IDENTIFICATION OF MicroRNAs INVOLVED IN CAMPTOTHECIN-INDUCED APOPTOSIS IN JURKAT T CELL LINE

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ABSTRACT

IDENTIFICATION OF MicroRNAs INVOLVED IN CAMPTOTHECIN-INDUCED APOPTOSIS IN JURKAT T CELL LINE

MicroRNAs which are non-coding RNAs 19-25 nt in length regulate gene expression at post-transcriptional and translational stages. Although it is known that they play a role in critical processes such as development and differentiation of T cells, a major component of the immune system, the function of miRNAs in T cell apoptosis is unknown. This study has aimed to identify miRNAs' involvement in camptothecin-induced T cell apoptosis in the Jurkat T cell leukemia cell line model. Following the enrichment of the apoptotic population by magnetic seperation, the negative and apoptotic fractions were profilled and compared according to the expression levels of microRNAs. Out of the 866 miRNAs in the miRBASE, 37 and 58 of them were down- and up-regulated in the apoptotic fraction, respectively. 7 miRNAs were members of the clusters that have predicted targets as anti-apoptotic genes and tumour suppressor proteins. 66 miRNAs have no known function. Candidate miRNAs, selected based on their higher differential expression levels with predicted apoptotic/antiapoptotic targets, will be verified by qPCR. These candidate then will be further characterized by overxpression and knock-down studies.

ÖZET

JURKAT T HÜCRE HATTINDA KAMPTOTESİN İLE İNDÜKLENMİŞ APOPTOZDA ROL OYNAYAN MikroRNALARIN TANIMLANMASI

Post-transkripsiyonel ve translasyonel aşamada gen regulasyonunu gerçekleştiren, kodlamaya katılmayan ve 19-25 nükleotid uzunluğa sahip miRNA'ların, immun sistemin önemli bileşenlerinden olan T hücrelerinin gelişimi ve farklılaşması gibi kritik proseslerde görev aldığı anlaşılmasına rağmen T hücrelerinin apoptozunda hangi miRNA'ların rol oynadığı bilinmemektedir. Bu çalışmada T hücre apoptozunda rol oynayan mikroRNAların belirlenmesi amaçlanmıştır. Jurkat T hücresi lösemi hattının model olarak kullanıldığı bu çalışmada apoptoz indüksiyonu kamptotesin ile sağlanmış ve apoptotik populasyonun manyetik ayrım ile zenginleştirilmesi sonrasında negatif ve apoptotik fraksiyon mikroRNA array analizi ile ekspresyon seviyeleri bakımından profillenerek karşılaştırılmıştır. Tarama yapılan 866 miRNA arasından 37 tanesinde ekspresyon seviyesinde azalma, 58 tanesinde artma gözlenmiştir. Literatür taraması sonucunda ekspresyon seviyesi bakımından fark gösteren 7 miRNAnın birlikte görev yapan ve tahminlenen hedefleri arasında antiapoptotik genler ve tumor baskılayıcı proteinler olduğu görülen grupların üyeleri olduğu ortaya çıkmıştır. 66 tanesine literatürde rastlanamamıştır. Ekspresyon seviyeleri ve apoptozda rol oynadığı bilinen moleküllerin hedeflenme olasılığı göz önünde bulundurularak seçilen aday miRNAların qPCR ile doğrulaması yapıldıktan sonra overexpression ve knockdown çalışmaları ile apoptozdaki olası rolleri ortaya çıkarılmaya çalışılacaktır.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
UTR	Untranslated region
rRNA	Ribosomal RNA
miRNA	microRNA
pri-miRNAs	Primary microRNAs
pre-miRNAs	Precursor microRNAs
RISC	RNA-induced silencing complex
RIN	RNA integrity number
Nt	Nucleotide
TBE	Tris/Borate/EDTA electrophoresis buffer
DGCR8	DiGeorge syndrome critical region gene 8
Caspase	Cysteine-aspartic acid proteases
FasL	Fas Ligand
TNF	Tumour necrosis factor
TRAMP	Transgenic adenocarcinoma of the mouse prostate
TRAIL	TNF-related apoptosis-inducing ligand
Apaf-1	Apoptotic protease activating factor 1
BCL2	B-cell lymphoma 2
Bid	BH3 interacting domain death agonist
Bax	Bcl-2-associated X protein
Bak	The Bcl-2 homologous antagonist/killer
FADD	Fas-Associated protein with Death Domain
IAP	Inhibitor of apoptosis
Smac	Second mitochondria-derived activator of caspases
DIABLO	Direct inhibitors of apoptosis binding protein with low pH
HtrA2	High temperature requirement factor
TOP1	Topoisomerase 1
HeLa	Cell line derived from cervical cancer
A549	Carcinomic human alveolar basal epithelial cells

B-CLL	B cell chronic lymphocytic leukemia
MEG-01	Human megakaryoblastic leukemia cell line
HCT116	Colon cancer cell line
H1299	Lung cancer cell line
PE	Phycoerythrin
7AAD	7-Amino-Actinomycin D
FBS	Fetal bovine serum
JNN	Jurkat negative control negative fraction
JNP	Jurkat negative control positive fraction
JAN	Jurkat apoptotic negative fraction
JAP	Jurkat apoptotic positive fraction

CHAPTER 1

INTRODUCTION

1.1. miRNAs: Biogenesis and Function

MicroRNAs (miRNAs) are non-coding small RNAs of about 19-25 nucleotides in lenght that negatively regulate gene expression by translational inhibition or destabilization of mRNAs of protein coding genes by binding to complementary target sequences in 3' untranslated region (UTR) (Stefani and Slack 2008, Miska 2005, Lawrie 2007). They were originally discovered in *Caenorhabditis elegans* as post-transcriptional regulators of genes involved in developmental timing and are now known to have effects on gene expression in all multicellular eukaryotes (Nilsen 2007). miRNAs can be encoded in independent transcription units, in polycistronic clusters or within the introns of protein-coding genes (Stefani and Slack 2008). A single microRNA can target several hundred genes. It is estimated that between 10–30% of all human genes are a target for microRNA regulation (Lawrie 2007). A single target gene also contains putative binding sites for multiple microRNAs that can bind cooperatively and form complex regulatory control networks.

miRNAs are generated from precursor molecules known as primary miRNAs (pri-miRNAs), which are transcribed from either intergenic or intronic transcripts by RNA polymerase II (Figure 1.1). Pri-miRNAs are cleaved by Drosha/DGCR8 complex in nucleus to form pre-miRNAs of about 70 nt hairpin structure (Filipowicz, et al. 2008; Stefani and Slack 2008). Following this cleavage pre-miRNAs are transported to cytoplasm by exportin-5 and processed into 19-25 nt mature miRNA duplex by Dicer. After helicase activity, one strand is selected to incorporate into functional RNA-induced silencing complex (RISC) which contains miRNA, an Argonaute protein and other protein factors and is the effector complex of the miRNA pathway (Stefani and Slack 2008). The RISC is directed to mRNAs that are complementary to its miRNA component. The strand loaded into the complex prefentially has the lowest free energy at its 5' terminus (Kloosterman, et al. 2006, Lawrie 2007).

