image analysis: used the Agilent's DNA micro array scanner and the Feature Extraction Software.

The experiments were repeated three times each. The analysis of the microarray result used the Agilent's GeneSpring Software to sort the miRNAs that showed increase or decrease by normalization, clustering and filtering. And to identify the function of the target genes of these miRNAs, DAVID's program from the website was used to analyze the biological function.

## RESULTS

As a result of treating Jurkat cell line with various durations and concentrations of FK506, 10 μM of FK506 lowered the viability of the cell to 92.2% after 24 hours of treatment, 77.3% after 48 hours, 62.5% after 72 hours, and 46.2% after



**Fig. 1.** Regulation of microRNAs expression in Jurkat T cell after FK506 treatment. Differentially expressed microRNAs were analyzed by hierarchical clustering of the log<sub>2</sub> value of each microRNA microarray signal at 24, 48, and 72 hours. Red, up-regulation; Green, down-regulation; Black, no change.

96 hours. With 20 μM of FK506, the viabilities were 87.2% after 24 hours, 68.4% after 48 hours, 45.7% after 72 hours, and 22.45% after 96 hours. Also, 30 μM of FK506 showed 78.08%, 56.52%, 33.49%, and 12.91% viability at above mentioned durations, respectively. Therefore, the treatment with FK506 decreased the viability of human Jurkat T-cell in dose- and time-dependent manners. Thus in this study, the cell line was treated with 20 μM of FK506 for 72 hours, the combination of which shows roughly 50% viability, for the microRNAs microarray analysis.

### Change in the microRNA expression by the effect of FK506 during apoptosis of Jurkat cell

MicroRNA array was performed to the experimental group of Jurkat cell lines, which were each treated with 20 μM FK506 for 24, 48, and 72 hours, and the changed signal intensity was analyzed after eliminating the background signal. The groups of miRNAs that were expressed more than twice, or less than a half compared to the control group were classified and marked as a cluster tree (Fig. 1). The group of miRNAs that were expressed more than twice the control group was marked red, and the group that showed the expression of less than a half the control group was marked green, and thereby sorted the miRNAs according to the pattern of expression in a time-

dependent manner.

### Analysis of the miRNAs that decreased in expression during FK506-mediated apoptosis of Jurkat cell

As a result of the microarray performed to the Jurkat cells treated with 20 μM FK506 for 24, 48, and 72 hours, the number of miRNAs that decreased in expression to less than a half compared to the control group was 21 in total, and by searching for any relationship between these miRNAs and the genes, the miRNAs miR-19a, -19a\*, -19b, -20a\*, -106a, -142-5p, -301a, -301b, and -487a were found at the end of mRNA of *NFATC3* (NFAT4) gene, which regulates the apoptosis of Jurkat cell mediated by FK506 (Table 1, Fig. 2). Also, the entire genes that are regulated by these miRNAs were analyzed using DAVIDs bioinformatics, and it was found that the genes regulated by these microRNAs, which showed decreased expression, were related to the biological processes, such as p53 signaling pathway and the regulation of cell cycle (Table 2).

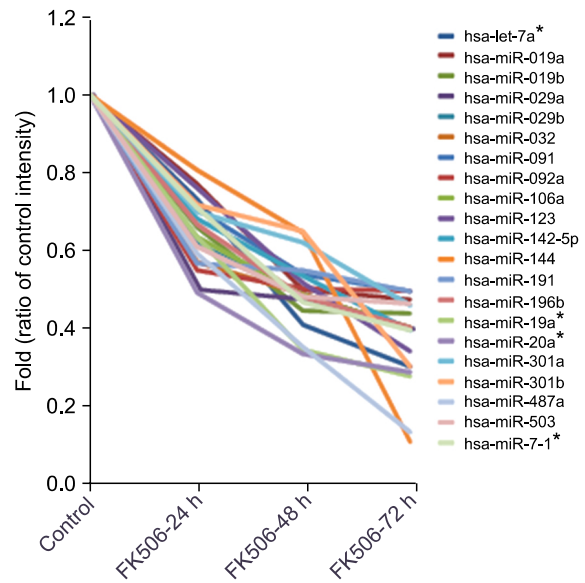
### Analysis of the microRNAs that increased in expression during FK506-mediated apoptosis of Jurkat cell

