INVESTIGATION OF THE FUNCTIONS OF CANDIDATE miRNAs IN CAMPTOTHECIN-INDUCED APOPTOSIS IN HUMAN CELLS

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ABSTRACT

INVESTIGATION OF THE FUNCTIONS OF CANDIDATE miRNAs IN CAMPTOTHECIN INDUCED APOPTOSIS IN HUMAN CELLS

MicroRNAs are non-coding 19-25nt long, small RNAs that regulate expression of about 30% of human genes by inhibiting mRNA translation or inducing its degradation. MicroRNAs play important role in cell growth, differentiation, apoptosis. miRNAs regulate apoptosis by targeting genes involved in apoptotic pathway as a pro or anti-apoptotic genes. This study has aimed to identify whether candidate miRNAs (miR-17* and miR-425) have a regulatory role in camptothecin induced apoptosis or not in Human cells and Hela cells that derived from cervical cancer were used as a model cell line. These candidates were selected based on deep sequencing data that showed some miRNAs differentially expressed after camptothecin treatment as compared with non-treated control group.

To show candidate miRNAs whether have a role or not in regulation of camptothecin induced apoptosis, first Hela cells were transfected with candidate miRNAs then candidate miRNA over-expressed cells were treated with camptothecin eventually level of apoptosis was measured by flow cytometry and the results were evaluated by comparing miRNA over-expressed cell group with un-transfected control group. Active caspase-3 level also was measured by using flow cytometry and the data showed miR-17* and miR-425 function as pro-apoptotic regulator in camptothecin induced apoptosis in Hela cells.

ÖZET

ADAY microRNA' LARIN İNSAN HÜCRELERİNDE KAMPTOTESİN İLE İNDÜKLENEN APOPTOZDAKİ FONKSİYONLARININ ARAŞTIRILMASI

miRNA'lar kodlanmayan 19-25 nükleotid uzunluğundaki küçük RNA lardır ve insan genomunun %30 unu mRNAların translasyonunu durdurarak yada yıkımına yol açarak düzenlerler. miRNAlar hücre gelişimi, farklılaşması ve apoptoz gibi önemli biyolojik olaylarda önemli bir yere sahiptir. Apoptotik mekanizmada yer alan anti yada pro-apoptotik gen hedeflerini baskılayarak apoptozu düzenlerler.

Bu çalışmada amaç aday miRNAların (miR-17* and miR-425) kamptotesin ile apoptozu aktive edilen insan hücrelerinde apoptozu düzenleyip düzenlemediğinin belirlenmesidir. Bu çalışmada Hela rahim ağzı kanseri hücre hattı model olarak kullanılmıştır. Bu miRNA adayları kamptotesin ile indüklenen hücrelerin ve indüklenmeyen kontrol grubu hücrelerinin derin sekans sonuçlarının karşılaştırması sonucu kontrol grubuna göre ifade edilme seviyesinde farklılık olan miRNA ların seçilmesiyle belirlenmiştir.

Bu miRNA adaylarının kamptotesin ile apoptozu indüklenen insan hücrelerinde apoptozu düzenleyip düzenlemediğini belirlemek için önce hücreler aday miRNA lar ile transfekte edilmiş ve 24 saat sonra transfeksiyon ile bu miRNA ların aşırı ifadadesinin sağlandığı hücreler kamptotesin ile 48 saat inkübe edilmiştir. 48 saat sonra apoptoz oranları Flow Sitometre ile ölçülmüş ve transfekte edilen hücre gruplarının apoptoz oranı transfekte edilmeyen kontrol grubuyla karşılaştırılarak sonuçlar değerlendirilmiştir. Ayrıca aktif kaspaz-3 miktarı flow sitometre ile ölçülmüştür. Sonuçlar miR-17* and miR-425'in apoptozu kamptotesin ile indüklenen Hela hücrelerinde pro-apoptotik bir fonksiyona sahip olduğunu göstermiştir.

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
UTR	Untranslated region
miRNA	microRNA
pri-miRNAs	Primary microRNAs
pre-miRNAs	Precursor microRNAs
RISC	RNA-induced silencing complex
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
TOP1	Topoisomerase 1
HeLa	Cell line derived from cervical cancer
PE	Phycoerythrin
7AAD	7-Amino-Actinomycin D
FBS	Fetal bovine serum
CPT	Camptothecin

CHAPTER 1

INTRODUCTION

1.1. Apoptosis

1.1.2. Definition of Apoptosis

Apoptosis is programmed of cell death and it was first described in 1972 by Currie and colleagues (Kery and Wyllie 1972). Apoptosis is carried out by caspases that are a family of cysteine proteases and expressed as a inactive precursors. When death-inducing stimuli comes, precursor caspases are activated by cleavage at specific aspartic residues. The caspases are divided into initiator and executionar caspases. Initiator caspases activate executionar caspases by cleavage them and executionar caspases execute apoptosis. (Yaron and Hermann 2011) Programmed cell death plays an important role in development and tissue homeostatis. During development many cells are produced in excess then lots of them are eliminated by apoptosis to contribute to forming organs and tissues (Jin and El-Deiry 2005). Defect in cell death pathway result in pathological conditions such as developmental defects, autoimmune diseases, neurodegeneration or cancer (Jin and El-Deiry 2005, Blank and Shiloh 2007).

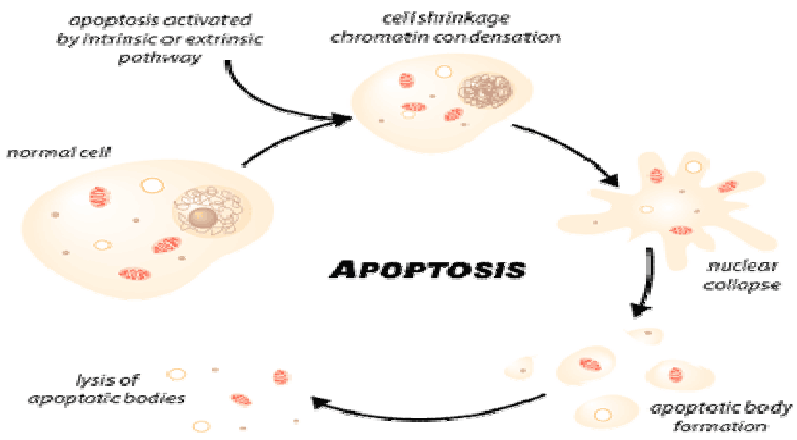


Figure 1.1. Changes in cell during apoptosis

(Source: Raff 1998).

