ANALYSIS OF TNFRSF10B-AS LONG-NONCODING RNA'S EFFECTS ON VARIOUS CANCER CELL PROPERTIES

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ABSTRACT

ANALYSIS OF TNFRSF10B-AS LONG-NONCODING RNA'S EFFECTS ON VARIOUS CANCER CELL PROPERTIES

Long noncoding RNAs (lncRNAs) being longer than 200 nucleotides constitute a different class of RNA molecules. Several studies indicated that they have regulatory role in cellular processes including cancer development. Some of them have exclusively high expression in particular cancer types and regulate certain cancer cell properties. This renders them potential biomarker or therapeutic target in cancer. In this study, effects of a candidate lncRNA TNFRSF10B-AS and lncCAMTA1 on cancer cell properties were investigated. Candidate lncRNAs from Doxorubicin, Fas mAB, TNF-alpha and Cisplatin treated HeLa cell line were chosen and their expression level was measured in different cell lines including healthy (BEAS2B and MCF10A), metastatic (H1299 and MDA-MB-231) and non-metastatic cell lines (A549 and MCF-7) by qPCR. From a few candidates lncCAMTA1 and TNFRSF10B-AS were selected for further analysis. qPCR results obtained from comparison of different cancer cell lines showed that their expression differs at least in one comparison of cell lines. TNFRSF10B-AS silencing decreased proliferation of HeLa cells. lncCAMTA1 was silenced or overexpressed in HeLa cells but phenotypic effect couldn't be detected by apoptosis and cell proliferation assay. Additionally, phenotypic effect also couldn't be observed in other cell lines when TNFRSF10B-AS was silenced.

Keywords: cancer cell property, long non-coding RNA

ÖZET

TNFRSF10B-AS UZUN KODLANMAYAN RNA'SININ DEĞİŞİK KANSER HÜCRE ÖZELLİKLERİ ÜZERİNE ETKİLERİNİN İNCELENMESİ

Uzun kodlanmayan RNA'lar 200 nükleotid uzunluğundan daha fazla olup RNA moleküllerinin farklı bir sınıfını oluşturmaktadırlar. Birçok çalışma gösteriyor ki bu RNA'lar kanser gelişiminin de içinde bulunduğu hücresel proseslerde düzenleyici görev almaktadırlar. Bu özellik onların kanseri teşhis ve tedavi etmede birer potansiyel biyoişaret olmasına olanak sağlar. Bu çalışmada TNFRSF10B uzun kodlanmayan RNA'sının değişik kanser hücre özellikleri üzerine etkilerinin incelenmesi amaçlanmıştır. Doxorubicin, Fas mAB, TNF-alpha and Cisplatin ile muamele edilmiş HeLa hücre hattından seçildi ve seçilen aday uzun kodlanmayan RNA'ların ifade düzeyleri metastatic (H1299 and MDA-MB-231), metastatik olmayan (A549 and MCF-7) ve sağlıklı (BEAS2B and MCF10A) hücre hatları üzerinde qPCR ile tespit edildi. Birkaç aday arasından TNFRSF10B-AS ve lncCAMTA1 seçildi ve bu uzun kodlanmayan RNA'ların etkileri hücre proliferasyon analizi yapılarak araştırıldı. qPCR sonuçlarından bu RNA'ların hücre karşılaştırmalarından en az birinde farklı ifade edildiği görüldü. Buna ek olarak TNFRSF10B-AS'in susturulmasının HeLa hücre hattında proliferasyonun azalmasına neden olduğu tespit edildi. lncCAMTA1'in susturulmasının veya fazla ifadesinin herhangi bir fenotipic etkiye neden olmadığı yapılan apoptoz ve proliferasyon çalışmalarıyla tespit edildi. Ayrıca, TNFRSF10B-AS'nin HeLa dışındaki diğer hücre hatlarında susturulması sonucu da herhangi bir fenotipik değişiklik gözlemlenemedi.

Anahtar Kelimeler: kanser hücresi özellikleri, uzun kodlanmayan RNA

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CHAPTER 1

INTRODUCTION

1.1. Overview of Cancer

According to estimation of The International Agency for Research on Cancer (IARC) and data obtained from GLOBOCAN database including tables and graphics about 185 countries and 36 cancers, 18.1 million novel cancer incidents and 9.6 million cancer deaths have been reported in 2018[1]. Additionally, a tool developed by IARC involves estimates about upcoming cancer incidence and death globally from 2018 up to 2040. The predictions of the tool show that new cancer incidents will reach to 29.5 million globally by 2040[2].

Cancer is a complicated disease in which cells grow uncontrollably and spread into the surrounding tissue due to several factors such as inherited genetic mutations, epigenetic factors, hormones and defects in immune system[3,4]. In 2000, complicated cancer cell properties were separated into six group and reported as the hallmarks of cancer by Hanahan and Weinberg[5]. These hallmarks can be grouped as; (i) self-sufficiency in growth signals, (ii) insensitivity to anti-growth signals, (iii) evading apoptosis, (iv) limitless replicative potential, (v) sustained angiogenesis, and (vi) tissue invasion and metastasis[6]. In 2011, Hanahan and Weinberg enhanced the number of the hallmarks by addition of two hallmarks which are reprogramming energy metabolism and evading immune response as well as addition of two facilitating characters including genome instability and mutation, and tumor-promoting inflammation[6,7]. By means of transcriptome profiling utilizing next generation sequencing, it has been detected that a large number of long non coding RNAs (lncRNAs) have abnormal expression or mutation in diverse cancer types[3]. Additionally, significant correlation between lncRNAs and the hallmarks of cancer has been noticed[8].

1.2. Long Non-Coding RNA

Torbjörn Caspersson and Jean Brachet indicated separately abundance of RNA inside the cytoplasm in 1939 which was the first clue reflecting the connection between DNA and proteins[9,10]. First ncRNA composing part of the ribosome was described by Georges Palade in 1955[11]. By virtue of central dogma theory announced by Francis Crick, mechanism of storage and transmission of genetic information resulting in one useful protein production was clarified[12,13]. Nevertheless, the central dogma explanation was refuted following invention of ncRNAs which are functional RNA molecules instead of coding for protein products[14,15]. Examples of these ncRNAs are ribosomal rRNA, transfer RNA and small nucleolar RNAs[13]. Invention of the ncRNAs which do not code for proteins deciphered the C-value paradox as well. C-value paradox is that amount of DNA in a haploid genome does not account for organismal complexity[16–18]. Less complex animals like salamander can possess a genome 15 times longer than higher complex animals like humans[16]. By means of developments of genomic technologies like DNA–RNA hybridization experiments and measure of genome-wide mutation load, scientists put forward a new idea in the 1970s that humans possess protein-coding genes not more than 30,000[19,20]. Although transcription is known as pervasive procedure and large part of the genome is transcribed, the number of protein coding genes which is ~21,000 in humans and resembles to the number in less complex species *Caenorhabditis elegans* astonished the scientists[21,22]. The rest of the genome existing as noncoding DNA was named as ‘’junk DNA’’ because of containing transposons, pseudogenes, and simple repeats[19,23]. High throughput sequencing technologies and improvements in bioinformatics tools contributed to development of the Encyclopedia of DNA Elements (ENCODE) project which offered archive of human transcripts broadly. ENCODE project detected that human genome yields myriads of small and long noncoding RNA transcripts[24–26]. This project proposes that this junk DNA makes human and mammals physiologically more complex. By means of current genome-wide researches, it was comprehended that numerous genomic regions yield long noncoding RNAs (lncRNAs) possessing vital biological functions (Figure 1.1) [21,27].

lncRNAs are transcribed RNA molecules which do not code for proteins and have more than 200 nucleotides. Open reading frames (ORFs) carried by them don’t have any function and also shorter than ORFs carried by coding RNAs. Additionally, some

lncRNAs don't have any ORFs [28–30]. Level of lncRNAs expression is relatively low and favorably tissue-specific [27]. According to RNA-seq results obtained on tetrapods, greatest part of lncRNAs exhibit poor conservation in terms of DNA sequence but lncRNAs whose DNA sequence was highly conserved was also found [31]. 5' cap and alternative splicing which are remarkable feature of protein coding genes can also be seen in lncRNAs. Two or more exons can generate lncRNAs. Additionally, lncRNAs having polyA+ tails comprise 60% of lncRNAs[32].

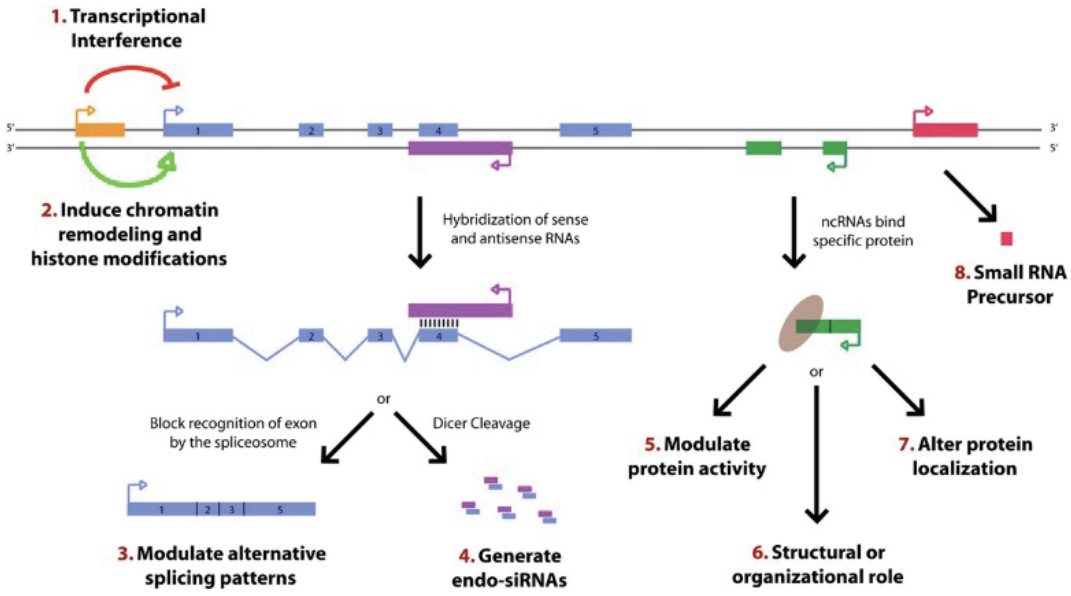

Figure 1.1. Diagram representing functional ways of lncRNAs. (Source: Wilusz, J. E.; Sunwoo, H.; Spector, D. L. Long Noncoding RNAs: Functional Surprises from the RNA World. *Genes Dev.* **2009**, *23* (13), 1494–1504. https://doi.org/10.1101/gad.1800909.

1.2.1. lncRNA Classification

Advances in sequencing technology, entailed measures for classification of lncRNAs. The categorization of lncRNAs includes different classes depending on length, transcription properties, position in terms of known genomic labels, regulator elements and function [33].

