IDENTIFICATION OF LONG NON-CODING RNAS THAT REGULATE APOPTOSIS IN HUMAN

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ABSTRACT

IDENFICATION OF LONG NON-CODING RNAS THAT REGULATE APOPTOSIS IN HUMAN

Apoptosis is essential for cellular homeostasis and normal development. Aberrant apoptosis (too much or too less) is associated with many important diseases such as autoimmune diseases and cancer. Studies have led to the identification of a number of proteins and microRNAs involved in the regulation of apoptosis. However, the role of long non-coding RNAs (lncRNAs) is still unclear. In this study, two cancer therapeutics drugs, cisplatin and doxorubicin, and two ligands, Fas mAb and TNF-alpha, were used in identification of differentially expressed pathway-drug specific and/or global lncRNAs in apoptotic HeLa cells. Following dose-kinetics experiments the level of apoptosis was measured by Flow Cytometry and was further verified by Fluorescence Microscopy and Western Blotting via measurement of Caspase 3, 8 and 9 protein levels. Three replicates of total RNAs (control and drug/ligand-treated cells) were sent to deep-sequencing using the Illumina platform. The resulting reads matched to the human genome greater than 95%. Under our experimental setting, treatments with cisplatin, doxorubicin, Fas mAb and TNF-alpha led to the differential expression of 1644, 506, 584 and 807 lncRNAs, respectively (2-fold or higher, $P < 0.01$). Two of identified lncRNAs common for all inducers was in antisense position to TRAIL-R2 receptor and FasR associated factor which play directly in apoptosis. Results suggest that many lncRNAs are differentially expressed upon treatment with the indicated agents. Functional characterization of candidates might provide an interesting insight into regulation of apoptosis.

Keywords: apoptosis, long non-coding RNA, deep sequencing

ÖZET

İNSANDA APOPTOZU DÜZENLEYEN UZUN KODLAMAYAN RNA'LARIN BELİRLENMESİ

Hücre içi homeostazinin sağlanması açısından çok önemli olan apoptoz normal gelişimin yanı sıra otoimmun ve kanser gibi önemli hastalıklarla da bağlantılıdır. Biyokimyasal ve genetik analizler sonucu apoptozun kontrol mekanizmasında görev alan bir dizi protein ve mikroRNA'lar belirlenmiştir. Post-genomik çağdaki son çalışmalar genomda bir dönem 'çöp' DNA olarak belirlenen bölgelerden çok sayıda uzun kodlanmayan RNA'ların (ukmRNA) keşfine yol açmıştır. Bu çalışmada, apoptozun tetiklendiği HeLa hücrelerinde farklı ifade edilen ukmRNA'ların belirlenebilmesi için iki anti-kanser ilaç, sisplatin ve doksorubisin, ve iki ligant, TNFalpha ve Fas monoklonal antikoru, kullanılmıştır. Doz - ve zaman - kinetik deneylerini müteakip apoptoz seviyesi akış sitometresiyle ölçülmüş ve floresan mikroskopuyla sonuçlar teyit edilmiştir. Apoptozun tetiklendiğini doğrulamak için biyokimyasal olarak kaspaz 3, 8 ve 9 proteinlerinin seviyeleri ölçüldü. İllumina platformunu kullanarak derin sekans analizi yapabilmek için kontrol ve ilaç ile muamele edilmiş hücrelerden üçer replika toplam RNA örnekleri elde edildi. Sekans sırasında örnekler insan genomu ile %95 eşleşmiştir. Doksorubisin, sisplatin, TNFalpha ve Fas monoklonal antikoru muamelesi hücrelerde sırası ile 1644, 506, 584 and 807 adet ukmRNA'nın farklı ifade edilmesine neden olmuştur (an ez 2 kat, $P < 0.05$). Tüm ilaç muamelerinde ortak olarak faklı ifade edilen ukmRNA'lardan ikisi apoptozda önemli rol oynayan TRAIL-R2 reseptör ve FasR reseptöre bağlı öğe 1'ye (FAF1) antisens olarak bulunmuştur. Deneysel şartlarımız çerçevesinde sonuçlar ukmRNA'ların yukarıda belirtilmiş ilaçla muamele sırasında farklı ifade edildiğini göstermektedir. Adayların fonksiyonel karakterizasyonu ukmRNA'ların apoptozdaki rollerinin moleküler düzeyde anlaşılmasına yardımcı olacaktır.

Anahtar Kelimeler: apoptoz, ukmRNA, derin sekanslama

To the idol of my life – to my Grandfather
Yusif AHMADOV

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CHAPTER 1

INTRODUCTION

1.1. Apoptosis

The balance between cell death and survival of normal cells is mediated by programmed cell death (PCD) which leads to cell death in pathological events once it is mediated by an intracellular program as well as playing key roles in concluding decisions of cancer cell fate (Hanahan and Weinberg 2011; Laubenbacher et al. 2009). There are three main forms of PCD: apoptosis, autophagy and programmed necrosis. These three forms are easily distinguished due to their morphological differences (Tan et al. 2009; Bialik et al. 2010). Apoptosis is referred as type I PCD and it was first described by Kerr et al. (1972). Apoptosis is crucial for normal development, aging and a part of homeostatic mechanism to preserve cells in tissue as well as a defence mechanism in case of immune response or cellular damage due to disease or harmful agents (Norbury and Hickson 2001). Inappropriate apoptosis, either too much or low, can cause some serious problems like neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer (Elmore et al. 2007)

Several studies identified various morphological changes during death of cells, apoptosis. During the early stage of apoptosis, cell shrinkage (smaller cell size, condensation of cytoplasm and tight packaging of organelles) and pyknosis (chromatin condensation) occurs which can be easily observed by light microscope (Kerr et al. 1972). Next, extensive plasma membrane blebbing takes place which is then followed by karyorrhexis (nuclear fragmentation) and budding, separation of cell fragments into apoptotic bodies that consist of cytoplasm with tightly packed organelles which can be with or without a nuclear fragment. These bodies have intact membrane and organelles. They are phagocytosed by macrophages, parenchymal or neoplastic cells which then degraded within phagolysosomes (Savill and Fadok, 2000; Kurosaka et al, 2003). In addition to morphological changes, apoptosis has some biochemical changes taking place as well: chromosomal DNA is cleaved into internucleosomal fragments, extensive

protein cross-linking, and phosphatidylserine are externalized and some proteolytic cleavage of a number of intracellular substrates (Cohen et al. 1994, Martin et al. 1995).

1.2. Mechanism of Apoptosis

1.2.1. Caspase-dependent Mechanism

The known mechanisms of apoptosis are mediated by energy-dependent cascade of molecular events and they are highly complex. Up to date, two main and one additional pathway have been identified. One of the main pathways is extrinsic pathway which is mediated by death receptor pathway. The other main pathway is intrinsic pathway and it is also called as mitochondrial pathway. The additional pathway is mediated by perforin/granzyme pathway via granzyme A or granzyme B. Despite differences among stimuli for those pathways, extrinsic, intrinsic and granzyme B pathways converge on the same terminal-execution pathway: cleavage of caspase 3 which results in fragmentation of DNA, cytoskeletal and nuclear protein degradation, cross-linking of proteins, apoptotic body formation and expression of ligands for phagocytosis (Igney and Krammer, 2002).

Caspases (cysteine-aspartic proteases or cysteine-dependent aspartate-directed proteases) are a member of cysteine protein family function in apoptotic and inflammatory signaling pathways and they are present in most cells. They are inactive proenzyme until they are cleaved. Once a procaspase is cleaved, it gets activated and this leads to the activation of other procaspases. Thus once caspases are activated, a signaling caspase cascade occurs which serves as amplification of apoptotic signals and ends with irreversible rapid cell death. Up to date, fourteen caspases have been identified and categorized into three major classes: apoptotic initiator caspases (caspase-2, -8, -9 and -10), executioner (effector) caspases (caspase-3, -6 and -7) and inflammatory caspases (caspase-1, -4 and -5) (Cohen 1997; Rai et al. 2005). Caspase-11, -12 and -14 play role in apoptosis under specific conditions and tissues. Caspase-13 is a bovine gene (Hu et al. 1998; Nakagawa et al., 2000, Koenig et al., 2001; Kang et al., 2002).

1.2.1.1. Extrinsic Pathway

Apoptosis could be initiated via transmembrane receptors – dead receptors like tumor necrosis factor (TNF) receptor superfamily which then is called as extrinsic signaling pathway (Figure 1.1) (Locksley et al. 2001). The members of this family share cytoplasmic 80 amino acids "death domains" (DD), which transmit death signals from cell surface to intracellular signaling pathways, and similar cysteine-rich extracellular domains (Ashkenazi and Dixit 1998). Up to date, six death domain containing receptors have been identified: fatty acid synthetase receptor (FasR), tumor necrosis factor receptor 1 (TNFR1), TNF receptor-related apoptosis-mediating protein (TRAMP), TNF-related apoptosis-inducing ligand receptor I and II (TRAIL-R1 and TRAIL-R2), and death receptor-6 (DR6) (Pan et al. 1998; Wiley et al. 1995; Pitti et al. 1996; Ashkenazi et al. 1998; Song et al. 2008).

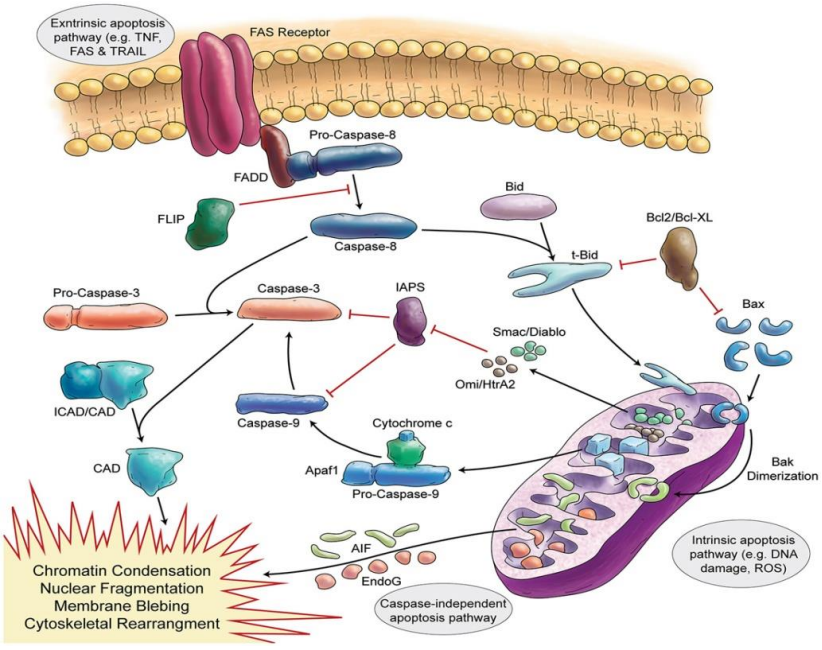

Figure 1.1. Schematic Representation of Apoptosis. In general apoptosis divides into two pathways: extrinsic and intrinsic pathway. Death receptors (like FAS&TRAIL) are involved in extrinsic pathway, which later can activate caspase-8. Caspase-8 activates caspase-3 in two separate ways (direct activation or activation via caspase-9). Stress signals and DNA damage triggers intrinsic apoptosis pathway via mitochondria. Mitochondria mediated intrinsic apoptosis is divided to caspase-dependent (activation of caspase 9) or caspase-independent pathways (AIF and EndoG). (Source: Marzban et al. 2015)

The best characterized ligand/receptor models are between FasL and FasR, and tumor TNF-α and TNFR1. Fas and TNF receptors cluster at the cell surface and binding of trimeric Fas and TNF ligands to the corresponding receptors results recruitment of adaptor proteins having death effector domains (DED): FADD (Fas-associated death domain) recruits to FasR upon FasL binding; TRADD (TNF receptor-associated death domain) is recruited to the TNFR1 due to TNF-α binding. TRADD itself mediates recruitment of FADD and RIP (Receptor-interacting protein) and a death-inducing signaling complex (DISC) is formed after association of procascase-8 with FADD via dimerization of the death effector domain (Hsu et al. 1995; Kelliher et al. 1998; Wajant 2002). Formation of complex results with autocatalytic activation of procaspase-8 and trigger of apoptotic execution pathway (Kischkel et al. 1995). However, the pathway can be inhibited by c-FLIP (FLICE-inhibitory protein) binding to FADD and caspase-8 and turn them inactive (Kataoka et al. 1998; Scaffidi 1999).

1.2.1.2. Intrinsic Pathway

The intrinsic pathway of apoptosis arises from various non-receptor mediated stimuli which produce intracellular signals and mitochondrial-initiated series of events (Figure 1.1). Those stimuli-initiated signals act directly on targets either in a positive or a negative fashion. The stimuli acting in a positive mode happens due to presence of free radicals, radiation, toxins, viral infections, and hypoxia and so on. Negative mode of acting happens after failure of suppressing death pathways and subsequent activation of apoptosis due to absence of growth factors, cytokines and hormones (Saelens et al. 2004).