During apoptosis cells undergo morphological changes such as exposure of phosphotidylserine on membrane surface, membrane blebbing, cytoplasmic shrinkage and reduction of cellular volume , condensation of the chromatin, and fragmentation of the nucleus , all of them eventually lead to formation of apoptotic bodies that are engulfed by neighbouring cells and macrophages (Blank and Shiloh 2007, Jin and El-Deiry 2005).

1.1.3 Pathways of Apoptosis

Apoptosis is induced by two signaling pathways; extrinsic pathway is initiated by death receptors and intrinsic pathway is induced by signals from within the cell. Extrinsic pathway of apoptosis is induced when a death ligand binds to death receptor at cell surface and this binding activates caspases that leads to apoptotic cell death. These death receptors are members of tumour necrosis factor (TNF) receptor superfamily, including Fas, TRAMP, TNF receptor 1 and 2, TRAIL receptor 1 and 2 (Xu and Shi 2007, Blank and Shiloh 2007).

Intrinsic pathway of apoptosis is initiated by intrinsic signals including DNA damage induced by irradiation or chemicals, growth factor deprivation or oxidative stress. These apoptotic signals triggers pro-apoptotic Bcl-2 proteins translocation to outer membrane of mitochondria which leads to mitochondrial outer membrane permeabilization (MOMP) that leads to release of cytochrome c into cytosol from intermembrane space of mitochondria and binding of cytochrome c to apoptotic protease-activating factor 1 (Apaf-1) leads to formation of a caspase activating complex called apoptosome that evokes activation of initiator and executioner caspases, usually caspase 9 and 3 respectively and apoptotic death occurs (Xu and Shi 2007, Blank and Shiloh 2007, Jin and El-Deiry 2005).

Besides the release cytochrome-c, the intramembrane content released also contains apoptosis inducing factor (AIF) to facilitate DNA fragmentation, and Smac/Diablo proteins to inhibit the inhibitor of apoptosis (IAP). (Hague and Paraskeva 2004)

There are a variety of regulatory molecules that regulate apoptosis such as Bcl-2 (B cell lymphoma 2) proteins, IAPs (Inhibitor of apoptosis), c-FLIP proteins

Bcl-2 family members that regulate intrinsic pathway of apoptosis consist of pro-apoptotic(Bax, Bak, Bad, Bim, Bid, Puma, Noxa) and anti-apoptotic(Bcl2, Bcl-X_L) members. Pro-apoptotic proteins induce apoptosis by increasing release of cytochrome c whereas anti-apoptotic proteins inhibit apoptosis by suppress release of cytochrome c. IAPs regulate apoptosis by inhibiting activation of caspases. c-FLIP proteins inhibit death receptor induced apoptosis by inhibiting procaspase 8 activation (Chowdhury, et al. 2008) and also miRNAs have an important role in regulation of apoptosis.

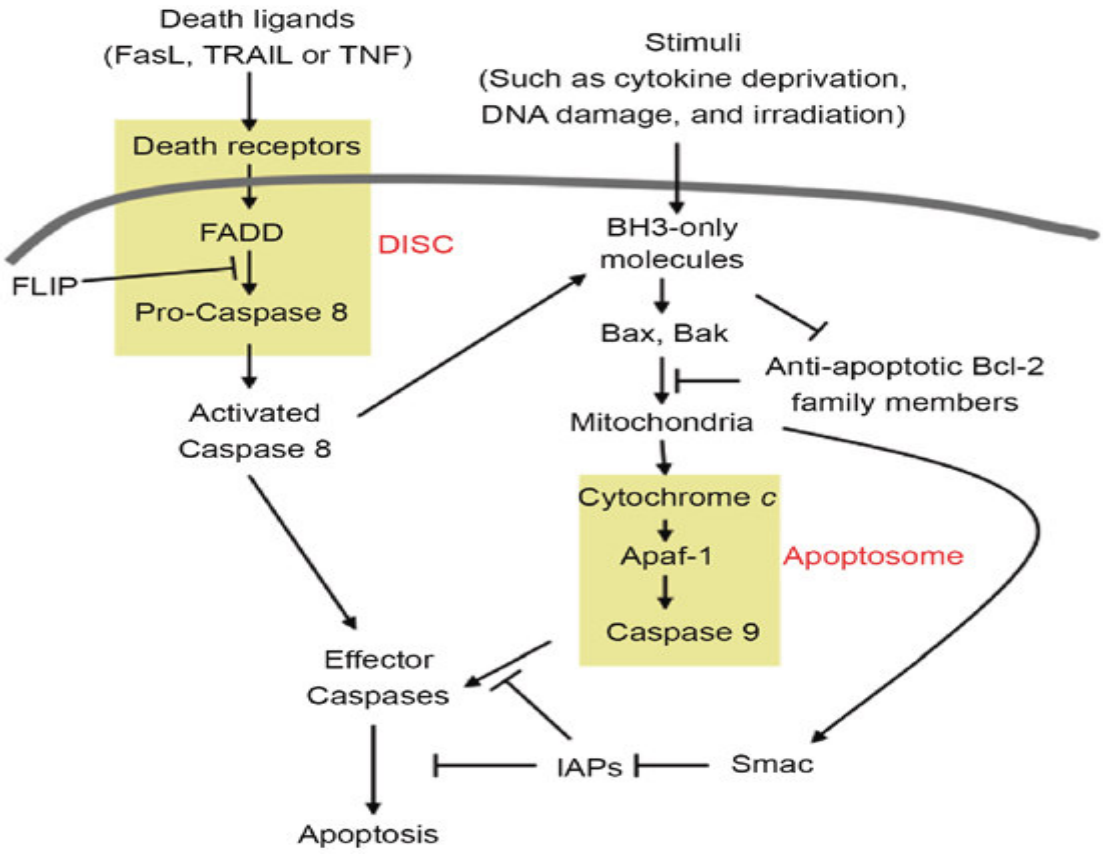


Figure 1.2. Pathways of apoptosis
(Source: Xu and Shi 2007).