As a result of the microarray performed to the Jurkat cells treated with 20 μM FK506 for 24, 48 and 72 hours, the

**Table 1. Decreased microRNAs regulate the specific target genes by the clustered localization**

Gene_name	Human miRNAs name	Chr	Strand	Transcript_ID	Transcript link	Target link
<i>CAPRN2</i>	hsa-miR-106a, -142-5p, -144, -19a, -19b, -29a, -29b, -301a, -301b	12	-	ENST00000251071	ENST00000251071	ENST00000251071
<i>TACC2</i>	hsa-let-7a*, hsa-miR-7-1*, -19a, -19b, -32, -92a, -191, -487a	10	+	ENST00000369005	ENST00000369005	ENST00000369005
<i>MRAP</i>	hsa-miR-19a, -19b, -29a, -32, -92a, -301a, -301b, 503	21	+	ENST00000303645	ENST00000303645	ENST00000303645
<i>IL20</i>	hsa-let-7a*, hsa-miR-19a, -19b, -301a, -301b, 487a, -503	1	+	ENST00000367098	ENST00000367098	ENST00000367098
<i>NFE2L2</i>	hsa-miR-7-1*, -19*, -142-5p, -144, -301a, -301b	2	-	ENST00000361004	ENST00000361004	ENST00000361004
<i>HMGB2</i>	hsa-let-7a*, hsa-miR-20a*, -191, -301a, -301b, -487a	4	-	ENST00000296503	ENST00000296503	ENST00000296503
<i>TNFSF5IP1</i>	hsa-let-7a*, hsa-miR-19a, 142-5p, 196b, -487a	18	+	ENST00000317615	ENST00000317615	ENST00000317615
<i>NFATC3</i>	hsa-miR-19a, -19b, -20a*, -142-5p, -487a	16	+	ENST00000346183	ENST00000346183	ENST00000346183
<i>NFATC3</i>	hsa-miR-19a*, -106a, -301a, -301b	16	+	ENST00000379165	ENST00000379165	ENST00000379165
<i>HSPA2</i>	hsa-miR-7-1*, -19a, -19b, -92a, -106a	14	+	ENST00000247207	ENST00000247207	ENST00000247207
<i>HMG1L1</i>	hsa-miR-7-1*, -20a*, -29a, -29b, -487a	20	-	ENST00000322901	ENST00000322901	ENST00000322901
<i>BECN1</i>	hsa-miR-19a, -19b, -29a, -29b, -144	17	-	ENST00000246933	ENST00000246933	ENST00000246933
<i>NFATC2</i>	hsa-miR-19a*, -29a, -29b, -191	20	-	ENST00000371567	ENST00000371567	ENST00000371567
<i>NFATC2</i>	hsa-miR-29a, -29b, -191	20	-	ENST00000371564	ENST00000371564	ENST00000371564
<i>IL2</i>	hsa-miR-19a*, -196b, -487a	4	-	ENST00000226730	ENST00000226730	ENST00000226730
<i>IL3</i>	hsa-miR-19a*, -19b, -503	5	+	ENST00000379197	ENST00000379197	ENST00000379197
<i>CD40</i>	hsa-miR-29a, -29b, 503	20	+	ENST00000279061	ENST00000279061	ENST00000279061
<i>CAMKK1</i>	hsa-miR-19a, -19b, 106a	17	-	ENST00000381763	ENST00000381763	ENST00000381763
<i>CAMK2B</i>	hsa-miR-7-1*, -29a, -29b	7	-	ENST00000258682	ENST00000258682	ENST00000258682