Both modes of action give rise to permeabilization of the outer mitochondrial membrane: an opening of the mitochondrial permeability transition (MPT) pore due to changes in the inner mitochondrial membrane, loss of the mitochondrial transmembrane potential. Upon formation of MPT, a series of pro-apoptotic protein members of the Bcl family are released which are normally sequestered in the space between the inner and outer mitochondrial membranes (Saelens et al. 2004; Green and Kroemer 2004). Proapoptotic Bcl family members involve cytochrome c, Smac/DIABLO, Omi/HtrA2, AIF, CAD and endonuclease G, and they trigger the apoptosis by caspase-dependent or caspase-independent death effectors (Saelens et al. 2004). Cytochrome c,

Smac/DIABLO and Omi/HtrA2 function in a caspase-dependent fashion. Released cytochrome c binds and activates Apaf-1 and procaspase-9, and they altogether form an "apoptosome" which leads to activation of caspase 9. On the other hand, Smac/DIABLO and HtrA2/Omi are stated to induce apoptosis by inhibiting inhibitors of apoptosis proteins (IAP) activity (Chinnaiyan 1999; van Loo et al. 2002a; Hill et al. 2004; Schimmer 2004).

Bcl-2 family of proteins play very important role in the control and regulation of mitochondria-derived apoptotic events and these proteins are regulated by p53, the tumor suppressor protein (Schuler and Green 2001; Cory and Adams 2002). The family members can be either pro-apoptotic or anti-apoptotic and they regulate mitochondrial membrane permeability. There are several well-known anti-apoptotic BCL-2 family members like Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w and BAG; and some pro-apoptotic proteins like Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk. Presence of these proteins determine between apoptosis and survival through regulation of cytochrome c release from the mitochondria via alteration of mitochondrial membrane permeability (Schuler and Green 2001). Interestingly, there is enough evidence suggesting "cross-talk" between the extrinsic pathway and the intrinsic pathway via mitochondrial damage by the caspase-8 cleavage of Bid after induction of extrinsic pathway of apoptosis (Li et al. 1998; Esposti, 2002; Igney and Krammer 2002).

Either singly or doubly phosphorylated pro-apoptotic Bad (BCL2 antagonist of cell death) protein on Ser-112 and Ser-136 binds to 14-3-3, a member of a family of multifunctional phosphoserine binding molecules, and it is trapped within the cytosol. However, unphosphorylated Bad translocates to the mitochondria and mediates release of cytochrome C (Zha, et al. 1996). Another mechanism for induction of apoptosis is neutralization of anti-apoptotic Bcl-Xl or Bcl-2 activity, inhibition of cytochrome C release, via forming heterodimer with Bad (Yang et al., 1995). There is another protein else, Aven, that binds to Bcl-Xl and Apaf-1 and inhibits procaspase 9 activation (Chau et al. 2000).

Other pro-apoptotic members of Bcl2 family Puma and Noxa play roles in p53-mediated apoptosis. Overexpression of Puma *in vitro* results with up-regulation and conformational change of BAX, which translocates to the mitochondria and mediates cytochrome c release and reduction in the mitochondrial membrane potential (Liu et al. 2003). Noxa itself can localize to the mitochondria and activate caspase-9 via interaction with anti-apoptotic Bcl-2 family members (Oda et al. 2000). p53-dependent

Puma and Noxa arise by genotoxic damage or oncogene activation like the Myc oncoprotein which can induce apoptosis through both p53-dependent and -independent mechanisms (Meyer et al. 2006).

1.2.1.3. Execution Pathway

The executioner or effector caspases are responsible for the final pathway of apoptosis – execution pathway which is the end point of both the extrinsic and intrinsic pathways. Activated execution caspases, caspase-3, caspase-6, and caspase-7, activate cytoplasmic endonuclease and proteases: cleaving cytokeratins, PARP, alpha fodrin, the nuclear protein NuMA and so on. As a result, they govern degradation of nuclear material, and the nuclear and cytoskeletal proteins leading the morphological and biochemical changes in apoptotic cells – hallmarks of apoptosis (Slee et al. 2001).

Among execution caspases, caspase 3 has the most critical role in execution phase of apoptosis and initiator caspases, caspase-8, caspase-9, or caspase-10, are responsible for its activation. The role of caspase 3 in apoptosis is cleaving the inhibitor of endonuclease CAD, ICAD, and cause release of CAD that degrades chromosomal DNA within the nuclei and causes chromatin condensation (Sakahira et al. 1998). In addition to ICAD, caspase 3 has another target, gelsolin, which has very role in actin nucleation and signal transduction. Caspase 3 cleaves gelsolin that ultimately causes disruption of the cytoskeleton, intracellular transport, cell division, and signal transduction (Kothakota et al. 1997)

The following phase after caspase cascade is phagocytic uptake of apoptotic cells which is characterized with phospholipid asymmetry and externalization of phosphatidylserine by scramblase on the cell surface (Wang et al. 2003). Phosphotidylserine on the cell surface governs early uptake and disposal of apoptotic cells via noninflammatory phagocytic recognition. The inflammatory response is not induced due to any release of cellular components after process of early and efficient uptake (Fadok et al. 2001).

1.2.1.4. Induction of Apoptosis via Drugs and Ligands

In this study, two anti-cancer drugs, doxorubicin and cisplatin, and two ligands, TNFalpha and Fas mAb, were used to induce cell death. Doxorubicin is one of the first anti-cancer drugs and has been used in chemotherapy in many different cancer types over 30 years. The ability to overcome rapid cell division and slow down cancer progression make it one of the most potent of the FDA (Food and Drug Administration)-approved chemotherapeutic drugs (Carvalho et al. 2009). It is known to intercalate DNA, bind to DNA-associated enzymes like topoisomerase enzymes I and II, and target various molecular targets to produce a range of cytotoxic effects – anti-proliferation and DNA damage (Buchholz et al. 2002). The apoptosis pathway is induced once cell fails to repair lesions on DNA and cell cycle is inhibited. However, studies have shown that doxorubicin can result in autophagy and necrosis (Tavar et al. 2012). Cytoprotective mechanism can induce autophagy by poly (ADP-ribose) polymerase-1 (PARP-1) activation as response to DNA damage. PARP-1 activation/deactivation decides whether the cell will undergo autophagy or necrosis (Minotti et al. 2004). Thus, doxorubicin mediated cell death type is strongly dependent on the concentration of drug, treatment duration and cell-cancer type. The effect of doxorubicin on HeLa cells is conditional – depending on concentration. Apoptosis is induced by only a particular concentration of doxorubicin; induction of necrosis is a higher probability (Tomoki and Robertson 2004).

The doxorubicin mediated apoptosis pathway is induced via AMPK activation (Shaw et al. 2004) or activation of various signals to alter the Bcl-2/Bax ratio (Leung and Wang 1999) which results in downstream activation of different caspases – induction of apoptosis. Downstream targets of AMPK are c-JunN-terminal kinase (JNK), p53 and mTORC1 (Meisse et al. 2002; Cao et al. 2008; Gwinn et al. 2008). Bcl-2/Bax ratio is affected due to p53-independent down-regulation of Bcl-2 mRNA levels. There is also a conflict about doxorubicin-mediated apoptosis. Some groups claim about existence of the Fas/Fas ligand apoptosis pathways; however, some groups have shown contradictory results (McGahon et al. 1998; Adams and Cory 1998).

Cisplatin has also been used as a chemotherapy drug over 30 years. Cisplatin is a platinum based drug. Chemotherapy with cisplatin often comes along with toxic side effects and tumor resistance, which leads to secondary malignancies (Chen et al. 2009).

Cytotoxicity of cisplatin depends on cell type, duration of treatment and drug concentration. Cisplatin damages tumor cells via activation of various signal transduction pathways. Cellular interaction of cisplatin includes reactive oxygen species (ROS), DNA, mitochondria, TNF, p53, caspases, calcium signaling and multidrug resistant proteins (Florea and Büsselberg 2011). Cisplatin-mediated inhibition of DNA synthesis, failure in DNA repair and cell cycle arrest lead to apoptosis (Desoize and. Madoulet 2002). Generation of ROS and interaction with DNA might introduce DNA damages and cytotoxicity which then results with cell death – death type depends on (cancer) cell type. (Che et al. 2010; Brozovic et al. 2010). Interestingly, cisplatin activates extrinsic apoptotic pathway via activating the *tumor necrosis factor-related apoptosis-inducing ligand* (TRAIL) receptor-mediated signal-transduction pathway. TRAIL receptor, DR4 and/or DR5, aggregation leads to death-inducing signaling complex (DISC) formation and results caspase activation. However, caspase 8 activation is mitochondria-dependent (Shamimi-Noori et al. 2008).

Both extrinsic and intrinsic pathways are induced in cisplatin mediated apoptosis in HeLa cells (Sui et al. 2015). Although cisplatin is known to induce mitochondria-mediated apoptosis, still interaction between cisplatin and mitochondria is less known. In several specific cases it was shown that cisplatin leads to mitochondrial depolarization, cytochrome c release, translocation of Bax and tBid to mitochondria and decrease in Bcl-2 expression, which results induction of apoptosis (Muscella et al. 2008). p53 and Protein kinase C (PKC) δ play role in cisplatin mediated cell death. It was shown that PKCδ is a positive regulator of cisplatin-induced cell death and in cooperation with p53, they mediates caspase-3 dependent apoptosis (Karger et al. 2005). In addition, cisplatin was shown to have effect on post-translational modifications like histone methylation and acetylation (Wang or Lippard 2004).

TNF-alpha and Fas ligands are commercially available for research and development projects. Monoclonal antibodies of both ligands are available as well, which exert the same function – bind to the ligand-specific death receptors and induces apoptotic extrinsic pathway. In type I cells, which have enough caspase 8 to activate effector/executioner caspases, TNF-alpha and FasL can induce executioner caspases directly. However, in type II cells like HeLa, caspase 8 amount is insufficient in caspase 3 activation, thus proapoptotic signals mediate release of cytochrome c from the mitochondria, activation of caspase 9 and caspase 3– induction of intrinsic apoptotic pathway (Kuwana et al. 1998). TNF-alpha cannot induce apoptosis alone in HeLa cells,

however, in the presence of metabolic inhibitors like cycloheximide (CHX) TNF-alpha can induce apoptosis (Miura et al. 1995). Several studies have stated that many FasR expressing tumor cells are completely insensitive to FasR-induced apoptosis (O'Connell et al. 1996). HeLa cells are not completely insensitive; however, sensitivity increases with the cellular stress due to ionizing agents and etc. (Park et al. 2003)

1.2.1.5. Perforine/Granzyme Pathway

The sensitized CD8+cells, cytotoxic T lymphocytes (CTLs), kill antigen-bearing cells by T-cell mediated cytotoxicity via extrinsic pathway, predominantly the FasL/FasR interaction to induce apoptosis (Brunner et al. 2003). However, in addition to extrinsic pathway, there is a novel pathway to show cytotoxic effects on virus-infected and tumor cells that is used by these cytotoxic cells. This pathway involves secretion of perforin, a transmembrane pore-forming molecule, with following release of cytoplasmic granules, the serine proteases granzyme A and granzyme B, through the pore and into the target cell (Trapani and Smyth 2002; Pardo et al., 2004).

Granzyme B can activate pro-caspase 10 via cleaving it at aspartate residue and can cleave ICAD either to induce apoptosis (Sakahira et al. 1998). Although it is shown that granzyme B can use intrinsic pathway via cleaving Bid and inducing cytochrome c release to amplify death signal (Barry and Bleackley 2002; Russell and Ley 2002), granzyme B can directly activate caspase 3 via bypassing the upstream signaling pathways to induce the execution phase of apoptosis directly (Goping et al. 2003). Granzyme A is inducing caspase-independent meachanism to induce apoptosis (Martinvalet et al. 2005).

1.2.2. Caspase-independent Mechanism

There is another group of mitochondrial released pro-apoptotic proteins, such as AIF, endonuclease G and CAD that act in a caspase-independent manner. They are released as a late event during apoptosis - after the cell has committed to die. Endonuclease G and AIF translocate to the nucleus and they work in a caspase-independent way (Susin et al., 2000; Li et al. 2001). Endonuclease G causes oligonucleosomal DNA fragmentation while AIF causes DNA fragmentation into ~50–

300 kb pieces. They cause “stage I” peripheral nuclear chromatin condensation (Joza et al. 2001). The released CAD translocates in nucleus where it leads to oligonucleosomal DNA fragmentation, advanced chromatin condensation and afterward “stage II” condensation (Enari et al. 1998, Susin et al. 2000).

The granzyme A pathway activates apoptosis in a caspase-independent manner via single strand DNA breaks (Martinvalet et al. 2005). The single stranded DNA break is mediated by the DNase NM23-H1, a tumor suppressor gene product, which has an important role in preventing cancer by inducting apoptosis in tumor cells (Fan et al., 2003). The granzyme A cleaves inhibitor of NM23-H1, a nucleosome assembly protein SET, thus releasing NM23-H1 to induce apoptosis via DNA degradation. The SET complex proteins, SET, Ape1, pp32, and HMG2, work together and they have vital roles in chromatin structure and DNA repair via protecting chromatin and DNA structure. Thus, granzyme A induces apoptosis by inhibiting the functions of the SET complex proteins (Lieberman and Fan 2003).

1.3. Long Non-Coding RNA

The scientists discovered in 1950s that the C-value - amount of DNA in the haploid genome has little correlation with organism size or developmental complexity (Mirsky and Ris 1951; Thomas 1971; Gall 1981). This meant that many less developed animals can have a bigger genome than more developed animals, including humans. The “C-value paradox” was partially solved after discovery of noncoding portion of genome, which is much more than protein coding portion in eukaryotes (Lewin 1980). The noncoding portion was then called “junk DNA” due to presence of transposons, pseudogenes, and simple repeats. It is estimated that total transposons, pseudogenes, and simple repeats are about 50–70% of the human genome (de Koning et al. 2011). Today the contradiction in genome size is no longer a paradox, but became more a “C-value enigma” (Gregory 2001). Morphologically similar and phylogenetically close species can have different genome size and thus noncoding content may indicate correlation between noncoding content and complexity (Ricroch et al. 2005; Taft et al. 2007).