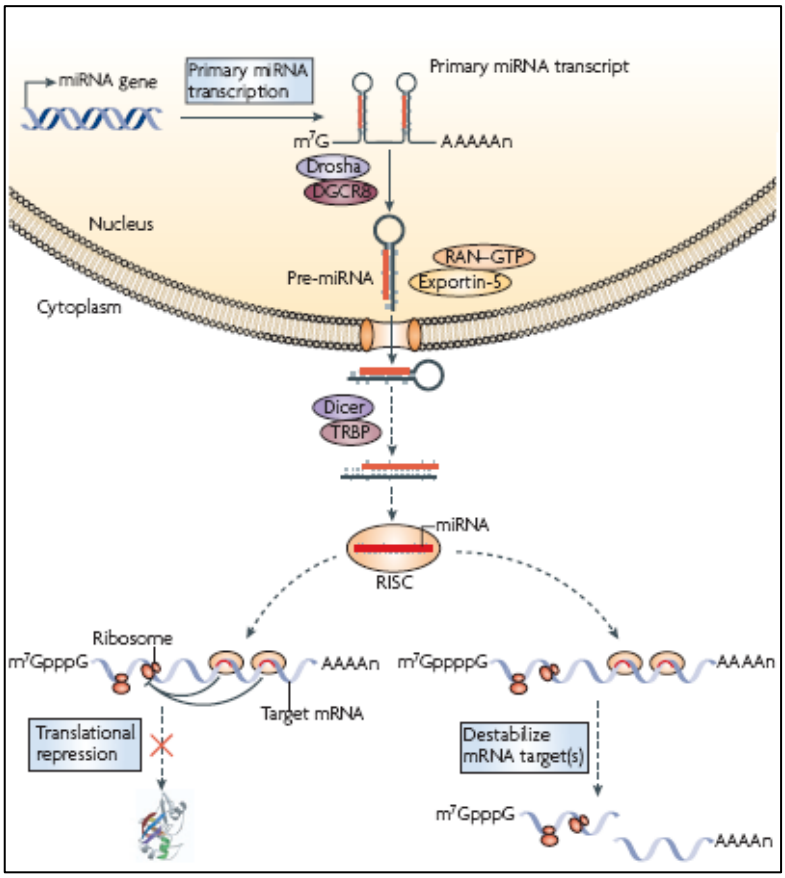


Figure 1.1. miRNA biogenesis steps

(Source: Lodish, et al. 2008)

RISC inhibits mRNA expression in two ways, depending on the degree of complementarity between miRNA and the target. If there is a perfect match between target mRNA and miRNA, mostly in plants, the target mRNA is cleaved and degraded (Stefani and Slack 2008, Shyu, et al. 2008). Another mode of inhibition is translational repression, as in animals whose miRNAs generally show a lower degree of complementarity to their target (Filipowicz, et al. 2008, Yu, et al. 2007). In animals miRNAs recognize their targets by interactions of seed region consisting of nucleotides 2 to 7 in the 5' terminus of miRNA and 3' UTR of target mRNA (Neilson, et al. 2007, Kloosterman, et al. 2006).

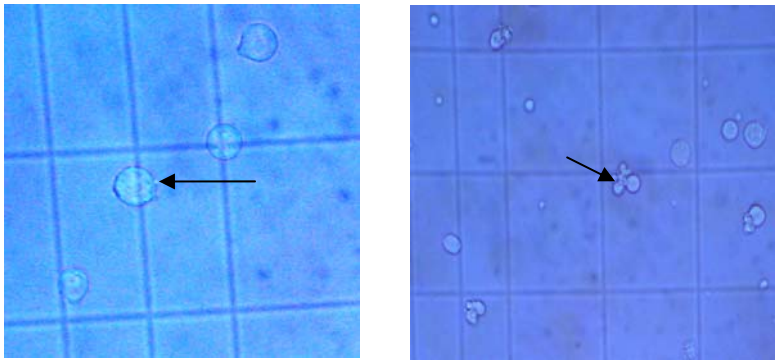
miRNAs have capacity to regulate many crucial developmental and physiological decisions such as developmental timing, differentiation, cell proliferation, organ development, maintenance of stem cell potency and apoptosis as well as many types of cancers (Nilsen 2007, Kloosterman, et al. 2006).

1.2. Apoptosis

1.2.1. Definition and Morphological Features

Apoptosis is programmed cell death performed by activation of cysteine proteases known as caspases in a chain reaction manner triggered by various stimuli from outside or inside the cell such as ligation of cell surface receptors, treatment with cytotoxic drugs or irradiation (Blank and Shiloh 2007). It has a regulatory role in the development, the maintenance of tissue homeostasis and repair of tissues. Defects in this regulation results in pathological conditions such as developmental defects, autoimmune diseases, neurodegeneration or cancer (Jin and El-Deiry 2005, Blank and Shiloh 2007, Baumann, et al. 2002).

Cells undergoing apoptosis show characteristic morphology, including exposure of phosphotidylserine on membrane surface, membrane blebbing, cellular shrinkage, chromatin condensation and fragmentation of nucleus leading to formation of apoptotic bodies (Figure 1.2) (Blagosklonny 2000, Blank and Shiloh 2007, Baumann, et al. 2002).



(a) (b)

Figure 1.2. Morphological features of apoptosis. Trypan-blue staining of (a) Round-shaped live jurkat cells with membrane integrity (b) Apoptotic jurkat cells with membrane blebbing and apoptotic body formation indicated with the arrows.

1.2.2. Pathways of Apoptosis

Apoptosis is induced via two main routes (Figure 1.3) involving either activation of death receptors (the extrinsic pathway) or mitochondria (the intrinsic pathway) (Taylor, et al. 2008, Kiechle and Zhang 2002). The extrinsic pathway involves binding

of extracellular ligands (e.g., FasL) to death receptors on cell surface which are members of tumour necrosis factor (TNF) receptor superfamily, including Fas, TRAMP, TNF receptor 1 and 2, TRAIL receptor 1 and 2 (Osthoff 1998, Blank and Shiloh 2007, Baumann, et al. 2002). As a result initiator caspases (caspase-8) and downstream effector caspases (caspases-3, 6, 7) are activated in a cascade effect that results in apoptosis (Blank and Shiloh 2007).

In the intrinsic pathway, stress-induced apoptosis is initiated by release of protein cytochrome c from the inter-mitochondrial membrane space. Once released, cytochrome *c* triggers a caspase activating complex called apoptosome by binding its component, apoptotic protease-activating factor 1 (Apaf1) with caspase 9. Upon activation, effector caspase 3 is activated in a chain reaction manner (Blank and Shiloh 2007, Taylor, et al. 2008). Cytochrome *c* release is regulated by Bcl2 family, composed of pro-apoptotic, anti-apoptotic and BH3-only members. As seen in Figure 1.3, Bid is a member of BH3-only protein family that promotes pro-apoptotic proteins Bax-Bak assembly and release of cytochrome *c* from mitochondrial intermembrane. On the other hand, antiapoptotic Bcl2 blocks apoptosis by preventing Bax-Bak assembly and pore formation (Taylor, et al. 2008, Baumann, et al. 2002).