1.2. Camptothecin (CPT)

As mentioned that before, intrinsic pathway of apoptosis is induced when DNA damage occurs and this damage can be carried out by several reagents such as camptothecin (CPT), an antitumor extract of plant *Camptotheca acuminata*, which is a human topoisomerase I (TOP I) inhibitor (Li and Liu 2001, Pommier, et al. 2003)**.** TOP I relaxes DNA supercoils by making single strand breaks (nicks) that facilitate separation of DNA strands to serve as a templates during replication, transcription, recombination, repair. In normal conditions, when the DNA is relaxed, TOP I religates the breaks but in the presense of camptothecin religation step is inhibited because CPT binds to the TOP I-nicked DNA complex and this prevents the re-ligation of the nicked strand (Pommier, et al. 2006). So, DNA damage occurs and this activates p53 that is tumor suppressor then p53 increases level of pro- apoptotic Bcl-2 proteins that enhance mitochondrial outer membrane permeabilization result in releasing of cytochrome c from mitochondria followed by caspase activation and apoptosis (Shen, et al. 2001).

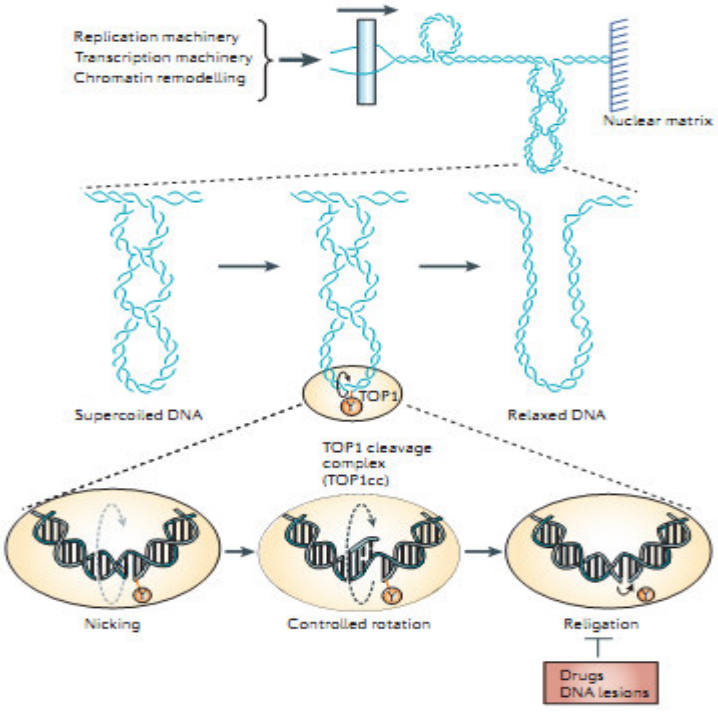


Figure 1.3. Camptothecin mechanism of action. TOP I relaxes DNA supercoil during replication, transcription by introducing single-strand breaks and after relaxation the breaks are relegated but in the presence of CPT, this religation is inhibited (Source: Pommier 2006).

1.3. MicroRNAs

MicroRNAs (miRNAs) are endogenous non-coding small RNAs and regulate gene expression negatively by targeting 3'UTR of its target gene mRNA. (Julia Starega-Roslan et al. 2011). miRNAs are 20 to 24 nucleotide long and control numerous physiological processes including cell proliferation, differentiation, development and play a significant role in regulating programmed cell death (Subramanian and Steeer 2010).

1.3.1 Biogenesis of miRNAs

miRNAs are transcribed from intergenic or intronic region by RNA polimerase II as a primary miRNAs (pri-miRNAs) that is a long transcript with one or more hairpin structures. In nucleus pri- miRNA is cleaved by Drosha /DGCR8 complex to form precursor miRNA (pre-miRNA) that is about 60-70 nt hairpin structure and pre-miRNA is transported to cytoplasm by Exportin-5 in Ran-GTP dependent pathway and in cytoplasm pre-miRNA is further cleaved by Dicer/TRBP complex thus mature miRNA dublex is generated then, one of strands that is named as a guide is selected to incorporate into RISC (RNA Induced Silencing Complex) and RISC is directed to target mRNAs that are complementary to its miRNA and this causes translational repression or degradation of target mRNA and the other strand that is named as a passenger is degraded but this does not happen always like this sometimes passenger strand incorporates a RISC complex and this is called as 'the star miRNA' (Michael Ladomery, et al. 2011, Julia Starega-Roslan, et al. 2011).