miRNA name	Control	FK506-24 h	FK506-48 h	FK506-72 h
hsa-let-7a*	1	0.733	0.416	0.305
hsa-miR-7-1*	1	0.714	0.470	0.400
hsa-miR-19a	1	0.772	0.503	0.477
hsa-miR-19b	1	0.664	0.448	0.442
hsa-miR-29a	1	0.503	0.478	0.404
hsa-miR-29b	1	0.621	0.492	0.401
hsa-miR-32	1	0.573	0.490	0.404
hsa-miR-91	1	0.708	0.543	0.498
hsa-miR-92a	1	0.553	0.506	0.499
hsa-miR-106a	1	0.637	0.487	0.407
hsa-miR-123	1	0.763	0.520	0.346
hsa-miR-142-5p	1	0.684	0.537	0.398
hsa-miR-144	1	0.808	0.649	0.115
hsa-miR-191	1	0.569	0.553	0.499
hsa-miR-196b	1	0.670	0.483	0.405
hsa-miR-19a*	1	0.637	0.350	0.283
hsa-miR-20a*	1	0.496	0.340	0.293
hsa-miR-301a	1	0.703	0.622	0.465
hsa-miR-301b	1	0.721	0.651	0.307
hsa-miR-487a	1	0.596	0.358	0.138
hsa-miR-503	1	0.613	0.486	0.466



**Fig. 2.** The microRNAs down regulated in the time dependent manners by FK506 treatment.

**Table 2.** The specific biological pathways were regulated by decreased microRNAs

Term	Genes	Count	P-value
Cell cycle	<i>PLK1, CDKN1A, TP53, CDKN2D, WEE1, CDC34, RASGRF1, RPS6KA1, YWHAH, GADD45A, CHEK1, MDM2, ATR, BRCA1, NPAT, MYT1, EP300, CHEK2, CCNB1</i>	19	6.5E-4
Role of BRCA1, BRCA2 and ATR in cancer susceptibility	<i>FANCD2, RAD1, FANCE, TP53, RAD17, TREX1, HUS1, RAD50, RAD91, CHEK1, ATR, FANCA, BRCA1, NPAT, RAD51, CHEK2, RANCG</i>	17	4.7E-3
RB tumor suppressor/checkpoint signaling in response to DNA damage	<i>RB1, CDK2, CHEK1, TP53, WEE1, NPAT, RASGRF1, MYT1, CDK4, YWHAH</i>	10	3.6E-2
Spliceosomal assembly	<i>SNRPD3, U2AF2, SNRPE, SNRPA, U2AF1, SNRPC, SNRPA1, SNRPB, SNRP70, SNRPD2, SNRPD1</i>	11	3.8E-2
Fibrinolysis pathway	<i>TAF1, CDKN1A, ABCB1, TP53, FHL2, CSNK1A1, HSPA1B, GADD45A, MDM2, HIC1, RPA1, HIF1A, NPAT, BAX, EP300</i>	8	6.0E-2
TGF beta signaling pathway	<i>APC, TGFBI, SKIL, MAP2K1, CDH1, MAP3K7IP1, ZFYVE9, EP300, MAPK3, SMAD4, SMAD7</i>	11	6.5E-2
p53 signaling pathway	<i>RB1, GADD45A, CDK2, CDKN1A, MDM2, PCNA, TP53, CCNE1, NPAT, BAX, CDK4</i>	11	6.5E-2
Hypoxia and p53 in the cardiovascular system	<i>TAF1, CDKN1A, ABCB1, TP53, FHL2, CSNK1A1, HSPA1B, GADD45A, MDM2, HIC1, RPA1, HIF1A, NPAT, BAX, EP300</i>	15	8.9E-2
Cyclins and cell cycle regulation	<i>CDC25A, CDK2, CDKN1A, CCNA1, CDK7, CDKN2A, CDKN2D, CCNE1, CCNH, RB1, CCND3, CDKN2C, CCNB1, CDK4</i>	14	9.2E-2
ATM signaling pathway	<i>CDKN1A, TP53, NFKBIA, RAD50, GADD45A, CHEK1, MDM2, TP73, BRCA1, NPAT, RBBP8, RAD51, CHEK2</i>	13	9.6E-2

number of microRNAs that showed increased expression to more than twice of the control group was 18 in total, and by searching for any relationship between these microRNAs and the genes, the microRNAs miR-486-5p and miR-518c\* were detected at the end of mRNA of TP53I3 gene, which is related