Although the lowest size limit of lncRNAs comprises 200 nucleotides providing separation of them from short ncRNAs, the upper size limit of them differs and constitutes the different lncRNA groups. Very long intergenic (vlinc) RNAs and macro lncRNAs

includes lncRNAs which are longer than 10 kb. These groups of lncRNAs differ from others by means of distinctive features as well. One of the features is that splicing of them is either inadequately or not existing. They have also low polyadenylation at 3' end and generated from particular genomic loci [34].

Another classification includes lncRNAs which are diverged based on lncRNA location with respect to protein coding gene (PCG). Initially transcripts have class named as either intergenic or intragenic. Long intergenic non-coding RNA (lincRNA) do not overlap with annotations belonging to protein-coding and ncRNA genes [35]. Transcription of lincRNAs is carried out by RNA polymerase II. They have shorter size than PCGs have [36]. Intragenic lncRNAs intersect with PCG annotations and have different lncRNA groups divided as antisense, bidirectional, intronic, and overlapping sense lncRNAs [9]. Antisense lncRNAs have been found as a primary attribute of eukaryotic transcriptomes [37,38]. In this class, natural antisense transcripts (NATs) are involved and they are furthermore separated into two groups as cis-NATs, having impact on the expression of the related sense transcripts, and trans-NATs controlling the expression of unrelated distant genes [39,40]. Bidirectional lncRNAs are produced from the opposed strand of a PCG strand and they do not intersect or moderately intersect with the 5′ region of paired PCGs [41–44]. It was understood from genetic researches that bidirectional promoters possess extraordinary sequences and epigenetic features [43,45]. Restrictions of intronic lncRNAs are PCG introns. These lncRNAs are generated either as distinctive transcripts or produced by the virtue of pre-mRNA process [46]. LncRNAs originated from pre-mRNA include circular intronic (ci)RNAs, whose sources are lariat introns, and snoRNA gene derived sno-lncRNAs [46,47]. Additionally, the types of intronic transcripts, whose expressions occur as host gene transcription free, are more prevalent ones [48]. Overlapping sense lncRNAs are produced from exons or entire PCGs within their introns devoid of intersecting any sense exon. Their transcription occurs similar to sense direction [49]. Furthermore, another type of lncRNAs include circular lncRNAs (circRNAs) derived from intronic and overlapping sense lncRNAs as a result of head-to-tail non-canonical splicing [50,51]. Stimulation of non-canonical splicing and production of a circular RNA molecule are assigned in case repetitive elements within introns and some other sequence features exist [52]. circRNAs are so stable that their usage as diagnostic marker and therapeutic agent are appropriate [53].

An additional type of lncRNAs is classified based on their position with reference to DNA regulator elements. One of them includes pseudogene-derived lncRNAs. Pseudogenes are derived from ancient coding genes and lack coding capacity. Their transcription occurs in lncRNA sense and antisense directions [54,55]. Ultra-conserved regions (UCRs) contribute to generation of T-UCR lncRNAs. UCRs include the part of the genome displaying 100% sequence conservation between humans, mice and rats [56–58]. Retinoid treatment or hypoxia like stress situations which are related to tumor development stimulates the expression of T-UCRs [56,59]. Telomere repeat-containing non-coding RNAs (TERRAs) are transcribed from telomeres which exist at the ends of chromosomes to shield nucleoprotein structures. Watson and Crick strands contribute to production of these lncRNAs by depending on cell cycle [60,61]. Furthermore there is another type of lncRNAs produced from centromeric repeats throughout the late phases of mitosis and the early G1 phase [62–65]. These centromeric lncRNAs constitute interaction with diverse centromere-specific nucleoprotein fragments [33]. Cis-regulator elements including promoters and enhancers also provide generation of lncRNAs. Enhancer RNAs (eRNAs) are transcribed freely in sense and antisense directions. Since eRNAs involve a upper level of the H3-K27ac and H3K4me1 histones in contrast with other lncRNA and PCGs, they differ from other lncRNAs at histone level. They are vastly unstable because of integrator complex which provides cleavage of 3′-end of eRNA transcripts lacking polyadenylation process[66]. Transcription of promoter-associated lncRNAs (PALRs) occur in sense and antisense directions as well as appearing at promoter level and partially intersecting with the 5’ extremity of the gene [67]. PROMPTs being very unstable and antisense RNA located upstream of the promoters (uaRNA) which are clearly noticeable in case the nuclear exosome is depleted [41,68,69].

Another type of lncRNAs is classified based on their biogenesis and degradation pathways. This type of classification is generally done in budding yeast since their numerous lncRNAs are greatly unstable or cryptic. Nevertheless, some so-called stable unannotated transcripts (SUTs) were recognized in a wild-type genetic contextual [70]. Unstable transcripts are divided into three groups such as cryptic unstable transcripts (CUTs), which have sensitivity to the nuclear RNA decay pathway [70,71]; Nrd1-unterminated lncRNAs (NUTs) [72]; and Xrn1-sensitive unstable transcripts (XUTs), that the cytoplasmic 5′–3′ exoribonuclease (Xrn1) degrades [73,74]. Some vastly unstable lncRNAs have been informed in further eukaryotes. PROMPTs and eRNAs are assumed

as human analogues of CUTs due to their stability upon RNA exosome inactivation [68,75]. Whether XUTs exist in mammals has not been clarified yet [33].

Alternative classification of lncRNAs is done depending on their location in the cell. Defining the location of a lncRNA gives clue about its function in the cell. They can be found in cytosol or nucleus or both regions [76]. Furthermore, mitochondrial genome produces mitochondrial ncRNA named as ncmtRNA whose biogenesis occurs basing on the mitochondrial proteins coded by the nucleus [77–80].

1.2.2. Mechanism and Functions of lncRNAs

Having enormous numbers of annotated transcripts and taking part in diverse biological contexts, lncRNAs are hardly classified in terms of their function [81–84]. Nevertheless, lncRNAs are classified into four groups with respect to functional mechanisms. Besides, one lncRNA may be included in more than one lncRNA groups [85].

First class comprises lncRNAs acting as molecular signals since they are transcribed at a particular time and location to act in response to stimuli. While some of them have regulatory role, rest of them are generated as a by-product following transcription or can be related to chromatin. According to latest researches, lncRNAs such as AIR, KCNQ1OT1, or XIST transcriptionally silence many genes by interplay of chromatin and lncRNAs, and providing recruitment of chromatin modifying machinery [86]. Long ncRNA H19 takes part in allelic imprinting. Its expression level from the IGF2 locus of the maternal allele throughout the blastocyst stage is immense. Additionally, it is expressed from mesodermal and endodermal tissues, however, only from skeletal tissues in adults [87]. Furthermore, some lncRNAs play role in designation of the anterior–posterior body axis and decision of the positional identity of separate cells. HOTAIR, FRIGIDAIR and HOTTIP are examples of these type of lncRNAs [88]. Another role of lncRNAs includes regulation of gene activity in reply to stimuli coming from environment. PANDA and LINC-p21 whose expressions are stimulated by p53 in the event of DNA damage cause cell cycle arrest [42,89].

Second class includes lncRNAs acting as decoys. These lncRNAs obstruct protein targets containing transcription factors, chromatin modifiers, and other regulatory factors by providing binding sites[90]. The regions where they function are nuclear subdomains

and the cytoplasm. One of the examples of decoy lncRNAs is telomeric repeat-containing RNA (TERRA). It is produced from telomerase transcription. Telomeres which are tiny assemblies and include DNA–protein composites protect ends of chromosomes against instability. TERRA binds to telomerase by a repetitive sequence which is complementary to the template sequence of RNA telomerase and so that it forms an integral segment of telomeric heterochromatin[91]. Second example of decoy lncRNA is PANDA. In case of DNA damage, expression of PANDA is stimulated through cyclin-dependent kinase inhibitor 1A (CDKN1A) and so PANDA prevents expression of nuclear transcription factor Y subunit alpha (NF-YA) which has positive regulatory role in apoptosis [42]. Current study indicated another decoy lncRNA GAS5 which competes for interaction with DNA-binding domain of the glucocorticoid receptor. This interaction controls steroid hormone activity in tissues desired [92].

Third class comprises guide lncRNAs. These lncRNAs connect with ribonucleoprotein complexes (RNP) and forward them to certain target genes. These lncRNAs regulate genes by linking with two types of components which are repressive (polycomb) or activating complexes [93]. One of the example of these lncRNAs is lncRNA AIR. This lncRNA binds to its promoter region in chromatin and this interaction results in silence of transcription of its target genes. Once AIR accumulates, it recruits G9a resulting in H3K9 methylation and gene silencing [94]. Xist distribution leads to recruiting PRC2 and matrix protein heterogeneous nuclear ribonucleoprotein (hnRNP) U to the inactive X chromosome [95]. On the contrary, lncRNA HOXB-LINC provides recruitment of the SET1/MLL complex and so that it triggers the transcription of HOXB gene [96]. Furthermore, transcriptional co-activator and co- repressor complexes including p300 histone acetyltransferase or cyclic AMP response element binding protein which are formed through lncRNAs plays important role in controlling protein expression [97]. Example of this lncRNA is seen in bladder cancer in which UCA1 lncRNA interacts with p300 and its co- activator CREB and, thus, it controls improvement of cell cycle [98].

Fourth class includes lncRNAs which behave as scaffolds. These lncRNAs provide constitution of RNP complexes. Since they have flexible structure and have diverse domains which is able to attach to different molecules simultaneously, the transcriptional activators or repressors unite at the same time and region [88,99]. An example of these scaffold lncRNAs is HOTAIR. It attaches with PRC2 which stimulates gene repression through methylation of H3 on lysine 27 [88]. Moreover, its 3'end attaches with

the lysine-specific demethylase 1 (LSD1)/ CoREST/ repressor element 1 silencing transcription factor (REST) complex which contributes to demethylation of H3 on lysine 4. Considering these features, HOTAIR was assumed as scaffold lncRNA and it was understood that it suppresses expression of genes through gathering diverse molecules [100].

In addition to classes of lncRNAs mentioned above, there are other types of lncRNAs acting as competing endogenous RNAs (ceRNAs) or precursor lncRNAs. CeRNAs are also called as lncRNA sponges. Their sequence moderately resembles to PCG transcripts. They compete for miRNA binding and regulation of post-transcription[101]. Equilibrium between ceRNA, miRNAs, and mRNA targets provides a complicated system which offers fine adjustments to control gene expression throughout adaptation, stress response, and development [102,103]. Precursor lncRNAs are derived from small genes and included in the RNAi pathway (mi/si/piRNA). Functions of several lncRNAs were recognized before precursor state function of them was identified. An example of this includes H19 lncRNA. H19 lncRNA includes two preserved microRNA, miR-675 and miR-675-5p. In case of undifferentiated cells, ribo-activator role of H19 emerges. It cooperates with and embolden the action of the KSRP (K-type splicing regulator protein) RNA-binding protein to inhibit myogenic differentiation [104]. During the maturity and the differentiation of skeletal muscle, Smad anti-differentiation transcription factors are regulated post-transcriptionally by means of miRNA which is mature form of H19 [105].