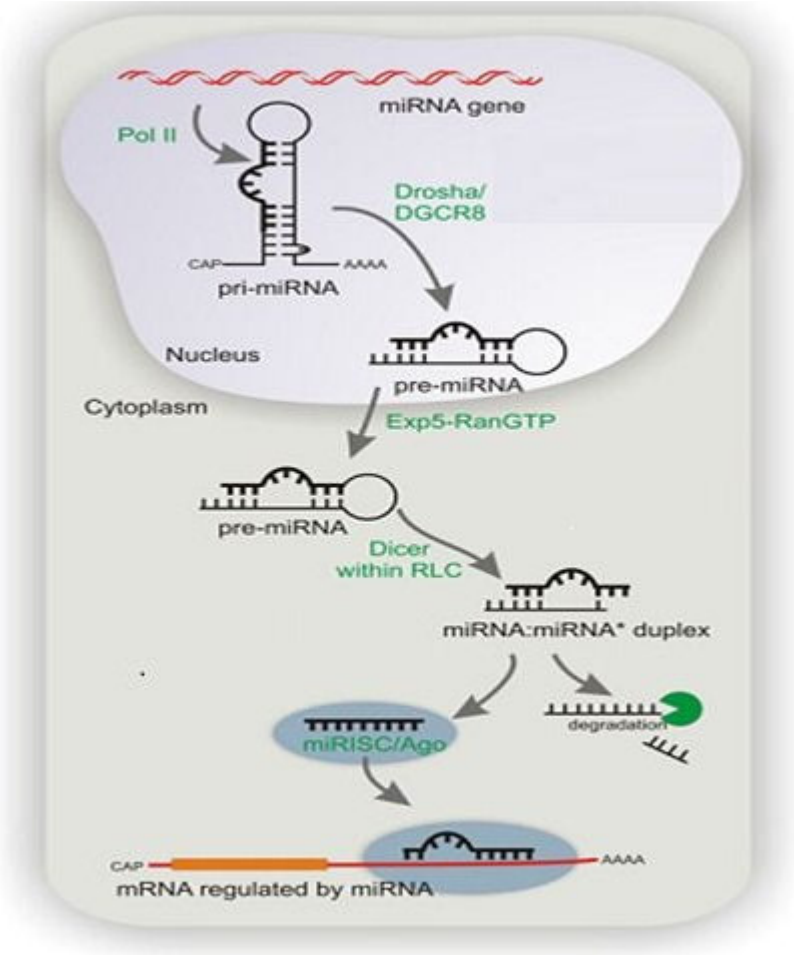


Figure 1.4. Biogenesis of miRNA

(Source: Starega-Roslan, et al. 2011)

1.4. miRNA-Mediated Regulation of Apoptosis

More than 1000 miRNA have been identified in humans and these miRNA have regulatory role on hundreds of mRNAs (Starega-Roslan, et al. 2011 and Friedman, et al. 2009). Many studies showed relationship between miRNAs and apoptosis. miRNAs can function as anti-apoptotic (oncomir) or pro-apoptotic (tumor suppressor) in programmed cell death (Kumar, et al. 2007).

miR-34a induces apoptosis in malignant peripheral nerve sheath tumor cell line (Subramanian, et al. 2010). miR-15 and miR-16 regulate anti-apoptotic members of Bcl-2 protein family, so they induce apoptosis in chronic lymphocytic leukemia (CLL) and other tumor cell lines (Cimmino, et al. 2005). Let-7 behaves as a tumor suppressor

by targeting HMGA2 and RAS oncogene expression and also regulates apoptosis by targeting caspase-3 (Mayr, et al. 2007, Tsang and Kwok 2008). Mir-106b-25 cluster is up-regulated in gastric cancer cases. They suppress pro-apoptotic p21 and BCL2L11 genes (Petrocca, et al. 2008). Recent studies showed that miR-21 is upregulated in various cancer types by targeting tumor suppressor genes, so these studies suggested that knocking down of miR-21 expression can be therapy for cancer treatment (Papagiannakopoulos, et al. 2008). miR-221 and miR-222 promote apoptosis by targeting p27 that is tumor suppressor gene and most targets of these miRNA are known as a pro- apoptotic, so up-regulation of miR-211 and miR-222 lead to cell proliferation and survival (Fornari, et al. 2008, Felli, et al. 2005). Activation of p53 is carried out by miR-29 family by suppressing expression of p85, thus it can regulate pathways of apoptosis (Park, et al. 2009).

In human cancer cells, over-expression of miR-206 suppresses expression of Notch3 by decreasing levels of both Notch3 mRNA and protein. Notch3 has anti-apoptotic activity, so when mir -206 targets Notch3 gene, apoptotic cell death is induced (Song, et al. 2009). MicroRNA-128 down-regulates Bax and induces apoptosis in human embryonic kidney cells. And also over-expression of mir-128 up-regulates Bcl-2 in SH-SY5Y neuroblastoma cells (Guidi, et al. 2010). miR-125b inhibites apoptosis in camptothecin induced human cancer cell lines so, down-regulation of miR-125b may be a novel approach for the treatment of cancer (Zeng, et al. 2012).

miR-425 and miR-17* regulate apoptosis in Hela cells are unknown. In this study, it is aimed to identify the function of these miRNAs in CPT-induced apoptosis in HELA cells. These candidate miRNAs were selected from deep sequencing data that includes screening of 1481 miRNAs after cells were treated with CPT when the miRNAs are compared with un-treated control group, it was observed that some miRNAs showed strikingly up or down-regulation and these candidates were selected among them because of their unknown function in CPT induced apoptosis in HELA cells.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Cervical cancer (HeLa) cell lines were used in this study were purchased from the American Type Culture Collection. Camptothecin was obtained from Sigma. Annexin V PE and 7AAD were provided from Becton Dickonson (BD). Metafectene-Pro transfection kit was purchased from Biontex Laboratories GmbH. miRNA mimics (mir-17* and mir-425) were obtained from Ambion. And PE conjugated Rabbit anti-active caspase-3 antibody was provided from BD Pharmingen. Perm and fix buffer were purchased from biolegend.

2.2. Cell Culture

Hela cell lines were used in this study and cultured 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. $3x10^5$ cells were seeded for camptothecin (Sigma–Aldrich) treatment and the cells were treated at two time points (32, 48 hours) and for five different concentration (2μM, 4μM, 8μM, 16μM, 32μM). 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 and CPT was added and pipeting one time for homogenization and incubated at 37^{o}C, in 5% CO_2 incubator.

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 500xg, +4^{o}C for 5 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 (Annexin diluted 1:5 and 7AAD 1:10 diluted in 1XPBS containing 0,1% NaN_3) for 20 minutes at room temperature in a dark place. Then, 160 µL of Ca^{++} 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.

2.4. Transfection

Hela cells were transfected with 100nM miRNA mimics by using Metafectene-pro transfection kit (Biontex Lab). For transfection $3x10^5$ cells were seeded before the day of transfection and on day of transfection cells were transfected with miRNA mimics according to instruction of Metafectene-pro and after 24h incubation for over-expression, cells were harvested. Then some group of cells were used to measure apoptosis ratio and the other transfected cells were seeded at $3x10^5$ cells per dish in 500

µl medium for CPT treatment and treated with 16µM CPT for 48h at 37°C in 5% CO_2 incubator. After 48h, cells were harvested and labeled with Annexin V PE and 7AAD (BD Pharmingen) to measure apoptosis ratio by flow cytometry.