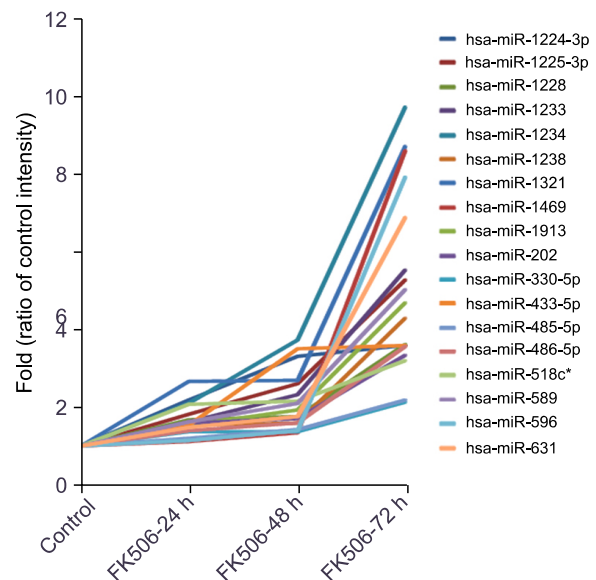
to triggering p53 associated cell death. As for FKBP8 gene, the intracellular calcineurin inhibitor, the microRNAs miR-330-5p and miR-631 were detected (Table 3, Fig. 3).

Also by using DAVIDs bioinformatics, the group of genes that are regulated by these 18 miRNAs were found to be

**Table 3.** The specific target genes were regulated in the clustered localization by increased microRNAs

Gene_name	Human miRNAs name	Chr	Strand	Transcript_ID	Transcript link	Target link
ZFPL1	hsa-miR-330-5p, -485-5p, -589, -586, -631	11	+	ENST00000294258	ENST00000294258	ENST00000294258
TREX2	hsa-miR-202, -486-5p, 596, -631	X	-	ENST00000370212	ENST00000370212	ENST00000370212
TNFRSF4	hsa-miR-202, -330-5p, -486-5p, -596	1	-	ENST00000379236	ENST00000379236	ENST00000379236
TNFAIP8L2	hsa-miR-330-5p, -486-5p, -518c*, -589	1	+	ENST00000368910	ENST00000368910	ENST00000368910
WNT6	hsa-miR-330-5p, -485-5p, -486-5p	2	+	ENST00000233948	ENST00000233948	ENST00000233948
TGFB1	hsa-miR-330-5p, -485-5p, -518c*	5	+	ENST00000305126	ENST00000305126	ENST00000305126
RELB	hsa-miR-202, -485-5p, -596	19	+	ENST00000221452	ENST00000221452	ENST00000221452
NOS3	hsa-miR-330-5p, -518c*, -631	7	+	ENST00000297494	ENST00000297494	ENST00000297494
JAK3	hsa-miR-330-5p, -485-5p, -518c*	19	-	ENST00000317306	ENST00000317306	ENST00000317306
HDAC8	hsa-miR-202, -330-5p, -518c*	X	-	ENST00000373573	ENST00000373573	ENST00000373573
CARD10	hsa-miR-202, -589, -631	22	-	ENST00000251973	ENST00000251973	ENST00000251973
TP53I3	hsa-miR-486-5p, -518c*	2	-	ENST00000313482	ENST00000313482	ENST00000313482
SOD2	hsa-miR-202, -518c*	6	-	ENST00000337404	ENST00000337404	ENST00000337404
SIRT3	hsa-miR-330-5p, -486-5p	11	-	ENST00000324945	ENST00000324945	ENST00000324945
SIRT6	hsa-miR-330-5p, -486-5p	19	-	ENST00000337491	ENST00000337491	ENST00000337491
NFE2	hsa-miR-330-5p, -631	12	-	ENST00000312156	ENST00000312156	ENST00000312156
HMOX1	hsa-miR-518c*, -589	22	+	ENST00000216117	ENST00000216117	ENST00000216117
GSTM2	hsa-miR-330-5p, -589	1	+	ENST00000326729	ENST00000326729	ENST00000326729
FKBP8	hsa-miR-330-5p, -631	19	-	ENST00000222308	ENST00000222308	ENST00000222308