1.2.3. Biomarker Potential of lncRNAs That Modulate Cancer Cell Properties

lncRNAs are highly stable during their circulation in body fluids, remarkably when they are carried in exosomes or apoptotic bodies. This feature makes them appropriate molecule to be cancer diagnostic and prognostic biomarker [106]. There are other specific characteristics of lncRNAs making them cancer biomarker. First one is their high specificity and sensitivity to the tumoral progression. Secondly, sample of lncRNAs having relative stability in body fluids can be obtained. Thirdly, due to their

predictor performance on survival time, metastasis possibility and relapse, lncRNAs can be valuable to use for the and purpose of diagnosis and/or prognosis in oncology [107].

As mentioned above lncRNAs have specificity and sensitivity to tumoral development. Studies showed that lncRNAs can have regulation role during cancer progression pathways including proliferation, apoptosis, migration, invasion and maintenance of stemness (Figure 1.2) [108–110]. Tumor development requires regulation in important progressions such as genomic mutation, DNA damage, immune escape, and metabolic disorder. LncRNAs exist in the nucleus and cytoplasm to have role in post-transcriptional and translational regulation in these processes [111]. Many lncRNAs having role in cancer cell proliferation , migration and invasion were investigated [112]. One of the well-known lncRNA is MALAT1. By means of several studies it was detected that it stimulates cell proliferation and migration while inhibiting cell apoptosis. It regulates these process by upregulating cyclinD1, cyclinD2 and CDK [113,114].

Numerous lncRNAs alone or in combination have had equal or better diagnostic capacity than usual cancer biomarkers, for diverse cancer forms. Through Kaplan-Meier analysis, lncRNA MALAT1 has been recognized as a successful prognostic factor to recover patients in stage I non-small cell lung cancer [115]. Additionally, lncRNA PCA3 found in patient urine samples has been recognized as more sensitive and particular diagnostic marker for prostate cancer than prostate-specific antigen (PSA) serum levels which is extensively applied [116–118]. In breast cancer, CEA, CA125, CA153, and AFP are used as usual biomarkers. Biomarker capacity of these markers is less than lncRNA RP11-445H22.4 since it is overexpressed in breast cancer and have 92% sensitivity and 74% specificity in serum samples [119].

The developments in diagnostic techniques are not sufficient to efficiently diagnose patients with cancer. This situation is seen in patients with non–small cell lung cancer NSCLC. When these patients are diagnosed, 40% of them exists as high-grade tumors or as metastatic cancer which prevents healing and recover [120]. For this reason, new diagnostic approaches ,allowing early recognition and preventing overtreatment, are immediately required. Studies have been conducted with lncRNAs showed functions of lncRNAs in tumorigenesis, tumor progression, invasion, and metastasis. By utilizing this information, lncRNAs can give clue about the dynamical status of the tumor. By this way, cancer may be efficiently diagnosed and prognosed.

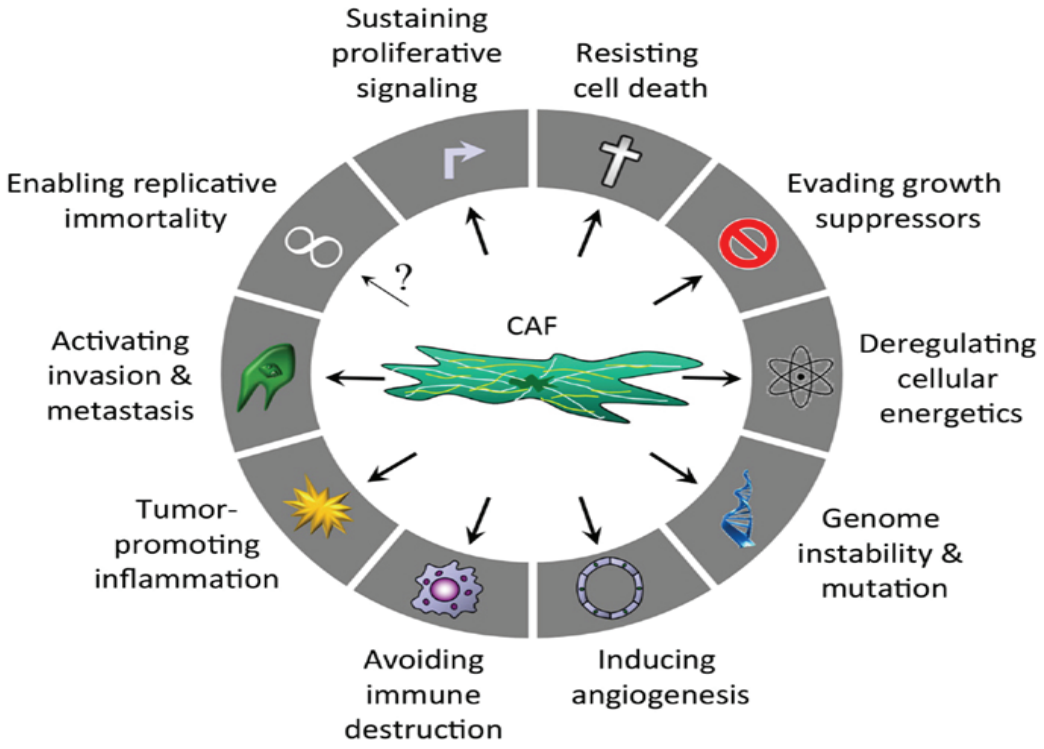

Figure 1.2. Cancer cell properties. (Source Tommelein, J.; Verset, L.; Boterberg, T.; Demetter, P.; Bracke, M.; De Wever, O. Cancer-Associated Fibroblasts Connect Metastasis-Promoting Communication in Colorectal Cancer. *Front. Oncol.* **2015**. https://doi.org/10.3389/fonc.2015.00063.)

1.2.4. lncRNA Expression in Apoptotic HeLa Cells

By project submitted by Prof. Bünyamin AKGÜL and numbered as 113Z371, differentially expressed lncRNAs under apoptotic conditions were defined. Apoptotic conditions were provided by anti-cancer drugs in HeLa cell line. RNA-seq data obtained from these drug treated cells provided differentially expressed lncRNAs after bioinformatic analysis mentioned later (Figure 3.2).

1.3. Aim

The aim of this study is to analyze candidate long-noncoding RNA's effects on various cancer cell properties.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell Culturing and Drug Treatment

NCI-H1299, Beas2B and HeLa cells were cultured in RPMI 1640 (with L-Glutamine, Gibco) supplemented with 10% fetal bovine serum. MDA-MB-231, A549 and MCF-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum. MCF-10A cells were cultured with F12/DMEM (1:1) medium containing 20 ng/ml EGF, 5% horse serum, 100 ng/ ml cholera toxin, 0.5 μg/ml, hydrocortisone and 10 μg/ml insulin. 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco) were added to all cultures and all cultures were incubated at 37°C in a humidified 5% CO2 atmosphere.

Drug treatments of HeLa cells were performed with cisplatin, doxorubicin, FAS mAB and TNF-alpha ligands in 6 well plate (Sarstedt). Each well contained 0.3 x 10^6 cells and overnight incubation was performed. Time- and dose- kinetics experiments were completed in earlier studies to gain significant apoptosis rate (TUBITAK Project 113Z371).

Fresh cisplatin was dissolved in DMSO as 83.2 mM stock in every drug treatments since it is chemically instable. It was exposed to cells as 80 μM for 16 hours. Since DMSO (dimethyl sulfoxide) is toxic for cells, it was used as negative control at a concentrate of % 0.1. Doxorubicin (Cell Signaling) was dissolved in DNase and RNase free water. Its stock preparation was done as 5mM stock. It was aliquoted and kept at -20 °C. It was exposed to cells as 4 μM for 4 hours. Fas mAb (Cell Signaling) was exposed to cells as 0.5 μg/ml for 16 hours. TNF-alpha ligands (Millipore) were prepared in DNase and RNase free water. Its stock was prepared as 100 ng. It was aliquoted and kept at -20 °C. TNF-alpha was exposed with Cycloheximide (CHX) (Applichem) since HeLa has type II cell feature. Minimum cytotoxic concentration experiments were done in previous study (TUBITAK Project 113Z371) and concentration was determined as 125 ng/ml TNF-alpha with 10 μg CHX for 8 hours. Negative controls include Cycloheximide alone and TNF- alpha alone.

2.2. Total RNA Isolation

Cells were cultured overnight in 75 cm2 Flasks. After removal of cells from flask surface through Trypsin-EDTA, they were washed with cold PBS. Following removal of PBS, 0.75 mL of TRIzol Reagent (Life Technologies) per 5– 10 × 10^6 cells was added to the cell pellets to lyse cells. After that cell lysates were incubated 5 minutes at room temperature to allow complete nucleoprotein complex dissociation. Then 0.2 ml chloroform which is RNase free (Sigma) was added per 1 ml of TRIzol. After the tubes were inverted 15-20 times, their incubation was done for 2-3 minutes at room temperature. Cells were centrifuged at 12,000 × g for 15 minutes at 4°C to allow phase separation. Aqueous phase containing RNA was transferred into new tube. Then 0.5 mL of 100% isopropanol which is RNase free (Sigma) was added per 1 mL of TRIzol to allow homogenization and they were incubated at RT for 10 minutes. After centrifugation of cells at 12,000 × g for 10 minutes at 4 °C, supernatant was removed. Washing of the pellet through 1 mL of 75% ethanol which is RNase free (Sigma) per 1 mL of TRIzol was performed. Samples were centrifuged at 7500 × g for 5 minutes at 4 °C and supernatant was removed. Pellet containing RNA were dried in air for 5-10 minutes. After drying, they were dissolved in DNase and RNase free water and stored at -80 °C.

RNA quality was assessed through NanoDrop (Thermo Scientific Spectrophotometry) and running samples on 1% agarose gel. For "pure" RNA 260/280 ratio is ~2, 260/230 ratio is ~2.0-2.2. For agarose gel control, 1 µg RNA was mixed with gel loading dye (6X). Following loading samples into the agarose gel, they were run in TBE buffer (Tris-borate-EDTA buffer, 1M Tris base, 1M Boric acid and 0.02M EDTA) for 45 min at 80V. Gels were visualized with Fusion Fx gel documentation system (Vilber Lourmat, Marne-la-Vallee, France) for 5 to 15 sec with UV light filter.