Although noncoding sequences were called “junk”, it received interest from 1970s to the present. And researchers even in 1970s started to speculate that more

portion of the genome is transcribed from repetitive and heterochromatic regions, as well as nonrepetitive regions other than coding sequences and known rRNAs and tRNAs. Those transcribed RNAs were named as "heterogeneous nuclear RNAs" (hnRNAs) and it was shown that 50% of them are restricted to the nucleus and they do not contain coding sequences (Holmes et al. 1972; Pierpont and Yunis 1977; Lewin 1980, Chap. 25). Discovery of introns was shown to account for a small portion of noncoding sequences (Berget et al. 1977; Chow et al. 1977).

Whole-genome technologies in the late 1990s and early 2000s helped to estimate the scale of "pervasive transcription". As much as 70–90% of human genome is transcribed at some point during development (Okazaki et al. 2002; Rinn et al. 2003; Bertone et al. 2004; Ota et al. 2004; Carninci et al. 2005; Birney et al. 2007; Kapranov et al. 2010; Mercer et al. 2011; Djebali et al. 2012). RNA sequencing analyses suggest that alternative splicing and/or extensions of known protein-coding genes may account for such amount of pervasive transcription (He et al. 2008; Mortazavi et al. 2008; Sultan et al. 2008; van Bakel et al. 2010, 2011).

The new findings support noncoding transcription in intergenic regions with a correlation with chromatin signatures, histone modifications or transcription factor binding at loci and dependence of expression level of those noncoding on these transcription factors (Guttman et al. 2009, 2011; van Bakel et al. 2010; Encode Project Consortium 2012). Although many novel, conserved long noncoding RNAs (lncRNAs) have been identified, the number of reported lncRNAs are only a few thousand, which is not enough to explain the "C-value enigma" - 70–90% of the genome. Although the idea of "transcriptional noise" (Hüttenhofer et al. 2005) is still more powerful in the field, even in the early 1990s several lncRNAs involved in epigenetic regulation, H19 (Brannan et al. 1990) and Xist, were discovered (Brockdorff et al. 1992; Brown et al. 1992). Calculations have shown that up to 90% of Pol II transcription can initiate non-specifically and this transcription can be spurious (Struhl 2007). During transcription there is a tendency to fluctuate away from "legitimate" transcripts – there are leaky expressions of neighboring regions (Ebisuya et al. 2008)

1.3.1. Classification of lncRNAs

LncRNAs can be grouped into five large groups according to their genomic locations: stand-alone, natural antisense, pseudogenes, intronic and divergent (Figure 1.2). The stand-alone lncRNAs do not overlap protein-coding genes and also called as "lincRNAs" (large intergenic noncoding RNAs) (Guttman et al. 2009; Cabili et al. 2011; Ulitsky et al. 2011). The majority of identified members has an average length of 1 kb and transcribed by RNA Pol II, polyadenylated, and spliced. *Xist* (Brockdorff et al. 1992; Brown et al. 1992), *H19* (Brannan et al. 1990), *HOTAIR* (Rinn et al. 2007) and *MALAT1* (Ji et al. 2003) are the most known members.

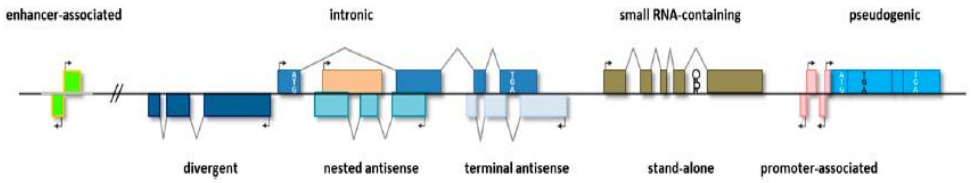

Figure 1.2. Genomic contexts of lncRNAs. lncRNAs may be stand-alone transcription units, or they may be transcribed from enhancers (eRNAs), promoters (TSSa-RNAs, uaRNAs, pasRNAs, and PROMPTs), or introns of other genes (in this case a protein-coding gene, with start codon ATG and stop codon TGA in white); from pseudogenes (shown here with a premature stop codon TGA in black); or antisense to other genes (NATs) with varying degrees of overlap, from none (divergent), to partial (terminal), to complete (nested). lncRNAs may also host one or more small RNAs (black hairpin) within their transcription units. (Source: Kung et al. 2013).

It is reported that up to 70% of sense transcripts have antisense counterparts (Katayama et al. 2005; He et al. 2008; Faghihi and Wahlestedt 2009). Sense-antisense (SAS) pairs can overlap completely, partially or nested within (Figure 1.2). Natural antisense transcript (NAT) lncRNAs tend to locate around the promoter or terminator regions (end) of the sense transcripts. However, there are known non-coding/ non-coding SAS pairs like *Xist/Tsix* (Lee et al. 1999a) as well as coding- non-coding pairs, such as *Kcnq1/Kcnq1ot1* (Kanduri et al. 2006) and *Igf2r/Air* (Lyle et al. 2000). Unlike stand-alone lncRNAs fewer of the newly discovered NATs are spliced or polyadenylated. And the expression of SAS pairs is rather intercorrelated than expected

by chance alone, however, whether all have any biological function or not still remains to be investigated (Kung et al. 2013).

Pseudogenes are the "relics" of coding genes that have lost their coding potential due to nonsense, frameshift, and other mutations (Balakirev and Ayala 2003; Pink et al. 2011). They are extra copies of genes by tandem duplication or retrotransposition and they are no longer transcribed, dead. However, a small portion (2-20%) is transcribed and rarely translated. It is thought that expressed pseudogenes are on the way to die, complete pseudogenization, or dead pseudogenes have gained new functions (Harrison et al. 2005; Bekpen et al. 2009). Interestingly, some transcribed pseudogenes have been shown to regulate expression of their ancestral coding genes. *Xist* is believed to be formed by the pseudogenization of *Lnx3* (a protein-coding gene) and integration of different transposon-derived repeat elements (Duret et al. 2006; Elisaphenko et al. 2008).

It was known that small ncRNAs such as snoRNAs and miRNAs are transcribed from introns. Recent studies show similar transcription of lncRNAs from annotated genes (Louro et al. 2009; Rearick et al. 2011). Although few of them have been studied detailed, many of them were differently expressed in various conditions and in cancer (Guil et al. 2012).

There are plenty of short transcripts (20 to 2500 nt) transcribed from neighborhood in the vicinity around transcription start sites in both sense and antisense directions (Buratowski 2008; Core et al. 2008; He et al. 2008; Preker et al. 2008; Seila et al. 2008). Transcription start site-associated (TSSa-)RNAs are the shortest transcripts among them and believed to be degradation products or they are processed from the longer upstream antisense (ua)RNAs or promoter upstream transcripts (PROMPTs). They are usually capped and polyadenylated, have low copy number (0.1 copy per cell), and are exposed to rapid degradation by exosomes. A subgroup called promoter-associated short (pas)RNAs were shown to interact with epigenetic factors such as Polycomb proteins. It is still unclear whether these transcripts are transcriptional by-products, whether they help maintain open chromatin, or whether they all play a regulatory role like (pas)RNAs (Kanhere et al. 2010). In addition to promoters, short bidirectional transcripts are also shown to be transcribed from enhancers. However, up to date no known biological function has been associated with them (Kim et al. 2010; Wang et al. 2011a).

1.3.2. Functional roles of lncRNAs

Currently, our understanding about functional roles of lncRNAs is very limited although some lncRNAs were functionally characterized. A very limited number of lncRNAs has shown important roles in various processes and studies showed that differential expressed lncRNAs are associated with developmental processes and disease states. However, a majority of lncRNAs require further investigation (Kung et al. 2013).

Currently, the best-studied biological function for lncRNAs is epigenetic regulation of allelic expression. Some of them play role in the processes of dosage compensation and genomic imprinting. The 17-kb X (inactive)-specific transcript (*Xist*) is highly expressed from a cluster of lncRNA loci, the X-inactivation centre (Xic), in inactive X (Xi) chromosome which coats the X chromosome, forms an "*Xist* cloud" and acts as a scaffold for the recruitment of silencing factors (Polycomb repressive complex 2 (PRC2) and etc.) during X chromosome inactivation (XIC) (Lyon 1961; Brown et al. 1991; Brown et al. 1992; Clemson et al. 1996; Zhao et al. 2008; Lee 2011).

Another important role of lncRNAs is in genomic imprinting, a phenomenon when a gene is expressed monoallelically compared to its parent of origin (Edwards and Ferguson-Smith 2007; Wan and Bartolomei 2008). There are specific genome loci, imprinting control regions, like in XIC where many lncRNAs are expressed. Both protein coding and lncRNAs are reciprocally expressed from many of such regions and lncRNAs may control the imprinted expression of neighbouring coding genes by recruiting epigenetic factors (Nagano et al. 2008; Pandey et al. 2008; Zhao et al. 2010).

Other than epigenetic regulation, lncRNAs play role during other aspects of development, from the control of pluripotency to lineage specification. Pluripotency transcription factors (e.g., Oct4, Sox2, and Nanog) are regulated by lncRNAs (Hawkins and Morris 2010; Ng et al. 2011). A number of lncRNAs (HOTAIR, HOTTIP and Mistral) are encoded within Hox genes, which are important for anterior–posterior pattern formation, regulate expression of Hox genes (either the host or a distant cluster) (Pearson et al. 2005; Rinn et al. 2007; Bertani et al. 2011; Wang et al. 2011b).

Numerous lncRNAs are associated with several diseases, especially cancer (Gutschner and Diederichs 2012). *PCAT-1*, *ANRIL*, *HOTAIR* and *MALAT1* lncRNAs are upregulated in several cancer cell types and contribute to cancer progression (Ji et

al. 2003; Gupta et al. 2010; Kotake et al. 2011; Lin et al. 2011; Prensner et al. 2011). There are several lncRNAs that play role in DNA damage and eventually in apoptosis, *lincRNA-p21* and *PANDA*, upregulated by p53 upon DNA damage (Huarte et al. 2010; Hung et al. 2011).

1.3.3. LncRNAs: Act of Mechanism

There are very few lncRNAs characterized in mechanistic details although we know many of them. According to current knowledge, they are categorized into some groups. However, in future, due to new discoveries in the field, we can have additional groups and themes about them. One of the major themes of lncRNAs is playing role in epigenetics as recruiters, tethers, and scaffolds. They mediate recruitment of protein factors for regulation of chromatin states via acting *cis*, acting on neighbor genes in the periphery of their site of synthesis; or acting in *trans*, acting on distant genes in the same or even in another chromosome (Campos and Reiberg 2009). Chromatin-modifying complexes, such as PRC2, are shown to interact with massive number of lncRNAs (Khalil et al. 2009; Kanhere et al. 2010; Zhao et al. 2010; Guil et al. 2012).

Due to some features of lncRNAs, they are excellent candidates for *cis*-acting tethers, but still *trans*-action is not defined yet. In X chromosome inactivation (XCI), tethering *Xist* RNA to the *Xic* is an example for tethering (Jeon and Lee 2011). Some other epigenetic complexes, other than PRC2, may interact with lncRNAs and some lncRNAs act as scaffold where multiple proteins can assemble (Yap et al. 2010; Kotake et al. 2011). Beside the epigenetic complexes, lncRNAs may recruit transcription factors to activate certain genes in *cis* (Bertani et al. 2011; Wang et al. 2011b). Long ncRNAs may modulate DNA methylation at CpG dinucleatides during epigenetic regulation for the stable repression of genes (Law and Jacobsen 2010). DNA methylation of ribosomal (r)DNA, which some remain always silenced by heterochromatic histone marks and DNA methylation, is also directed by certain lncRNAs (McStay and Grummt 2008).

Regulation of gene expression by lncRNAs can be directly affecting the process of transcription. They can act as decoys for TFs or competing for TF binding, and even affect the cellular localization of TFs (Willingham et al. 2005; Hung et al. 2011). Long ncRNAs can act as transcriptional coregulators and they recruit regulators which in turn

carry out their function on downstream targets by recruiting additional factors (Lanz et al. 1999, 2002). In addition to TFs, lncRNAs can affect gene expression by directly interfering with Pol II activity by preventing formation of preinitiation complexes via DNA:RNA triplex formation on promoter or binding with general transcription factors (Yakovchuk et al. 2009; Martianov et al. 2007).

Long ncRNAs play role as key regulators of nuclear compartments - "nuclear bodies" that exert important functions (Mao et al. 2011b). Long ncRNAs are linked to the function and structure of the members of nucleolus, paraspeckles and other nuclear compartments (Zhang et al. 2007; Chen and Carmichael 2009). Certain lncRNAs (MALAT1 or NEAT2) mediate proper localization of splicing factors to nuclear speckles and thus may have role in alternative splicing of certain mRNA precursors (Bernard et al. 2010; Tripathi et al. 2010). Thus, there is a complex interaction among lncRNAs, cell-signaling pathways, chromatin-modifying factors, and nuclear bodies in regulating gene expression.