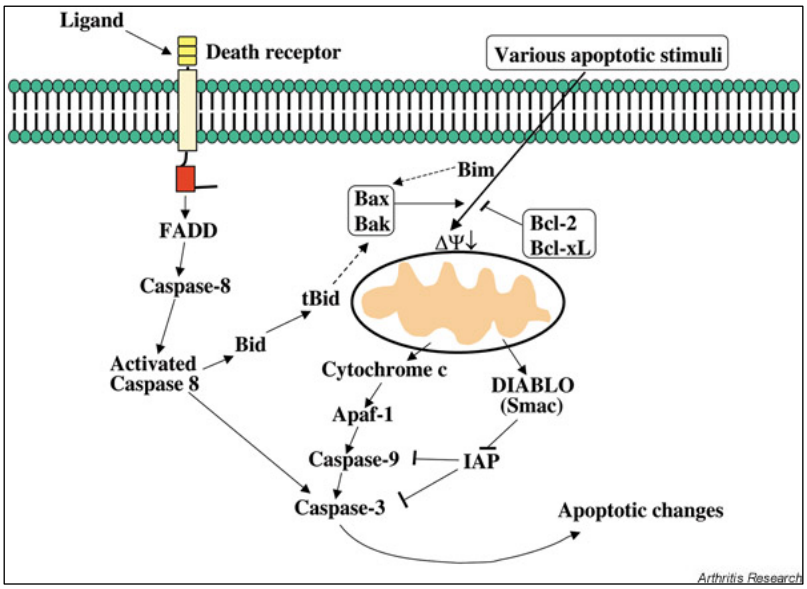


Figure 1.3. Major pathways of apoptosis. As a result of receptor-ligand interaction or by effect of apoptotic stimuli, a cascade of events occur to result in apoptosis whereas regulator molecules also take place within (Source: Mak and Yeh 2002).

Apoptosis can also be induced by several reagents such as camptothecin, an antitumor extract of plant *Camptotheca acuminata*, which is a human topoisomerase I (TOP I) inhibitor (Li and Liu 2001, Pommier, et al. 2003). Topoisomerase I relaxes DNA supercoils by single strand breaks which allows DNA double helix to rotate around intact strand during transcription and chromatin replication (Figure 1.4). When DNA is relaxed, Topoisomerase I ligates strand breaks and regenerates double helix. Camptothecin specifically inhibits the religation step by interaction with topoisomerase I-DNA complex generating DNA strand breaks which trigger elevations in p53 protein levels (Nelson et al., 1994). The p53 tumor suppressor serves as a positive regulator of pro-apoptotic Bcl-2 family members such as Bax and BH3-only member Bid (Blank and Shiloh 2007, Li and Liu 2001, Pommier, et al 2003, Taylor, et al. 2008). p53 also functions by neutralizing anti-apoptotic proteins such as Bcl-xL and activating pro-apoptotic regulators Bax or Bak to initiate mitochondrial outer membrane permeabilization. Followed by p53 activation cytochrome c is released from mitochondria; caspases are activated and results in DNA fragmentation (Sanchez, et al. 2000).

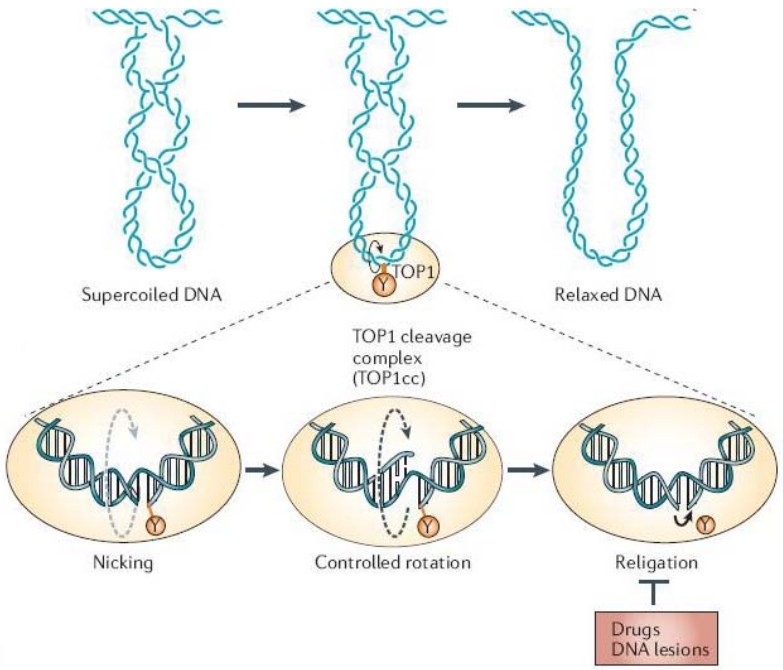


Figure 1.4. Topoisomerase I action on supercoiled DNA. TOP I introduces transient single strand nicks on DNA and enables rotation. DNA lesions and drugs such as camptothecin inhibits religation (Source: Pommier 2006).

The induction of mitochodrial membrane permeabilization also leads to the release of other pro-apoptotic molecules such as Smac (second mitochondrial activator of caspases)/Diablo (direct inhibitors of apoptosis binding protein with low pH) and Omi/HtrA2 (high temperature requirement factor) (Blank and Shiloh 2007). They can enhance apoptosis by inactivating IAPs, which function upon binding and inhibiting active forms of caspases. Overexpression of Smac/Diablo or Omi/HtrA2 in cells has been shown to markedly increase their sensitivity to apoptosis induction by DNA damage and other signals.

Apoptosis plays a key role in regulation of immune system where one of the major components is T lymphocytes. Dysregulation of immune system causes immune-related disorders such as autoimmune diseases and malignancies (Lodish, et al 2008). Studies revealed that homeostasis and function of immune system is maintained by growth factors, cell surface receptors, intracellular signaling molecules and transcription factors. Recent studies have introduced new mediators having role in post-transcriptional regulation of gene expression in function and development of immune cells known as microRNAs (miRNAs).

1.3. miRNA-Mediated Regulation of Apoptosis

Due to homology between *Drosophila* and mammalian system (Figure 1.4), studies performed on *Drosophila* show that miRNAs are involved in apoptosis (Miska, 2005). *Drosophila* caspase Drice, is a target of *miR-14* which has antiapoptotic activity (Bergmann and Lane 2003). Hid, Reaper, Grim and Sickle proteins inhibit function of DIAP1 (inhibitor of apoptosis), cell death regulator (Jovanovic and Hartgarner 2006; Hwang and Mendell 2006). As *Bantam* negatively regulates Hid protein expression, members of *miR-2* family, *miR-2* and *miR-13* are also thought to regulate proapoptotic genes *hid, reaper, grim* and *sickle* result in downregulation of apoptosis (Jovanovic and Hartgarner 2006; Wienholds and Plasterk 2005).

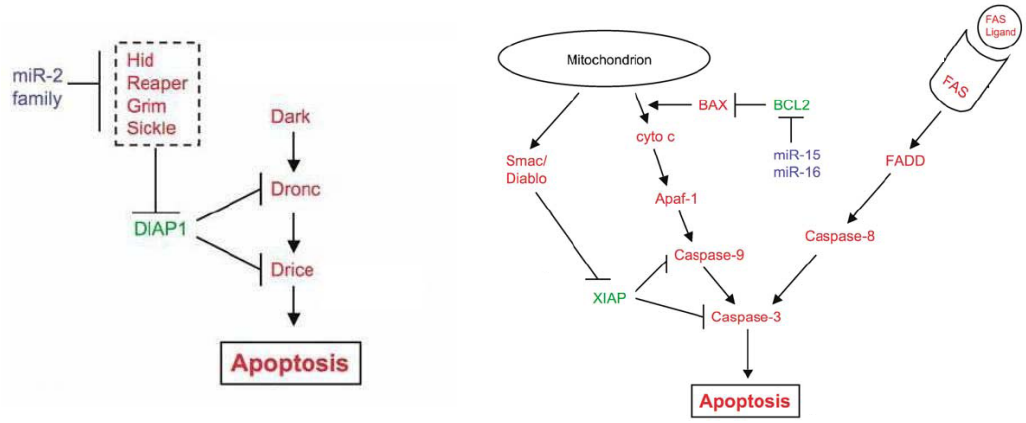


Figure 1.5. Pathway of apoptosis in a) *Drosophila* and b) mammalian system
(Source: Jovanovic, et al. 2006)

In addition to studies performed on *Drosophila*, researchers focused on miRNA-apoptosis relationship also by *in vitro* studies. Cheng et al. (2005) used an antisense RNA library to knock down 90 human miRNAs in HeLa (cervical carcinoma) and A549 (lung carcinoma) cell lines, testing for changes in apoptosis. Caspase-3 level was measured as the indicator. It was found that inhibition of seven miRNAs (*miR-1d, 7, 148, 204, 210, 216* and *296*) led to increased caspase-3 activity, suggesting potential role of miRNAs in apoptosis.

