IDENTIFICATION OF LONG NON-CODING RNAS OVERCOMING TAMOXIFEN RESISTANCE IN ESTROGEN RECEPTOR ALPHA POSITIVE BREAST CANCER

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By

Hilal Bal

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We certify that we have read this dissertation and that in our opinion it is fully adequate in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

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Hilal Bal

M.S. in Molecular Biology and Genetics

Advisor: Özgür Şahin

September, 2017

Most of the breast cancer incidences all over the world fall into Estrogen Receptor alpha (ERα)-positive breast cancer subtype, which are treated with endocrine therapy. Tamoxifen, a selective ER modulator drug, is the most prescribed endocrine therapy option for the patients, providing a decreased mortality rate. Although patients respond to tamoxifen well initially they may lose their sensitivity to tamoxifen and develop resistance which is a major obstacle when tackling ERα-positive breast cancer. Global transcriptome analyses performed in recent years demonstrated that most parts of the genomic DNA that are transcribed into RNA are not further translated into proteins. RNA molecules that are not converted into proteins and are therefore called non-coding RNAs (ncRNA) were found to be involved in cellular processes like sequence-specific chromosome modifications, gene silencing and regulation of protein signaling pathways. While the roles of protein and microRNA (miRNA) regulators in the tamoxifen resistance have been identified, the roles of long non-coding RNAs in tamoxifen resistance are still elusive.

To elucidate the impact of the long non-coding transcripts in tamoxifen resistance, I have developed acquired tamoxifen resistant ERα-positive cell line models and examined alterations in their transcriptome with respect to long non-coding RNA expression. The results of whole genome RNA-Seq analysis showed that 330 long non-coding transcripts were differentially expressed in the tamoxifen resistant cell line compared to its parental counterpart. I filtered-out ncRNAs according to criteria based on fold change, cancer-

association, and being a validated lncRNA, and I ended up with two candidate lncRNAs. Here, I continued with the upregulated candidate lncRNA and confirmed its elevated expression by qRT-PCR in both of the *in vitro* acquired tamoxifen resistant cell line models I used. Moreover, I showed that knockdown of the candidate lncRNA using antisense oligonucleotide (ASO) re-sensitizes resistant cells to tamoxifen. This sensitization effect of candidate lncRNA was achieved *via* induction of autophagy shown by increased LC3 II/LC3 I ratio followed by apoptosis evidenced by cleaved Caspase 7 when the lncRNA was targeted. Finally, analysis of tamoxifen-treated, ERα-positive breast cancer patient data sets suggested that higher expression of the candidate lncRNA was associated with poor overall, relapse-free and disease-free survival of the patients. Overall, in this thesis, I identified a novel lncRNA regulator of tamoxifen resistance and a potential biomarker of therapy response.

Keyword: Tamoxifen, drug resistance, lncRNA, ER positive, breast cancer

ÖZET

ÖSTROJEN RESEPTÖRÜ ALFA POZİTİF MEME KANSERİNDE TAMOKSİFEN DİRENCİNİ KIRAN UZUN KODLANMAYAN RNA'LARIN BELİRLENMESİ

Hilal Bal

Moleküler Biyoloji ve Genetik, Yüksek Lisans

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Eylül, 2017

Tüm dünyadaki meme kanseri vakalarının büyük çoğunluğunu, ERα pozitif meme kanseri alt tipi oluşturmaktadır. Seçici ER modülatörü bir ilaç olan tamoksifen, hastalara önerilen en yaygın endokrin tedavi seçeneğidir ve hastalığın ölüm oranını önemli ölçüde düşürmektedir. Hastalar başlangıçta tamoksifene iyi derecede yanıt verseler de, zamanla direnç geliştirebilirler. Tamoksifen direnci olarak da bilinen bu durum, endokrin tedavi ile ERα-pozitif meme kanseri mücadelesinin önünde önemli bir engel teşkil etmektedir. Son yıllarda gerçekleştirilen global transkriptom analizleri, RNA'ya çevirilen genomik DNA bilgisinin çoğunun proteinlere dönüştürülmediğini göstermiştir. Proteinlere dönüştürülmeyen ve dolayısıyla kodlanmayan RNA'lar (ncRNA) olarak isimlendirilen bu RNA moleküllerinin, dizi-spesifik kromozom modifikasyonları, gen susturma ve protein sinyal yolaklarının düzenlenmesi gibi birçok hücresel süreçlerde yer aldıkları belirtilmiştir. Tamoksifen direncinde, protein ve mikroRNA (miRNA) moleküllerinin rolleri geniş ölçüde tanımlanmış olsa da, uzun kodlamayan RNA'ların (lncRNA, long non coding RNA) rolleri ise henüz yeterince anlaşılmış değildir.

Bu tez çalışmasında, tamoksifen direncindeki uzun kodlamayan transkriptlerin etkisini anlamak amacıyla tamoksifen dirençli ERα-pozitif hücre hattı modelleri geliştirilmiş olup; duyarlı ve dirençli hücre modellerinin tüm transkriptom ebadında uzun kodlanmayan RNA ifade profilleri değerlendirilmiştir. Tüm genom RNA-Seq dizileme analizinin sonuçları, 330 tane uzun kodlamayan RNA'nın, tamoksifene dirençli hücre hattında parental hücre hattına

kıyasla farklı şekilde ifade edildiğini göstermektedir. İfade kat değişimi, kanserle ilişkilendirilmesi ve geçerliliği onaylanmış uzun kodlanmayan RNA kriterleri dikkate alınarak bir filtreleme uygulanmış ve iki aday uzun kodlanmayan RNA çalışma için seçilmiştir. Çalışmaya bu iki lncRNA'dan hedeflenebilme potansiyeli ile ifadesinde artma görülen ile devam edilmiş olup; ifade seviyesindeki artış eş zamanlı PCR metodu ile doğrulanmıştır Daha sonra, bir antisens oligonükleotid (ASO) ile aday lncRNA'nın ifadesinin susturulmasının dirençli hücreleri tamoksifene yeniden duyarlı hale getirdiği gösterilmiştir. Aday lncRNA'nın tamoksifen direncinin kırılması üzerindeki potansiyel etkisi lncRNA hedef alındığında önce artmış LC3 II / LC3 I oranı ile gösterilen otofaji indüksiyonu, ardından kesilmiş Kaspaz 7 ile desteklenen apoptoz ile moleküler seviyede açıklanmaya çalışılmıştır. Ayrıca, tamoksifen ile tedavi edilen ERα-pozitif meme kanseri hasta veri setlerinin analizi, aday lncRNA'nın yüksek ifadesinin hastaların genel, nükssüz ve hastalıksız sağkalımlarını olumsuz yönde etkilediğini göstermiştir. Sonuç olarak, bu tez çalışması kapsamında ER pozitif meme kanserinde tamoksifen direncini düzenleyen ve tedavi yanıtının değerlendirilmesinde potansiyel bir biyobelirteç özelliği taşıyan yeni bir aday lncRNA tanımlanmıştır.

Anahtar Sözcükler: Tamoksifen, ilaç direnci, lncRNA, ER pozitif, meme kanseri

I dedicate this work to my family, friends and partner who were always there for me, lifted me up when I was down and supported me.

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En son olarak, aileme, arkadaşlarıma ve hayat arkadaşıma bitmez tükenmez destekleri ve sevgileri için en derin, en içten minnetimi sunarım.

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Abbreviations

AKT1	V-akt murine thymoma viral oncogene homolog 1
AKT2	V-akt murine thymoma viral oncogene homolog 2
APS	Ammonium peroxodisulfate
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
CDH1	E-cadherin
CDH2	N-cadherin
DMEM	Dulbecco's Modified Eagle Medium
dNTP	deoxynucleotide triphosphate
ECL	Enhanced chemiluminescence
EGFR	Epidermal growth factor receptor 1
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
ERK1	Mitogen-activated protein kinase 3 (MAPK3)
ERK2	Mitogen-activated protein kinase 1 (MAPK1)
ESR1	Estrogen receptor alpha gene
FBS	Fetal bovine serum
GEO	Gene Expression Omnibus
HER2	Human epidermal growth factor receptor 2
kDA	Kilo Dalton
LINC00152	Long intergenic non-coding RNA 152
lncRNA	Long non-coding ribonucleic acid

METABRIC	Molecular Taxonomy of Breast Cancer International Consortium
P/S	Penicilllin/Streptomycin
p27	Cyclin-dependent Kinase Inhibitor 1B (CDKN1B)
p38	Mitogen-activated protein kinase 14
p62	Sequestosome 1
PARP	Poly[ADP-ribose] polymerase 1
PBS	Phosphate buffered saline
PR	Progesterone receptor
qRT-PCR	Quantitative real time polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
TAE	Tris-acetate EDTA
TamR	Tamoxifen resistant
TBS-T	Tris buffer saline Tween20
Thr	Threonine
TNBC	Triple negative breast cancer
ZEB1	Zinc finger e-box binding homeobox 1
ZO1	Zona occludens-1 (Tight junction protein 1, TJP1)

CHAPTER 1

INTRODUCTION

1.1. Breast Cancer

Breast cancer is the most common cancer in women, and it is the second leading cause of cancer-related death among women. Annually, about 1.7 million people are diagnosed with breast cancer (World Health Organization (WHO), Globocan 2012 data). The disease is categorized into 5 subtypes according to the gene expression profiles: Luminal A, Luminal B, HER2-enriched, Basal-like and Normal-like [1]. While both Luminal A and B subtypes have distinctive overexpression of estrogen receptor alpha (ERα), the latter one expresses elevated levels of another receptor, human epidermal growth factor receptor 2 (HER2). In this respect, Luminal B and HER2-enriched subtypes have a common trait. Normal-like and Basal-like subtypes, on the other hand, are characterized by loss of ERα and HER2 expressions (***Figure* 1**).