2.3. cDNA Synthesis and Quantitative PCR (qPCR)

RNA samples was transcribed into cDNA by using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. cDNA reaction contains 1 µl random primer, 3 µg total RNA, 4 µl 5X reaction buffer, 1 µl RiboLock RNase Inhibitor, 2 µl 10 mM dNTP Mix, 1 µl RevertAid

M-MuLV RT and nuclease free water up to 20 µl. After mixing the reagents, first they were incubated at 25°C for 5 min. Secondly, they were incubated at 42°C for 1 hour. Finally, the reaction was terminated by incubation at 70°C for 5 min. cDNA was used for amplification in quantitative real- time PCR (qPCR). q-PCR reaction was performed with GoTaq q-PCR Master Mix (Promega) in Roche Lightcycler® 96 real- time PCR instrument. q-PCR reaction contains 0.5 µl forward and reverse primer mix (10 µM) obtained from Qıagen, 6.25 µl GoTaq q-PCR Master Mix, 3 µl nuclease free water and 2.75 µl (45 ng) cDNA template. The reaction was added to 96 well multi-well plate. The plate was sealed and centrifuged at 1000 x g for one minute. After centrifugation, the q-PCR set to 95 °C for 2 min for initial denaturation, (40 cycle) 95°C for 15 sec for denaturation and 60°C for 1 min for annealing/extension. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene for normalization. For calculation of relative quantifications of genes $2^{-\Delta\Delta CT}$ method was applied.

Table 2.1. List of lncRNAs used in qPCR assay.

Number	Ensembl ID	Type	Gene name
1	ENSG00000171522	lncRNA	PTGER4
2	ENSG00000099860	Retained intron	GADD45B
3	ENSG00000050748	lncRNA	MAPK9
4	ENSG00000070808	Retained intron	CAMK2A
5	ENSG00000273783	lncRNA	AL136040.1 (GTF2A1-AS)
6	ENSG00000246130	lncRNA	AC107959.2 (TNFRSF10B-AS)
7	ENSG00000237436	lncRNA	CAMTA1-DT (lncCAMTA1)

2.4. Transfection

Cells were transfected at 60% to 80% confluence with 50 nM locked nucleic acid (LNA) GapmeR (Exiqon, Qıagen) targeting lncRNA genes listed in the table 1 and control GapmeR for silencing. Additionally, cells were transfected at same confluence with pcDNA3.1(+) vector including our candidate genes (prepared by Dilek Cansu

GÜRER for her master thesis, unpublished) as 50 ng for 96 well plate and 1500ng for 6 well plate. For transfection, nonliposomal FuGENE® HD transfection reagent (Promega) was used. One day before transfection, cells were seeded in 96 well plates or 6 well plates. All cell lines were seeded at 1000 or 3000 cells/well in 96 well plate in 100 µl complete growth medium. All cell lines were seeded at 60000 cells/well in 6 well plate in 2 ml complete growth medium. On the day of transfection, wells were washed with PBS. Then for transfection reaction, reaction mixture per well containing medium without FBS and antibiotic, FuGENE® HD Transfection Reagent and DNA were incubated for 10 minutes at room temperature. After that, the mixtures were added to each well containing medium supplemented with 10% FBS. Plates were put to the incubator for 72 hours.

2.5. Apoptosis Assay

In order to detect apoptotic cell fraction, Annexin V and 7AAD (BD) were used. Following cell trypsinization (Trypsin-EDTA, Gibco, 0.25%), cells were washed with ice cold 1X PBS. Then 50 µl 1X annexin binding buffer (BD) was added to cell pellets. Following buffer addition, cells were labeled with 10 µl of Annexin V and 7AAD. After 15 min incubation of cells in dark at room temperature, 150 µl 1X PBS was added to the samples and they were analyzed using MUSE cell analyzer. Annexin V positive cells indicated early apoptotic cells. Cells which were positive for Annexin V and 7AAD indicated late apoptotic cells. Cells which were 7AAD positive and Annexin V negative indicated dead cells. Cells which were 7AAD and Annexin V negative showed alive cells. DMSO (5%) used as positive control to show increased apoptosis rate.

2.6. WST-1 Cell Proliferation Assay

Cell proliferation was measured with a WST-1 Cell Proliferation Assay kit (Roche Molecular Biochemicals, Mannheim, Germany) which measures the enzymatic conversion of WST-1 to formazan through cell mitochondrial dehydrogenases existing in viable cells. For proliferation assay, 72 hours post-transfection, 10 µL WST-1 reagent

was added to the treated and untreated control cells. After addition of the reagent, lung cell lines (Beas2B, NCI-H1299 and A549) were incubated at 37°C for 30 minutes, 5% CO2 and breast cell lines (MCF-10A, MDA-MB-231 and MCF-7) were incubated at 37°C for 1.5 hours, 5% CO2. HeLa cells were incubated at 37°C for 3 hours, 5% CO2. The absorbance was read at 450 nm with a microplate reader (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer). DMSO (5%) used as positive control to show decreased cell proliferation.

2.7. Bioinformatic Analysis

For selection of candidate lncRNAs, RNA-seq raw data ,obtained from total RNAs of HeLa cell lines treated with four drugs, cisplatin and doxorubicin, and two ligands, Fas mAb and TNF-alpha, were analyzed by using OmicsBox bioinformatic tool. Omicsbox provided quality control of data existing in FASTQ format, adaptor and quality trimming, mapping reads to the reference genome which is human GRCh38 genome, counting reads, differential expression analysis (Osama SWEEF Ph.D. thesis,unpublished). Gene set enrichment analysis was applied through a bioinformatic tool, Gene Ontology to see whether the genes expressed differentially are related with an assured biological process or molecular function. These biological processes searched includes apoptosis, cell proliferation and migration. Selected lncRNAs was listed in the table 2.1.

2.8. Statistical Analysis

The relative changes in the candidate expressions were analyzed from qPCR experiments by $2^{-\Delta\Delta Ct}$ method [121]. The statistical analyses were calculated by GraphPad Prism V.7.0. Numerical data were represented as mean ± standard error of the mean. Experiments include 3 biological replicates. Student's t-test was used to determine the difference between two groups. $P<0.05$ was considered as implying a statistically significant difference.

CHAPTER 3

RESULTS

3.1. Apoptosis Assay and RNA Quality Control

Induction of apoptosis in HeLa cells was triggered with treatments with doxorubicin, fas mAB, TNF-alpha, cisplatin. DMSO was used for control as 0.1 % and incubation lasted 16 hours. Anti Fas was treated as 0.5 µg/ml for 16 hours. Doxorubicin was treated as 0.5 µM for 4 hours. TNF-alfa with CHX was treated as 0.125 ng/ml for 8 hours. Additionally, CHX was used for control as 10 µg/ml during 8 hours since it activates TNF- alpha, Fas mAB, Cisplatin, Doxorubicin induced apoptosis as 36.1, 34.1, 17.2, 26.7, respectively (Figure 3.1).

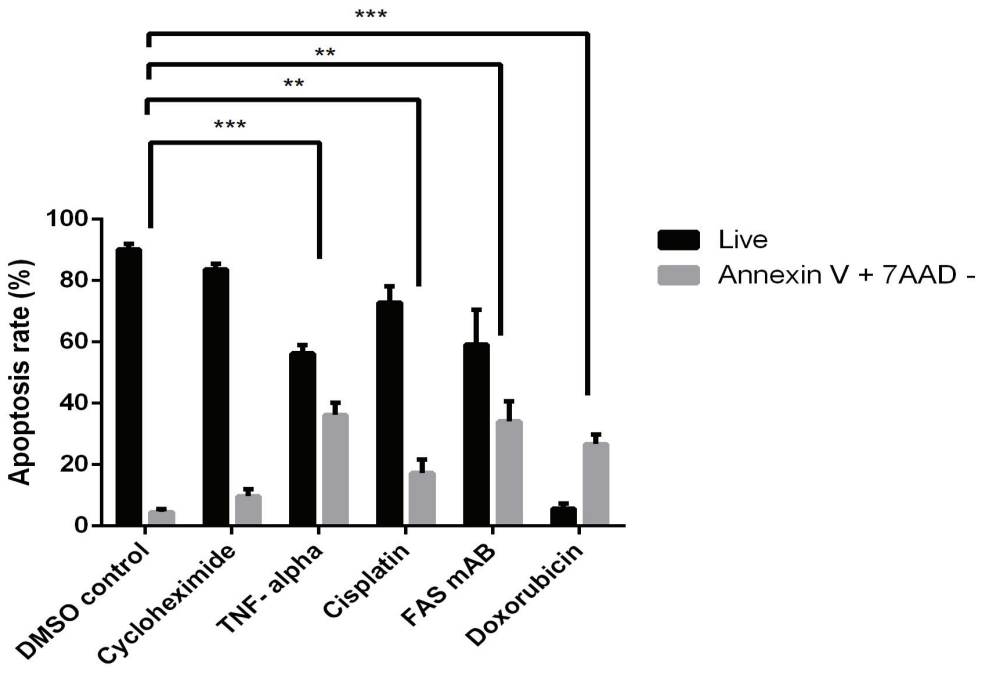

Figure 3.1. Apoptosis rate in apoptosis induced HeLa cells after drug treatments such as Doxorubicin, Fas mAB, TNF-alpha, Cisplatin, (n=3). ***P<0.001, **P<0.01, *P<0.05 vs. DMSO (student's t-test).

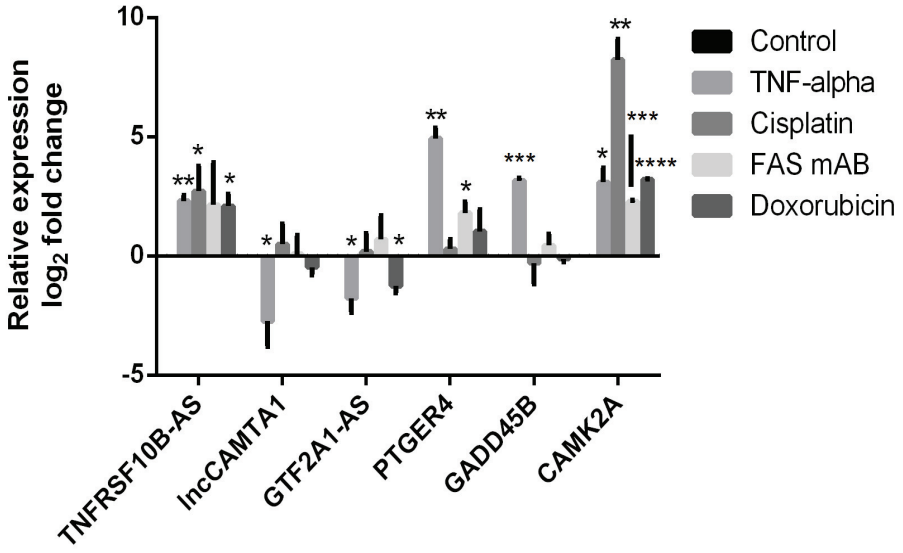

Figure 3.2. Validation of RNA-seq data with qPCR. (n=3). ***P<0.001, **P<0.01, *P<0.05 vs. DMSO (student's t-test). GAPDH was used for internal reference. Expression values were calculated applying the $-2^{\Delta\Delta CT}$ algorithm.