Calin et al. (2003) identified *miR-15a* and *miR-16-1* cluster in loss of chromosomal region 13q14 which is associated with B cell chronic lymphocytic leukemia (CLL) but no protein coding genes. Cimmino et al. (2005) showed that *miR-15a* and *miR-16-1* expression is indeed inversely correlated with Bcl2 expression in chronic lymphocytic leukemia (CLL) cells. Bcl2 is an antiapoptotic gene that blocks the mitochondrial release of cytochrome c and inhibits the activation of caspase 9 through sequences that are imperfectly complementary to the target mRNA, mainly to the 3'UTR. The Bcl2 3'UTR region contains one potential binding site for both miRNAs. miRNA expression in cell lines with high Bcl2 levels caused a reduction in Bcl2 protein levels, without affecting Bcl2 mRNA levels, indicating that the regulation of expression is post-transcriptional.

Cimmino et al. (2005) also reported that *miR-15a/16-1* cluster induces apoptosis of human acute megakaryoblastic leukemia cell line, MEG-01 by activating the intrinsic apoptosis pathway as identified by activation of the APAF-1–caspase 9 pathway. Calin et al. (2008) investigated the mechanism of miR-15a and miR-16-1 as tumor

suppressors in leukemias by analyzing the transcriptome and proteome in MEG-01 acute megakaryocytic leukemic cells. Tumour suppressor function was tested by *in vitro* transfection of nude mice with *miR-15a/16-1*- transfected MEG-01 cells and after 28 days, tumour growth was completely suppressed in three of the five (60%) mice. Microarray analysis of MEG-01 cells transiently transfected with pRS15/16 vector containing the genomic region encoding both miRNAs showed that after transfection 3,307 genes were down-regulated and 265 were up-regulated which of none were predicted as a target of miR-15a/16. MEG-01 cells transfected with pSR15/16 were also analyzed by proteomics analysis to investigate effects at the protein level and 27 different proteins grouped in four according to functions as regulation of cell growth and cell cycle, antiapoptotic proteins, proteins involved in human tumorigenesis, as oncogenes, or as tumor-suppressor genes were isolated.

Recent studies have also identified miR-34 family as a microRNA component of the p53 network (Hannon et al. 2007). As mentioned before, p53 is a transcription factor coordinating cellular responses to DNA damage and oncogene activation and altering expression of genes leading to cell-cycle arrest, apoptosis and increased DNA repair upon induction (Chang, et al. 2007). p53 may also regulate the expression of specific miRNAs. miRNA profiling of p53-proficient cells differs from cells lacking p53 (Xi et al., 2006).

Relationship between p53 and miRNAs has been investigated using different profiling approaches. Chang et al. (2007) compared miRNA profiles of p53+/+ and p53-/- HCT116 colon cancer cells in response to DNA damage. After treatment with adriamycin, a DNA-damaging agent, seven miRNAs, miR-23a, miR-26a, miR-34a, miR-30c, miR-103, miR-107, and miR-182 were found upregulated. Shapira et al. (2007) and Tarasov et al. (2007) probed miRNA levels in lung carcinoma cells (H1299). P53 null H1299 cells were transfected with temperature sensitive p53 mutant (H1299-ts) that could express active p53 when the temperature shifted to 32^{o}C. RNA from H1299 and H1299-ts cells was hybridized on array and elevated expression miRNAs *638, 373*, 492, 126, 140, 491*, and *296* was observed. miR-34a elevation was more than 10-fold.

The expression of *miR-34a, miR-34b* and *miR-34c* is induced by DNA damage in a p53-dependent manner. When overexpressed, *miR-34* leads to apoptosis or cellular senescence, whereas reduction of *miR-34* function attenuates p53-mediated cell death

showing that miRNAs can affect tumorigenesis by working within tumor suppressor pathways (Hannon, et al. 2007)

The predicted targets of *miR-34a* also include mRNAs whose inhibition might mediate p53 functions. A mild decrease in Bcl2 protein upon *miR-34a* overexpression was also observed (Shapira, et al. 2007). It is possible that *miR-34a* can act together with other miRNAs such as *miR-15* and *miR-16* (Cimmino, et al. 2005) to suppress effectively Bcl2 protein levels.

Studies on T lymphocytes have revealed miRNAs' role in development and differentiation stages such as *miR-150* and *miR-146* in differentiation (Monticelli, et al. 2005) and *miR-155* (Neilson, et al. 2007) in regulatory T cell formation and function (Lodish, et al. 2008). Li et al. (2007) Although studies on miRNA expression relative to developmental stages reveal their role in establishing and/or maintaining cell identity in lymphocytes in stage specific manner; miRNAs that regulate apoptosis in T lymphocytes are unknown. In this study it is aimed to identify and understand the function of miRNAs having role in T lymphocyte apoptosis. As miRNA expression differs according to development, activation and pathologic stages of an organism; deciphering changes in miRNA profile may have potential role in defining new pathogenic pathways and novel treatments.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

The human leukemic T cell line, Jurkat, was obtained from American Type Culture Collection (clone E6.1). Camptothecin was purchased from Sigma. Annexin V PE and 7AAD were provided from Becton Dickonson (BD). Cell separator, kits and columns were from Miltenyi Biotech. Kits used in total RNA isolation and DNase treatment were obtained from Ambion.

2.2. Cell Culture

Jurkat cell line, originated from human T cell leukemia, was maintained in RPMI 1640 (Gibco) supplemented with L-glutamine, 10% fetal bovine serum (GIBCO), 2% penicillin-streptomycin (Biochrom AG) in an atmosphere of 5% CO_2 at 37^{o}C. Cells were passaged every 48 hours.

Camptothecin dose and treatment time were optimized to capture cells at the early apoptotic stage. 10^6 cells were treated with camptothecin (Sigma–Aldrich) at three time points (16, 6 and 4 hours respectively) and concentrations between 0,5-1024 μM (Johnson et al., 1997). Camptothecin was dissolved in DMSO (Applichem) at a concentration of 10 mg/ml (28700 μM) as stock solution. Working concentrations were prepared by serial dilution in culture media composed of RPMI 1640 supplemented with 10% FBS. Cell culture was set up in a 500 μl volume. In culture setting camptothecin dilutions were added to the formerly distributed media. Lastly cells were distributed into culture tubes, vortexed for homogenization and incubated at 37^{o}C, 5% CO_2.