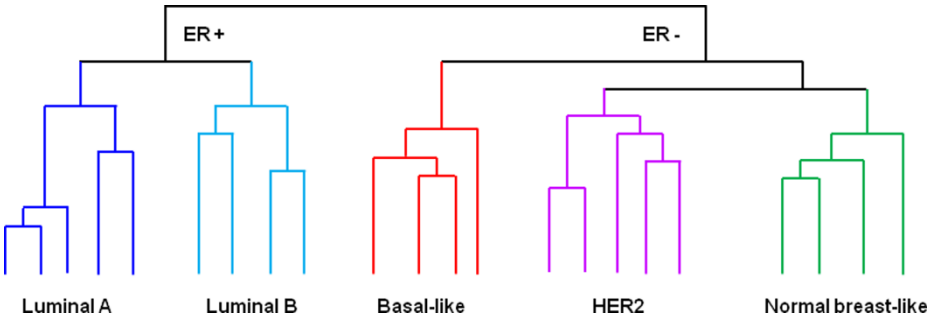


Figure 1: Representative scheme of breast cancer classification. Luminal A and Luminal B subtypes belong to ERα-positive group while HER2-enriched, Basal and Normal breast-like are ERα-negative (Taken from: Colombo et al., 2011, Breast Cancer Res [2]).

ERα-positive subtype constitutes around 70% of the all breast cancer incidences [1]. Thus, ER expression has a key role in clinical diagnosis of breast cancer. Moreover, contributions of other genes together with receptor status should be taken into account when therapy option will be decided. Patient stratification based on gene expression profile does not only help decide on the treatment, but also predicts clinical outcome of the patient and of the therapy. Thus, from the gene expression profiles prognosis and diagnosis can be evaluated [3].

1.1.1 Estrogen and Estrogen Receptor

Estrogen is a steroid hormone synthesized from cholesterol. It has an important role in female fertility and regulates physiological processes in the body, mainly in reproductive system and mammary glands. While estrogen can be found in several different structures, estradiol (E2), also called 17β-estradiol, is the most abundant form of estrogen in mammary glands. Main source of its production is ovaries in premenopausal women, yet it also produced by converting androgen in several other tissues including breast [4]. Estradiol executes its action *via* binding to its receptor, estrogen receptor.

Human ER is coded by two relatively similar genes: *ESR1* (ERα) and *ESR2* (ERβ). They share about 80% sequence similarity. ERβ is generally overexpressed in ovaries, while ERα is mostly located in breast tissues [5]. They both can modulate gene expression in either ligand- dependent or ligand-independent manner. In ligand-dependent gene modulation, ER is required to be activated by estrogen binding. Activated ER could either directly act as a transcription factor or recruit other transcription factors to promoter of its target genes. On the other hand, ligand-independent action of ER involves its phosphorylation *via* kinases. Phosphorylated ER acts as if it was estrogen-bound and functions in transcription of different genes (***Figure 2***).

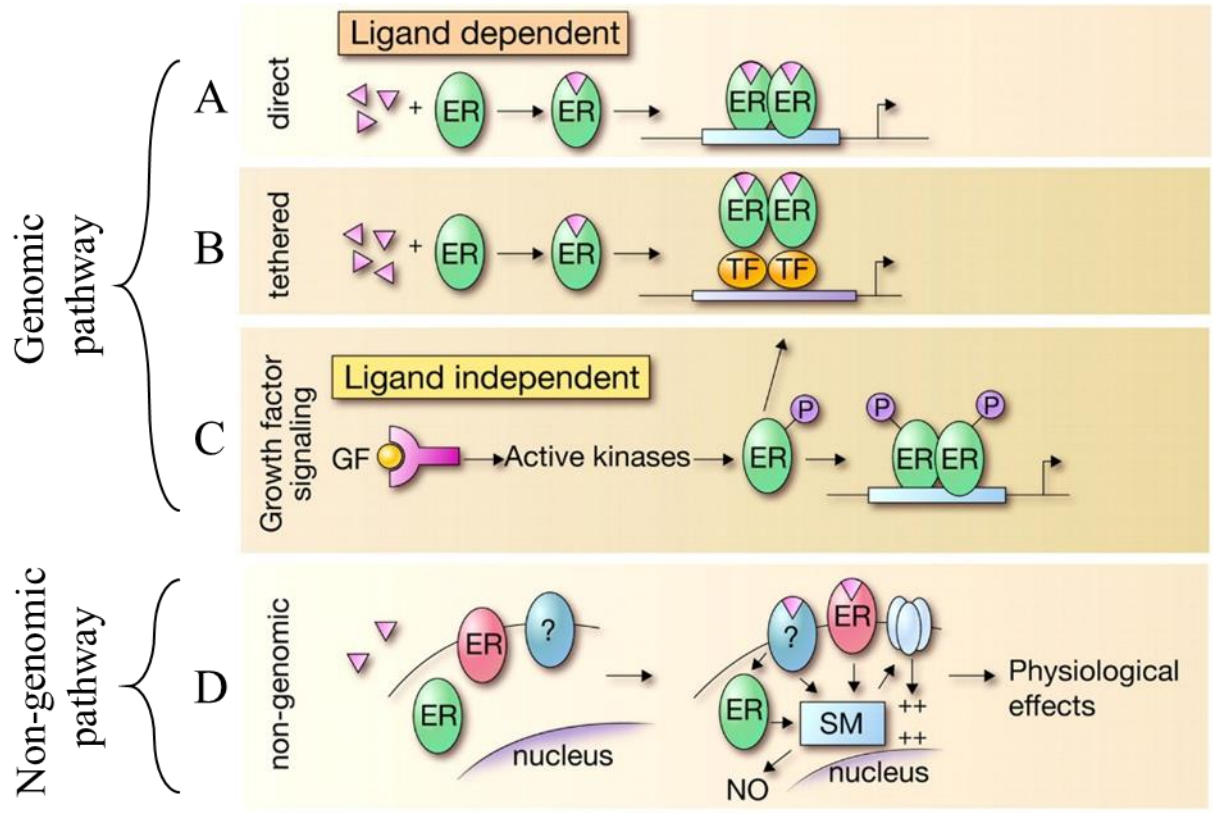


Figure 2: Schematic representation of the ER action. Genomic (A, B and C) and non-genomic pathways (D) of ER is shown. Genomic pathway can be exerted through ligand-dependent (A and B) or -independent (C) actions. In A, ER is directly initiates transcription activation after ligand binding. In contrast, ligand-bound ER initiates transcription *via* recruiting other transcription factors in B. Furthermore, ER activation can be enabled *via* phosphorylation by kinases leading to the conformational change of ER (C). Lastly, membrane-bound ERs can interact with other proteins within cell and cause physiological effects (D) (Modified from: Heldring et. al., 20067, Physiol Rev [6]). See Appendix for the copyright permission.

1.1.2 ERα-Positive Breast Cancer

Luminal subtypes (Luminal A and Luminal B) of the breast cancer are the most common among all subtypes comprising roughly 70% of the all breast cancer patients. This corresponds to approximately 1.2 million new cases annually for the estrogen receptor positive (ERα-positive) breast cancer. [7]. ERα-positive breast cancer has a less aggressive, slow developing profile compared to ERα-negative subtypes, and may or may not possess progesterone receptor (PR) overexpression. However, elevated PR levels are shown to have a positive effect on patient prognosis [8] [9] [10]. Moreover, while Luminal A patients are

regarded having low-risk, Luminal B patients are regarded as high-risk in terms of prognosis. The difference is potentially originated from HER2 expression level that is shown to effect clinical outcome negatively [10] [11]. Because of the fact that ERα is a transcription factor and able to modify a very wide range of genes downstream, overexpressed or overactive ERα state is the main tumor-promoting factor in this subtype. Therefore, Targeting ERα and, thus, its downstream is the major approach to treat ERα-positive breast cancer.

1.1.3 First-line Treatment of ERα-Positive Metastatic Breast Cancer

In general, first-line therapy for cancer is defined as the primary choice of treatment of the disease. In case of ERα-positive metastatic breast cancer, first-line therapy is endocrine therapy. Endocrine therapy can be divided into 3 groups based on the mechanism of the action of the drugs. First group consists of selective ER modulators (SERMs) like tamoxifen and raloxifene, second group is aromatase inhibitors (AI) such as letrozole, anastrozole, and exemestane and last of the endocrine therapy groups is the selective ER down-regulators (SERDs) like fulvestrant. While raloxifene can be used in any prevention of invasive breast cancer and AIs can only be used in postmenopausal women [12] [13], tamoxifen has been used for the treatment of both premenopausal and postmenopausal, high-risk ER-positive breast cancer patients.

Both Luminal A and B patients can be assigned to anti-estrogens or aromatase inhibitors. Another crucial point which is worth considering before determining the treatment regimen is initial pathological examinations for molecular markers of breast cancer. Herein, immunohistochemistry results of the biopsy samples are reliable indicators. For instance, Luminal B patients can benefit from HER2-targeted therapies or cytotoxic chemotherapies together with endocrine therapies [14]. If patients do not show any clinical benefit, which is defined as complete or partial response or stable disease, they can be assigned to second-line therapies [15].

1.1.4 Selective Estrogen Receptor Modulators and Aromatase Inhibitors

The mechanism of the action of AI drugs, such as Letrozole, Anastrozole and Exemestane, is to cease all ER-mediated activities *via* blocking the main enzyme for estrogen production; aromatases. As a result, not only the ERα overactive cancer cells but the whole body deprives

of estradiol hormones. Estrogen is the main fertility hormone, thus patients prescribed with AI generally are post-menopausal women or have advanced stage of breast cancer [16] [17]. In a meta-analysis of 30 independent studies, it was shown that aromatase inhibitors as cross-over therapy resulted in a better outcome than drug alone [18]. Apart from aromatase inhibitors, anti-estrogen modulators are used commonly in breast cancer. The most known estrogen modulator drug is tamoxifen.