miRNA name	Control	FK506-24 h	FK506-48 h	FK506-72 h
hsa-miR-1224-3p	1	2.187	3.326	3.607
hsa-miR-1225-3p	1	1.825	2.607	5.286
hsa-miR-1228	1	1.702	1.762	3.633
hsa-miR-1233	1	1.618	2.329	5.541
hsa-miR-1234	1	2.108	3.741	9.728
hsa-miR-1238	1	1.555	1.606	4.311
hsa-miR-1321	1	2.666	2.700	8.710
hsa-miR-1469	1	1.125	1.345	8.593
hsa-miR-1913	1	1.537	1.931	4.694
hsa-miR-202	1	1.600	1.708	3.345
hsa-miR-330-5p	1	1.365	1.366	2.147
hsa-miR-433-5p	1	1.539	3.513	3.588
hsa-miR-485-5p	1	1.201	1.449	2.180
hsa-miR-486-5p	1	1.414	1.592	3.583
hsa-miR-518c*	1	2.072	2.169	3.198
hsa-miR-589	1	1.629	2.110	5.032
hsa-miR-596	1	1.150	1.406	7.923
hsa-miR-631	1	1.494	1.776	6.894



**Fig. 3.** The microRNAs up regulated in time dependent manner during FK506 treatments.

associated with various biological processes, including calcium signaling pathway (Table 4).

## DISCUSSION

Most of the studies regarding FK506 have focused primarily

**Table 4.** The specific biological pathways regulated by up-regulated microRNA

Term	Genes	Count	P-value
Alanine and aspartate metabolism	<i>GPT, PDHA2, GAD2, CAD, ADSL, PC, PDHB, CRAT, GAD1, ASPA, PDHA1, ADSSL1, NARS2, AARS, DARS, ASS1</i>	16	1.1E-02
Pyrimidine metabolism	<i>CTPS2, CAD, POLR2I, CDA, POLR2L, NP, NT5C2, POLR3GL, POLD4, POLR1A, POLE, POLR2H, POLD2, POLR2A, NT5C, ENTPD5, CANT1, ENTPD6, DPYS, DHODH, NT5C1A, POLR2G, POLR2E, DTYMK, UPB1, NT5C1B, ECGF1, NUDT2, ITPA, TXNRD2, POLR1C, PRIM1</i>	32	2.8E-02
Adherens junction	<i>ACTN2, FGFR1, ACTN4, PVRL1, PTPN6, IGF1R, ACTN3, LMO7, ERBB2, FYN, PTPRM, PTPRJ, BAIAP2, PTPRF, FARP2, PVRL3, PVRL2, CTNNA3, CSNK2A2, RAC3, TCF7L1, MLLT4, CTNNA2, ACTN1, TCF7, PARD3, SORBS1</i>	27	4.4E-02
Pyruvate metabolism	<i>LDHD, PDHA2, ACSS2, ACAT2, GRHPR, LDHAL6B, PC, AKR1B1, PDHB, MDH1, HAGHL, PKM2, PDHA1, ACAT1, HAGH, PCK1, ME3</i>	17	5.1E-02
Fructose and mannose metabolism	<i>MTMR1, PMM1, PFKFB2, PFKM, PFKFB3, UGCGL2, AKR1B1, HSD3B7, KHK, FUK, PHPT1, ALDOA, GMDS, FPGT, GMPPA, FBP1, TSTA3</i>	17	5.1E-02
Purine metabolism	<i>APRT, ADA, ENPP3, PFAS, PDE9A, POLR2I, ATIC, ADSL, ENTPD2, NUDT9, POLR2L, NP, NT5C2, PDE6D, POLR3GL, POLD4, GUCY2D, PRPS2, GDA, ADSSL1, POLR1A, POLE, POLR2H, ADCY5, POLD2, POLR2A, NT5C, GUCY1A2, ENTPD5, CANT1, ENTPD6, POLR2G, PDE4C, NT5C1A, GART, PDE4A, ADCY1, POLR2E, PKM2, GUCY2C, NUDT5, NT5C1B, ECGF1, NUDT2, ITPA, POLR1C, PRIM1</i>	47	5.7E-02
Basal cell carcinoma	<i>DVL3, AXIN1, PTCH1, TP53, FZD9, WNT7A, DVL1, WNT6, SMO, GLI1, WNT5B, WNT3, TCF7L1, PTCH2, WNT7B, FZD5, WNT10A, TCF7, AXIN2, STK36, WNT9B</i>	21	6.1E-02
Calcium signaling pathway	<i>SPHK2, PTAFR, MYLK2, GNA11, P2RX3, PLCG2, CCKBR, PRKACG, PLCB3, PLCD1, P2RX5, TBXA2R, SPHK1, PHKG2, TNNC1, ITPKA, PPP3R1, F2R, CACNA1S, ADRA1B, P2RX2, GRM5, CACNA1G, ADORA2B, CAMK2A, CACNA1I, CACNA1F, ATP2A3, VDAC1, ATP2A2, CACNA1H, PLCB2, GRM1, HTR4, VDAC2, SLC25A4, ERBB2, PDE1B, GNAS, P2RX4, PHKG1, HTR6, NOS2A, AVPR1B, PDGFRB, ADCY1, CALML3, PLCG1, CACNA1C, ATP2B2, PTGER1, NOS3, ADORA2A, TACR1, CACNA1D</i>	55	6.1E-02
Arginine and proline metabolism	<i>DAO, PYCR2, GAMT, NOS2A, P4HA2, PYCRL, PYCR1, PARS2, OAT, ALDH4A1, CKM, P4HA3, NOS3, ASS1</i>	14	7.3E-02
Riboflavin metabolism	<i>PHPT1, MTMR1, ENPP3, ACP5, TYR, FLAD1, ACP6, ACP2</i>	8	8.9E-02
Glycerophospholipid metabolism	<i>CDIPT, AGPAT4, ACHE, ETNK2, LYPLA2, PPAP2C, NAT5, PLA2G2F, PISD, CDS2, CHPT1, PPAP2A, PLA2G4A, PEMT, ARD1A, PLD2, CRIS1, LCAT, DGKZ, CHAT, PLA2G5, CDS1, PLA2G2E, DGKG</i>	24	9.40E-02