2.3. Immunoflorescent Labeling

Apoptosis was measured by flow cytometry after labeling with Annexin V PE and 7AAD (BD Pharmingen). Phosphotidylserine layer of the cell membrane was flipped inside out in apoptotic cells providing binding site for annexin V. Upon apoptosis progression, membrane integrity was lost and 7AAD was bound to DNA as DNA was fragmented. Following treatment with camptothecin, culture media was removed by centrifugation at 1400 rpm, +4^{o}C for 6 minutes. The supernatant was aspired by vacuum and cells were washed with 1XPBS (Gibco) and centrifuged as before. The pellet was re-suspended in 20 µL of Annexin-binding buffer (BD Pharmingen) followed by incubation with 10 µL Annexin V PE and 10 µL 7AAD (both diluted 1:5 in 1XPBS containing 0,1% NaN_3) for 20 minutes at +4^{o}C. Then, 300 µL of Annexin binding buffer was added to the samples and loaded on 96-well plates for analysis by flow cytometry (BD FacsArray). Cells were monitored in dot-blot and histogram graph. In dot blot analysis cells were distributed in four parts indicative of various stages of apoptosis. Early apoptotic annexin-positive, 7AAD-negative cells were distributed in Q1 part whereas double positive (annexin positive 7AAD positive) apoptotic cells in Q2. Q3 part showed annexin and 7AAD negative live population. Membrane integrity was completely distorted in late apoptotic population which was annexin negative 7AAD positive and monitored in Q4 part. Data analysis was performed by MS Excel. Optimum camptothecin concentration and time point was determined to collect early stage apoptotic jurkat T cells.

2.4. Cell Sorting

Apoptotic cells were seperated by VarioMacs magnetic cell seperation system composed of magnetic columns and Annexin V Microbead Kit (Miltenyi Biotech). The choice of column depended on the approximate number of annexin-positive cells. MS column was used up to 10^7 annexin-positive cells whereas LS column was used when there were 10^8 annexin-positive cells. The culture was set in large scale between 5-7*10^7 cells as a starting number. Cells were treated with camptothecin at optimum concentration and incubation time determined by changing concentrations at fixed time points. Camptothecin treated and non-treated cells were collected and centrifuged at

300xg, at 9^{o}C for 10 minutes. Cells were resuspanded in 1X Binding Buffer in 80 µl buffer/10^7 cells and incubated with Annexin V MicroBeads as 20 µl bead/10^7 cells for 15 minutes at 9^{o}C in dark. Following incubation, cells were washed with 1X binding buffer (2 ml buffer/10^7 cells) and the pellet was resuspended in 1X binding buffer. Buffer volume varied between 0,5-10 ml with the capacity of the column. The column was prepared by washing with 1X binding buffer. The annexin negative fraction was collected as the cell suspension was applied through the column. The column was washed four times with 1X binding buffer to collect the annexin-negative fraction completely. The annexin-positive population retained in column was passed through by 21 G needle as the column was removed from the magnetic zone and washed with 1X binding buffer.

Four group of cells have been obtained as two subfractions for each of treated and untreated population termed as annexin-positive and annexin-negative. The cells in each fraction were counted by trypan blue staining. 10^6 cells of each fraction were analysed in flow cytometry after immunoflorescent labeling with Annexin V PE and 7AAD. Apoptosis percentage and distribution of cells in dot blot analysis of each fraction were used to assess the purity and efficiency of the sorting process.

2.5. Sample Preparation

2.5.1. RNA Isolation

Total RNA was isolated from apoptotic and non-apoptotic cells by mirVana miRNA isolation kit (Ambion). Sorted cells were pelleted at 1400 rpm, +4^{o}C for 6 minutes and washed with 1X PBS. Cell pellets were lysed by pipetting and vortexing with lysis buffer (300 µl/10^2-10^6 of cells, 600 µl/10^6 of cells). After incubation with homogenate additive for 10 minutes on ice, phenol-chloform extraction was performed. Phenol-chloroform was added as equal to lysate volume. The sample was then vortexed for 30-60 seconds and centrifuged at 10000xg, at room temperature for 5 minutes to separate organic and aqueous phases. Following centrifugation, aqueous phase containing RNA was transferred to a fresh tube and 1,25x volume of 100% ethanol at room temperature was added to the aqueous phase. Samples were passed through filters to retain nucleotides in washing steps by centrifuging at 10000xg, room temperature

according to manufacturer's instructions and eluted in 100 µl pre-heated DEPC-treated water. RNA concentration, 260/280 and 260/230 ratios of the recovered RNA was measured by Nanodrop (NanoDrop® ND-1000).

2.5.2. DNase Treatment and Quality Control

Total RNA samples were treated with TurboDNase DNA-free kit (Ambion) to remove genomic DNA contamination. The reaction volume was fixed at 50 µl. 1 µl DNase and 5 µl 10X reaction buffer was used up to 10 µg RNA. Volume was completed to 50 µl by adding DEPC-treated water and reaction was incubated at 37^{o}C for 30 minutes. DNase was inactivated by the inactivation reagent included in the kit. Following incubation at room temperature for 2 minutes, the samples were centrifuged at 13000 rpm at room temperature and the aqueous phase containing DNA-free RNA was collected in a fresh tube without disturbing the interface.

Concentration and purity of the samples were measured and compared with the untreated group by Nanodrop spectrophotometer. Absorbance ratio of 260 nm/280 nm and 260 nm/230 nm between 1,8-2,1 refers to RNA sample free of contaminants such as ethanol, phenol, thiocyanate and protein. RNA integrity was also checked by Bioanalyzer (Agilent 2100) using RNA 6000 Nano Kit. A RIN higher than 7 on a scale of 0-10 indicates RNA of good quality for the array analysis.

2.6. Microarray Analysis

Samples sets for the array analysis included 2 replicates of annexin-negative fraction of negative control (JNN), 3 replicates of annexin positive (JAP) and negative fractions (JAN) of the camptothecin treated group. After fulfilling the quality control requirements with regard to the 260/280 and 260/230 ratios and RIN, 1 µg of each sample was aliquoted for the array analysis. The miRNA array analysis was conducted by Febit Biomed Gmbh, Germany.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Dose Response and Time Point Assay for Camptothecin-induced Apoptosis

Camptothecin-induced apoptosis in Jurkat cell line was optimized in order to capture cells at the early apoptotic stage. First cells were exposed to camptothecin at concentrations between 0,5-8 μM for 16 hours. The flow cytometric analysis showed that 64% of cells were annexin positive. Concentrations higher than 0,5 μM did not show any significant difference during the 16 h time period (Figure 3.1) Dose dependence could not be observed in 0,5-8 μM camptothecin concentration range.