1.2 Tamoxifen

For more than 3 decades, tamoxifen has been marketed as the best treatment option for patients with early-stage breast cancer with roughly 10% recurrence rate in first 5 years of the treatment [19]. Tamoxifen is an anti-estrogenic drug that was first prescribed as a contraceptive drug in 1960s [20]. However, in 1971 a study showed that, tamoxifen is a potent breast cancer drug and proven to improve progression-free survival [21]. Since then, tamoxifen has been used extensively for ERα-positive breast cancer. However, it is also well recognized that not all ERα-positive patients respond to tamoxifen well in clinics due to *de novo* or acquired resistance

1.2.1 Mechanism of Action of Tamoxifen

Proposed mechanism of action for tamoxifen is its binding to ER, subsequent activating its dimerization, resulting in its translocalization into nucleus and altering its downstream gene expression profile [15]. Tamoxifen could act either as an ER antagonist or as an ER agonist. ER is normally activated by binding of E2 to the ligand-binding domain of ER. This activation stimulates dimerization of ER. With the help of co-activators, active ER translocates into nucleus and binds specific sequences called estrogen responsive elements (ERE) located on DNA. ERE is a palindromic sequence, and it is found generally upstream of E2-inducible genes [22] [5]. Tamoxifen binds to ER with a lower affinity and does not impede activation of ER, but it alters its conformational dynamics and leads to co-repressor recruitment rather than co-activators. Hence, it inhibits proliferation promoting E2-indicible gene transcription. In other words, tamoxifen modifies ER transcription landscape (***Figure 3***) [23] [24] [25].

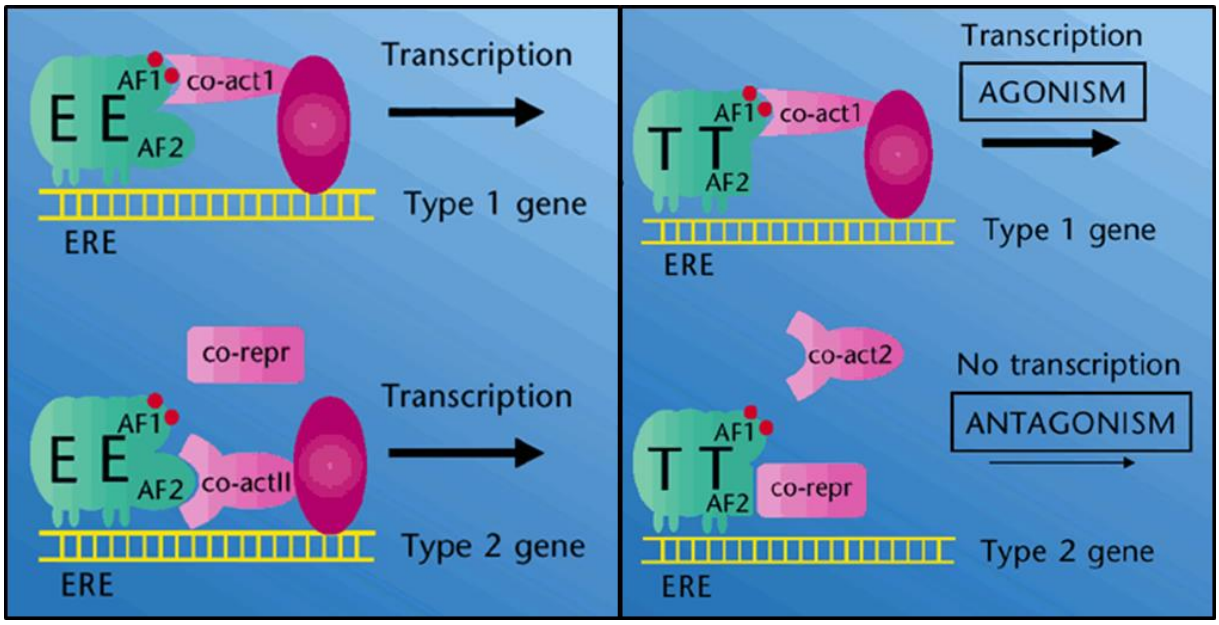


Figure 3: Tamoxifen does not inhibit but alters transcriptional activity of ER. On the left, figure illustrates binding of estradiol (E) to ER, which subsequently synergizes with activation factor 1 and 2 (AF1 and AF2) upon dimerization to further translocate dimer structure to recognized ERE site. Co-activators attracted by AF1 or AF2 resulting in transcription of different genes. In the presence of the tamoxifen (T) conformational change is seen in activation factors, followed by recruitment of the co-repressors instead of co-activators. This conformational change led by tamoxifen results transcriptomic alteration (Modified from: Dowsett and Howell, 2002, Nat Med) [26]. See Appendix for the copyright permission.

1.3 Drug Resistance

Drug resistance is divided into two categories: First one is called intrinsic or *de novo* drug resistance, which is defined by pre-existing resistance factors and elements before initiation of the treatment. Because of pre-existing conditions, drug never becomes truly effective. The second one is called acquired drug resistance. Here, patients respond to treatment initially; however, response is lost over the course of treatment [27] [28]. Regardless of the type, resistance is a main issue in cancer therapies, and great number of studies is ongoing to tackle this problem.

1.3.1 Therapy Options for ERα-Positive Breast Cancer after Resistance Development

Although 5 years of adjuvant tamoxifen therapy has been demonstrated to reduce mortality rate by 30-40% in the first 15 years [20] [29], unfortunately about half of patients develop intrinsic (*de novo)* or acquired resistance and metastasis in long-term [29] [30] [31]. After failure of tamoxifen treatment, patients with early stage of breast cancer are prescribed with other ER antagonists as well as aromatase inhibitors [18]. In advanced stages, they are treated with combination of aromatase inhibitors and ER antagonists [32]. When cancer is more aggressive and in metastatic stage, drugs with different mechanisms of action are used, such as an mTOR inhibitor Everolimus [17], a CDK4/6 inhibitor Palbociclib [33] or a VEGF modulator Bevacizumab [34]. In addition to those targeted drugs, chemotherapy is also an option for those patients [32].

Patients are generally prescribed with endocrine therapy rather than chemotherapy due to the fact that endocrine therapy is much more tolerated than the latter. On the other hand, in rapid progression and metastasis breast cancer situation, patients are given a chemotherapy regimen [33]. Lapatinib, a kinase inhibitor, showed a great potential to overcome tamoxifen resistance especially in patients with reduced or no ER expression [35] [36]. Several studies suggested that if EGFR, a commonly upregulated receptor in tamoxifen resistance, is targeted, it would benefit patients. Indeed, clinical studies showed that gefitinib, an EGFR inhibitor, combined with tamoxifen resulted in better outcome than tamoxifen alone in advanced and metastatic settings [37] [38].

1.3.2 Resistance Mechanisms Against Tamoxifen

The well-known mechanisms of tamoxifen resistance are loss of ER expression lost over time and simultaneous activation of other oncogenic signaling pathways. Hence, tamoxifen cannot target ER or modify its downstream events. Drug becomes ineffective and cancer cells in this stage are considered as “tamoxifen resistant” [16]. In the tamoxifen responsive tumors, tamoxifen alters transcriptomic effect of ER by competing estradiol, subsequently alters transcription effect of it and consequently pro-apoptosis genes dominate pro-survival genes. On the other hand, in the tamoxifen irresponsive tumors, growth factor receptor-mediated pro-survival signaling pathways take place, eventually leading ER depletion. In the absence of ER, tamoxifen cannot regulate its transcriptional activity and pro-survival gene expression is restored by GFR-mediated signaling. (***Figure 4***)

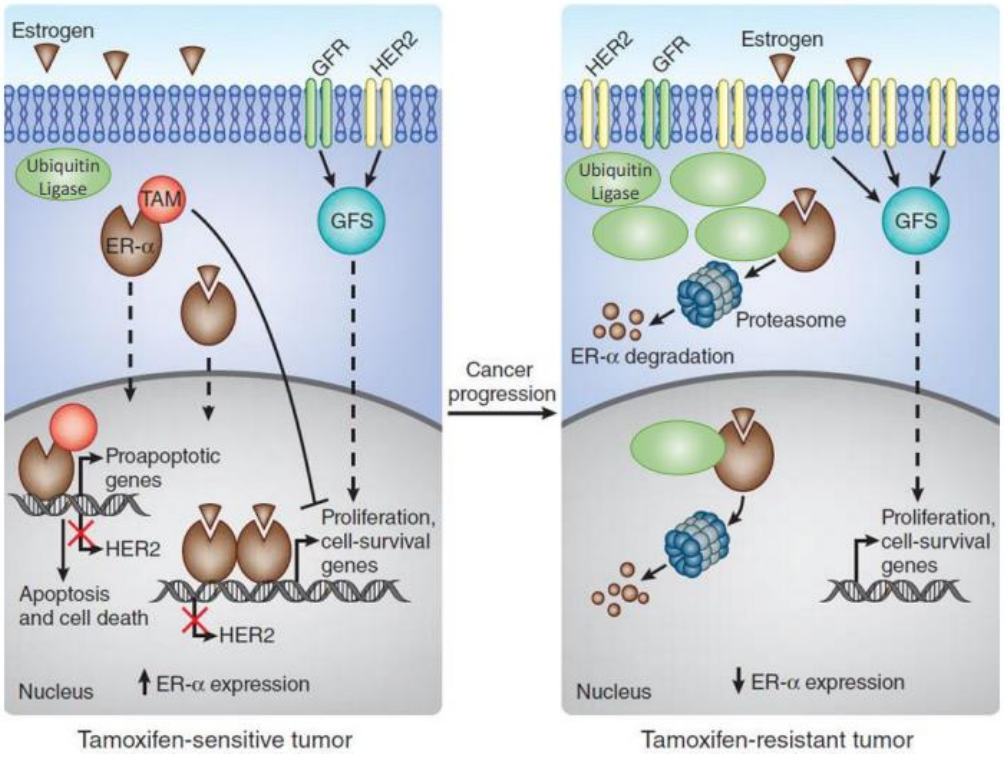


Figure 4: Schematic representation of over-simplified tamoxifen sensitiveness and resistance mechanisms. On the left, tamoxifen alters transcriptomic effect of ER and switches its downstream from proliferative gene expression to apoptosis-related genes. On the right, HER2 and GFR mediated tamoxifen resistance mechanism is shown which eventually leads to decreased ER levels. Thus, tamoxifen cannot control its transcriptional activity. (Modified from: Thomas & Gustafsson, 2011, Nat Med [39]). See Appendix for the copyright permission.