Several studies have shown diverse functions of lncRNAs in mRNA processing, stability and translation including alternative splicing. Natural antisense (NAT) lncRNAs may affect alternative splicing of overlapping transcripts by forming RNA duplexes that inhibit splicing which is a type of post-transcriptional regulation (Beltran et al. 2008; Annilo et al. 2009). NATs, mainly produced from the 3'-UTR, may be involved in stability of its antisense by recruiting factors that lead to stabilization or destabilization of the transcripts (Kim et al. 2005, 2007; Barreau et al. 2006). LncRNAs, specifically NATs, may even play role in translational regulation of their targets, specifically on their sense mRNAs, by competing for binding to the certain translation initiation factors (Ebralidze et al. 2008).

It is not surprising that long non-coding RNAs are intertwined with small non-coding RNAs. Certain lncRNAs were shown to interfere with miRNA-mediated mRNA destabilization by masking miRNA-binding sites or competing for the miRNAs themselves (Faghihi et al. 2010, Wang et al. 2010). Some lncRNAs have miRNA-binding sites in their 3'-UTRs which can serve as "sponge" to keep miRNAs away from their mRNA targets (Franco-Zorrilla et al. 2007). On the other hand, lncRNAs may themselves be host genes for small RNAs, such as miRNA and snoRNAs (Smith and Steitz 1998; Cai and Cullen 2007; Keniry et al. 2012).

1.3.4. Long-Coding RNAs in Apoptosis

As stated above, certain lncRNAs are differentially expressed in several diseases and cancer. Some of them are playing role in apoptosis as well. There are known negative and stimulatory regulators of apoptosis - anti- and pro-apoptotic lncRNAs. lncRNAs like PCGEM1 (Fu et al. 2006), LincRNA-EPS (Paralkar et al. 2011), PANDA (Puvvula et al. 2014), AFAP1-AS1 (Wu et al. 2013), SPRY4-IT1 (Khaitan et al. 2011), PlncRNA-1 (Cui et al. 2013) and HOXA-AS2 (Zhao et al. 2013) are anti-apoptotic. They are upregulated in cancer cells and often play a role in tumor survival and progression. On the other hand, certain lncRNAs, such as lincRNA-p21 (Wu et al. 2014), GAS5 (Pickard et al. 2013), ncRNA CCND1 (Wang et al. 2008a), MEG3 (Zhang et al. 2014), INXS (DeOcesano-Pereira et al. 2014), LOC401317 (Gong et al. 2014), are pro-apoptotic and are down-regulated in certain cancer types.

Although numerous lncRNAs playing role in apoptosis are known, however, there is not any systematic study intended to identify the total number of lncRNAs playing role in apoptosis. The current known anti- and pro-apoptotic lncRNAs were discovered under specific conditions. Pathway-specific or master regulators require a systematic approach to demonstrate lncRNA function in apoptosis.

1.4. Aim

The aim of the project is to identify differentially expressed lncRNAs via deep sequencing under apoptotic conditions in HeLa cells.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell Culturing

HeLa cells were obtained from DSMZ GmbH and were cultured in RPMI 1640 (with L-Glutamine, Gibco) in a humidified incubator with 5% CO2 in air at 37°C. The cell culture medium was supplemented with 10% inactivated fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco). HeLa cells were seeded every two days with 1/3 or 1/4 ($2.0 – 2.5 \times 10^6$ cells).

Drug treatments were performed using 6-well plate (Sarsted). HeLa cells were seeded 0.3×10^6 cells/well density. Overnight grown HeLa cells in 6-well plates were incubated with agents in time- and dose-kinetics experiments. The entire drug screening experiments were performed at least three times and the results were analyzed with student's t-test to show whether changes statistically significant.

Cisplatin (SantaCruz) was freshly prepared in DMSO as 83.2 mM stock in every drug screening experiment due to its chemical instability. Cisplatin concentration varying 2 μM up to 320 μM were screened for 4 hours up to 24 hours. Subsequent experiments were set to 80 μM for 16 hours. Due to toxic effect of DMSO, one more control was set as DMSO control.

Doxorubicin (Cell Signaling) was dissolved in DNase and RNase free water and was prepared as 5mM stock, aliquoted and stored at -20 °C. Doxorubicin concentration varying 0.0625 μM up to 32 μM were screened for 4 hours up to 24 hours. Subsequent experiments were set to 4 μM and 4 hours.

Fas mAb (Cell Signaling) concentration ranging from 0.125 μg/ml up to 2 μg/ml for 4 hours up to 24 hours were tested and subsequent experiments were set up for 0.5 μg/ml and 16 hours.

TNF-alpha ligands (Millipore) were dissolved in DNase and RNase free water and prepared 100 ng stock, was aliquoted and stored at -20 °C. Cycloheximide (CHX) (Applichem) was coupled with TNF-alpha due to type II cell feature of HeLa. Less cytotoxic concentration of cycloheximide was determined by screening cycloheximide

concentration ranging 5 µg to 80 µg for 4 to 24 hours. TNF-alpha coupled with cycloheximide with different concentrations, 1 ng/ml up to 125 ng/ml for 4 hours up to 24 hours were screened and subsequent experiments were set to 125 ng/ml TNF-alpha with 10 µg CHX for 8 hours. In addition to negative untreated control, TNF-alpha and cycloheximide alone were used as negative controls as well.

2.2. Measurement of Apoptosis

Time- and dose-kinetics were carried out for all drugs and were analyzed with Flow Cytometry. Annexin V and 7AAD (BD) were used in detection of apoptosis and all experiments/doses were repeated at least 3 times. Annexin V was diluted 1:5 with PBS and 7AAD was diluted 1:10 with PBS as well. Drug treated and untreated cells were harvested with Trypsin-EDTA (Gibco, 0.25%) and washed twice with ice-cold PBS. After removal of PBS from last wash, cells were suspended in 200-300 µl annexin binding buffer (BD) and 50 µl of each cell suspension was added into eppendorf. Further, 5 µl from Annexin V and 7AAD were added into eppendorf as well, after 15 min incubation in dark, cells were suspended again in 200 µl PBS prior to analysis with Flow Cytometry (Applied Biosystems or BD FACS). Cells with Annexin V signal were considered to be at the early stage of apoptosis. Cells with both Annexin V and 7AAD signal were considered to be at the late onset of apoptosis. Dead cells were only 7AAD positive and live cells were both Annexin V and 7AAD negative.

The efficiency of fluorescent labeling was further verified with Fluorescence Microscope by the help of same markers, Annexin V and 7AAD. The same procedure for Flow Cytometry was followed, however, cells were analyzed under microscope. After incubation in the dark, 10 µl of cell suspension was spread on clean chamber and covered with cover slide and analyzed with Fluorescence Microscope (Filter 2 and 4, Olympus IX70)

2.3. Total Protein Purification

Total protein extracts were prepared by using RIPA lysis buffer (Cell Signaling). Drug treated and untreated cells were harvested with Trypsin-EDTA and washed twice with ice-cold PBS. Cells were lysed with RIPA (50 µl per 10^6 cells) , Protease Inhibitor

Cocktail (100X) (SantaCruz) was added immediately after and cell lysates were kept on ice up to 20 min with vortexing every 5 min. Lysates were centrifuged for 10 minutes at 14.000 rpm at 4 °C. Supernatants aliquoted into two or three eppendorf tubes and stored at -80 °C.

In order to determine protein concentration, Bradford Assay was used. Standard curve standard was drawn with 40 µl of different BSA (bovine serum albumin) concentrations ranging between 20 and 200 µg/ml in Bradford reagent (0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, 8.5% (w/v) phosphoric acid) in order to relate protein concentration with absorbance (595 nm). Equation obtained from standard curve was used to estimate protein concentration from absorbance reads of samples. Protein samples were diluted 1:10, 4 µl diluted with RIPA to 40 µl and added into 1.5 ml of Bradford reagent into cuvettes. After 5 min dark incubation, OD of each sample was detected immediately with spectrophotometer.

2.4. Western Blotting

The potential effect of all drug treatments on caspase activation was determined biochemically by western blotting. Caspase 3, 8 and 9, and β-Actin monoclonal (mouse) antibodies were purchased from Cell Signaling. Caspase 3 activation shows induction of apoptosis; specifically, caspase 8 activation indicates induction of extrinsic apoptotic pathway while caspase 9 activation indicates induction of intrinsic pathway.

Protein amount was fixed to 20 µg per well and protein samples with protein loading dye was heated for 5 minutes at 90 °C. Protein samples were run in two 15% separating and 5% stacking SDS gel [dH20, separating/stacking buffer, 30% Acrylamide (Sigma), 10% SDS (Applichem), TEMED (Sigma), 10% APS (Applichem)] vertically for two hours at 100V in running buffer (25 mM Tris, 192 mM Glycine, 1% SDS (w/v)).

One of the gels was used to stain with Coomassie Blue solution [1 mg/ml Coomasie Blue (Sigma), 10% acetic acid (v/v), 30% methanol (v/v)] in order to check proper running and verify presence of proteins. Incubation of gel at room temperature with Coomassie Blue for an hour followed incubation with Coomassie Destaining solution [10% acetic acid (v/v) and 30% methanol (v/v)] for an hour in order to visualize total protein bands. On the other side, the other gel was run for blotting; gel

was placed between Whatman paper and methanol activated PVDF membrane (Millipore). Proteins were transferred to PVDF membrane at 30V overnight. Transfer of proteins onto PVDF membrane was tested with Ponceau S [0.1% (w/v) Ponceau S in 5% (v/v) acetic acid] staining for 30 min and destaining with ultrapure water for 30 min.

Membrane was blocked with blocking buffer (1X TBS, 0.05% Tween20, 0.5% non-fat dry milk) for an hour at room temperature. Caspase 8 and 9, and β-Actin primary antibodies were diluted 1:5000 whereas Caspase 3 mAb was diluted 1:7000 in wash buffer (1X TBS, 0.05% Tween20) including 10% blocking buffer and incubated for an hour at room temperature with gentle shaking with shaker. After primary antibody incubation, membrane was washed with wash buffer 5 times for 45 min and incubated with gentle shaking for an hour with HRP-conjugated anti-mouse secondary antibody (Cell Signaling) with 1:20000 ratio in wash buffer including 10% blocking buffer. The wash step was repeated after the addition of secondary antibody as well. The membrane was then prepared for visualization via adding SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) on membrane; 500 µl enhanced chemiluminescent substrate for HRP and 500 µl enhancer solution were mixed and spread on membrane, and incubated for 2 minutes. Chemiluminescence from membranes was visualized with VersaDoc MP 4000 Molecular Digital Imaging System (BioRad) in Biotechnology and Bioengineering Research and Application Center at Izmir Institute of Technology or Fusion SL (PEQLAB).

2.5. Total RNA Isolation and RNA-Seq

Cells were grown overnight in 75cm^2 Flasks prior to treatment. Drug treated and untreated cells were harvested with Trypsin-EDTA and washed twice ice cold PBS. After the complete removal of PBS from the last wash step, 1 ml TRIzol (Life Technologies) was used to dissolve each pellet and cell lysate was stored at -80 °C. RNA isolation from the cell lysate was performed within one week and the protocol from manufacturer was followed.

Frozen cell lysates were thawed and was incubated for 5 minutes at room temperature to facilitate complete dissociation of the nucleoprotein complex. 0.2 ml of RNase free chloroform (Sigma) was added per 1 ml of TRIzol for homogenization. After vigorous shaking by hand for 15 seconds, tubes were incubated for 2-3 min at

room temperature (RT). Centrifugation of samples at 12,000 × g for 15 minutes at 4°C led phase separation; aqueous phase was pipetted out into new eppendorf by angling the tube at 45° without disturbing middle and down phase. 0.5 mL of 100% RNase free isopropanol (Sigma) was added per 1 mL of TRIzol for homogenization and incubated at RT for 10 minutes. Further, samples were centrifuged at 12,000 × g for 10 minutes at 4°C and supernatant was removed after. Pellet was washed with 1 mL of 75% RNase free ethanol (Sigma) per 1 mL of TRIzol in the initial homogenization. After a short vortex, samples were centrifuged at 7500 × g for 5 minutes at 4°C and supernatant wash was discarded. Pellet RNA samples were dried in air for 5-10 minutes, were dissolved with DNase and RNase free water, aliquoted and kept at -80 °C.

Initial RNA quality control was checked by NanoDrop (Thermo Scientific) and by running on 1% agarose gel. 1 µl from each RNA sample was used in NanoDrop and 260/280 and 260/230 ratios were obtained for initial RNA purity and quality (for "pure" RNA 260/280 ratio is ~2, 260/230 ratio is ~2.0-2.2). 1 µg RNA was mixed with gel loading dye (2X), heated for 2 min at 85 °C and directly was kept on ice for next 2 min. RNA was run in TBE buffer (Tris-borate-EDTA buffer, 1M Tris base, 1M Boric acid and 0.02M EDTA) for 30 min at 100V. Gels were visualized with AlphaImager (The AlphaImager High Performance Gel Documentation and Image Analysis System, Model IS-2200) for 5 to 15 sec with UV light filter.

Total RNAs from three replicates of selected doses from four drugs with control untreated cells were sent for deep sequencing by Fasteris SA (Switzerland) using Illumina Platform. Totally 5 µg from each RNA sample was sent for initial Quality Check (QC) and RNA-Seq was performed using a specific method based on identification of long non-coding RNAs.

2.6. Bioinformatics Analyses

The output from RNA-Seq was analysed by Allmer lab (collaboration). Firstly, the output (fastq) files were subjected to Quality Control via FasQC. Then, adaptor and quality trimming was done via cutadapt and Sickle tools, respectively. The output was then mapped to the human GRCh38 genome as a reference. Further, read counting was done via using HTSeq Count, and normalization of reads, RPKM (Reads per kilo base

per million) values and detection differentially expressed genes were obtained via DESeq2 tool.