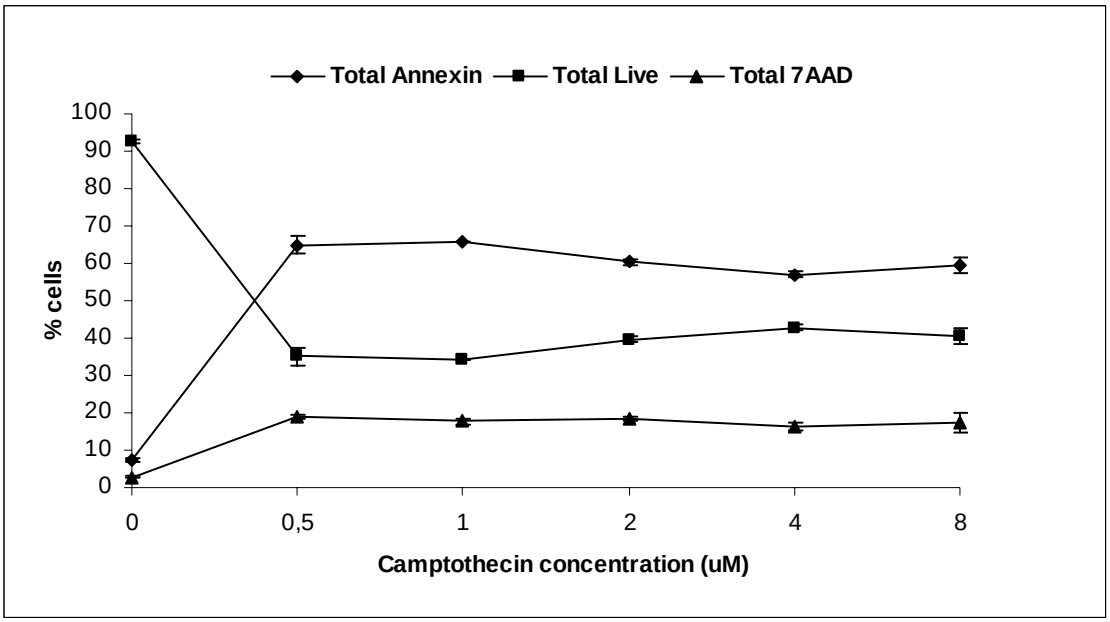


Figure 3.1. Camptothecin dose response graph for 16 hours between concentrations 0,5-8 μM. 64% of cells were annexin positive when treated with 0,5 μM camptothecin. Apoptosis percentage did not change significantly at concentrations higher than 0,5 μM.

The camptothecin concentration was fine-tuned (0,025-2 μM) to observe a dose effect before reaching a plateu. The percentage of annexin-positive cells increased

proportionally up to 0,5 μM camptothecin. At higher concentrations, the dose-dependence was lost. As seen in Figure 3.2, 63,7% of cells became annexin positive at 0,5 μM concentration and no significant difference of dose effect was seen at higher concentrations.

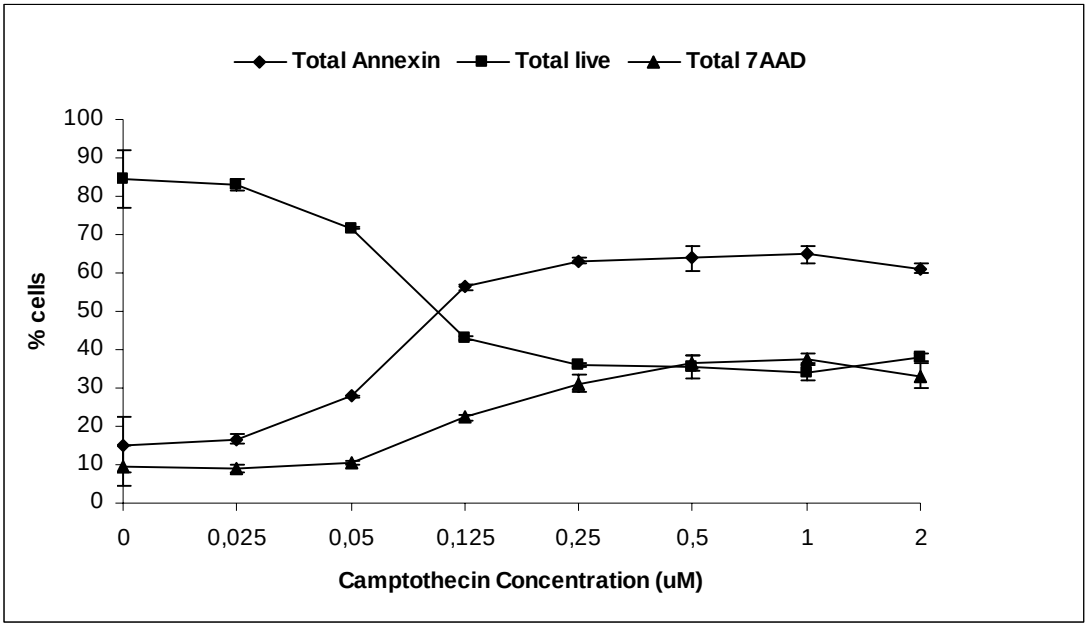


Figure 3.2. Camptothecin dose response for 16 h between concentrations 0,025-2 μM. Percentage of annexin-positive cells gradually increased up to 0,5 μM. Highest apoptotic percentage was 63,7%.

Dot blot analysis showed that cells passed to apoptotic region, Q2 in 16 h treatment as seen in Figure 3.3. To retain cells at the early apoptotic phase, the treatment time was reoptimized to 4 h. The concentration range was broadened from 0,5 to 1024 μM to see the full plateu and the decrease of annexin-positive cells in dose response. Figure 3.4 shows that the highest apoptotic ratio was 41% at 8 μM camptothecin in 4 h and 37% of the apoptotic cells were at the early apoptotic phase as can be seen in Figure 3.3.b. In Figure 3.3.a it can also be seen that 63% of the cells were apoptotic treated with 0,5 μM camptothecin in 16 h and only 37% of the cells were at the apoptotic phase. Cells could be captured at early apoptotic phase in 4h treatment with 8 μM camptothecin.

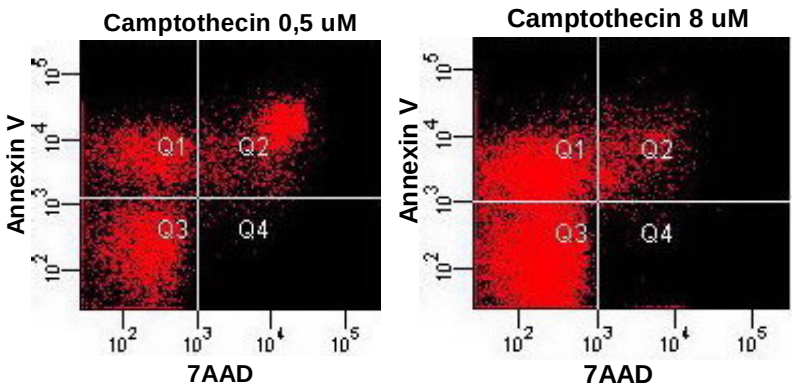


Figure 3.3. Dot blot analysis of dose response assay a) 16 h treatment with 0,5 μM camptothecin b) 4 h treatment with 8 μM camptothecin. Jurkat cells were labeled with Annexin PE and 7 AAD as explained in Materials and Methods. Annexin-positive cells distributed in Q1 part represents early apoptotic phase whereas annexin and 7AAD-positive cells in Q2 part are at apoptotic phase. 7AAD-positive cells in Q4 are at the late apoptotic phase. Q3 part shows live cells. Apoptosis percentage was 63,7% in 16 h treatment with 8 μM camptothecin and 37% of the apoptotic cells were at the early apoptotic phase. For 4 h treatment with 8 μM camptothecin, 41% of the cells were apoptotic and 37% were at the early apoptotic phase.