Expression levels of ER and PR decrease

Decrease in ERα expression level is a common phenomenon in tamoxifen resistance. Studies have shown that both patients and *in vitro* models lose expression of ERα for some extent upon tamoxifen treatment [40] [24]. Epigenetic silencing through hypermethylation in the promoter of ER is reported in tamoxifen treated patient [24] [41] and *in vitro* samples [42] [43] samples. Moreover, loss in ER expression was also suggested to be consequence of stimulation of MAPK/ERK and PI3K/AKT/MTOR signaling pathways through over-activated HER2 and other GFRs [40] [44] [25] [45] .In addition to decrease in ERα expression levels, cells could also decrease Progesterone Receptor (PR) levels as well. Since PR is a direct transcriptional target of ER, loss of PR expression clinically validated to be additive factor on tamoxifen resistance [9].

GFR upregulation

Not only steroid hormone regulated receptors, but also growth factor mediated receptors were shown to contribute tamoxifen resistance by both *in vitro* and clinical studies. EGFR and HER2 are the most frequently altered non-steroidal receptors in the breast cancer. Both of them are generally associated with basal and HER2-enriched subtypes which are considered to be rather advanced. Yet, luminal patients whose ER expression decreased or lost in the course of treatment, were known to have increased EFGR and HER2 expression which would not only give them more advanced breast cancer properties, but also compensate for ER lost and cell cycle block. Afterwards, cancer cells would have another abnormally active pathway to avoid the anti-cancer effects of tamoxifen [16] [33] [46].

Non-coding RNAs

Global transcriptome analyses performed in recent years demonstrated that most parts of the genomic DNA that are transcribed into RNA are not further translated into proteins. RNA molecules that are not converted into proteins and are therefore called non-coding RNAs (ncRNA) were found to be involved in cellular processes like sequence-specific chromosome modifications, gene silencing and regulation of protein signaling pathways. They are classified into 2 major classes: short and long non-coding RNAs. Long non-coding RNAs (lncRNAs) are further divided into several subclasses, and lincRNA or long intergenic non-coding RNA, most abundant member, is spanning a length of more than 200 nucleotides [47].

Recently, the functions of non-coding RNAs, thanks to increasing studies, are uncovered. Several of these studies proved the potential of the non-coding RNAs in disease progression. They can either take an active part in disease progression or may act as a biomarker. Short non-coding RNAs, including miRNAs, siRNAs, piwiRNAs etc., are relatively more studied compared to long non-coding RNAs. MicroRNAs (miRNAs) are 20-22 nucleotide long RNA molecules that inhibit protein synthesis by binding to mRNAs. There are few well-known miRNAs associated with cancer, such as miR-200 family and let-7. Furthermore, in terms of tamoxifen resistance in breast cancer, ectopic expression of miR-375 was shown to overcome tamoxifen resistance *via* modulating EMT, while another miRNA, miR-519, was proven to cause tamoxifen resistance [48] [49]. These are just few examples of many small non-coding RNAs associated with tamoxifen resistance.

1.4 Long Non-Coding RNAs in Human Diseases

Non-coding part of the human genome has seem shown to have functional roles in various biological processes. Genome-wide association studies (GWAS) mainly focusing on single nucleotide polymorphism (SNP) showed that about 40% of a trait or a disease- associated SNPs are found in non-coding regions of the genome [50]. Those studies played important roles to reveal functions of non-coding RNAs. The list of diseases associated by lncRNAs are long and in progress, yet it includes Prader-Willi Syndrome of neuronal system [51], fragile X syndrome [52], possibly Alzheimer's disease [53], coronary diseases [54] [55], and lastly, cancer [56] [57] [58].

1.4.1 Effect of Long Non-Coding RNAs in Breast Cancer

GWAS and later on functional studies demonstrated that non-coding RNAs are functional and take a role in various biological processes. Homeobox antisense intergenic RNA or widely known as HOTAIR, is one of the most well-known lncRNAs in cancer biology. While it is more commonly studies in breast cancer, its influence is not limited to a single type of cancer. Rather, includes gastric, colorectal and cervical cancers [59]. The reason behind how it affects different cancers is that it regulates chromatin methylation status of a neighbor gene, HOXD, which belongs to homeobox polycomb complex protein family regulating developmental processes [60]. Another such example is Metastasis Associated in Lung Adenocarcinoma Transcript 1, shortly MALAT1. MALAT1 is located in nucleus and its increased expression is linked to metastatic capacity of the both lung and breast tumors [61] [62]. Higher expression of both lncRNAs predicts worse survival, and also is suggested to be a biomarker in terms of metastasis and survival [63] [64] [65].

One of the lncRNAs important as being the first lncRNA identified to contribute to anti-estrogen resistance is Breast Cancer Anti-Estrogen Resistance 4 (BCAR4) This lncRNA, previously named as *LOC400500,* is initially reported to contribute to anti-estrogen resistance when ectopically expressed in *in vitro* settings [66]. Although initial study was focused on a functional screen using cDNA libraries, a clinical study conducted with 280 ERα-positive, first-line tamoxifen-treated patients confirmed the important role of this lncRNA in tamoxifen resistance where higher BCAR4 expression is correlated with lower RFS and poor clinical benefit rate [67]. Later, characterization studies were performed to solve underlying

mechanism of BCAR4 [68] [69]. The studies suggested that BCAR4 expression contributes to therapy failure regardless of ER function, possibly through modulating non-canonical Hedgehog signaling pathway. However, the role of lncRNAs in tamoxifen resistance is at its infancy.

1.5 Autophagy and Cancer

Autophagy is the catabolic degradation of cellular components in response to starvation or stress [70]. Autophagy is believed to influence tumor development. In one study, basal levels of the autophagy was found to be much less in cancer cells than their normal counterparts [71]. However, autophagy has a dichotomous role in cancer. In early stage of cancer, it was shown to have tumor suppressor effect. In a study conducted with mice bearing deletion in a major autophagy protein, Beclin1, manifested lung cancer in early ages. On the other hand, at a later stage of cancer, autophagy is believed to have tumor-promoting effect, and there are a number of clinical studies combining autophagy inhibitors with cancer drugs (***Figure 5***) [72] [73]. Currently, both activation and complete inhibition of autophagy approaches are being tested in cancer treatment [74].

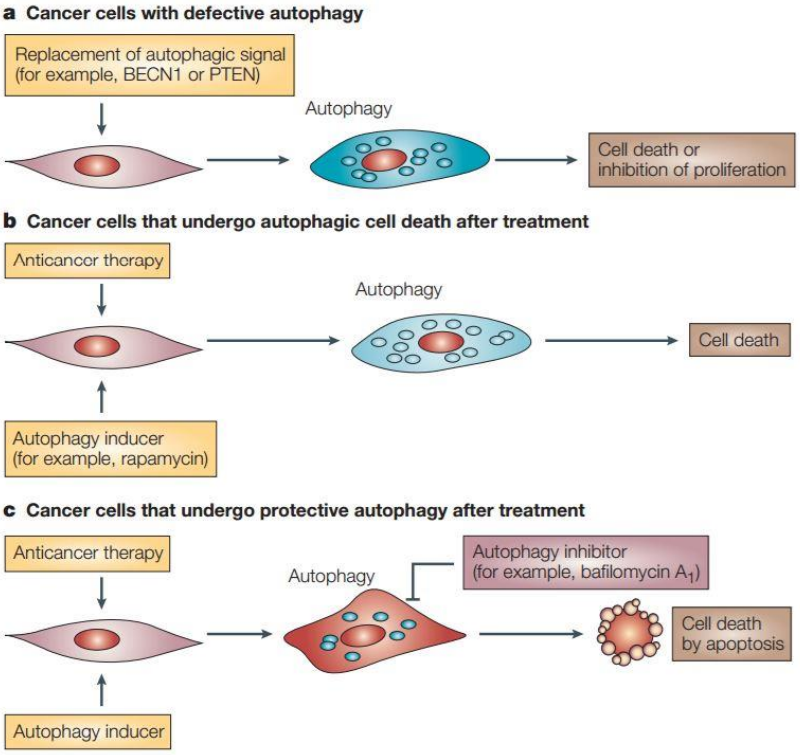


Figure 5: Schematic representation of modulation of autophagy in cancer. (A) When cancer cells are re-introduced with absent or defective autophagic proteins, autophagy is induced and results in cell death. (B) Anticancer therapy along with other autophagy inducers can enhance autophagy and cell death. (C) Cancer cells may use protective autophagy as tumor-promoting process. Those types of cells could then be treated with autophagy inhibitors to switch autophagy to apoptosis. (Taken from: Kondo et al., 2005, Nat Rev Cancer [72]) See Appendix for the copyright permission.