2.5. Caspase Activitiy Assay

Activities of caspase-3 was measured by using PE conjugated Rabbit anti-active caspase-3 antibody (BD Pharmingen) with Flow Cytometry. Firstly Hela cells were centrifuged to removed their medium at 500xg, +4°C for 5 minutes. Then washed with 1XPBS and 100µl fix buffer (1:10 diluted in 1XPBS) was added and incubated 10 minutes in the dark. After incubation 300µl 1XPBS was added and centrifuged at 500xg, +4°C for 5 minutes. Then 100µl permeabilization buffer (1:10 diluted in 1XPBS) was added and incubated for 15 minutes after incubation, 300µl 1XPBS was added and centrifuged at 500xg, +4°C for 5 minutes. And 5µl anti-active caspase-3 antibody was added to each well and incubated for 30 minutes in the dark. After incubation cells were washed 2 times with 1XPBS and the pellets were re-suspended in 200µl 1XPBS and the samples loaded on 96-well plates for analysis by flow cytometry.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Dose Response and Time Point Assay for Camptothecin-induced Apoptosis in Hela cell lines

Camptothecin-induced apoptosis in Hela cell line was optimized in order to capture cells at the early apoptotic stage for this, Hela cells were treated with CPT at concentrations between 2-32 µM for 32 and 48h and the apoptosis ratio were detected using flow cytometry. For 32h CPT treatment, up to 20% of annexin positive cells were observed (Figure 3.1).

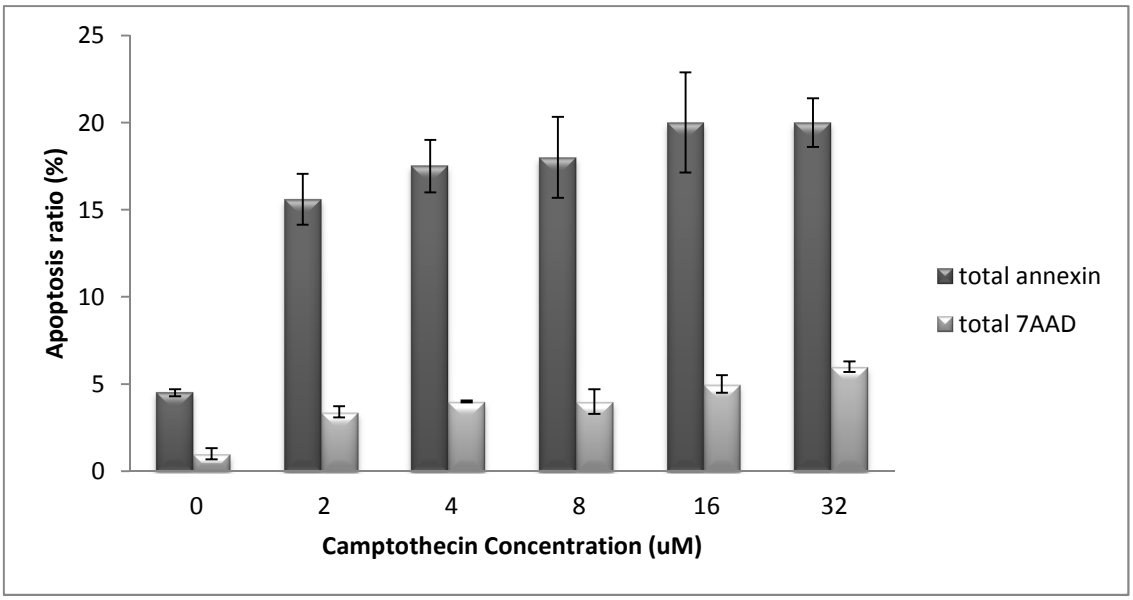


Figure 3.1. Camptothecin dose response graph for 32 hours between concentrations 2-32 µM. Hela cells were treated with CPT (camptothecin) at concentration between 2-32µM for 32h and after 32h apoptotic rate was detected by Flow Cytometry. Observed highest annexin positive cells was 20%.

In 32h CPT treatment, we observed that 20% annexin positive cells as a highest apoptotic percentage at 16 and 32µM CPT concentration. But we aimed to reach at least 40-50% apoptotic ratio so, 48h treatment was carried out because 24h CPT treatment

showed that there is no significant differences in apoptotic ratio between these CPT concentration and dose dependency was not observed (Figure 3.1).

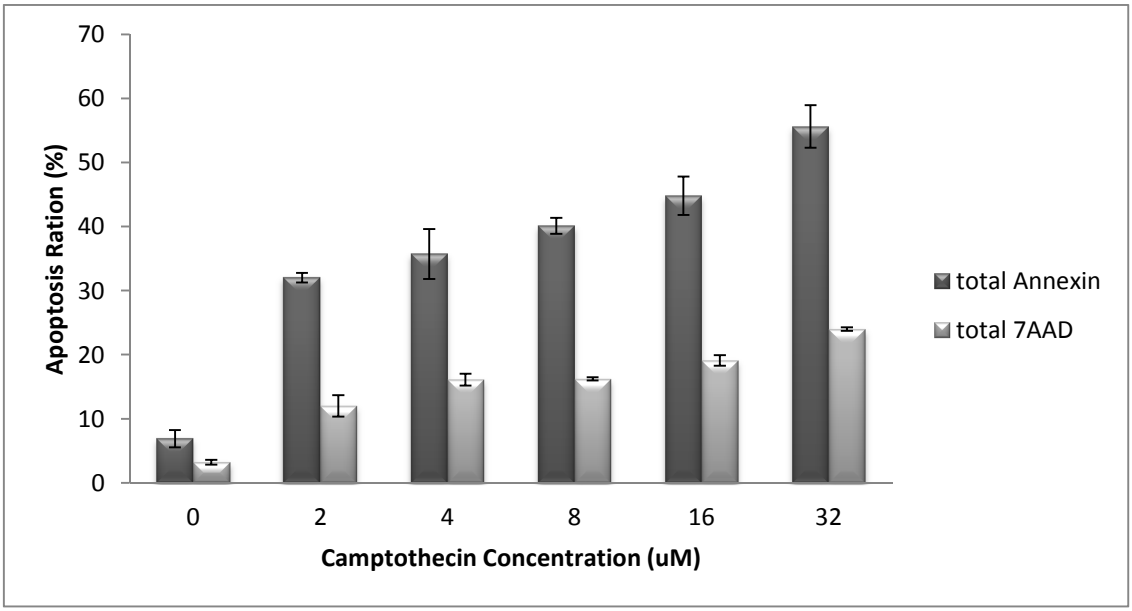
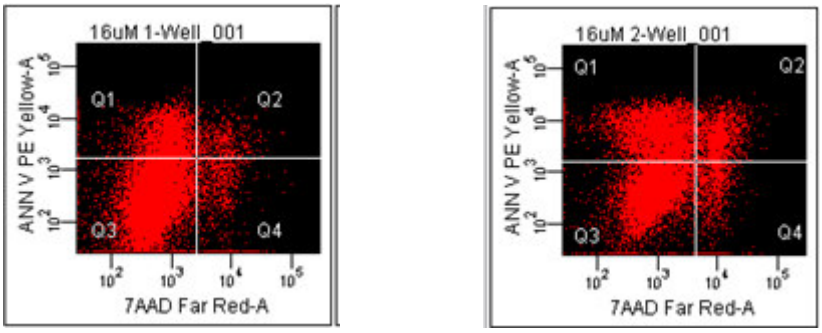