Validation of expression levels for selected candidate lncRNAs including TNFRSF10B-AS, lncCAMTA1, GTF2A1-AS, lncRNA PTGER4, retained intron GADD45B and retained intron CAMK2A was carried out by qPCR. Control samples include DMSO treated cells for cisplatin, cycloheximide treated cells for TNF-alpha and untreated cells for FAS mAB and Doxorubicin. Expression levels obtained from qPCR (Figure 3.2) were compared to expression levels obtained from RNA-seq data (Table 3.1). After comparison, it was seen that expression levels were similar for 5 candidates (TNFRSF10B-AS, lncRNA PTGER4, retained intron GADD45B and retained intron CAMK2A). Also it was seen that all candidates were differentially expressed under apoptotic conditions.

Total RNAs obtained from 4 different drug treated cells and untreated cells were run on 1% Agarose gel to control quality of them. 28S and 18S rRNA bands were prominently detected (Figure 3.3).

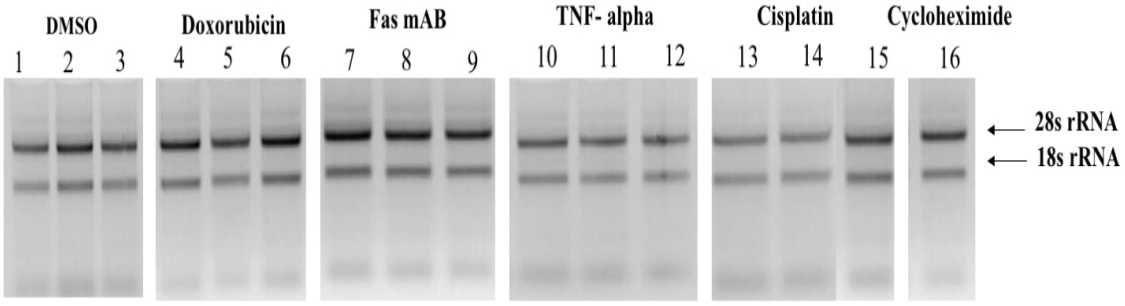

Figure 3.3. RNA Gel and Quality Check (QC) Analysis of Total RNAs. First three line include total RNAs obtained from untreated cells containing DMSO only as control with three replicates. Last line includes total RNA which was used as another control and obtained from cycloheximide added cells. From 4. line to 15. line includes total RNAs obtained from Doxorubicin, Fas mAB, TNF-alpha, Cisplatin treated cells with three replicates for each drug. Total RNAs were run at 80V for 40 min in 1% Agarose gel as 500 ng/well.

3.2. Candidate lncRNA Selection by Bioinformatic Analysis

Candidates were selected by using two different bioinformatic tools, OmicsBox and Gene Ontology. OmicsBox provided the quality control of raw data offered in FASTQ format, trim adaptors and very low quality bases, map reads to the human reference genome GRCh38, count reads and conduct differential expression analysis. After getting some list of lncRNAs for all drugs (Figure 3.4) (Osama SWEEF Ph.D. thesis, unpublished), the list was analyzed on Gene Ontology. From Gene Ontology results, the lncRNAs which have potential role in apoptosis, cell proliferation and cell migration were chosen. After selection of them, they were also filtered to get lncRNAs having highest fold change. Finally lncRNA candidates on Table 3.1 were determined for investigation. From the results it was detected that transcript ENST00000592937 was seen only in TNF alpha treated cells, ENST00000514343 was seen only in Anti-FAS treated cells, ENSG00000070808 was seen only in Doxorubicin treated cells. On the other hand, ENST00000524170, ENST00000501897, ENST00000618431, ENST00000442889 was detected in all drug treated cells.

Table 3.1. Selected candidate genes chosen as a result of OmicsBox and Gene Ontology analyses.

Transcript ID	Gene ID	Biotype	Fold change	Drug
ENST00000592937	ENSG00000099860	Retained Intron	9,383	TNF alpha
ENST00000514343	ENSG00000171522	lncRNA	6,132	Anti-FAS
ENST00000508662	ENSG00000070808	Retained Intron	5,119	Doxorubicin
ENST00000524170	ENSG00000050748	lncRNA	1,022	TNF alpha
			0,556	Anti-FAS
			3,447	Doxorubicin
			2,808	Cisplatin
ENST00000501897	ENSG00000246130	lncRNA	2,736	TNF alpha
			2,656	Anti-FAS
			3,053	Doxorubicin
			4,508	Cisplatin
ENST00000618431	ENSG00000273783	lncRNA	3,341	TNF alpha
			3,689	Anti-FAS
			1,502	Doxorubicin
			5,148	Cisplatin
ENST00000442889	ENSG00000237436	lncRNA	3,182	TNF alpha
			3,377	Anti-FAS
			1,078	Doxorubicin
			3,699	Cisplatin

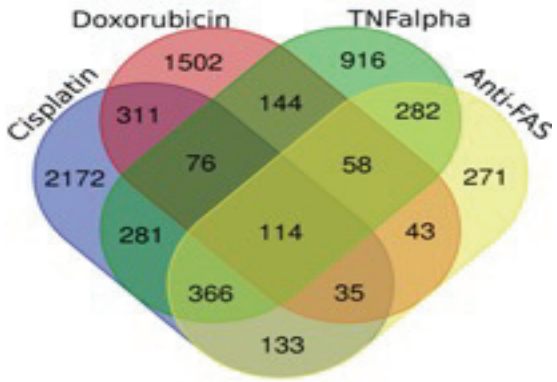

Figure 3.4. Venn Diagram. Differently expressed transcripts in drug treatments. (Source: Osama SWEEF Ph.D. thesis, unpublished)

3.3. Differential Expression of lncRNAs in Different Cell Lines

Expression levels of three different selected lncRNAs (GTF2A1-AS, lncCAMTA1, TNFRSF10B-AS) were determined by qPCR in 3 different lung cell lines (BEAS2B, A549, NCI-H1299) and 3 different breast cell lines (MCF10A, MCF-7, MDA-MB-231). If $p < 0.05$ and fold change ≥ 2.0, relative expression was determined as statistically and physiologically significant for lncRNAs. Relative expressions was calculated based on comparing healthy (BEAS2B and MCF10A) and cancer cell lines in addition to metastatic (H1299 and MDA-MB-231) and non-metastatic cell lines (A549 and MCF-7).

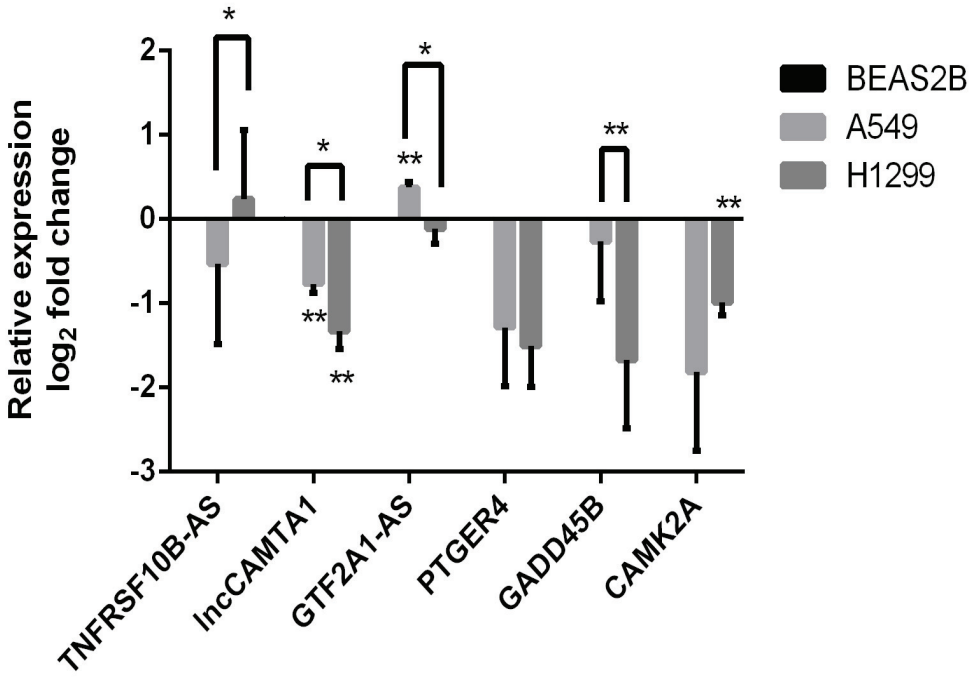

Figure 3.5. qPCR analysis results for gene expression. Expression levels of TNFRSF10B-AS, lncCAMTA1, GTF2A1-AS, lncRNA PTGER4, retained intron GADD45B, retained intron CAMK2A in BeaS-2B, A549 and NCI-H1299 cell lines (n=3). **P<0.01, *P<0.05 vs. BeaS2B (student's t-test). GAPDH was used for internal reference. Expression values were calculated applying the $-2^{\Delta\Delta CT}$ algorithm.

In the qPCR results, it was seen that lncCAMTA1 was significantly downregulated in A549 cells by 2 fold and in H1299 cells by 3.3 fold in comparison to

BEAS2B cells (Figure 3.5) . In case of breast cell lines it was seen that, TNFRSF10B-AS was upregulated in MDA-MB-231 cells by 2.64 fold in comparison to MCF-7 cells. While this upregulation was physiologically significant, it wasn't statistically significant (Figure 3.6). lncCAMTA1 was upregulated in MCF-7 cells by 2.3 fold in comparison to MDA-MB-231 cells. This regulation was also only physiologically significant (Figure 3.6). For GTF2A1-AS, it was seen that this lncRNA was significantly upregulated in MCF-7 cells by 7.2 fold in comparison to MCF10A cells. Also, GTF2A1-AS was upregulated in MDA-MB-231 cells by 2.2 fold in comparison to MCF10A cells. This regulation was only physiologically significant. Additionally, it was seen that GTF2A1-AS was significantly upregulated by 3.2 fold in MCF-7 cells in comparison to MDA-MB-231 cells (Figure 3.6). It was detected that retained intron GADD45B was upregulated in both non-metastatic cell lines MDA-MB-231 and H1299 as 2.1 and 2.85 fold respectively (Figure 3.5 and Figure 3.6).