1.5.1 Autophagy in Breast Cancer

One of the well-studied autophagy regulator proteins, Beclin1, was reported to be decreased in breast cancer tissues compared to their normal counterparts. A key study which was conducted with MCF7 xenografts to validate the tumor suppressor role of Beclin1 in breast cancer showed that when Beclin1 expression was restored to normal levels, tumor growth was suppressed [75]. Besides Beclin1, mTOR (mechanistic target of rapamycin) has an important role in controlling autophagy. Active mTOR blocks autophagy induction while in the case of starvation, mTOR is inhibited allowing autophagy to be activated. Therefore, mTOR inhibitors are proposed to induce autophagy in breast cancer [76] [77].Importantly, tamoxifen was shown to be an autophagy inducer drug [78].

1.6 Hypothesis and Aim

ERα-positive breast cancer patients treated with tamoxifen initially have good response, but after a long latent period, tumors relapse. Even though there have been numerous studies on tamoxifen resistance, not all the players, especially lncRNAs, in this process are identified. A large, heterogeneous patient population with different molecular backgrounds, adverse side effects of the second-line and third-line therapeutic agents, advances in the field of personalized medicine and accumulation of the cancer biology knowledge give motive to investigate and understand more on drug resistance and enhance therapy options for the tamoxifen resistant patients.

Thus, non-coding portion of the genome has great potential to bring up new targets and/or biomarkers overcoming tamoxifen resistance. In this thesis, I aimed to take an unbiased transcriptome approach to identify novel lncRNA regulators of tamoxifen resistance. In this line, whole transcriptome analysis was performed in acquired tamoxifen resistant cell line models (MCF7-TamR) and their sensitive counterparts (MCF7-WT). After refining whole RNA-Seq results, I selected validated lncRNAs and narrowed down the list by choosing previously cancer-associated lncRNAs. LINC00152 was the most prominent candidate among 330 lncRNA transcripts in the list. It was previously shown to be prognostic marker for gastric, hepatocellular and lung carcinoma. Furthermore, a recent study by Grembergen et. al., showed that LINC00152 expression was elevated in breast cancer [79]. In line with its oncogenic potential, LINC00152 was also upregulated in TamR-resistant cell line models (both MCF7-TamR and T47D-TamR). Knockdown of LINC00152 sensitized both resistant cell lines to tamoxifen. Importantly, this sensitization was potentiated by autophagy induction and apoptosis. Finally, higher expression of LINC00152 was associated with worse survival in tamoxifen-treated ER alpha positive breast cancer patients showing potential role of LINC00152 in tamoxifen resistance.

CHAPTER 2

2 MATERIALS AND METHODS

2.2 Materials

2.2.1 Buffers

1x Anode Buffer I	300mM Tris, 20% (v/v) methanol
1x Anode Buffer II	25mM Tris, 20% (v/v) methanol
1x Cathode Buffer	40mM 6-aminocaparoic acid, 20% (v/v) methanol
1x SDS-PAGE Running Buffer	25mM Tris, 14.41g/l glycine, 1% (v/v) SDS
1x TBST	20nM Tris, 8g/l NaCl 0.2% (v/v) Tween20

2.2.2 Chemicals and Reagents

4x Protein Loading Dye	250mM Tris HCl (pH:6.8), 10% (w/v) SDS, 0.1% w/v), Bromophenol Blue, 50% (v/v) Glycerol, 25% (v/v) Betamercaptoethanol
6-aminocaparoic acid	Sigma Aldrich, St Louis, MO, USA
Acrylamide/bisacrylamide	Applichem, Darmstadt, Germany
Ammonium persulfate (APS)	Carlo Erba, Cornaredu, Italy
Bovine Serum Albumin (BSA)	Santa Cruz, Dallas, TX, USA
ElectroChemoLuminescence (ECL) Detection Reagent	Amersham Pharmacia Biotech, Amersham, UK
Ethanol	Sigma Aldrich, St Louis, MO, USA
Isopropanol	Sigma Aldrich, St Louis, MO, USA
LightCycler 480 SYBR Green I Master Mix	Roche Applied Science, Mannheim, Germany

Lipofectamine 2000	Invitrogen, Carlsbad, CA, USA
Methanol	Sigma Aldrich, St Louis, MO, USA
Milk Powder	Sigma Aldrich, St Louis, MO, USA
Nuclease free water	Applied Biosystems/Ambion, Austin, TX, USA
Page Ruler Protein Ladder	Thermo Ficher Scientific, Waltham, MA, USA
Phosstop	Roche Applied Science, Mannheim, Germany
Ponceu S	Sigma Aldrich, St Louis, MO, USA
Protease inhibitor cocktail	Roche Applied Science, Mannheim, Germany
Sodium Chloride (NaCl)	Merck, Darmstandt, Germany
Sodium Dodecyl Sulfate (SDS)	Merck, Darmstandt, Germany
Tetramethyl ethylenediamine (TEMED)	Serva, Heidelberg, Germany
TRIsure	Bioline, Luckenwalde, Germany
Triton X-100	Sigma Aldrich, St Louis, MO, USA
Trizma Base (Tris)	Sigma Aldrich, St Louis, MO, USA
Tween-20	VWR, Radnor, PA, USA

2.2.3 Media and Supplements

DMEM	Lonza, Basel, Switzerland
Fetal bovine serum (FBS)	Biowest, Nuaille, France
Insulin	Sigma Aldrich, St Louis, MO, USA
Non-essential aminoacids (NEAA)	Lonza, Basel, Switzerland

optiMEM	Invitrogen, Carlsbad, CA, USA
Penicillin/Streptomycin (P/S)	Lonza, Basel, Switzerland

2.2.4 Kits

BCA Protein Assay Kit	Pierce, Rockford, IL, USA
Cell Titer-Glo cell viability assay kit	Promega, Madison, WI, USA
MycoAlert detection kit	Lonza, Basel, Switzerland
Revert Aid first strand cDNA synthesis kit	Fermantas, St Leon-Roth, Germany

2.2.5 Equipment

Cell Culture Hood	Nüve, Ankara, Turkey
Cell Culture Incubator	Nüve, Ankara, Turkey
Centrifuges	Thermo Fisher Scientific, Waltham, MA, USA Beckman, Pasadena, CA, USA
Counting Chamber	Marienfeld, Könighofen, Germany
Freezer (-20C)	Bosch, Stuttgart, Germany
Freezer (-80C)	Hettich, Geldermansen, Germany
Fridge	Bosch, Stuttgart, Germany
Horizontal shakers	FinePCR, Seoul, South Korea Bellco, Vineland, NJ, USA
LightCycler 96	Roche Applied Science, Mannheim, Germany
Mini-PROTEAN Gel casting module	Biorad, Hercules, CA, USA

Mini-PROTEAN Tetra Cell	Biorad, Hercules, CA, USA
Multichannel Pipette	Thermo Fisher Scientific, Waltham, MA, USA
NanoDrop 1000	Thermo Fisher Scientific, Waltham, MA, USA
Nikon TS300 Inverted Microscope	Nikon, Tokyo, Japan
Power supplies for electrophoresis	Biorad, Hercules, CA, USA
Semidry western blot transfer unit	Biorad, Hercules, CA, USA
Synergy HT Multireader	Biotek, Winooski, VT, USA
Vortex	Isolab, Wertheim, Germany
Water Bath	Nüve, Ankara, Turkey
X-ray casette	Amersham Pharmacia Biotech, Amersham, UK
X-ray hyper processor	Amersham Pharmacia Biotech, Amersham, UK

2.2.6 Consumables

100mm dishes	Greiner bio-one, Frickenhausen, Germany
145mm dishes	Greiner bio-one, Frickenhausen, Germany
6-well plates	Greiner bio-one, Frickenhausen, Germany
96-well plates	Greiner bio-one, Frickenhausen, Germany
Cell scrapers	Greiner bio-one, Frickenhausen, Germany
Coverslips	Marienfeld, Königshofen, Germany
Cryovials	Greiner bio-one, Frickenhausen, Germany
Cuvettes	VWR, Radnor,PA, USA
Filtered pipette tips (10ul, 20ul,	Greiner bio-one, Frickenhausen, Germany

200ul, 1000ul)	
Microscope slides	Marienfeld, Königshofen, Germany
Parafilm	VWR, Radnor,PA, USA
PCR tubes	Axygen, Corning, NY, USA
Plastic pipettes (5 ml, 10ml, 25ml)	Corning Incorporated, Corning, NY, USA
PVDF Membrane	Biorad, Hercules, CA, USA10
qPCR Plates	Roche Applied Science, Mannheim, Germany
Reaction tubes (500ul, 1.5ml, 2ml)	Axygen, Corning, NY, USA
Storage bottles (250ml, 500ml, 1L)	Corning Incorporated, Corning, NY, USA
Whatmann paper	GE Healthcare, Little Chalfont, UK
White plates	Costar, Corning, NY, USA
-X-ray films	Kodak, Rochester, NY, USA

2.3 Methods

2.3.1 Cell Culturing

2.3.1.1 Culturing Human Breast Cancer Cell Lines MCF7 and T47D

MCF7-WT and MCF7-TamR cells are developed previously [48]. T47D cells were generous gift from Işık Yuluğ's group. Cells were grown in 100mm petri dishes at 37°C 5% CO_2 condition. Cells were passaged every 4-5 days in 1-to-3 ratio. Medium of the cells was composed of phenol-red-free DMEM or RPMI supplemented with 10% heat inactivated and filtered FBS, 1% NEAA, 1% P/S and 0.01 mg/ml of insulin. For cell splitting, growth medium was removed, and cells were briefly rinsed with 2 ml PBS, then 1.5ml trypsin was added onto cells and incubated in 37° C for 5 minutes. Trypsin was inhibited adding fresh culture medium, and cells were resuspended. Petri dish was filled up to 10 ml after splitting.