Figure 3.2. Camptothecin dose response for 48h. In this time point, cells were treated with camptothecin between concentration 2-32µM and the highest concentration of annexin positive cells was measured by Flow Cytometry as 55% at 32µM camptothecin.

In 48h CPT treatment, 45-55% annexin positive cells were observed at 16µM in 48h thus this concentration and time point were selected as a camptothecin dose to induce apoptosis in Hela cells.

Dot blot analysis showed that in 48h camptothecin treatment more cells passed to annexin positive region (Q1) from region that shows live cells percentage (Q3) as compared with Q1 region in 32h camptothecin treatment. Thus Q1 region in 48h camptothecin treatment had more cells than in Q1 region of 32h treatment. At 16µM camptothecin in 48h, Q1 region has 45% annexin positive cells whereas 20% annexin positive cells were detected in Q1 region in 24h camptothecin treatment. So, the data indicated that camptothecin induces apoptosis in a time dependent manner.



(a) 32h (b) 48h

Figure 3.3. Dot blot analysis of dose response assay. a) 32h treatment with 16μM camptothecin. b) 48h treatment with 16μM camptothecin. As explained in Materials and Methods part Hela cells were labeled with Annexin PE and 7AAD. 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. Whereas in 32h treatment, Q1 region had 20% annexin positive cells after 48h camptothecin treatment 45% annexin positive cells were observed in Q1 region also there was a time dependent increase in cell number of Q2 and Q4 region same as Q1.

3.2. miR-17* and miR-425 Response to Camptothecin Induced Apoptosis in Hela cells

To investigate the function of miR-17* and miR-425 in CPT induced apoptosis, Hela cells were transfected with miR-17* and miR-425 mimics individually for 24h and after transfection for 24h, some cells were used to measure apoptosis and remainder cells were treated with CPT for 48h.

After 24h transfection, figure 3.5 shows that miR-17* and miR-425 increase apoptosis in 7AAD positive cells approximately 2 fold as compared with Transfection Reagent controls (TR). In TR control group 7,5% 7AAD positive cells were observed whereas in miR-17* overexpressed cells had 14% and miR-425 overexpressed cells had 16%. This result showed that in miR-17* and miR-425 overexpressed cells, apoptotic rate increased from 7,5% to 14-16% (Figure 3.5).

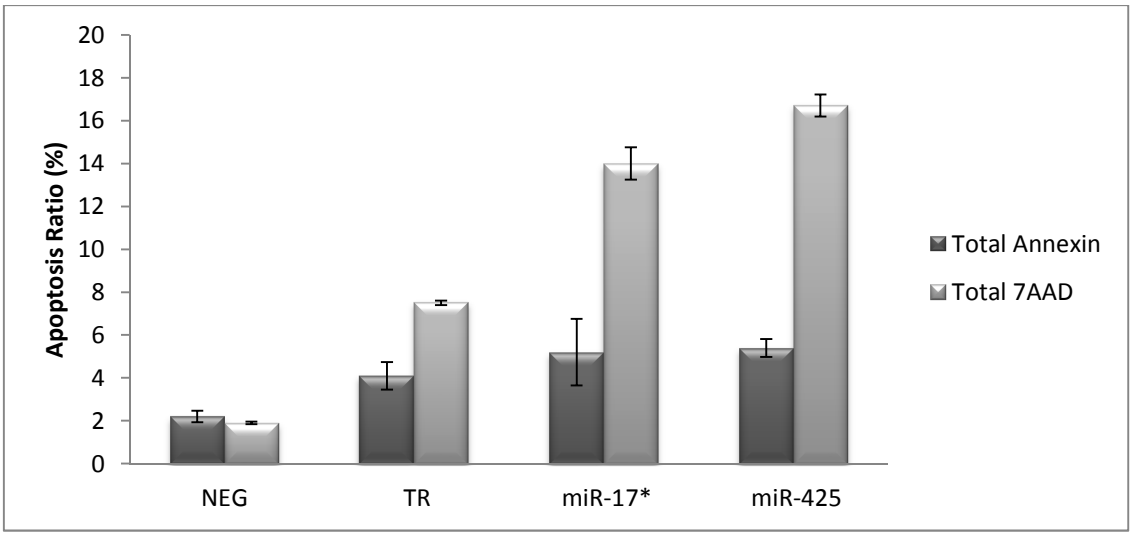


Figure 3.4. After 24h transfection of Hela cells with miR-17* and miR-425. In the figure, Negative (NEG) represents cells which is not transfeted and treated with transfection reagent , Transfection Reagent control group(TR) represents cells containing Transfection Reagent in their medium and miR-17* and miR-425 represent cells were transfected with miR-17* or miR-425.

To show function of these miRNAs in CPT-induced apoptosis in Hela cells, the miR-17* and miR-425 overexpressed cells were treated with 16μM camptothecin for 48h and after 48h cells were labeled with Annexin V PE and 7AAD to measure apoptosis by Flow Cytometry (Figure 3.6).