Stringent filters were set to detect meaningful expression differences: two-fold and upper, and P<0.01. Candidates were chosen from top 20 up- and down-regulated lncRNAs that were commonly differentially expressed upon treatment with four agents.

2.7. Flow Chart of Overall Approach

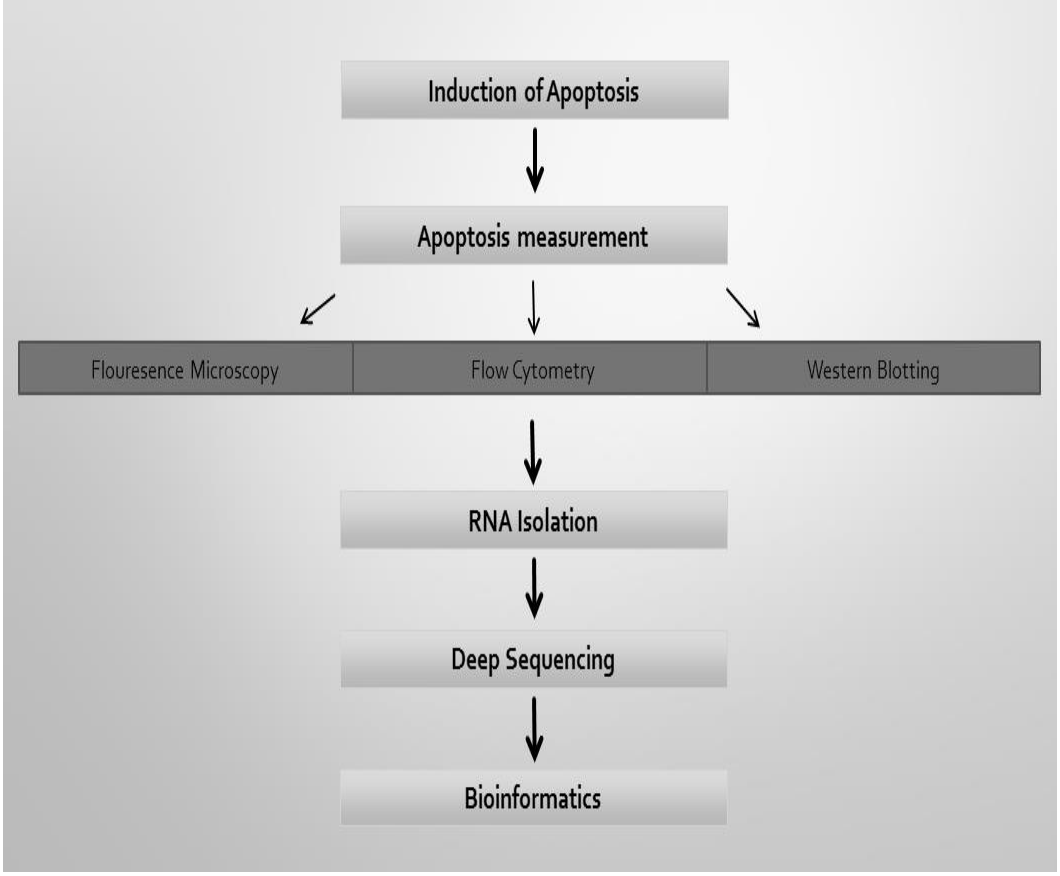

Figure 2.1. Flow Chart of Overall Approach. The figure illustrates the flow of the project.

CHAPTER 3

RESULTS

3.1. Drug Dose-kinetics

Cisplatin caused severe cell death at varying doses (Figure 3.1, a). Control and DMSO (0.4%) showed minimal cellular death (7%) whereas the 40 µM, 80 µM, 160 µM and 320 µM cisplatin doses caused 19%, 46%, 71% and 88% cell death. The moderate effect dose, 80 µM drug concentration, was selected for subsequent experiments.

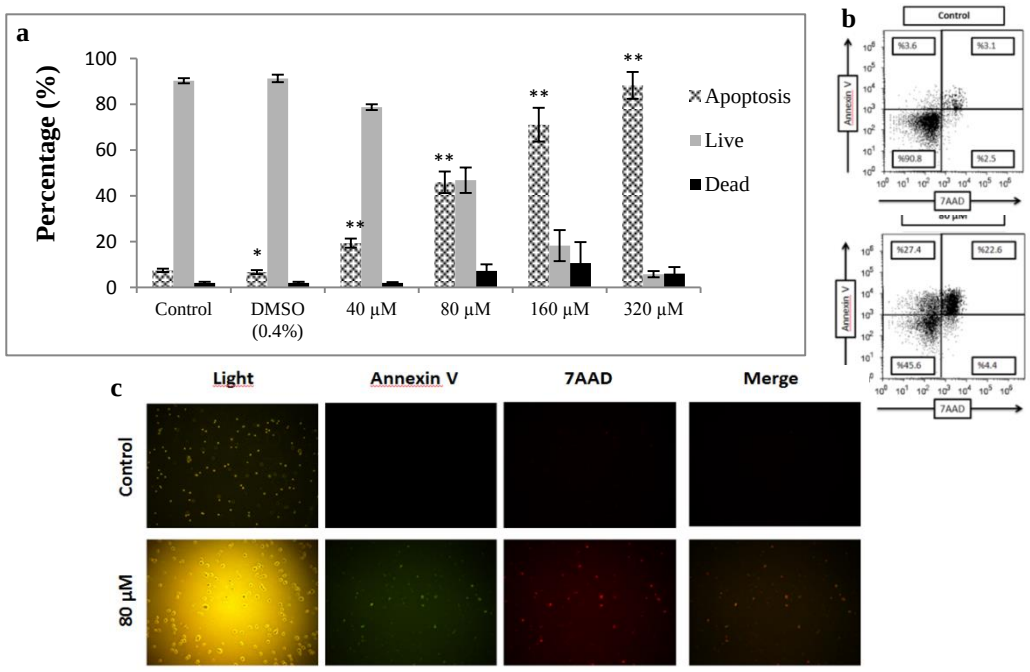

Figure 3.1. Cisplatin Dose-Kinetics. Cisplatin causes cell death with dose-dependent manner on HeLa cells for 16 hours. (a) Flow Cytometry analysis of cisplatin screening via Annexin V and 7AAD staining. Apoptotic cells were Annexin $V^+/7AAD^-$ and Annexin $V^+/7AAD^+$; dead cells were Annexin $V^-/7AAD^+$; and live cells were Annexin $V^-/7AAD^-$. (b) Dot Plot figures of cisplatin treated (80 µM) and control (0.4% DMSO) cells. (c) Fluorescence Microscopy analysis of cisplatin treated (80 µM) and control (0.4% DMSO) cells via Annexin V and 7AAD staining. Statistical analysis (student t-test) suggested that change in apoptotic rate between control and DMSO control was not significant - (*) $P>0.05$ unlike between control and doses or to each other - (**) $P<0.05$.

Doxorubicin treatment of HeLa was challenging due to its unique way of death induction. Cells were shifting directly from live cell quadrant (Annexin $V^-/7AAD^-$) to double positive late apoptosis quadrant (Annexin $V^+/7AAD^+$). At lower concentration of doxorubicin (below 1 μM), cells could not be gated properly due to shifting and overflow of population to other quadrants. At a drug concentration above 1 μM, cells became 98% double positive and hence showed no significant difference. The subsequent experiments were performed at 4 μM due to its clear shift to double positive quadrant (Figure 3.2, b-2).

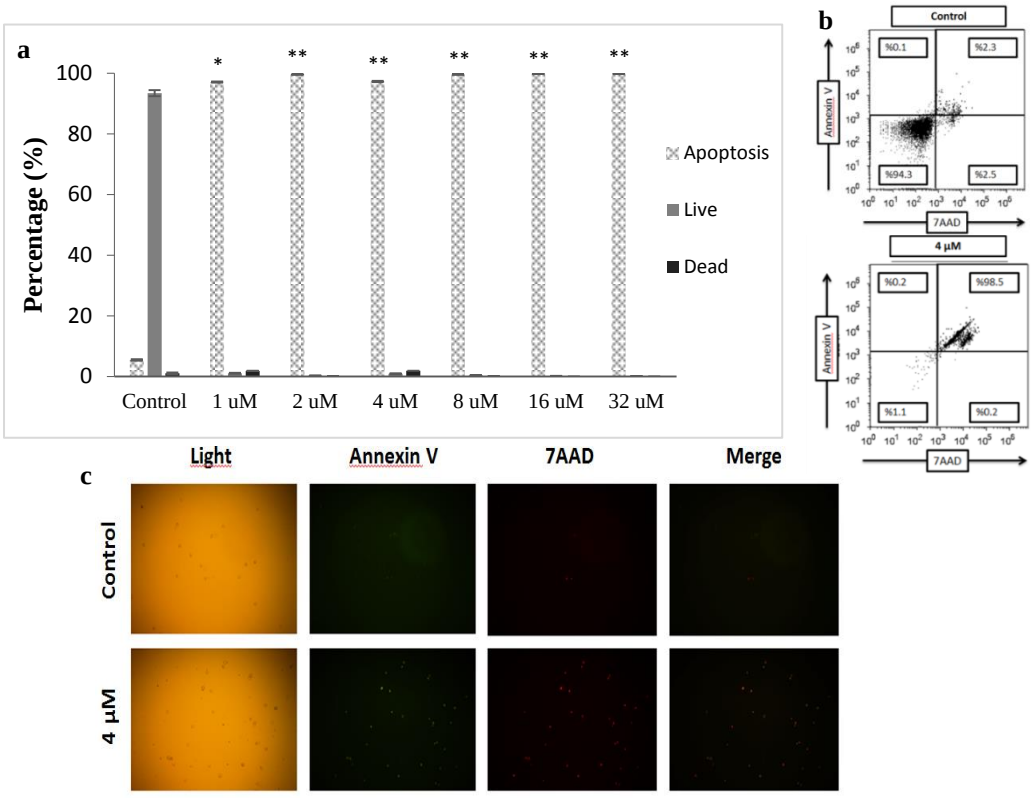

Figure 3.2. Doxorubicin Dose-Kinetics. Doxorubicin causes severe cell death with dose-independent manner between 1 μM and 32 μM for 4 hours on HeLa cells. In smaller doses, below 1 μM , cells could not be gated properly due to shifting and overflow of population to other quadrants Thus, subsequent experiments were set to 4 μM due to its clear shift between quadrants. (a) Flow Cytometry analysis of doxorubicin screening via Annexin V and 7AAD staining. Apoptotic cells were Annexin $V^+/7AAD^-$ and Annexin $V^+/7AAD^+$; dead cells were Annexin $V^-/7AAD^+$; and live cells were Annexin $V^-/7AAD^-$. (b) Dot Plot figures from Flow Cytometry analysis of control and doxorubicin treated (4 μM) cells. (c) Fluorescence Microscopy analysis of doxorubicin treated (4 μM) and control cells via Annexin V and 7AAD staining. Statistical analysis (student t-test) suggested the change in apoptotic/cell death rate between control and 1 μM was significant (*) $P<0.01$, however, not significant (**) $P>0.05$ among doses from 1 μM to 32 μM.

Anti-Fas treatment of HeLa cells resulted in dose-independent mild cell death due to limited expression of FasR (Figure 3.3). Doses varying from 0.125 µg/ml to 2 µg/ml showed very slight increase, from 16% to 23%, thus 0.5 µg/ml was selected for 16 hours in subsequent experiments.