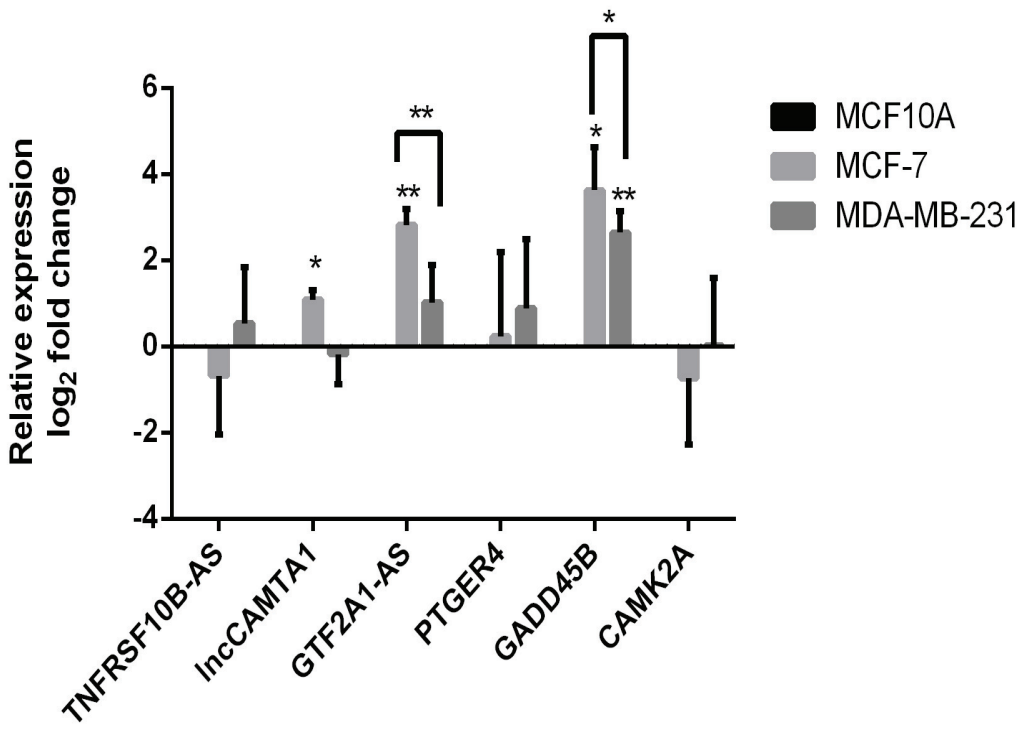

Figure 3.6. qPCR analysis results for gene expression. Expression levels of TNFRSF10B-AS, lncCAMTA1and GTF2A1-AS, lncRNA PTGER4, retained intron GADD45B, retained intron CAMK2A in MCF10A, MCF-7 and MDA-MB-231 cell lines (n=3). **P<0.01, *P<0.05 vs. MCF10A (student's t-test). GAPDH was used for internal reference. Expression values were calculated applying the $-2^{\Delta\Delta CT}$ algorithm.

3.4. Effect of Silencing and Overexpression of lncCAMTA1 and TNFRSF10B-AS on Cell Proliferation in Different Cell Lines

Effects of silencing or overexpression of lncCAMTA1 lncRNA on proliferation of HeLa cell lines were separately measured by WST-1 assay. WST-1 assay results showed that there was no significant difference between control cells and cells transfected with lncCAMTA1 GapmeR or lncCAMTA1+Vector in cell proliferation (Figure 3.7 and Figure 3.8).

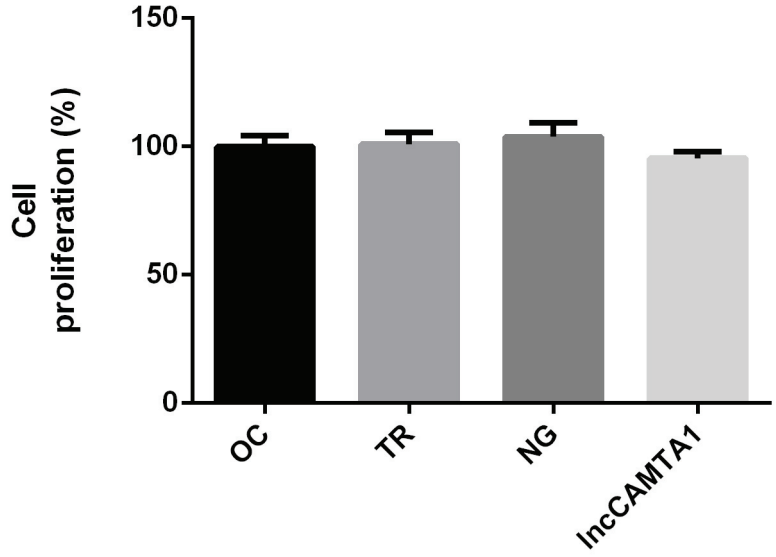

Figure 3.7. Cell proliferation analysis by WST-1 assay. Effect of silencing of lncCAMTA1 lncRNA on proliferation of HeLa cell lines. (1000 cells/well were seeded, 3 hours WST-1 incubation) *P<0.05, (student's t-test), OC: Only Cell, TR: Transfection Reagent, NG: Control Negative GapmeR, lncCAMTA1: lncCAMTA1 GapmeR.

Effect of silencing of TNFRSF10B-AS lncRNA on proliferation of HeLa cell lines was measured by WST-1 assay. Here it was used as positive control because it was conducted before in our laboratory (Dilek Cansu GÜRER thesis, unpublished). WST-1 assay results showed that TNFRSF10B-AS GapmeR transfected cells had significantly decreased proliferation rate compared to controls (Figure 3.9).

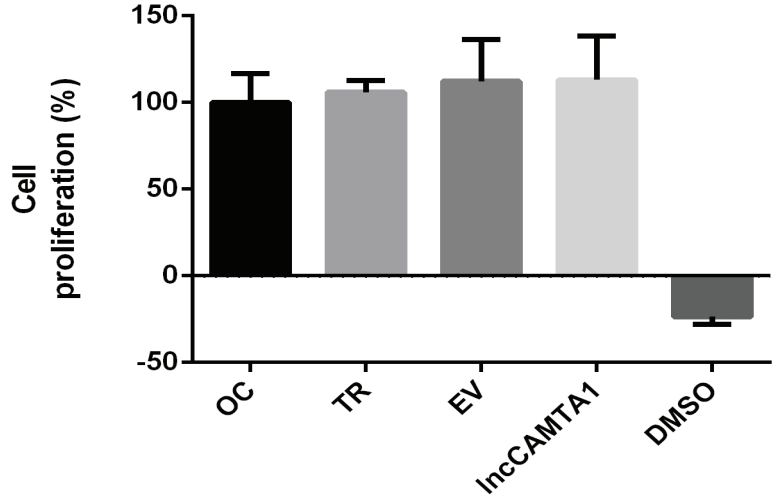

Figure 3.8. Cell proliferation analysis by WST-1 assay. Effect of overexpression of lncCAMTA1 lncRNA on proliferation of HeLa cell lines. (1000 cells/well were seeded, 3 hours WST-1 incubation) *P<0.05, (student's t-test), OC: Only Cell, TR: Transfection Reagent, EV: Empty Vector, lncCAMTA1: lncCAMTA1+Vector.

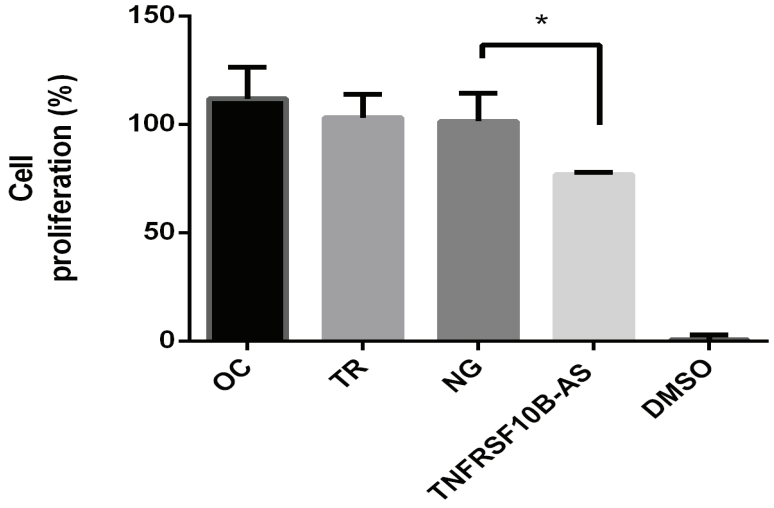

Figure 3.9. Cell proliferation analysis by WST-1 assay. Effect of silencing of TNFRSF10B-AS lncRNA on proliferation of HeLa cell lines. (1000 cells/well were seeded, 3 hours WST-1 incubation) *P<0.05, (student's t-test), OC: Only Cell, TR: Transfection Reagent, NG: Control Negative GapmeR, TNFRSF10B-AS: TNFRSF10B-AS GapmeR.

In addition to HeLa cell lines, effect of silencing of TNFRSF10B-AS lncRNA on proliferation of other cell lines including BEAS2B, MCF10A, H1299, MDA-MB-231, A549 and MCF-7 was measured by WST-1 assay (Figure 3.10-Figure 3.15). For each cell

lines, different conditions including cell number, WST-1 incubation time and duration of treatment were used for optimization. Cell proliferation results were not consistent for these cell lines used.

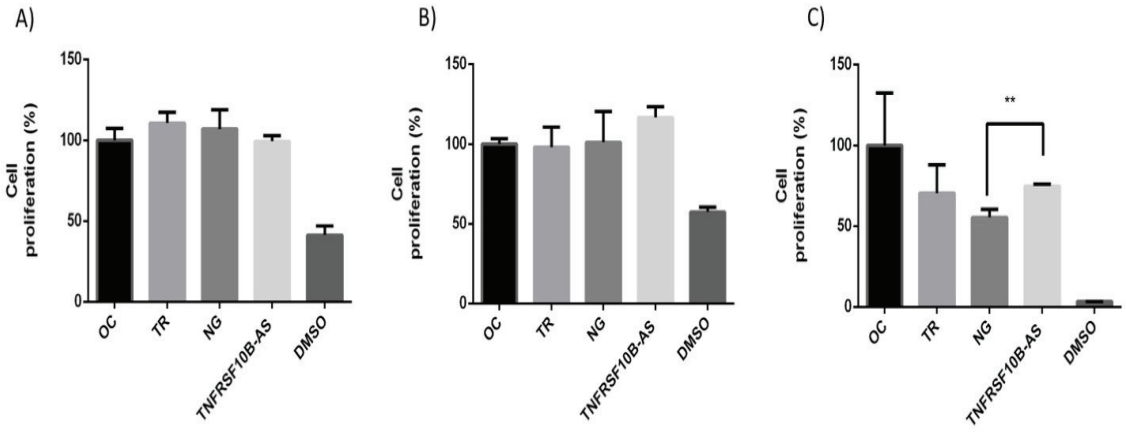

Figure 3.10. Cell proliferation analysis by WST-1 assay. Effect of silencing of TNFRSF10B-AS lncRNA on proliferation of MCF-7 cell lines. *P<0.05 (student's t-test). A) and B) 3000 cells/well were seeded. (3 hours WST-1 incubation) C) 1000 cells/well were seeded (2 hours WST-1 incubation). OC: Only Cell, TR: Transfection Reagent, NG: Control Negative GapmeR, TNFRSF10B-AS: TNFRSF10B-AS GapmeR.