2.3.1.1.1 Development of Tamoxifen Resistant Cell lines

T47D cells are first tested for mycoplasma contamination and tamoxifen response. TamR resistant T47D cells were developed under continuous tamoxifen exposure for more than 8 months. Briefly, 4-OH-tamoxifen was added into culture medium one day after splitting. Medium was kept with tamoxifen for 3-4 days, and then growth medium was replenished in order to allow cells to proliferate. After 2-3 days of recovery, cells were split and incubated with tamoxifen until cells develop resistance. All cell lines were tested for mycoplasma contamination periodically.

2.3.1.2 Antisense oligonucleotide (ASO) Transfections

ASO was designed according to manufacturer's recommendations. All nucleotides bear Phosphorothioate bonds (PS bonds). ASOs were aliquoted under the hood with nuclease-free, high grade water upon delivery. ASO transfections were carried out using Lipofectamine 2000^{TM} and OptiMEM reagents. Final concentrations of ASOs were 25nM/well. Sequence of the ASOs are given in Table 1. Cells were seeded at a number of 200,000 cell/well for 6-well-plate and 6,000 cell/well for 96-well-plate. After 24 hours of cell seeding, cells received 25nM final concentration of ASO. All transfections were done with P/S-free medium.

Table 1. List of ASO sequences used in the experiments (* stands for PS bonds)

Antisense Oligonucleotide Sequences		**Manufacturer**
LINC00152 ASO	G*T*G*T*G*T*C*A*T*A*G*A*G*C*T*T*C*C*T*G	IDT
Control ASO	G*C*G*T*A*T*T*A*T*A*G*C*C*G*A*T*T*A*A*C	IDT

2.3.2 Cell-based Assays

2.3.2.1 *In Vitro* Sensitization Assay

Viability of the cells was measured by Cell Titer-Glo cell viability assay kit according to manufacturer's instructions. Briefly, cells were seeded as 4 replicates in 96-well format where each of the wells harbors 6,000 cells. Seventy-two hours after treatment, cells were incubated

with Cell-Titer-Glo reagent about 15 minutes on orbital shaker. After incubation, equal volume of the media was transferred onto opaque white bottom 96-well plates, and luminescence was measured on Biotek Multireader machine. Readings were converted to percent inhibition, and Student's two-tailed t-test was used for statistical analysis.

2.3.3 Molecular Biology

2.3.3.1 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

2.3.3.1.1 RNA isolation

Cell seeding and treatments were done as described previously (2.2.1.2.). After 24, 48 and 72 hours of the incubation in cells were collected by trypsinization and rinsed once with PBS. RNA was isolated by using TRIsure, chloroform and isopropanol. After isolation, RNAs were solubilized with adequate volume of nuclease-free, high grade water and stored at -80° C freezer.

2.3.3.1.2 cDNA synthesis

RevertAid Reverse Transcription (RT) first strand cDNA synthesis kit was used for reverse-transcription of total RNA. Total of 1-2ug RNA from each sample was converted to cDNA by using random oligomer. Protocol for RT is given at Table 2

Table 2. Components of first step of RT reaction.

Reagent	**Volume**
RNA (1-2 ug)	X ul
Random primer	1ul
H2O	(11-X) ul

Samples were placed in ThermoCycler for 5 mins at 65° C. After first step was accomplished, proceeded with the last step.

Table 3. Components for last step of RT reaction.

Reagent	**Volume**
5x Revert Aid reaction buffer	4ul
dNTPs(10mM)	2ul
Revert Aid H Minus M-MuLV RT	1ul
Ribolock Ribonuclease Inhibitor	1ul

Samples were placed back in ThermoCycler and incubated as stated in Table 4.

Table 4. Thermocycler program for cDNA synthesis.

Temperature	**Time**
37º C	5 minutes
42º C	60 minutes
70º C	10 minutes
4º C	∞

All cDNA samples were diluted to 10 ng/uL of final concentration before proceeding with qRT-PCR.

2.3.3.1.3 qRT-PCR for RNA expression

For quantitative Real-Time-PCR, SYBR Green Master mix was used along with the primers listed on Table 5.

Table 5. List of qRT-PCR primers.

Name	**Gene ID**	**Forward Primer (5' to 3')**	**Reverse Primer (5' to 3')**
ACTB	60	CCAACCGCGAGAAGATGA	CCAGAGGCGTACAGGGATAG
CDH1	999	CCCGGGACAACGTTTATTAC	GCTGGCTCAAGTCAAAGTCC
CDH2	1000	ACAGTGGCCACCTACAAAGG	CCGAGATGGGGTTGATAATG
ESR1	2099	TTACTGACCAACCTGGCAGA	ATCATGGAGGGTCAAATCCA
FN	2335	CTGGCCGAAAATACATTGTAAA	CCACAGTCGGGTCAGGAG

GAPDH	2597	GCCCAATACGACCAAATCC	AGCCACATCGCTCAGACAC
HPRT	3251	TGACCTTGATTTATTTTGCATACC	CGAGCAAGACGTTCAGTCCT
KRT18	3875	GGCTTGTAGGCCTTTTACTTCC	GGCTTGTAGGCCTTTTACTTCC
LINC00152	112597	ATAACGGGAACCAGCGGAC	AGGGGGCTGAGTCGTGATTT
MMP9	4318	GAACCAATCTCACCGACAGG	GCCACCCGAGTGTAACCATA
SNAI2	6591	TGGTTGCTTCAAGGACACAT	GTTGCAGTGAGGGCAAGAA
ZEB1	6935	GGGAGGAGCAGTGAAAGAGA	TTTCTTGCCCTTCCTTTCTG
ZO1	7082	CAGAGCCTTCTGATCATTCCA	CATCTCTACTCCGGAGACTGC

Reaction was performed in 96-well LightCycler plates. Each well contained 20ng cDNA and 8 uL of MasterMix (total volume of 10 uL). Reaction components were given in Table 6.

Table 6. Mastermix components for qRT-PCR reaction

Reagent	**Volume**
dNTP	2,5 uL
Forward Primer (20uM)	0,25 uL
Reverse Primer (20uM)	0,25 uL
SYBR Green	5 uL

Tightly-sealed plate was briefly centrifuged and then placed into LightCycler® 96 qRT-PCR thermocycler (Roche Applied Science, Mannheim, Germany) machine. qRT-PCR incubation program was shown on Table 7.

Table 7. qRT-PCR incubation program.

Pre-incubation							
Target (°C)	**Acquisition Mode**	**Hold (hh:mm:ss)**	**Ramp Rate (°C/s)**	**Acquisitions (per °C)**	**Sec Target (°C)**	**Step Size (°C)**	**Step Delay (cycles)**
95	None	00:05:00	4.4	5	0	0	0
Amplification							
Target (°C)	**Acquisition Mode**	**Hold (hh:mm:ss)**	**Ramp Rate**	**Acquisitions (per °C)**	**Sec Target**	**Step Size**	**Step Delay**

			(°C/s)		(°C)	(°C)	(cycles)
95	None	00:00:10	4.4	5	0	0	0
58	Single	00:00:20	2.2	5	0	0	0
72	None	00:00:20	4.4	5	0	0	0
Melting Curve							
Target (°C)	**Acquisition Mode**	**Hold (hh:mm:ss)**	**Ramp Rate (°C/s)**	**Acquisitions (per °C)**	**Sec Target (°C)**	**Step Size (°C)**	**Step Delay (cycles)**
95	None	00:00:05	4.4	5	0	0	0
55	None	00:01:00	2.2	5	0	0	0
95	Continuous	00:00:00	0.11	5	0	0	0
Cooling							
Target (°C)	**Acquisition Mode**	**Hold (hh:mm:ss)**	**Ramp Rate (°C/s)**	**Acquisitions (per °C)**	**Sec Target (°C)**	**Step Size (°C)**	**Step Delay (cycles)**
4	None	00:00:30	2.2	5	0	0	0

2.3.3.1.4 qRT-PCR data analysis

All qRT-PCR plates had at least two housekeeping genes. Every sample had triplicates for one gene. Data analyses were done using ΔΔCt method and normalized to geometric average of the housekeeping genes. Statistical analysis was performed using Student's two-tailed t-test.

2.3.3.2 RNA Localization

The whole protocol was modified from study of Zhang et. al. (2014) [80]. In summary, the cells were harvested fresh using a scraper when they reached around 3 million cells, and were briefly centrifuged at 4° C. Pellet was re-suspended by Cell Lysis Buffer. After the suspension was incubated on ice up to 15 mins, it was transferred into a sterile Dounce homogenizer. Ensuring not to make any bubbles, the cells were homogenized. Later, the homogenate was transferred to a fresh tube while adding Triton X-100 with a final concentration of 0.1%. After mixing the new suspension by inversion, samples were centrifuged at 1500g for 5 minutes at 4° C. While the supernatant contains cytoplasmic extract, the pellet contains nuclei and cell debris. All the supernatant was placed into a new tube without disturbing the pellet.

The nuclear and cytoplasmic RNAs are isolated using Tri-Reagent as described above (2.3.3.1.), and qRT-PCR was performed.

2.3.3.3 Protein Biochemistry

2.3.3.3.1 Protein isolation

Cell seeding and treatments were done as described previously (2.2.1.2.). After 24, 48 and 72 hours of the incubation, first medium was collected and centrifuged at 5,000 rpm for 5 minutes for apoptotic body collection. Cells were then trypsinized and collected. Depending on cell pellet size, 50-100μL RIPA lysis buffer was added to the pelllet and mixed thoroughly by pipetting up and down. Suspension was then transferred to 1.5 ml Eppendorf tubes and vortexed for 5-10 seconds every 5 minutes for a total of 30 minutes. Later, this suspension was centrifuged at 13,000 rpm 4^{o}C for 30 minutes. Supernatant was collected as protein and stored at -20^{o}C for further experiments.