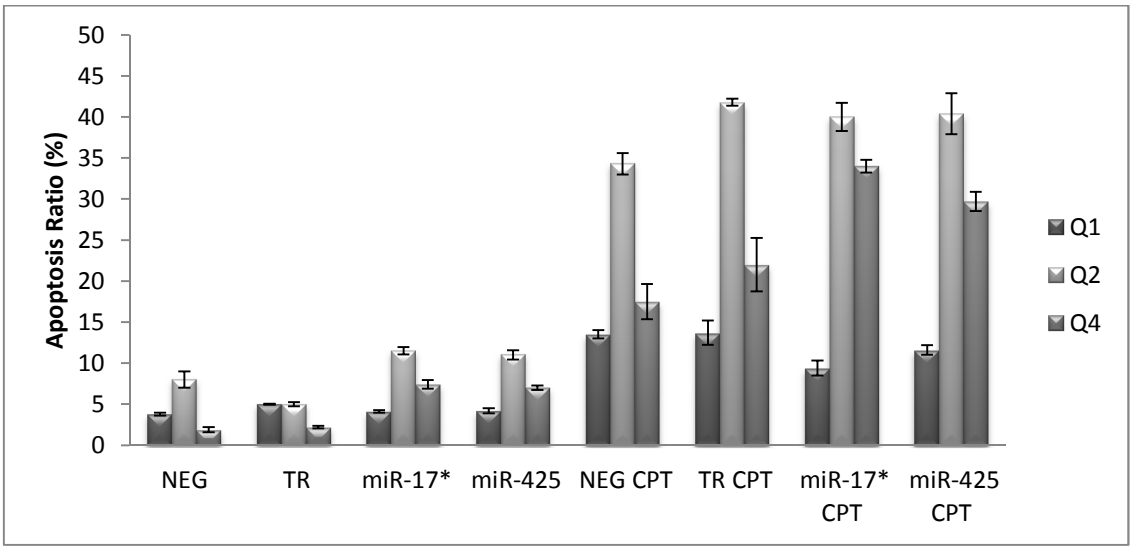


Figure 3.5. miR-17* and miR-425 function in CPT-induced apoptosis. miR-17* and miR-425 overexpressed cells were treated with 16μM camptothecin for 48h. NEG-CPT represents only CPT treated cells, TR- CPT represents both transfection reagent and CPT treated cells, miR-17* and miR-425 CPT represents miR-17* or mir-425 overexpressed and CPT treated cells.

After 48h of CPT treatment the results showed that in miRNA overexpressed cells, double positive (Annexin and 7AAD) cells number in Q2 region and 7AAD positive cell number in Q4 region higher than TR control group (Transfection Reagent treated control group). For CPT treated cells as a control group TR CPT and NEG CPT were used and for non - treated group with CPT, TR and NEG were used as a control group. When miR-17* overexpressed cells are compared with TR group, miR-17* overexpressed cells have 11,5% double positive cells in Q2 region and 7,4% 7AAD positive cells in Q4 region meanwhile TR group has 5% double and 7% 7AAD positive cells the data showed that there was 2 fold differences in double positive cells and 3,5 fold differences in 7AAD positive cells between miR-17* or miR-425 overexpressed cells and TR control groups. (Figure 3.6)

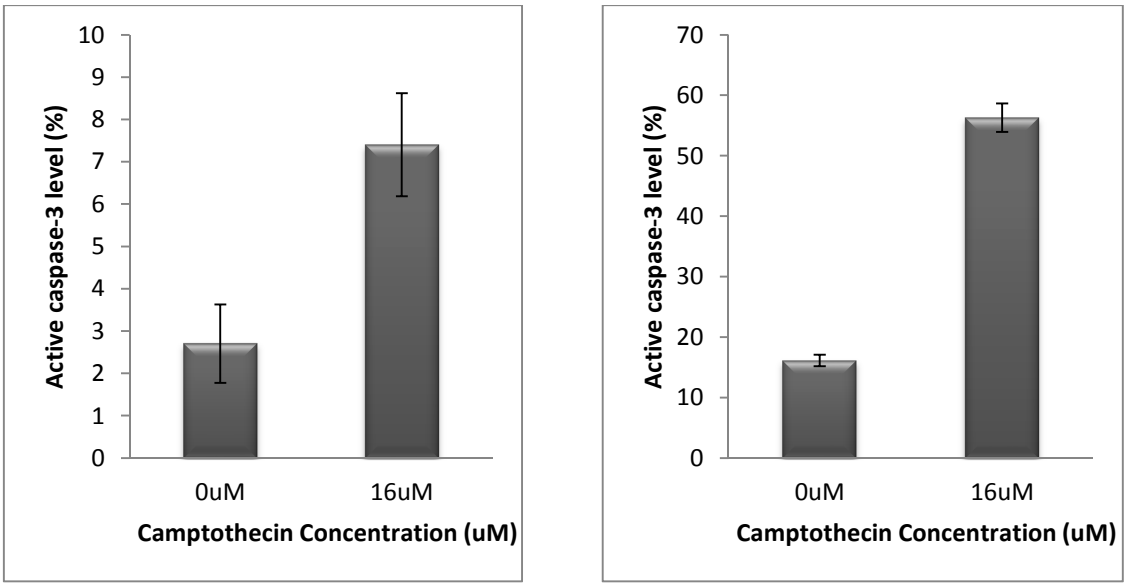
Cells which were miR-17* or miR-425 overexpressed and treated with CPT had respectively, 34% and 30% 7AAD positive cells in Q4 region as compared with TR CPT(treated with both transfection reagent and CPT) control group which had 21% 7AAD positive cells.

3.3. Active Caspase-3 Level in Camptothecin Induced Apoptosis in Hela Cells

Caspases are the executioners of apoptosis and the another evidence that shows induced caspase-dependent apoptosis. Caspases are synthesized as inactive pro-enzymes then activated and Caspase-3 is one of the key executioners of apoptosis. (Gerald M.C 1997).

Hela cells were treated with camptothecin for 24h and 48h to show increasing active caspase-3 level with apoptosis which results in camptothecin treatment.. Then cells were treated with anti-active caspase-3 antibody and the result was detected by using Flow Cytometry. Figure 3.7 shows the results.

In 24h camptothecin treatment, 7% active caspase-3 positive cells were observed whereas camptothecin un-treated control group had 2,7% active caspase positive cells.