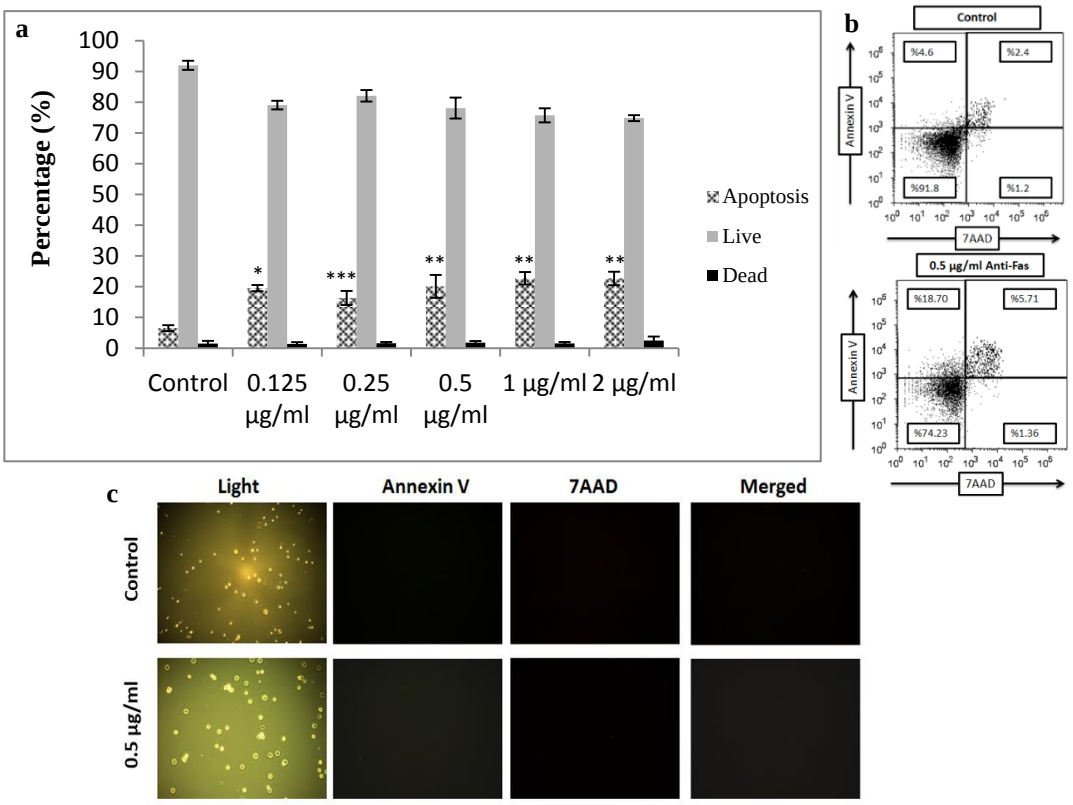

Figure 3.3. Anti-Fas mAb Dose-Kinetics. Anti-Fas mAb causes mild cell death with dose-independent manner between 0.125 µg/ml and 2 µg/ml concentrations for 16 hours on HeLa cells due to lower expression of FasR. Subsequent experiments were set to 0.5 µg/ml concentration. (a) Flow Cytometry analysis of Anti-Fas mAb screening via Annexin V and 7AAD staining. Apoptotic cells were Annexin $V^+/7AAD^-$ and Annexin $V^+/7AAD^+$; dead cells were Annexin $V^-/7AAD^+$; and live cells were Annexin $V^-/7AAD^-$. (b). Dot Plot figures from Flow Cytometry analysis of anti-Fas treated (0.5 µg/ml) and control cells. (c) Fluorescence Microscopy analysis of anti-Fas treated (0.5 µg/ml) and control cells via Annexin V and 7AAD staining. Statistical analysis (student t-test) suggests that the change in apoptotic rate between control and the 0.125 µg/ml is significant, (*) $P<0.05$, except the change between 0.25 µg/ml and 1 and 2 µg/ml, (***) $P<0.05$, the rest changes between doses are not significant, (**) $P->0.05$.

TNF-alpha alone had no effect on HeLa cells and coupling with cycloheximide sensitized HeLa cells to TNF-alpha. Cycloheximide concentration was adjusted to 10 µg/ml and TNF-alpha dose kinetics was done with varying doses of TNF-alpha (Figure 3.4). Compared to control, increase in drug concentration led to a slight increase in cell death; 1 ng/ml, 5 ng/ml, 25 ng/ml and 125 ng/ml showed 10%, 25%, 29% and 36%, respectively. 125 ng/ml was chosen due to moderate and effective efficacy on cell death.

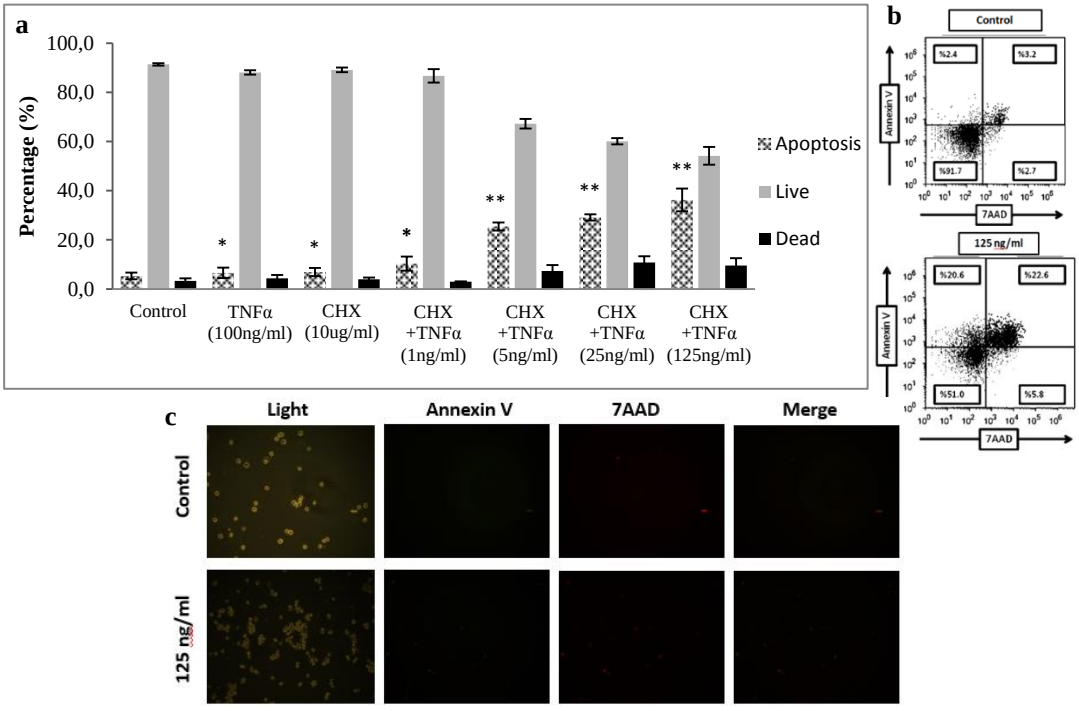

Figure 3.4. TNF-alpha Dose-Kinetics. TNF-alpha alone had no effect on HeLa cells and coupling with cycloheximide sensitized HeLa cells to TNF-alpha. TNF-alpha with cycloheximide (CHX, 10 µg/ml) causes moderate death with dose-dependent manner between 1 ng/ml and 125 ng/ml for 8 hours on HeLa cells. Subsequent experiments were set to 125 ng/ml due to its moderate cell death. Note that cycloheximide and TNF-alpha alone were chosen as negative controls and they did cause cell death compare to control. (a) Flow Cytometry analysis of TNF-alpha screening via Annexin V and 7AAD staining. Apoptotic cells were Annexin $V^+/7AAD^-$ and Annexin $V^+/7AAD^+$; dead cells were Annexin $V^-/7AAD^+$; and live cells were Annexin $V^-/7AAD^-$. (b) Dot Plot figures from Flow Cytometry analysis of TNF-alpha treated (125 ng/ml) and control (10 µg/ml CHX) cells. (c) Fluorescence Microscopy analysis of TNF-alpha treated (125 ng/ml) and control (10 µg/ml cycloheximide, CHX) cells via Annexin V and 7AAD staining. Statistical analysis (student t-test) suggests that the changes in apoptotic rate between control and TNF-alpha only, cycloheximide (CHX) and 1ng TNF-alpha-CHX were not significant as well as the change among 5ng TNF-alpha-CHX, 25ng TNF-alpha-CHX and 125ng TNF-alpha-CHX, (*) $P>0.05$. However, the change between control and 5, 25 and 125ng TNF-alpha-CHX is significant, (**) $P<0.05$.

3.2. Western Blotting

The western blotting analysis was performed to investigate the effect of each drug dose on initiator (8 and 9) and effector (3) caspase at specific times. According to our result and our experimental settings, 4 hours doxorubicin treatment (4 μM) showed very slight cleavage of effector caspase 8 and 9 caspases, but not executioner caspase 3. On the other hand, cisplatin (80 μM, CP, 16 hours), TNF-alpha (125 ng/ml, TNF, 8 hours) and anti-Fas (0.5 μg/ml, 16 hours) treatments led to the cleavage of caspase 8. Caspase 9 cleavage was detected upon TNF-alpha and anti-Fas treatment, but not cisplatin. Anti-Fas treatment dose and timing was sufficient to detect caspase 3 cleavage, however we could not detect active caspase 3 at given time and dose for cisplatin and doxorubicin. Neither procaspase 3 nor active caspase 3 could be detected upon TNF-alpha treatment under our experimental settings.

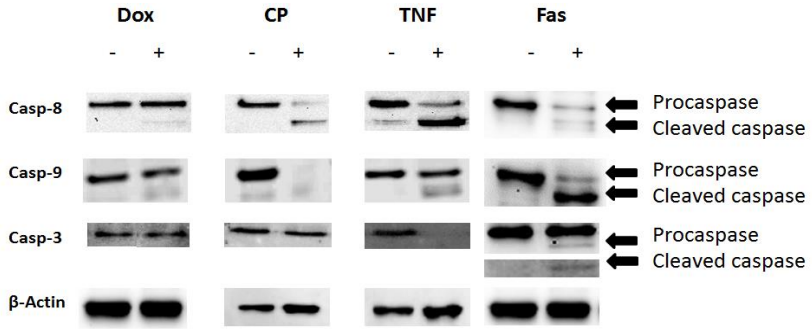

Figure 3.5. Western Blotting Analysis of All Agents. Western Blotting analysis was further performed for showing relationship between caspases and chosen dose and time for each drug [doxorubicin (4 μM, Dox, 4 hours), cisplatin (80 μM, CP, 16 hours), TNF-alpha (125 ng/ml, TNF, 8 hours) and anti-Fas (0.5 μg/ml, 16 hours)]. Activation of initiator caspases, procaspase-9 cleavage in activation of intrinsic pathway and procaspase-8 cleavage in activation of extrinsic pathway, and executor caspase, procaspase 3 cleavage, indicate overall induction of apoptosis. Under our experimental design, doxorubicin treatment led slight cleavage of activate effector caspases, but not executioner caspase. Cisplatin, TNF-alpha and anti-Fas treatments led cleavage of procaspase 8 and detection of active caspase 8 fragment, suggesting induction of apoptotic extrinsic pathway. TNF-alpha and anti-Fas treatments led cleavage of procaspase 9 and detection of active caspase 9 fragment, suggesting induction of apoptotic intrinsic pathway. However, neither procaspase 9 nor active caspase 9 detected upon cisplatin under our experimental setting. Caspase 3 activation was detected only upon anti-Fas treatment and neither procaspase 3 nor caspase 3 was detected upon TNF-alpha treatment. β-Actin was used as loading control in comparison of protein concentration in each well.

3.3. Total RNA Isolation and RNA-Seq Quality Control

Total RNAs from three replicates of each drug and control were first run in a 1% Agarose gel and 18S and 28S rRNA bands were detected (Figure 3.5). Prior to library preparation, QC scores (Table 3.1) were detected by BioAnalyzer and showed no degradation. Library preparation gave percentages of primary mapping which were quite high (~96%) (Figure 3.6).

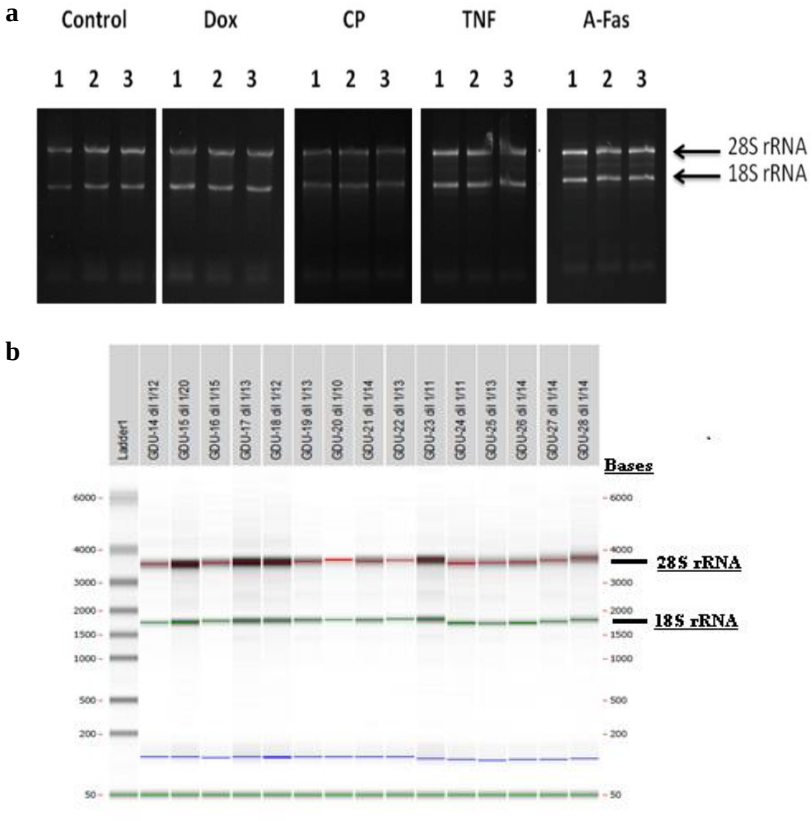

Figure 3.5. RNA Gel and Quality Check (QC) Analysis of Total RNAs. (a) Three replicates (1, 2 and 3) of total RNA extracts from untreated (control) cells, doxorubicin (4 µM, Dox, 4 hours), cisplatin (80 µM, CP, 16 hours), TNF-alpha (125 ng/ml, TNF, 8 hours) and anti-Fas (0.5 µg/ml, A-Fas, 16 hours) treated cells were run in 1% Agarose gel, 1 µg RNA per well. (b) Initial quality check (QC) for total RNA samples of treated and treated replicates. The QC was performed prior to Library Preparation by Fasteris SA Company. All RNA samples had proper quality for library preparation. Note that, RNA samples were analyzed by LabChip® GX Touch microfluidics technology which gives complete assessment of RNA quality for better gene expression data. GDU-14-16 indicates control RNA replicate samples; GDU-17-19 indicates cisplatin replicates; GDU-20-22 indicates doxorubicin replicates; GDU-23-25 indicates TNF-alpha replicates; GDU-26-28 indicates anti-Fas replicates.