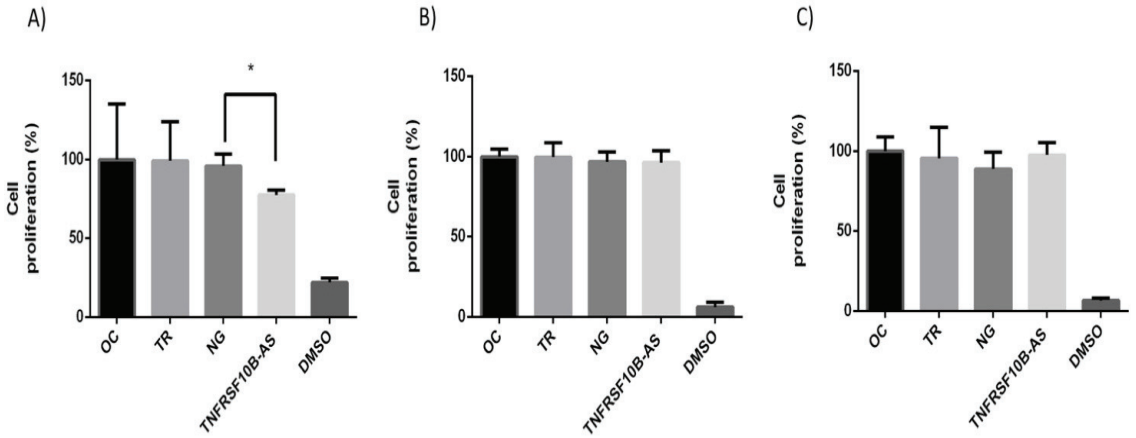

Figure 3.11. Cell proliferation analysis by WST-1 assay. Effect of silencing of TNFRSF10B-AS lncRNA on proliferation of MDA-MB-231 cell lines. *P<0.05 (student's t-test). A) and B) 3000 cells/well were seeded. (3 hours WST-1 incubation) C) 1000 cells/well were seeded. (2 hours WST-1 incubation). OC: Only Cell, TR: Transfection Reagent, NG: Control Negative GapmeR, TNFRSF10B-AS: TNFRSF10B-AS GapmeR.

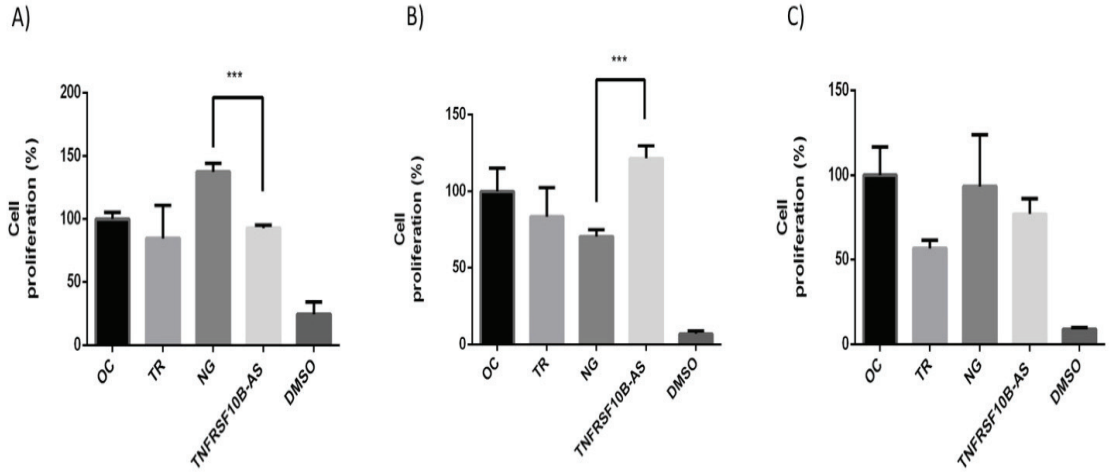

Figure 3.12. Cell proliferation analysis by WST-1 assay. Effect of silencing of TNFRSF10B-AS lncRNA on proliferation of MCF10A cell lines. *P<0.05 (student's t-test). (3000 cells/well were seeded, 3 hours WST-1 incubation). OC: Only Cell, TR: Transfection Reagent, NG: Control Negative GapmeR, TNFRSF10B-AS: TNFRSF10B-AS GapmeR.

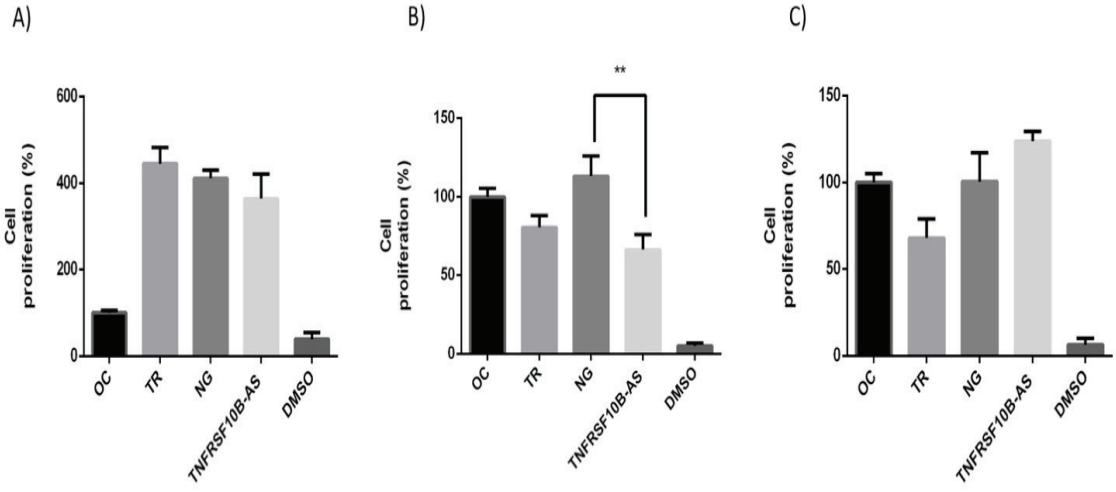

Figure 3.13. Cell proliferation analysis by WST-1 assay. Effect of silencing of TNFRSF10B-AS lncRNA on proliferation of NCI-H1299 cell lines. *P<0.05 (student's t-test). (3000 cells/well were seeded.) A) WST-1 incubation for 3 hours B) WST-1 incubation for 2 hours C) WST-1 incubation for 1 hour. OC: Only Cell, TR: Transfection Reagent, NG: Control Negative GapmeR, TNFRSF10B-AS: TNFRSF10B-AS GapmeR.

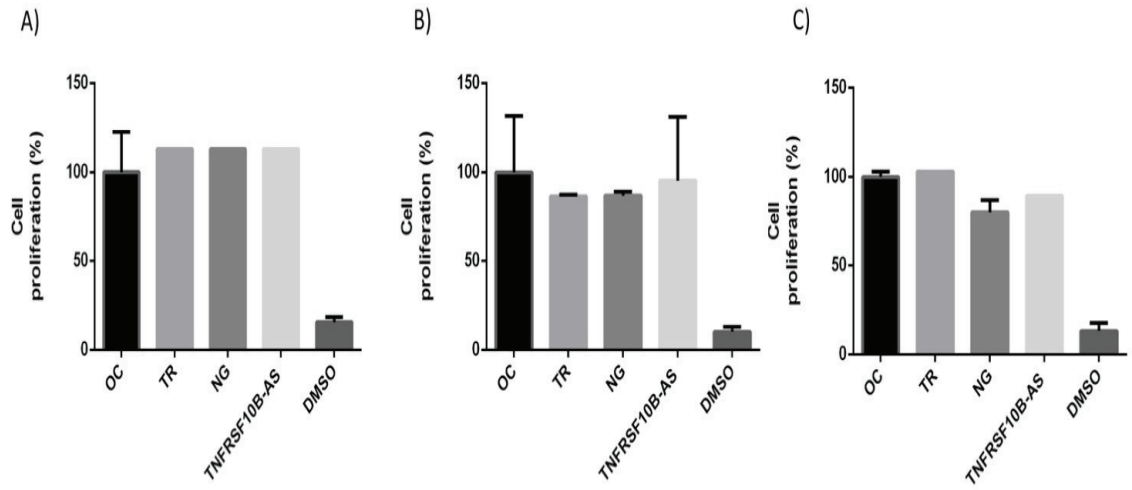

Figure 3.14. Cell proliferation analysis by WST-1 assay. Effect of silencing of TNFRSF10B-AS lncRNA on proliferation of A549 cell lines. *P<0.05 (student's t-test). (3000 cells/well were seeded.) A) WST-1 incubation for 3 hours B) WST-1 incubation for 2 hours C) WST-1 incubation for 1 hour. OC: Only Cell, TR: Transfection Reagent, NG: Control Negative GapmeR, TNFRSF10B-AS: TNFRSF10B-AS GapmeR.

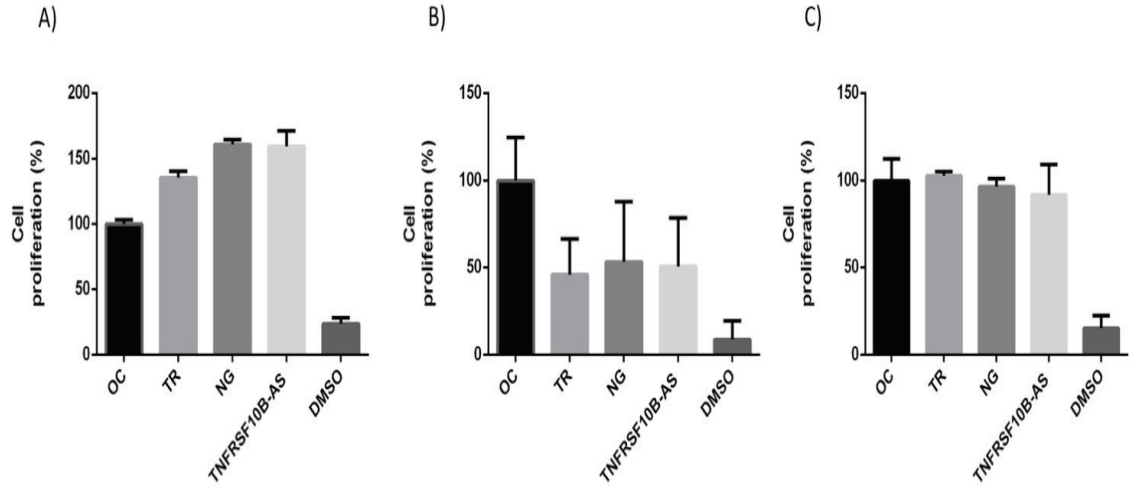

Figure 3.15. Cell proliferation analysis by WST-1 assay. Effect of silencing of TNFRSF10B-AS lncRNA on proliferation of BEAS2B cell lines. *P<0.05 (student's t-test). (3000 cells/well were seeded.) A) WST-1 incubation for 3 hours B) WST-1 incubation for 2 hours C) WST-1 incubation for 1 hour. OC: Only Cell, TR: Transfection Reagent, NG: Control Negative GapmeR, TNFRSF10B-AS: TNFRSF10B-AS GapmeR.