(a) 24h camptothecin treatment (b) 48h camptothecin treatment

Figure 3.6. Measurerment of active caspase-3 level after 24h and 48h camptothecin treatment. (a) after 24h CPT treatment, 7% active caspase-3 positive cells were observed as compared with un-treated control group. (b) after 48h CPT treatment approximately %55 active caspase-3 positive cells were observed whereas un-treated group had %16.

3.4. Active Caspase-3 Level in miR-17* and miR-425 Over-expressed Hela Cells after 48h Camptothecin Treatment

As it was explained before, Hela cells first were transfected with candidate miRNAs individually for over-expression then treated with camptothecin for 48h and some group of transfected Hela cells were not treated with camptothecin to used as a control.

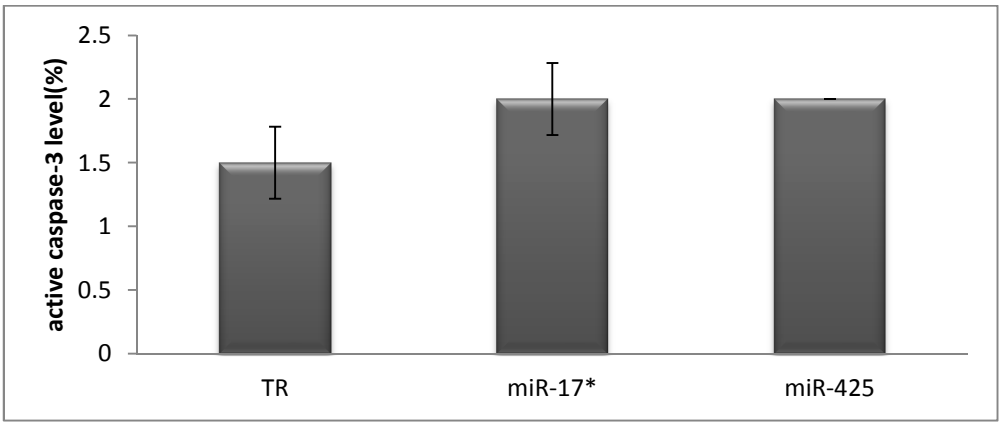


Figure 3.7. Active caspase-3 level in miR-17* or miR-425 transfected Hela cells. TR shows trasfection reagent control group, miR-17* shows miR-17* transfected cells and miR-425 shows miR-425 transfected cells.

In miRNA overexpressed Hela cells, active caspase-3 level was higher than un-transfected control but significant difference was not observed (Figure 3.8).

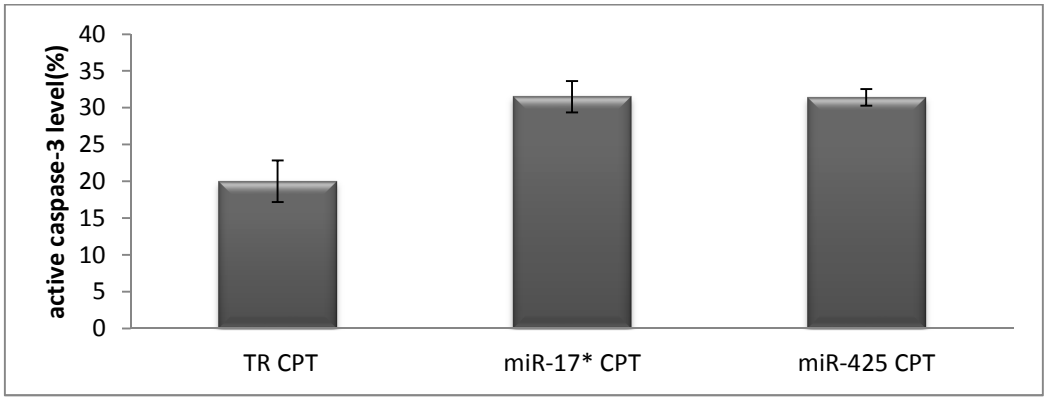


Figure 3.8. Active caspase-3 level in miR-17* or miR-425 over-expressed and 48h camptothecin treated cells.

After 48h camptothecin treatment in miR-17* or miR-425 over-expressed cells it was observed that miRNA over-expressed cells have higher active caspase-3 activity than un-transfected control (TR CPT). In this data a significant difference also was observed (P value =0,02) when miRNA transfected groups and control were compared (Figure 3.9).

So, the data showed that active caspase-3 level increases by camptothecin in a time dependent manner.

CHAPTER 4

CONCLUSION

In this study, we identified functions of miR-17* and miR-425 in CPT-induced apoptosis in Hela cells for the first time. To show functions of these miRNAs in CPT-induced apoptosis, firstly CPT dose was found to induce apoptosis in Hela cells and the results reported that CPT induces apoptosis in a time dependent manner. After that Hela cells were transfected with miR-17* and miR-425 individually and after 24h transfection for overexpression, cells were treated with camptothecin and the results demonstrated that apoptosis rate increased at late stage of apoptosis in miR-17* and miR-425 overexpressed cells as compared with negative controls. So, miR-17* and miR-425 showed pro-apoptotic function in CPT-induced Hela cells.

This data also was supported with measurment of active caspase-3 level. Caspases are the executioners of apoptosis and the increasing caspase level shows apoptosis. Thus, caspase-3 that is one of the key executioners of apoptosis level was measured and the results showed that active caspase-3 level is higher in miR-17* or miR-425 overexpressed and CPT treated cells as compared with their negative controls. So, the data demonstrated that miR-17* and miR-425 function as pro-apoptotic regulators in CPT-induced apoptosis in Hela cells.

miRNAs have a role in regulation of apoptosis by targeting genes involved in apoptotic pathways and these genes can be potential targets of one or more of these miRNAs (Subramanian, et al. 2010). For future aspects, targets of miR-17* and miR-425 will be identified by TargetScan which is target screening programme and targets related with apoptosis will be chosen and will be verified by qPCR. So we will learn which genes targeted by these miRNAs in regulation of apoptosis in Hela cells and with this study, we contributed to litreture about fuction of miR-17* and miR-425 in apoptosis and also this data may help in the development of potential therapeutics against cancer.

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