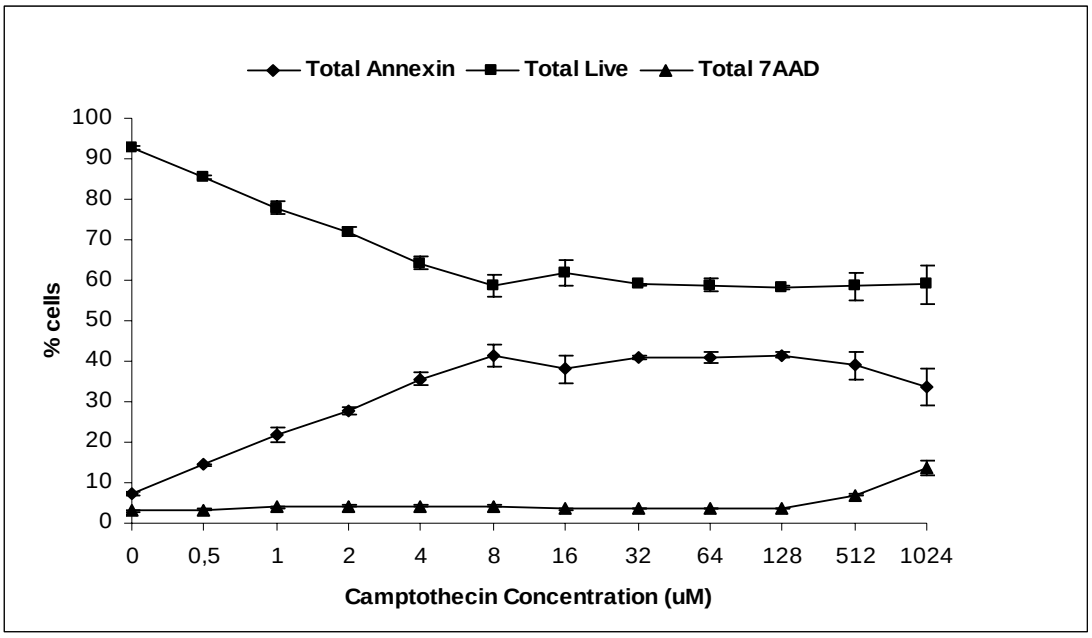


Figure 3.4. Camptothecin dose response for 4 h. Cells were treated with camptothecin between concentrations 0,5-1024 μM to see the full plateu. Highest apoptotic percentage was 41% at 8 μM camptothecin. After this point, dose dependence was lost.

3.2. Apoptotic Cell Sorting

Following optimization, cells were exposed to camptothecin in large scale up to 60 million cells, incubated with 20 µl of Annexin V microbeads per 10^7 cells in order to sort apoptotic and non-apoptotic populations. Both negative control and camptothecin-treated cells were sorted, each group had two subgroups within as annexin-negative and positive populations (Figure). Samples were taken before and after sorting for flow cytometric analysis to identify apoptotic percentage in negative control, camptothecin-treated population and sorted fractions. After sorting, camptothecin-treated population of 68% apoptotic was enriched by 89% in positive fraction. Dot blot analysis shows live and apoptotic population of fractions (Figure 3.5).

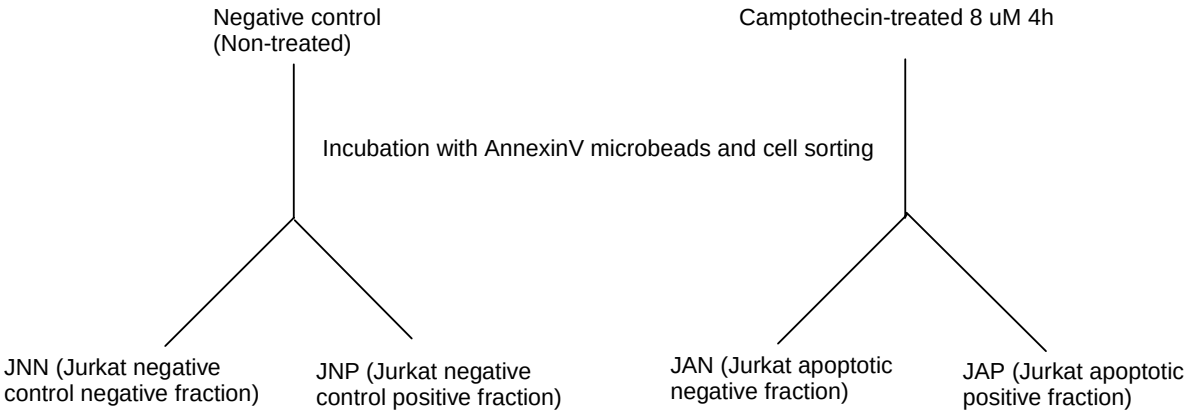
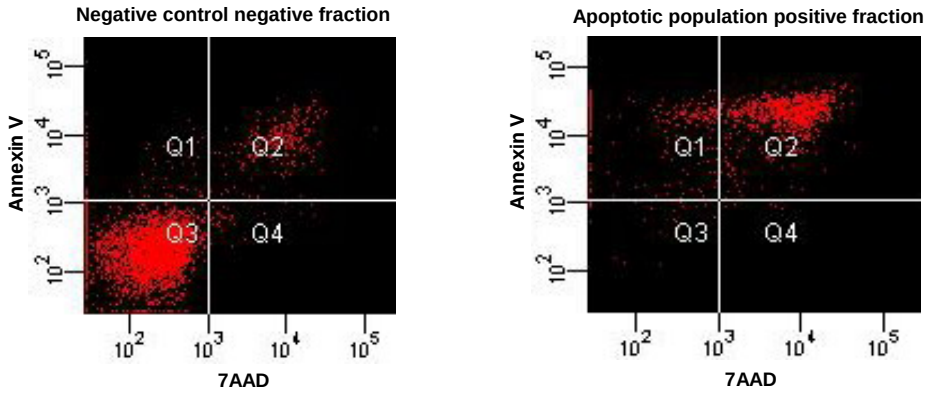


Figure 3.5. Apoptotic cell sorting. Non-treated and camptothecin-treated cells were incubated with Annexin V magnetic microbeads and sorted by magnetic columns. Each group have two subgroups as annexin-negative and positive fractions



(a) (b)

Figure 3.6. Dot blot analysis of a) Negative fraction of negative control b) Positive fraction of camptothecin-treated population. Distribution of the cells determine purity of the fractions. Percentage of live cells in negative fraction of the negative control was 90%, distributed in Q3 part. 58% of the cells were apoptotic in camptothecin-treated group. After sorting apoptotic population was enriched to 97% as seen in (b).

3.3. RNA isolation and DNase Treatment

Total RNA was isolated from cells by using mirVana miRNA isolation kit (Ambion). When samples were run on 1% agarose gel, genomic DNA bands were observed. DNA contamination was removed by TurboDNase (Ambion) to determine RNA concentration exactly. In Figure 3.6, gel photograph shows DNase treated and non-treated samples.