on its function as an inhibitor for the intracellular calcium-dependent calcineurin and the drug's target protein, NFAT, which is a transcription factor of T cells, as well as its function to regulate the expression of cytokines. But not enough studies were conducted regarding the role of FK506 to express the genes related to apoptosis and its mechanism.

According to the recent studies, the intervention of miRNAs in RNA molecules plays an important role in regulating the gene expression, and is phylogenetically conserved. The gene expression is regulated by disassembling or inhibiting the mRNA of the target gene with very small RNAs [6]. Hundreds of miRNAs are investigated in nematodes, plants and animals through computer analysis and Reverse Transcriptase-Polymerase Chain Reaction cloning [10,11]. Hence this study used Jurkat human T lymphocyte line to verify that the cell's viability decreases in a time and dose dependent manner when treated with FK506, the immunosuppressive drug that is used after the organ transplantation to prevent rejection.

In order to analyze the change caused by FK506 in the expression pattern of miRNAs and the signal pathway stimulated by the drug's target molecule, 20  $\mu$ M of FK506, which

shows about 50% of cell viability, was treated for 24, 48, and 72 hours and followed by miRNA microarray using the total RNA to analyze the expression of miRNAs associated with the activation of T cell by FK506 and the drop in cell viability.

As a result of treating 20  $\mu$ M of FK506, 21 kinds of miRNA including miR-20a\* decreased to less than a half in time-dependent fashion, and other 18 kinds of miRNAs including miR-518c\* increased to more than twice of the control group. Most of the decreased miRNAs are related to the immunosuppressive functions of FK506, including inhibition of the well-known cytokines, such as interleukin 2 and CD40 [12,13], and the regulation of calcium/calmodulin dependent kinases [14-16] and NFATs [17], which are the intracellular transcription factors. FK506 is known to release FKBP12, which is bound to the transforming growth factor (TGF)- $\beta$  receptor in a chronic lymphocytic leukemia B cell, and phosphorylates and activates the receptor, thereby causing phosphorylation of Smad and leading to apoptosis [18,19]. In a smooth muscle cell, however, it was found that the activation of the TGF- $\beta$  receptor by FK506 stimulates cell growth [1,18]. Ataxia telangiectasia-mutated (ATM) kinase is a multifunctional