3.5. Apoptosis Measurement After Transfection

Effect of silencing or overexpression of lncCAMTA1 lncRNA on apoptosis in HeLa cell lines was measured by MUSE cell analyzer. Results showed that there was no significant difference between control cells and cells transfected with lncCAMTA1 GapmeR or lncCAMTA1+Vector in apoptosis. (Figure 3.16 and 3.17).

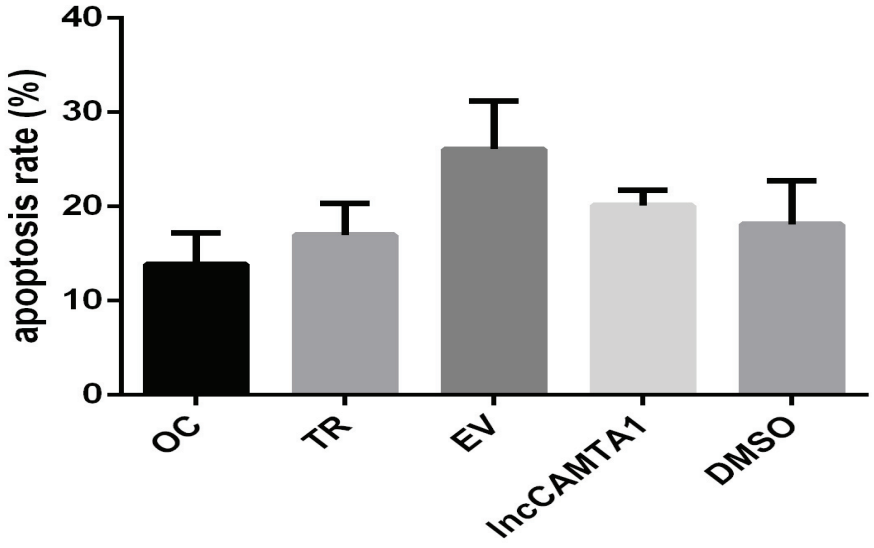

Figure 3.16. Apoptosis analysis by MUSE cell analyzer . Effect of overexpression of lncCAMTA1 lncRNA on apoptosis in HeLa cell lines. Annexin V and 7AAD staining. Apoptotic cells, Annexin V+/7AAD- and Annexin V+/7AAD+; dead cells, Annexin V-/7AAD+; and live cells, Annexin V-/7AAD-. 5%DMSO. *P<0.05, (student's t-test). OC: Only Cell, TR: Transfection Reagent, EV: Empty Vector, lncCAMTA1: lncCAMTA1+Vector.

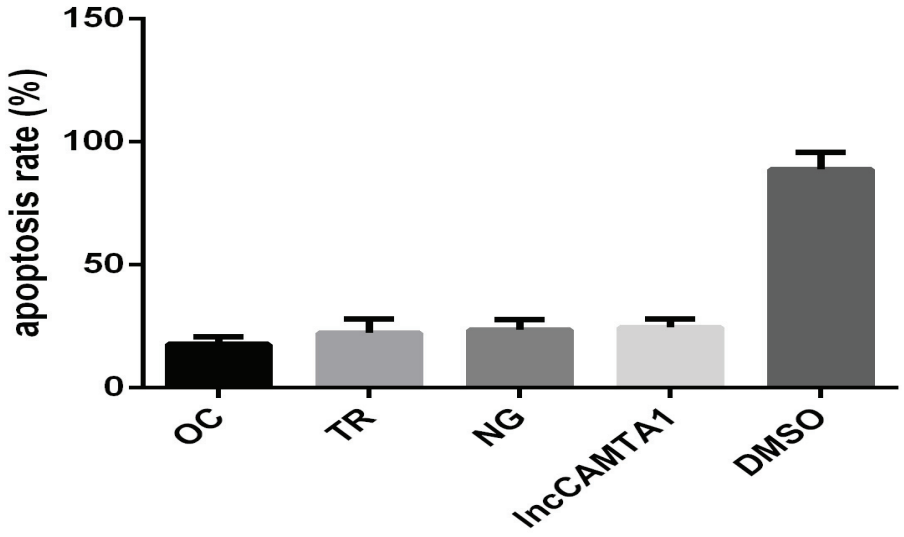

Figure 3.17. Apoptosis analysis by MUSE cell analyzer . Effect of silencing of lncCAMTA1 lncRNA on apoptosis in HeLa cell lines. Annexin V and 7AAD staining. Apoptotic cells were Annexin V+/7AAD- and Annexin V+/7AAD+; dead cells were Annexin V-/7AAD+; and live cells were Annexin V-/7AAD-. 5%DMSO.*P<0.05, (student's t-test). OC: Only Cell, TR: Transfection Reagent, NG: Control Negative GapmeR, lncCAMTA1: lncCAMTA1 GapmeR.

CHAPTER 4

DISCUSSION

Total human transcriptome includes more than 98% non-coding RNAs and irregular expression of them cause development of specific cancer phenotypes [103,122]. Although there are lots of studied lncRNAs, remaining unstudied lncRNAs which have role in cancer cell property and potential to be a biomarker are waiting to be discovered.

Bioinformatic analysis of RNA-seq data from previous research conducted in our laboratory helped us to determine candidate lncRNAs. For this analysis, qualitied and usable data was provided by OmicsBox tool. After that, Gene Ontology tool results gave lncRNAs which can be related to apoptosis, cell proliferation and cell migration. From these list, lncRNAs which have highest expression level were selected as candidates. (Table 2.1).

Validation of RNA-seq results and expression level of the selected candidates were detected by qPCR on drug treated cells. After drug treatments (Doxorubicin, Fas mAB, TNF-alpha, Cisplatin), RNAs were isolated and quality of them was checked on agarose gel (Figure 3.3). As seen in Figure 3.3, 28S and 18S bands can be clearly detected. Apoptosis and qPCR validations were successful for most of candidates.

Differential expression of the selected lncRNAs TNFRSF10B-AS, lncCAMTA1 and GTF2A1-AS in different cancer cell lines was detected by qPCR. Metastatic (H1299 and MDA-MB-231), non-metastatic (A549 and MCF-7) and healthy (BEAS2B and MCF10A) cell lines of breast and lung tissue were analyzed. It was seen that lncCAMTA1 was downregulated in both metastatic and non-metastatic cell line of lung tissue in comparison to healthy cell line as by 3.3 fold in non-metastatic line and 2 fold in metastatic line (Figure 3.5). This data may indicate that lncCAMTA1 may have role in lung cancer development . lncCAMTA1 was upregulated in non-metastatic MCF-7 cells by 2.3 fold in comparison to metastatic MDA-MB-231 cells. Although this regulation was not statistically significant, it was physiologically significant since fold change is $\geq$ 2.0. It may be supposed from this data that lncCAMTA1 may have role in development of non-metastatic breast cancer. GTF2A1-AS was upregulated in both metastatic and non-metastatic cell line of breast tissue in comparison to healthy cell line as by 7.2 fold

in non-metastatic line and 2.2 fold in metastatic line. This regulation was only physiologically significant. Furthermore, GTF2A1-AS was significantly upregulated by 3.2 fold in non-metastatic MCF-7 cells in comparison to metastatic MDA-MB-231 cells (Figure 3.6). It can be supposed that GTF2A1-AS may have role in development of non-metastatic breast cancer. TNFRSF10B-AS was upregulated in metastatic MDA-MB-231 cells by 2.64 fold in comparison to non-metastatic MCF-7 cells. While this upregulation was physiologically significant, it wasn't statistically significant. Nevertheless, it may be supposed that TNFRSF10B-AS may have regulatory role in metastatic cancer development (Figure 3.6). It was detected that retained intron GADD45B was upregulated in both non-metastatic cell lines MDA-MB-231 and H1299 as 2.1 and 2.85 fold respectively (Figure 3.5 and Figure 3.6). This may indicate that retained intron GADD45B can be a non-metastatic cancer cell marker.

Among candidates lncCAMTA1 and TNFRSF10B-AS was selected to see effects of this lncRNA on cancer cell properties. They were chosen because they were upregulated upon drug treatment as understood from RNA-seq data. In addition to this reason, effects of TNFRSF10B-AS in HeLa cell properties was promising in previous study conducted in our laboratory. For confirmation, cell proliferation assay repeated for HeLa cells and it was seen that proliferation of TNFRSF10B-AS silenced cells decreased. After HeLa cell line, it was silenced also in metastatic (H1299 and MDA-MB-231, non-metastatic (A549 and MCF-7) and healthy (BEAS2B and MCF10A) cell lines of breast and lung tissue. However, the results were not consistent under our experiment conditions. For this reason, further optimization experiments should be conducted.

Effect of lncCAMTA1 on cancer cell properties was analyzed by cell proliferation assay and apoptosis assay after overexpression and silencing of lncCAMTA1 in HeLa cells. Apoptosis assay results showed no significant difference between control cells and cells transfected with lncCAMTA1 GapmeR or lncCAMTA1+Vector in apoptosis, although effective silencing and overexpression were validated by qPCR (Figure 3.14 and 3.15). Likewise, there was no significant difference between them according to the proliferation results (Figure 3.5 and 3.6).

CHAPTER 5

CONCLUSION

In this study effects of candidate lncRNAs chosen from RNA-seq data on cancer cell properties were investigated. After selection of candidates basing on their relation to apoptosis, cell proliferation and cell migration, their validation with apoptosis assay and qPCR was done. Additionally, their expression level in 3 different lung cell lines (BEAS2B, A549, NCI-H1299) and 3 different breast cell lines (MCF10A, MCF-7, MDA-MB-231) were detected by qPCR. Among candidates, lncCAMTA1 and TNFRSF10B-AS were chosen to see their effects on cancer cell properties. For this purpose, lncCAMTA1 was silenced and overexpressed separately. Additionally, TNFRSF10B-AS was silenced only in these cell lines. Following transfection, apoptosis and cell proliferation assays were conducted.

In conclusion, differential expression analysis of candidates showed that expression levels of TNFRSF10B-AS, lncCAMTA1, GTF2A1-AS, retained intron GADD45B and retained intron CAMK2A have significant difference between at least one comparison of different cell lines. It was detected that lncCAMTA1 was efficiently silenced and overexpressed in HeLa cells but their phenotypic effect couldn't be recognized by both cell proliferation or apoptosis assays. They may require different phenotypic analyses such as cell migration and invasion. Phenotypic effect of TNFRSF10B-AS silencing was seen in HeLa cells. It was recognized that its silence decreased cell proliferation. However, the effects couldn't be realized in the other cell lines. Other cell lines need further optimization processes.

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