Table 3.1. Library Preparation of RNA Samples**.** Library of total RNA replicates of control and -treated cells were prepared. The read and mapping results indicated in the table. The percentage of primary mapping was around 96% percent which is good.

Library	RNA Quality Score	Reads	Primary Mappings	% of Primary Mappings	Alternate mappings
Control (1)	9.3	19,478,665	19,066,837	97.89%	5,031,257
Control (2)	9.2	13,977,569	13,636,817	97.56%	3,451,273
Control (3)	9.4	13,989,348	13,640,586	97.51%	3,476,351
Cisplatin (1)	9.0	16,695,258	15,975,390	95.69%	6,082,994
Cisplatin (2)	9.3	18,871,780	17,888,988	94.79%	7,554,017
Cisplatin (3)	8.9	16,922,803	16,051,158	94.85%	6,122,212
Doxorubicin (1)	7.7	13,255,959	12,888,311	97.23%	3,920,319
Doxorubicin (2)	8.8	13,635,427	13,293,528	97.49%	3,732,917
Doxorubicin (3)	7.9	15,485,199	15,148,239	97.82%	4,883,967
TNF-alpha (1)	8.9	15,720,221	15,243,443	96.97%	4,475,529
TNF-alpha (2)	8.3	19,665,744	18,901,417	96.11%	6,027,820
TNF-alpha (3)	8.6	20,958,445	20,121,618	96.01%	6,417,161
anti-Fas (1)	9.3	22,066,898	21,090,702	95.58%	7,061,540
anti-Fas (2)	8.6	19,332,230	18,460,235	95.49%	5,994,549
anti-Fas (3)	9.1	18,825,067	18,129,644	96.31%	6,015,316

3.4. Bioinformatics Analysis – lncRNA candidates

The bioinformatics analysis identified 24 antisense lncRNAs, 5 sense-intronic lncRNAs and 17 lincRNAs that were significantly up- or down-regulated. Cisplatin, doxorubicin, anti-Fas and TNF-alpha treatment triggered differential expression of drug specific 1644, 506, 584 and 807 lncRNAs, respectively (2-fold and upper, $P<0.01$) (Table 3.2). The HeatMap analysis of identified lncRNAs illustrated the similarity among expressional profiles of each drug. As a result, TNF-alpha and anti-Fas treatment showed a higher similarity and they together were similar to cisplatin. However, doxorubicin showed different lncRNA expression profile and was closer to the profile of control cells (figure 3.7). Top 20 up- and down-regulated lncRNAs common for all drugs were listed and among them four candidates were selected (Table 3.3).

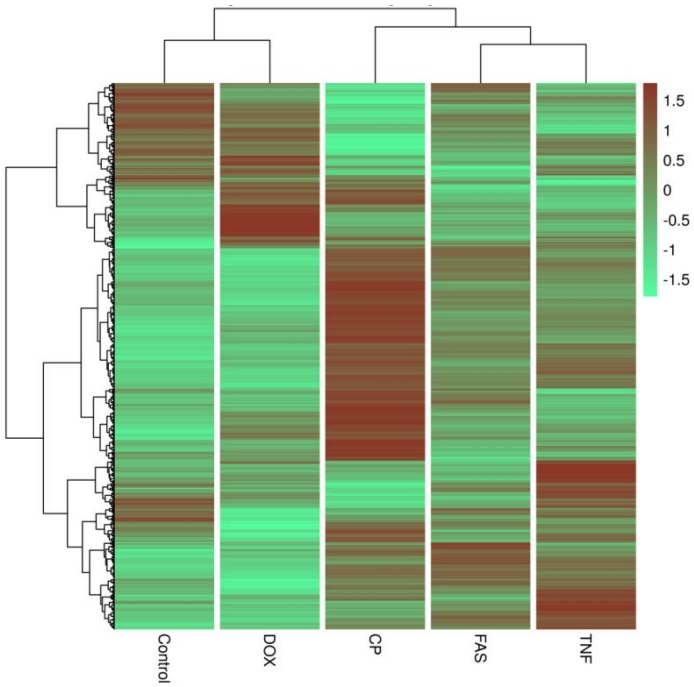

Figure 3.7. Heatmap Analysis of Differentially Expressed lncRNAs. Differentially expressed lncRNAs were clustered using Heatmap analysis; upregulated (dark red, >0) and downregulated (green, <0) lncRNAs were color coded. Clustering of the columns was based on similarity among drugs. Treatments with cisplatin (CP), anti-Fas (FAS) and TNF-alpha (TNF) led to similar differential expressed lncRNAs, so they are alike and anti-Fas and TNF-alpha treatments are even much closer. Treatment with doxorubicin showed difference in lncRNA expression profile compare to the rest three agents and is close to control group of lncRNAs. Clustering of rows was based on expression similarity of individual lncRNAs in each column.

Table 3.2. Types of Identified lncRNAs. The number of differentially expressed lncRNAs are listed in the table below.

	Cisplatin	**Doxorubicin**	**TNF-alpha**	**Anti-Fas mAb**	**Total (common)**
Antisense	851	225	368	284	124
Sense-intronic	121	71	47	26	15
Sense-overlapping	26	15	18	10	3
lincRNA	646	195	374	264	100
Total (drug-specific)	1644	506	807	584	242

Table 3.3. List of Top 20 Common Upregulated and Downregulated lncRNAs. lncRNAs that were significantly up- and down-regulated upon treatment with all agents is listed below with their antisense/sense partner coding genes. The majority top 20 upregulated (a) and downregulated (b) lncRNAs are antisense lncRNAs and their counter genes are protein coding genes playing role in different cellular events and pathways. The lncRNAs in red boxes were chosen as candidates due to their potential to regulate apoptosis.

a

Ensembl ID	Type	Coding Genes in Antisense/Sense Position
ENSG00000273783	antisense	GTF2A1 (general transcription factor IIA, 1, 19/37kDa (TFIIA))
ENSG00000261335	antisense	MXRA7 (matrix-remodelling associated 7)
ENSG00000246130	antisense	TNFRSF10B (tumor necrosis factor receptor superfamily, member 10b)
ENSG00000261360	antisense	OTULIN (OTU deubiquitinase with linear linkage specificity)
ENSG00000227527	sense-overlapping	FOXJ3 (forkhead box J3)
ENSG00000260898	antisense	ADPGK (ADP-dependent glucokinase)
ENSG00000233230	antisense	FBXO11 (F-box protein 11)
ENSG00000260708	antisense	TBC1D22A (TBC1 domain family, member 22A)
ENSG00000266680	antisense	PTP4A1 (protein tyrosine phosphatase type IVA, member 1)
ENSG00000260929	antisense	CYLD (cylindromatosis (turban tumor syndrome))
ENSG00000272969	antisense	CLOCK clock circadian regulator
ENSG00000274270	sense-intronic	DLEU1 (deleted in lymphocytic leukemia 1 (non-protein coding))
ENSG00000273010	antisense	LRIF1 (ligand dependent nuclear receptor interacting factor 1)
ENSG00000270068	antisense	COPS7A (COP9 signalosome subunit 7A)
ENSG00000237436	antisense	CAMTA1 (calmodulin binding transcription activator 1)
ENSG00000259818	antisense	C1orf123 (chromosome 1 open reading frame 123)
ENSG00000275457	antisense	XRN2 (5'-3' exoribonuclease 2)
ENSG00000224349	antisense	FAM135A (family with sequence similarity 135, member A)
ENSG00000261485	antisense	PAN3 (PAN3 poly(A) specific ribonuclease subunit)
ENSG00000232536	sense-intronic	TUFT1 (tuftelin 1)

b

Ensembl ID	Type	Coding Genes in Antisense/Sense Position
ENSG00000228237	antisense	EFCAB14 (EF-hand calcium binding domain 14)
ENSG00000264491	antisense	CACNG4 (calcium channel, voltage-dependent, gamma subunit 4)
ENSG00000236432	antisense	COL4A3 (collagen, type IV, alpha 3 (Goodpasture antigen)) and MFF (mitochondrial fission factor)
ENSG00000233848	antisense	CDKAL1 (CDK5 regulatory subunit associated protein 1-like 1)
ENSG00000244161	antisense	FLNB (filamin B, beta)
ENSG00000272627	sense-intronic	MCU (mitochondrial calcium uniporter
ENSG00000250354	antisense	NR3C2 (nuclear receptor subfamily 3, group C, member 2)
ENSG00000255176	antisense	PHLDB1 (pleckstrin homology-like domain, family B, member 1)
ENSG00000254453	antisense	NAV2 (neuron navigator 2)
ENSG00000231628	antisense	PREP (prolyl endopeptidase)
ENSG00000180139	antisense	ACTA2 (actin, alpha 2, smooth muscle, aorta)
ENSG00000273035	sense-intronic	MAP4K3 (mitogen-activated protein kinase kinase kinase kinase 3)
ENSG00000246859	antisense	STARD4 (StAR-related lipid transfer (START) domain containing 4) and NREP (neuronal regeneration related protein)
ENSG00000230433	antisense	EXOC2 (exocyst complex component 2)
ENSG00000235984	antisense	GPC5 (glypican 5)
ENSG00000227230	antisense	CEP170 (centrosomal protein 170kDa)
ENSG00000225767	antisense	FAF1 (Fas (TNFRSF6) associated factor 1)
ENSG00000250740	antisense	NPNT (nephronectin)
ENSG00000267690	antisense	LDLRAD4 (low density lipoprotein receptor class A domain containing 4)
ENSG00000257671	antisense	KRT7 (keratin 7, type II)

Two of selected candidate lncRNAs were antisense partner of receptors, that play a direct role in apoptosis (TRAIL-R2 and FAF1) (Figure 3.8) and two candidates lncRNAs were antisense partner of transcription factors (GTF2A1 and CAMTA1). All of the candidates are uncharacterized. The candidate lncRNA RP11-875O11.2 (ENSG00000246130) locates on chromosome 8 (8:23,068,229-23,083,619) and it is a known antisense partner of TRAIL-R2 (tumor necrosis factor receptor superfamily member 10b, TNFRSF10B). Another candidate lncRNA RP5-850O15.3 (ENSG00000225767) locates on chromosome 1 (1: 50,461,469-50,471,150) and it is a known antisense partner of FAF1 (Fas (TNFRSF6) associated factor 1). The other candidate lncRNA CTD-2506P8.6-001 (ENSG00000273783) locates on chromosome 14 (14: 81,221,218-81,222,460) and it is a known antisense partner of GTF2A1 (general transcription factor IIA, 1, 19/37kDa), which is a known general transcription factor. The last candidate lncRNA RP11-312B8.1 locates on chromosome 1 (1: 6,783,892-6,784,843) and it is a known antisense partner of CAMTA1 (calmodulin binding transcription activator 1).

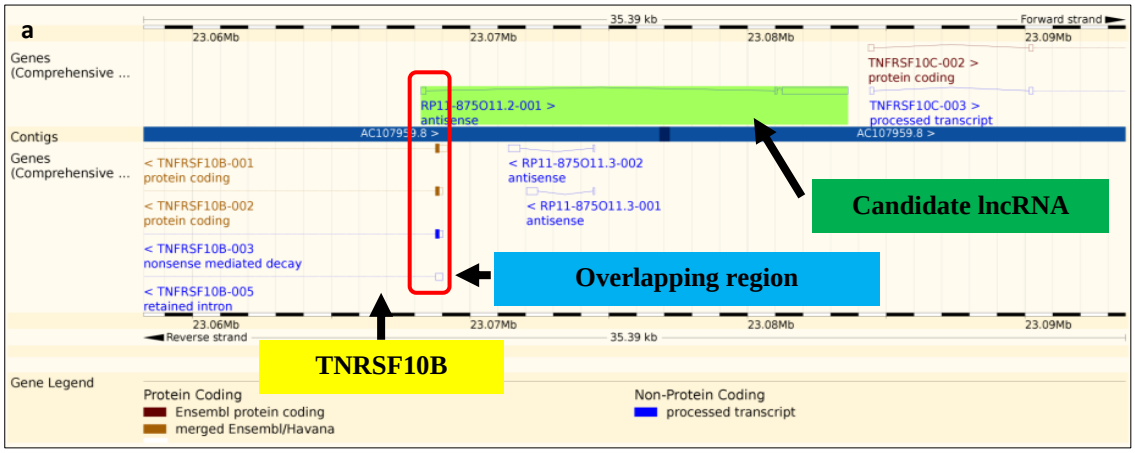

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Figure 3.8. Candidate lncRNAs. The figure shows genomic location of two of four selected candidate lncRNAs (green highlighted) which were obtained from Ensembl (http://www.ensembl.org/index.html). (a) Candidate lncRNA RP11-875O11.2 locates on the forward strand and is in antisense position (blue) to two coding and two noncoding (nonsense mediated decay and retained intron) isoforms of TNFRSF10B (reverse strand), which plays a direct role in receptor mediated apoptosis. The overlapping region (in the red frame) between candidate lncRNA and the antisense gene is 196 bp long. (b) Candidate lncRNA RP5-850O15.3 locates on the forward strand and is in antisense position to two coding and one noncoding (nonsense mediated decay) isoforms of FAF1 (reverse strand), which also plays a direct role in receptor mediated apoptosis. The overlapping region between candidate lncRNA and the antisense gene is 9700 bp long.