2.3.3.3.2 Protein quantification

Quantity of the proteins was determined by BCA Assay Kit according to manufacturer's instructions. Nine different standard protein solutions (BSA) with a range of 0-2 ug/uL were placed in 96-well clear plates in duplicates. Twenty uL of the standard solutions were used while only 5 uL of the unknown sample was used. Thus, standards and samples share a dilution factor of 5. A and B reagents were mixed 50:1 ratio, and total of 200 uL mixed working solution were added into each well. Proteins incubated in working solution for 30 minutes at 37° C. Colorimetric reading was obtained by BioTek Multiplate reader at 562 nm. A standard calibration curve was drawn based on absorbance readings from BSA standards, and sample concentrations were quantified from line graph of the curve.

2.3.3.3.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Concentrations and loading volumes of protein samples were equalized using 4X protein loading dye and RIPA buffer. Subsequently, samples were heated to 95°C for 3-5 minutes. Polyacrylamide-based stacking and resolving gels were prepared according to Table 8.

Table 8. Components of polyacrylamide gels.

Reagent	**8% Resolving gel**	**10% Resolving gel**	**12% Resolving gel**	**5% Stacking gel**
1 M Tris solution	(pH 8.8) 1.3ml	(pH 8.8) 1.3ml	(pH 8.8) 1.3ml	(pH 6.8) 260ul
10% APS	50ul	50ul	50ul	20ul
10% SDS	50ul	50ul	50ul	20ul
30% acrylamide mix	1.3ml	1.7ml	2ml	340ul
H2O	2.3ml	1.9ml	1.6ml	1.36ml
TEMED	5ul	5ul	5ul	2ul
Total volume	**5 mL**	**5 mL**	**5 mL**	**5 mL**

Using Mini-PROTEAN Gel casting module (Biorad, Hercules, CA, USA), resolving gel solution was poured initially and overlaid with isopropanol until gel polymerized. After removing isopropanol from gel casting system, stacking gel solution was poured on top of resolving gel, and 10 or 15 well comb was placed in it. 10-20 μg protein samples were loaded per well, and empty wells were filled with diluted protein loading dye. Electrophoresis was performed at 130V for 90 minutes.

2.3.3.3.4 Western Blotting

Whatmann papers with 3 mm thickness were cut in a dimension of 7cm X 9 cm. Four cut papers were soaked in anode buffer I, 2 of them in anode buffer II and 6 of them in cathode buffer. PVDF membranes were activated in 100% methanol for 3 minutes. Layers for a proper transfer from bottom to top were 4 papers for anode I, 2 for anode II, one activated PVDF membrane, polyacrylamide gel and lastly 6 papers for cathode. Semi-dry transfer was performed at 25V for 30-60 minutes. After transfer was completed, PVDF membrane was briefly stained with Ponceu S solution. Membranes were then washed with ddH_2O until Ponceu stain was completely removed and cut at specific molecular weight (kDa) of interest. Cut membranes were then blocked either in 5% (w/v) milk:TBST or in 5% (w/v) BSA:TBST for 1 hour room temperature Blocking solution was removed, and membranes were incubated with primary antibody either for 1-hour in room temperature or overnight in 4° C (Table 9). After primary antibody incubation, membranes were washed with 1X TBST and then

incubated with secondary antibody either for 1-hour at room temperature or overnight at 4° C. Later, membranes were again washed with 1X TBS-T three times for 10 minutes on shaker. After washing, enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech) was applied on membranes, and X-ray films were exposed to membranes for different time points ranging from 3 seconds to 30 minutes and then membranes were developed.

Table 9. List of Western Blot antibodies.

Gene Name	**Firm**	**Catalog Number**	**Dilution**
Beclin 1	Santa Cruz	sc-11427	1:2000
Beta-actin	MP Biomedicals	69100	1:5000
Bim	Santa Cruz	sc-11425	1:1000
Cleaved Caspase 7	Cell signaling	Sc-11427	1:1000
Cleaved PARP	Cell signaling	5625S	1:1000
EGFR	Cell signaling	2646S	1:1000
ER F10	Santa Cruz	sc-8002	1:2000
ERK1/2	Cell Signaling Technology	CST4695	1:1000
HER2	ThermoScientific	MA5-13105	1:1000
HRP-coupled anti-mouse IgG	Cell Signaling Technology	CST7076	1:10000
HRP-coupled anti-rabbit IgG	Cell Signaling Technology	CST7074	1:10000
LC3 A/B	Cell Signaling Technology	D3U4C	1:1000
p27/Kip1	BD Biosciences	610241	1:1000
p62	ThermoScientific	PA5-20839	1:4000
phospho-AKT (Ser473)	Cell Signaling Technology	CST4058	1:1000
phospho-AKT (Thr308)	Cell Signaling Technology	CST4056	1:1000
phospho-EGFR (Tyr1173)	Cell signaling	2244S	1:1000
phospho-ERK1/2 (Thr202/Tyr204)	Cell Signaling Technology	CST4376	1:1000
phospho-p38 (Thr180/Tyr182)	Santa Cruz	sc-17852	1:1000
phospho-Rb (Ser807/811)	Cell Signaling Technology	CST8516	1:1000
PR	Santa Cruz	sc-7208	1:2000
total-AKT	Cell Signaling Technology	CST9272	1:1000

2.3.4 Bioinformatics Analysis

2.3.4.1 Transcriptome Sequencing (RNA-Seq)

Whole Transcriptome sequencing was done by using Illumina HiSeq 2000 technology at McGill University, Canada. A paired-end library of 100 bp reads was produced for MCF7-WT and MCF7-TamR samples in triplicates. Total of 6 libraries were marked with different barcodes and afterwards pooled and read. Nearly 65 million paired-end 2×100bp reads/replicate were obtained.

2.3.4.1.1 RNA-Seq analysis

Data including the reads was sent to Bilkent *via* cloud as bmp format. Bmp was converted to FASTQ format in order to proceed with Tuxedo protocol [81]. Briefly, from tamoxifen resistant and sensitive samples were combined *via* TopHat v2.1.0, and mapped into human genome. As a reference genome, GH37(h19) and MiTranscriptome was used [82]. Then, *CuffLinks* was used to assemble transcripts, and *CuffDiff* identified differentially expressed transcripts between MCF7-WT and MCF7-TamR.

2.3.4.2 Patient Data Set Analysis

2.3.4.2.1 Patient Survival Analysis (Kaplan-Meier plot)

METABRIC patient data set along with NCBI Gene Expression Omnibus (GEO) database (GSE6532, GSE9195, GSE58644, GSE22220) were analyzed. By using Graphpad software (GraphPad software Inc., La Jolla, CA, USA) Kaplan-Meier patient survival graphs were generated for LINC00152 expression. Patient clinical information was given in GSE datasets. Patients with no survival information, "dead of non-cancerous cause" were censored. For each survival analysis, patients were separated from median. Log-rank (Mantel-Cox) test was applied as statistical analysis.

CHAPTER 3

RESULTS

Overall workflow of the study

In order to identify lncRNA regulators of tamoxifen resistance, I utilized MCF7 and T47D cell lines, both of which are well-known ERα-positive breast cancer cell lines. MCF7-WT and MCF-TamR cells were already available and used for identification of miRNA regulators of tamoxifen resistance in Şahin Lab [48]. Firstly, RNA-Seq analysis was performed using MCF7-WT and TamR cells in order to find differentially expressed lncRNAs associated with tamoxifen resistance. After well-refining steps for filtering cancer-associated and validated lncRNAs, I chose LINC00152 as a candidate potential mediator of tamoxifen resistance. In meantime, I developed a second model of tamoxifen resistance using T47D cell line *via* exposing cells to tamoxifen over 9 months which are named T47D-TamR. Using sensitization assays and protein biochemistry in both MCF7-TamR and T47D-TamR cell lines, I showed that LINC00152 sensitizes resistance cells to tamoxifen *via* inducing autophagy and apoptosis. Finally, these findings were corroborated with patient data analyses and LINC00152 was identified as a novel lncRNA regulator of tamoxifen resistance (***Figure 6***)