enzyme that is activated after birth and known to take part in suppressing the onset of ataxia–telangiectasia (AT) [20,21]. The cells that lack ATM show markedly increased activation of thiol redox and DNA synthesis compared to a normal cell. The regulation of ATM signal transduction by FK506 affects the cell cycle by controlling the intracellular thiol redox pathway and therefore is involved in suppressing the abnormal DNA synthesis and oncogenesis [22,23]. On the other hand, the target genes of the miRNAs that are increased by FK506 were identified and their biological functions were analyzed using DAVID's bioinformatics. The analysis detected numerous genes that are associated with the intracellular energy metabolism pathway, the production of nucleotides; pyrimidine and purine, the production of amino acids related to protein synthesis, the calcium signal transduction pathway and the signal transduction by T cell receptors. Benedini et al. [24], reported that in case of lung transplant patients, the immunosuppressive drugs increase metabolism of glucose and leucine. It was also reported that when FK506 was treated to a mouse after the liver resection for regeneration, the insulin receptors in the liver cell of mouse increased in number, and the energy metabolism and the amino acid synthesis were boosted [25,26]. It was also recently reported that calcineurin controls the synaptic plasticity and the transcription factor, NFAT in a synapse of a neuron. Furthermore, it was found that the dendritic branching could increase in number by suppressing the calcineurin molecule [27,28]. The FK506-binding proteins (FKBP12 and 12.6) are involved in a regulation of ryanodine receptors that control the calcium ion in the sarcoplasmic reticulum of a heart, and the treatment of FK506 loosens their binding and calcium is released from the sarcoplasmic reticulum as a result [29]. In addition, when IP3R is inhibited in association with the control of neurite outgrowth by calcium, the neurite outgrowth was suppressed in the early stage, whereas the inhibition of calcium-induced calcium release by ryanodine receptor suppresses the neurite outgrowth in the later stage. And when treated with FK506, the neurite outgrowth was suppressed in the early stage as well [28].

In conclusion, as a result of treating FK506 to a Jurkat cell line and running the miRNA microarray, it was found that FK506 not only took part in the suppression of T cell proliferation/activation by inhibiting calcineurin in Jurkat apoptosis, but also affected the miRNAs that are involved in the regulation of various signal transduction pathways that are related to the causes of apoptosis and the cardiovascular diseases, kidney problems, neurotoxicity, diabetes, and the acute hearing impairment triggered by a long-term use of FK506.

## CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

## REFERENCES

1. Arai S, Vogelsang GB. Management of graft-versus-host disease. *Blood Rev* 2000;14:190–204.
2. Assmann T, Homey B, Ruzicka T. Applications of tacrolimus for the treatment of skin disorders. *Immunopharmacology* 2000;47:203–13.
3. Hanauer SB, Dassopoulos T. Evolving treatment strategies for inflammatory bowel disease. *Annu Rev Med* 2001;52:299–318.
4. Pridohl O, Heinemann K, Hartwig T, Witzigmann H, Lamesch P, Fangmann J, et al. Low-dose immunosuppression with FK 506 and sirolimus after liver transplantation: 1-year results. *Transplant Proc* 2001;337:3229–31.
5. Ambros V. The functions of animal microRNAs. *Nature* 2004;431:350–5.
6. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281–97.
7. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* 2005;433:769–73.
8. Du T, Zamore PD. MicroPrimer: the biogenesis and function of microRNA. *Development* 2005;132:4645–52.
9. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* 2004;5:522–31.
10. Bentwich I. Prediction and validation of microRNAs and their targets. *FEBS Lett* 2005;579:5904–10.
11. Berezikov E, van Tetering G, Verheul M, van de Belt J, van Laake L, Vos J, et al. Many novel mammalian microRNA candidates identified by extensive cloning and RAKE analysis. *Genome Res* 2006;16:1289–98.
12. Kaminska J, Nowacki MP, Kowalska M, Rysinska A, Chwalinski M, Fuksiewicz M, et al. Clinical significance of serum cytokine measurements in untreated colorectal cancer patients: soluble tumor necrosis factor receptor type I—an independent prognostic factor. *Tumour Biol* 2005;26:186–94.
13. Luo C, Burgeon E, Carew JA, McCaffrey PG, Badalian TM, Lane WS, et al. Recombinant NFAT1 (NFATp) is regulated by calcineurin in T cells and mediates transcription of several cytokine genes. *Mol Cell Biol* 1996;16:3955–66.
14. Harding MW, Galat A, Uehling DE, Schreiber SL. A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase. *Nature* 1989;341:758–60.
15. Maki N, Sekiguchi F, Nishimaki J, Miwa K, Hayano T, Takahashi N, et al. Complementary DNA encoding the human