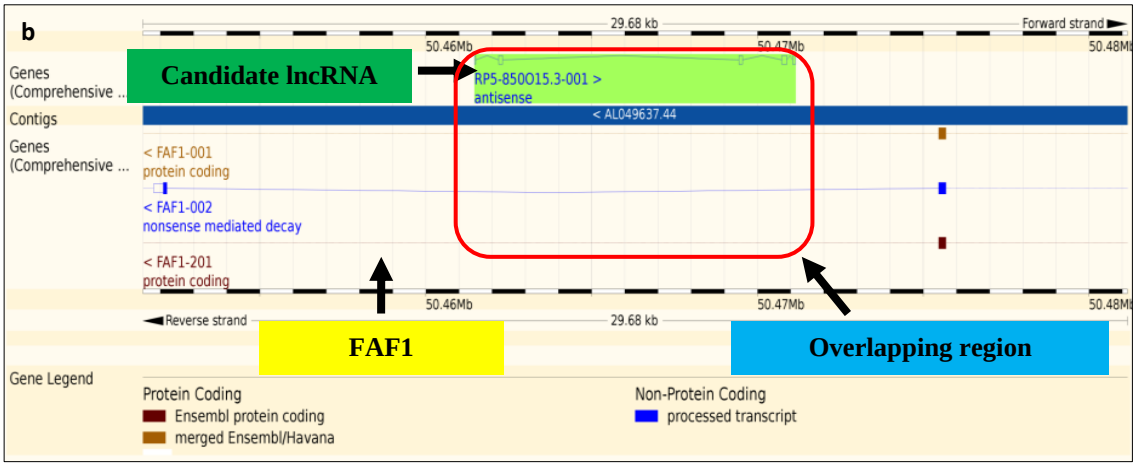

Figure 3.8. (cont.)

CHAPTER 4

DISCUSSION

The dose kinetics of cisplatin showed a significant increase in parallel to increasing in drug concentration. The reference dose prior to experiment was obtained from Wang et al. (2010) (30 μM for 24 hours). Under our experimental setup and reagents, 80 μM cisplatin was selected due to its moderate affect for 16 hours according to Flow Cytometry analysis. Compared to the reference dose (30 μ), we needed to increase concentration drug dose to find an optimum dose during drug dose kinetics. On the other hand, it should be noted that the experiment setting of apoptosis detection was different - Annexin V and 7AAD staining vs. DAPI staining.

Doxorubicin treatment of HeLa cells was challenging due to its unique way of drug response. Treated cells were shifting directly from live cell quadrant (Annexin V^- $/7AAD^-$) to double positive quadrant (Annexin $V^{+/}7AAD^+$), thus in a smaller doses overflow to other quadrants was observed. However, a drug concentration of 4 μM showed a clear shift of cell to double positive quadrant. The reference dose and time was obtained from Bien et al. (2010) (1 μM and 24 hours). However, we decided to increase the doxorubicin dose and decrease exposure time. Considering the difference in detection method of cell death between Bien et al. and us, doxorubicin dose was chosen high enough to see clear shifting of population despite the risk of causing necrosis. In such case, doxorubicin may be used as another control group; however, possible presence of necrosis should be detected by performing extra experiments – detection necrotic markers (like necrostatins), which was not in our experimental settings.

Anti-Fas mAb treatment showed similar apoptotic rates with our doses and the maximum apoptotic rate (~20%) due to having slight expression of FasR as mentioned in Chapter 1. The reference dose and time was 0.1 μg/ml for 24 hours (Holmstorm et al. 1999). Due to similarity among doses, we chose 0.5 μg/ml, the middle dose which had ~20% apoptosis in 16 hours.

TNF-alpha alone did not induce cell death in HeLa cells, which is expected as indicated in Chapter 1. Coupling with cycloheximide, TNF-alpha caused cell death in a dose-dependent manner. Our reference dose and time was 100 ng/ml and 6 hours (Wang

et al. 2008b). Although, the same apoptotic rate (35%) could be achieved by treating with 125 ng/ml for 8 hours, this could be explained by the difference in measurement technique of apoptotic rate.

Fluorescent Microscopy analysis of each chosen drug dose showed similar results (qualitatively) by using same stains and the same protocol. All the control cells were not stained any of dyes (Annexin V and 7AAD). However, the cisplatin treatment caused numerous single or double stained cells. Doxorubicin treated cells showed double stained pure population, just like in the Flow Cytometry results. Fas caused very few single (majority was Annexin V positive) stained cells. Treatment with TNF-alpha resulted with unstained and numerous single stained cells.

Western Blotting experiment gave us the state of mind of cells in a given drug dose and time with respect to apoptosis. As expected, cisplatin showed caspase 8 activation; however neither procaspase 9 nor active caspase 9 could be detected under our experimental settings, suggesting another time kinetics (less than 16 hours) to show procaspase 9 and active fragment. Similar time kinetics (more than 16 hours) should be performed to show cleavage of procaspase 3 and detection of active fragment.

Doxorubicin treatment led to detection of tiny active fragments of caspase 8 and 9, but not caspase 3, thus presumably no or too less apoptosis and existence of another type of cell death (like necrosis). Considering together with Flow Cytometry results, doxorubicin dose (4 μM) might be too much or exposure time (4 hours) might be less. Additional time kinetics (more than 4 hours) should be done to show cleavage of caspases. In case of no detected even after time kinetics, then smaller doses should be tested.

TNF-alpha treatment showed activation of caspase 8 and 9, which was expected and mentioned in Chapter 1. However, under our experimental settings - chosen time (8hours) and dose (125 ng/ml), neither procaspase nor active caspase 3 could be detected due to timing or too much drug. In our case, additional time kinetics (less than 8 hours) should be tested to show procaspase 3 and active caspase 3.

Anti-Fas treatment showed activation of both effector caspases and the executioner caspase. Compare to other selected drug doses, anti-Fas treatment showed the least apoptotic rate according to Flow Cytometry analysis, however, it showed the action of effector and executioner caspases. As it is mentioned in Chapter 1, anti-Fas binds to and activates CD95/Fas receptor, which play role activation of extrinsic apoptotic pathway. Unlike anti-Fas, doxorubicin, cisplatin and TNF-alpha induce

several pathways and can cause cytotoxicity. Thus, treatment with them might have additional effects.

Deep sequencing method was based on detection of lncRNAs, because not all lncRNAs have poly A tail. On the other hand, RNA Quality scores were very good and library preparation showed very high percentage of primary mapping.

Bioinformatics analysis by Allmer lab gave us to direct them and analyze the output together unlike paid services. Using the latest programs and articles and using very stringent filters was powerful side of the bioinformatics results.

The bioinformatics results show that lncRNAs are differentially expressed upon treatment with cisplatin, doxorubicin, anti-Fas and TNF-alpha. Heatmap analysis of differentially expressed lncRNAs showed similar fashion of drug screening and western blotting analysis: doxorubicin treated cells had a unique profile and were close to control, not other agents. On the other hand, anti-Fas and TNF-alpha treatments had close profiles and their profiles were close profile upon cisplatin treatment. Induction of extrinsic apoptotic pathway upon cisplatin treatment might contribute the similarity with TNF-alpha and anti-Fas treatment with respect to apoptosis. In addition, the expressional similarities of individual lncRNAs in each raw showed expressional difference between control (being alive) and treatments (dying cells): some lncRNAs were upregulated only in control and vice versa.

There are known pro- and anti-apoptotic lncRNAs that are differentially expressed and play role in apoptosis as indicated in Chapter 1 and literature. According to our data, there are several well-known lncRNAs differentially expressed as well. One of the well-known lncRNA is *GAS5*, and it was significantly upregulated upon cisplatin and anti-Fas treatment, but not upon doxorubicin and TNF-alpha treatment. Such a result is not surprising, because, expression of lncRNAs, including GAS5 (Ozgur et al. 2013), is cell type and drug/ligand/inducer dependent in different cases, including apoptosis due to difference in molecular mechanism of specific lncRNA. More interesting, newly discovered and uncharacterized antisense lncRNA of *GAS5*, *GAS5-AS1*, is upregulated upon cisplatin, TNF-alpha and anti-Fas treatment as well, however, it is significantly downregulated upon doxorubicin treatment. The expression of *GAS5-AS1* seems independent from GAS5 expression and still the impact of its transcription on GAS5 expression is unknown (Pickard and Williams 2015).

Expression of pro-apoptotic *INXS*, as indicated in Chapter 1, was elevated upon cisplatin, TNF-alpha and anti-Fas treatment, however, it is significantly downregulated

upon doxorubicin treatment. In a number of tumor types, *INXS* is downregulated and upon activation of intrinsic apoptotic pathway in those tumor cells, it is elevated (DeOcesano-Pereira et al. 2014). However, the effect of doxorubicin on *INXS* expression is currently unknown and downregulation of *INXS* in apoptotic HeLa cells may serve shifting *BCL-XS/BCL-XL* ratio to anti-apoptotic *BCL-XL* splice variant and thus blocking intrinsic apoptotic pathway.

Most agents, especially anti-cancer drugs, work different and influence various cellular processes and signaling pathways. There was a clear difference in cell the death mechanism among agents, especially between doxorubicin and the rest agents, according to Flow Cytometry and Western Blotting analysis. The same trend was observed in lncRNA expression levels as well, according to Heatmap Analysis, such as in *Linc-RoR* (Grammatikakis et al. 2014), *HOTAIR* and *AFAP1-AS1* expression. Consistent with literature, in our data, *Linc-RoR* expression is elevated; *HOTAIR* and *AFAP1-AS1* expressions are decreased upon cisplatin, TNF-alpha and anti-Fas treatment, however, their expression level did not change significantly upon doxorubicin treatment. On the other hand, anti-apoptotic *LUST* lncRNA (Rintala-Maki et al. 2009) is significantly decreased upon only doxorubicin treatment. Furthermore, pro-apoptotic *HOXA-AS2* expression is elevated upon treatment with all agents, which is expected during apoptosis.

Expression of uncharacterized MIR7-3HG lncRNA, which is a miR7-3 host gene that plays role in apoptosis, is elevated just in TNF-alpha and anti-Fas mediated cell death and may serve as an important insight about additional molecular mechanism of TNF-alpha and anti-Fas mediated cell death. Likewise, several lncRNAs like *ANRIL* (Huang et al. 2015) and *PVT1* (Ding et al. 2015) expression levels vary among agents as well according to our data. Anti-apoptotic *ANRIL* expression level decreased upon only cisplatin and anti-Fas treatment; anti-apoptotic *PVT1* expression decreased due to cisplatin and doxorubicin treatment, however, its level significantly increased unexpectedly upon TNF-alpha treatment.

Identified lncRNAs, especially antisense and sense intronic/overlapping lncRNAs, may serve us very important information about regulation of molecular mechanism behind apoptosis. The selected candidates are common in all agents and have top scores among differentially expressed lncRNAs. Thus, they can be very powerful candidates and may have a great impact on apoptotic phenotype. To be precise, possible regulation of apoptosis via TNFRSF10B (tumor necrosis factor

receptor superfamily, member 10) antisense and FAF1 (Fas (TNFRSF6) associated factor 1) antisense lncRNAs would not be a surprise and even that is what we have expected. TNFRSF10B and FAF1 directly play role in apoptosis and targeting those key apoptotic markers may have major impacts on downstream events and thus having a global effect on cell fate.

On the other hand, GTF2A1 (general transcription factor IIA, 1) antisense and CAMTA1 (calmodulin binding transcription activator 1) antisense candidate lncRNAs may provide unique information about transcriptional regulation of pro-apoptotic and or anti-apoptotic genes, because, *GTF2A1* gene play role in transcriptional regulation (Kobayashi et al. 1995) and is hypermethylated in ovarian cancer (Huang et al. 2009). *CAMTA1* gene is reported play role in apoptosis (Juhlin et al. 2015), acts as tumor suppressor (Henrich et al. 2011) and is deleted in adrenomedullary tumors (Edstrom et al. 2002).

CHAPTER 5

CONCLUSION

In this study, identification and functional characterization of differentially expressed lncRNAs during apoptosis was performed via deep sequencing method. After a successful drug screening experiments of each drug, Fluorescence Microscopy and Western Blotting analysis were further done for verification of selected doses and the results support each other.

Further, RNA samples from samples passed Quality Control and Library preparation of RNA samples was successful as well. Mapping of primary reads to human genome was very high. Bioinformatics analysis was done by Allmer lab (IzTech) and the thresholds and approaches in detection of differentially expressed genes were very stringent as well.

In conclusion, a systematic and comprehensive approach led to identification of differentially expressed 1644, 506, 584 and 807 lncRNAs upon treatments with cisplatin, doxorubicin, Fas mAb and TNF-alpha, respectively (2-fold or higher, $P < 0.01$). Among them 124 antisense lncRNAs, 15 sense-intronic lncRNAs, 3 sense-overlapping lncRNAs and 100 lincRNAs were significantly and commonly up- or down-regulated upon induction of cell death via cisplatin, doxorubicin, anti-Fas mAb and TNF-alpha. Interestingly, top scored differentially expressed antisense and sense intronic/overlapping lncRNAs in each drug were similar. Those lncRNAs might have functional role(s) and might play role in cell death, including apoptosis as regulator of apoptosis. Antisense and sense intronic/overlapping lncRNAs might regulate their antisense/sense positioned protein coding genes unlike lincRNAs as indicated in Chapter 1. According to our data, two of them were positioned as antisense to death receptors (TRAIL-R2 and FasR) which play role directly in apoptosis as key regulators.

As future direction, the candidates should be functionally characterized by knockdown and/or overexpression approach.

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