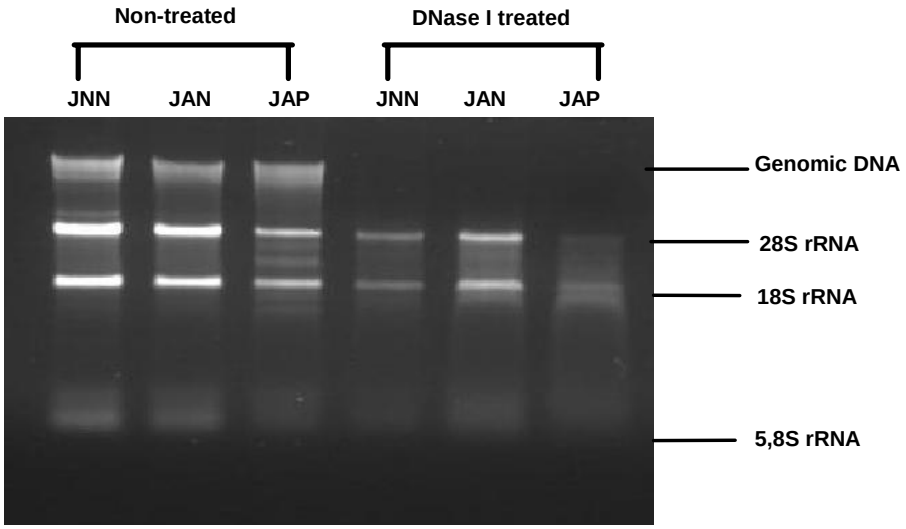


Figure 3.7. Gel photograph of DNase-treated and non-treated samples. Total RNA samples of 500 ng from each fraction were loaded on 1% agarose gel prepared in 0,5X TBE buffer (Tris/Borate/EDTA electrophoresis buffer). EtBr was added at the concentration of 0.5 µg/ ml from a 10 mg/ml stock. Samples were exposed to an electric constant at 80V for 30 min and visualized in gel documentation system. Genomic DNA was removed by DNase treatment.

3.4. RNA Quality Control

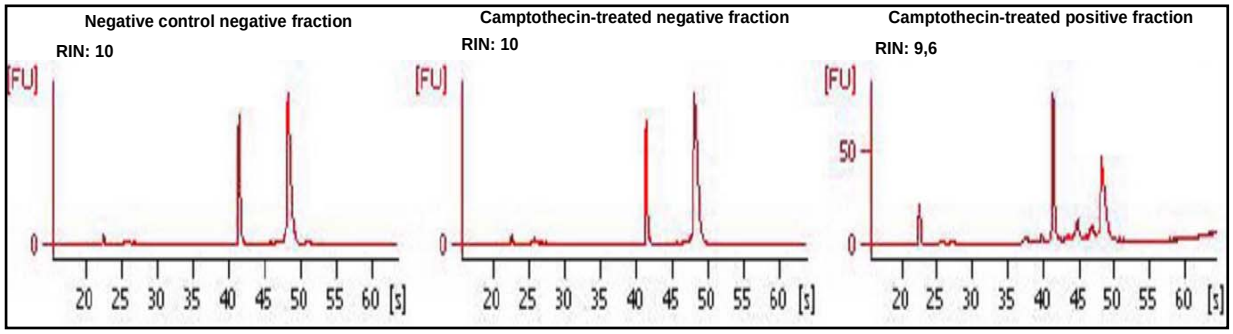
The RNA sample quality was determined by Nanodrop spectrophotometer and Bioanalyzer (Table 3.1). 260/280 and 260/230 ratio gave information about the purity whereas Bioanalyzer electropherograms represent the integrity of the samples generated by the ratio of 18S and 28S ribosomal subunits. RIN higher than 7 refers to RNA of good quality. The extent of degredation increases as RIN decreases.

Quality of DNase-treated and non-treated samples were controlled and compared by Bioanalyzer. As seen in Figure 3.8a and b change in RNA integrity due to DNase treatment is negligible and sample quality is enough for array analysis.

Table 3.1. Concentration and quality indicators of replicates after DNase treatment. 260/280 and 260/230 ratios also RINs show RNA of good quality.

Sample name	Concentration	260/280	260/230	RIN
JNN1	183,78	1,89	1,73	9,4
JNN2	194,63	1,85	1,77	9,8
JNN3	178,81	1,79	1,8	8
JAN1	272,6	1,91	1,96	10
JAN2	168	1,81	1,97	9,8
JAN3	127,4	1,87	2,16	8,6
JAP1	169,2	1,92	1,82	10
JAP2	192,9	1,88	1,82	N/A
JAP3	164,4	1,78	1,84	8,8

a)



b)

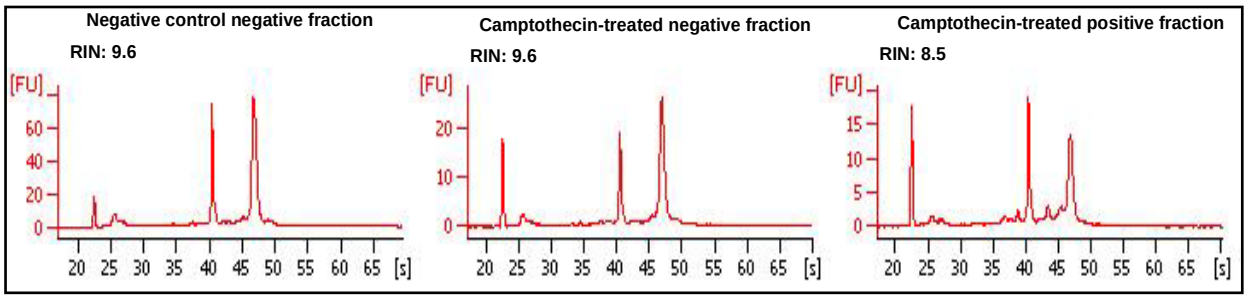


Figure 3.8. Bioanalyzer electrpherograms of a) non-treated and b) DNase-treated samples of each fraction.

3.5. MicroRNA Array Analysis

microRNA expression profiles of camptothecin-treated and non-treated cells were compared due to expression changes (1,8-fold change cutoff). 866 miRNAs were screened. 37 miRNAs were downregulated whereas 58 miRNAs were upregulated. Examples of profiled miRNAs screened in the literature are shown in Table 3.2. These

miRNAs are supposed to have regulatory role in cancer, cell cycle regulation, also have predicted targets in apoptosis machinery. There are also miRNAs without known function based on the literature. After determination of candidate miRNAs based on literature screening and verification by quantitative PCR, further characterization will be accomplished knock-down and overexpression studies.

Table 3.2. Representative list of miRNAs as a result of array analysis. These miRNAs are supposed to have role in cell cycle regulation, tumour formation and have predicted targets in apoptosis machinery. Some of them have no known function in the literature.

miRNA name	Up/down regulation	Fold Change	Known Function
miR-18b	Down-regulated	8,2	miR-18b has the potential to regulate multiple antiapoptotic genes, including Akt, Sp1, Bcl-2, E2F, and IGF1 (Wang et al. 2008)
miR-185	Down-regulated	7	miR-185 up-regulation is common in both kidney and bladder cancers (Gottardo et al. 2007)
miR-17, 18a, 20a, 19b	Down-regulated	3	Members of the miR17-92 cluster. Upregulation associated with MYC family which regulate expression of cell cycle transcription factor gene E2F1 resulting in tumour development (Takakura et al. 2008)
miR-634	Up-regulated	6	No known function
miR-1909	Up-regulated	6	No known function
miR-663	Up-regulated	5,4	Aberrant hypermethylation was shown for mir-663 in 34–86% of cases in 71 primary human breast cancer specimens where tumour suppressor genes are inactivated by aberrant hypermethylation (Lehmann et al. 2007)
miR-133a	Up-regulated	4	Targeting transcript of pyruvate kinase type M2 (PKM2), a potential oncogene in solid cancers. Overexpression of PKM2 in the cancer cells was partly caused by downregulation of miR-133a. It can also modulate the intrinsic apoptotic machinery as observed in tongue cancer cell lines (Wong et al. 2008)

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