- T-cell FK506-binding protein, a peptidylprolyl cis-trans isomerase distinct from cyclophilin. *Proc Natl Acad Sci U S A* 1990;87:5440-3.
16. Standaert RF, Galat A, Verdine GL, Schreiber SL. Molecular cloning and overexpression of the human FK506-binding protein FKBP. *Nature* 1990;346:671-4.
  17. Brown EJ, Albers MW, Shin TB, Ichikawa K, Keith CT, Lane WS, et al. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature* 1994;369:756-8.
  18. Akool el-S, Doller A, Babelova A, Tsalastra W, Moreth K, Schaefer L, et al. Molecular mechanisms of TGF beta receptor-triggered signaling cascades rapidly induced by the calcineurin inhibitors cyclosporin A and FK506. *J Immunol* 2008;181:2831-45.
  19. Romano S, Mallardo M, Chiurazzi F, Bisogni R, D'Angelillo A, Liuzzi R, et al. The effect of FK506 on transforming growth factor beta signaling and apoptosis in chronic lymphocytic leukemia B cells. *Haematologica* 2008;93:1039-48.
  20. Brown KD, Barlow C, Wynshaw-Boris A. Multiple ATM-dependent pathways: an explanation for pleiotropy. *Am J Hum Genet* 1999;64:46-50.
  21. Rolig RL, McKinnon PJ. Linking DNA damage and neurodegeneration. *Trends Neurosci* 2000;23:417-24.
  22. Barlow C, Liyanage M, Moens PB, Deng CX, Ried T, Wynshaw-Boris A. Partial rescue of the prophase I defects of Atm-deficient mice by p53 and p21 null alleles. *Nat Genet* 1997;17:462-6.
  23. Yan M, Kuang X, Qiang W, Shen J, Claypool K, Lynn WS, et al. Prevention of thymic lymphoma development in Atm<sup>-/-</sup> mice by dexamethasone. *Cancer Res* 2002;62:5153-7.
  24. Benedini S, Ruffini E, Terruzzi I, Mancuso M, Luzi L. Glucose and leucine metabolism in lung transplanted patients on low dose of steroids for immunosuppressive therapy. *Transplant Proc* 2008;40:1566-71.
  25. Escribano O, Fernandez-Moreno MD, Pina MJ, Fueyo J, Menor C, Roman ID, et al. Pretreatment with FK506 up-regulates insulin receptors in regenerating rat liver. *Hepatology* 2002;36:555-61.
  26. Nattrass M, Lauritzen T. Review of prandial glucose regulation with repaglinide: a solution to the problem of hypoglycaemia in the treatment of Type 2 diabetes? *Int J Obes Relat Metab Disord* 2000;24 Suppl 3:S21-31.
  27. Schwartz N, Schohl A, Ruthazer ES. Neural activity regulates synaptic properties and dendritic structure in vivo through calcineurin/NFAT signaling. *Neuron* 2009;62:655-69.
  28. Arie Y, Iketani M, Takamatsu K, Mikoshiba K, Goshima Y, Takei K. Developmental changes in the regulation of calcium-dependent neurite outgrowth. *Biochem Biophys Res Commun* 2009;379:11-5.
  29. Gomez AM, Rueda A, Sainte-Marie Y, Pereira L, Zissimopoulos S, Zhu X, et al. Mineralocorticoid modulation of cardiac ryanodine receptor activity is associated with downregulation of FK506-binding proteins. *Circulation* 2009;119:2179-87.