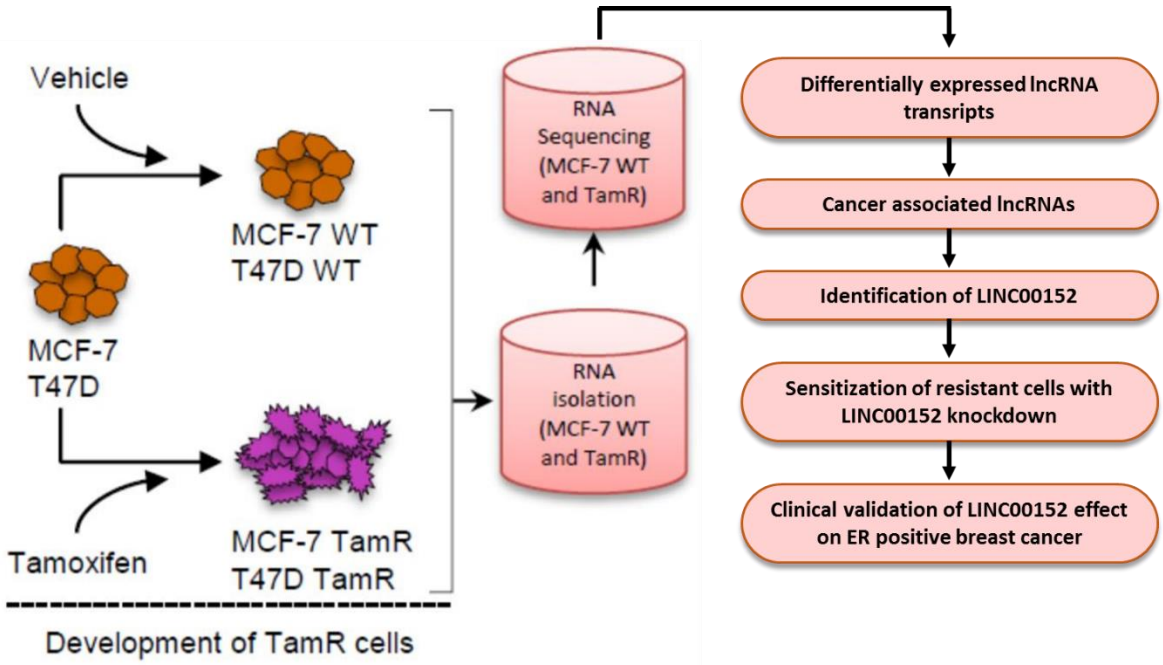


Figure 6. Overall workflow of the study. Acquired tamoxifen resistant cell lines were developed (in case of T47D-TamR) using ERα-positive breast cancer cell lines, MCF7 and T47D. Total RNA was

obtained and RNA-Seq was performed with MCF7-WT and MCF7-TamR cells. Differentially expressed lncRNA transcript lists were reduced according to criteria described in the text, and LINC00152 was selected. Later, effect of LINC00152 on tamoxifen resistance was examined in both MCF7 and T47D-TamR cells and also supported by clinical data.

3.1 Development and Characterization of Tamoxifen Resistant Cell Line Models

Tamoxifen has been the most commonly used endocrine therapy drug in the clinic to treat ER-positive breast cancer patients. However, intrinsic (*de novo*) or acquired resistance to tamoxifen is one of the important cause of the therapeutic failure, and it needs to be addressed. Potential mechanisms have been proposed so far in many studies, but the underlying molecular mechanisms of tamoxifen resistance are not fully understood yet. Our lab has previously studied different resistance mechanisms developed against targeted therapy agents for different subtypes of breast cancer. In this context, different resistant cell lines or animal models were developed and characterized to identify new targets that are involved in the acquisition of resistance [48] [49].

3.1.1 Developing Tamoxifen Resistant T47D Cell Line

In order to elucidate the molecular mechanisms for tamoxifen resistance, two different tamoxifen resistant cell line models were used in this thesis. Tamoxifen-resistant MCF-7 cells (MCF7-TamR) were previously developed and characterized [48] [49]. I generated a second tamoxifen resistant cell line model (Tamoxifen-resistant T47D cells (T47D-TamR)) to obtain a better representation of diverse patient background. Both cell lines are clinically classified as ER- positive, representing the luminal A molecular subtype. Therefore, these two cell lines are suitable models to study molecular events that are likely to be important in tamoxifen-treated ER-positive breast cancers.

As first step of resistance development, I assessed viability of T47D cells under tamoxifen treatment. The starting dose of tamoxifen for resistance development was selected as of 5 uM according to the dose response of T47D cells to ensure selection of resistant cell populations as well as maintenance of the well-being of the cells (***Figure 7***).

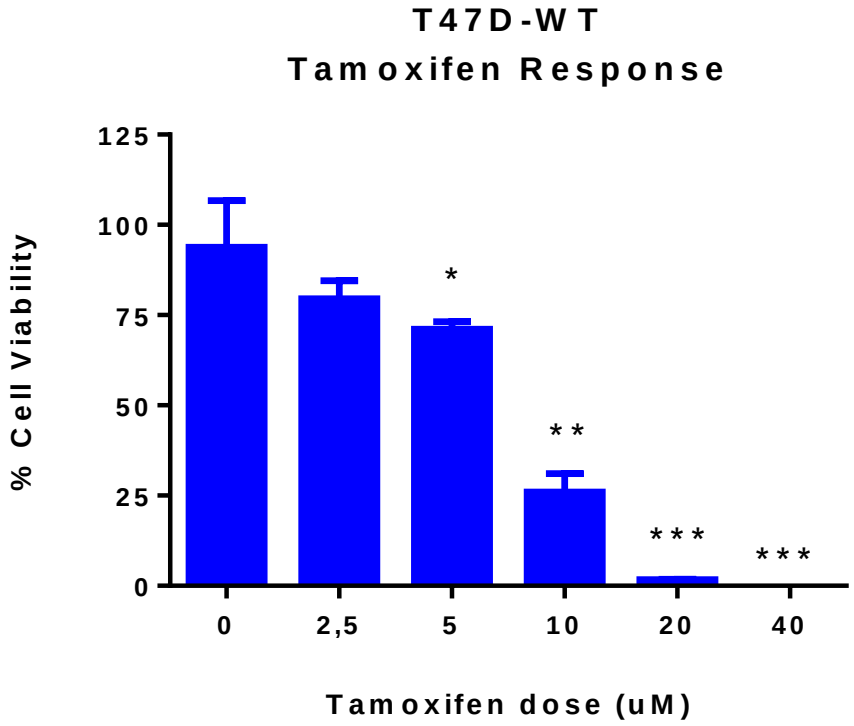


Figure 7: Dose response of the T47D cells to tamoxifen. Cells received increasing dose of 4-OH-Tam in triplicates, and cell viability assay was performed 3 days after treatment by Cell-Titer-Glo Reagent. At dose of 5 uM, cells showed 71% proliferation (*p<0.05). At higher doses, more than 50% percent of cell proliferation was inhibited (**p<0.01).

During the development of tamoxifen resistant T47D cell line, cells received the drug 1 day after passage, and medium was replenished with fresh medium after 3-4 days. I measured cell viability using Cell Titer Glo Reagent every second month to quantify degree of the resistance. T47D cell line was exposed to gradually increasing doses of tamoxifen for 9 months. At around the dose of 7.5 uM, parental cells reached their IC50 while for resistant cells it was only IC25 (***Figure 8A***). In order to confirm resistant profile of MCF7-TamR cells, I also examined the dose-response graph of MCF7-WT and TamR cells. Similar to T47D-WT and T47D-TamR profile, MCF7-TamR cells showed higher proliferation than WT

counterparts at same dose (MCF7-TamR IC50: 16.19 uM, MCF7-WT IC50: 11.99 uM) (***Figure 8B***). As a result, dose response graphs demonstrated that both cell lines have acquired tamoxifen resistance.

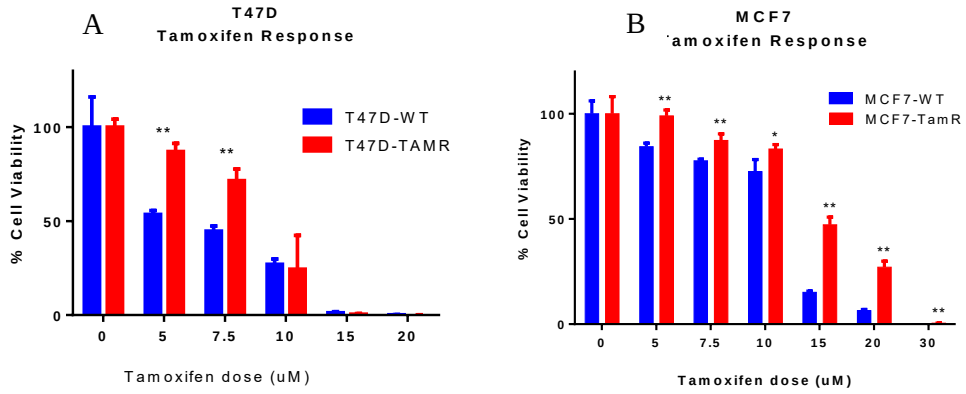


Figure 8: Dose response graphs of the tamoxifen resistant T47D and MCF7 cells and their sensitive counterpart. WT and TamR T47D (A) and MCF7 (B) cells received increasing doses of 4-OH-Tam in triplicates, and cell viability assay was performed 3 days after treatment by Cell-Titer-Glo Reagent. T47D-WT cells had IC50 of 6.55 uM whereas T47D-TamR cells had 8.62 uM (A). MCF7-WT cells had IC50 of 11.99 uM while MCF7-TamR cells had 16.19 uM (B). *(*p <0.05, **p<0.01)*

3.1.2 Phenotypic Characterization of the Tamoxifen Resistance

After confirmation of two different tamoxifen resistant cell line models, I performed the phenotypic characterization of the resistance profile. In order to identify altered cell processes which has been shown to important role in tamoxifen resistance, Western Blot analysis was done to examine the expression of key receptors in tamoxifen resistance mechanisms (EGFR and HER2) in T47D-WT and T47D-TamR cells. I also examined the activation status of proteins involved in cell proliferation (ERK) and cell survival (AKT). I also examined the expression of ERα and its target PR in these two TamR models compared to their parental counterparts. These experiments were also repeated in MCF7-TamR model.

Here, T47D-TamR cells almost completely lost their ERα protein while T47D-WT maintains its expression (**Figure 9**A). Loss of PR expression clinically validated to be additive factor in tamoxifen resistance [9]. Here, as a consequence of reduced ER expression, T47D-TamR cells drastically lost PR expression. Patients whose ER expression decreased or lost in the course of treatment were known to have increased EFGR and HER2 expression which

would not only give them more advanced breast cancer properties, but also compensation for ER loss and cell cycle block induced by tamoxifen. In line with this, I have observed a prominent increase in HER2 and EGFR protein expressions in TamR models which was completely absent in WT cells (**Figure 9**A). Similar to T47D model findings, MCF7 model also manifested similar profile (**Figure 9**B).

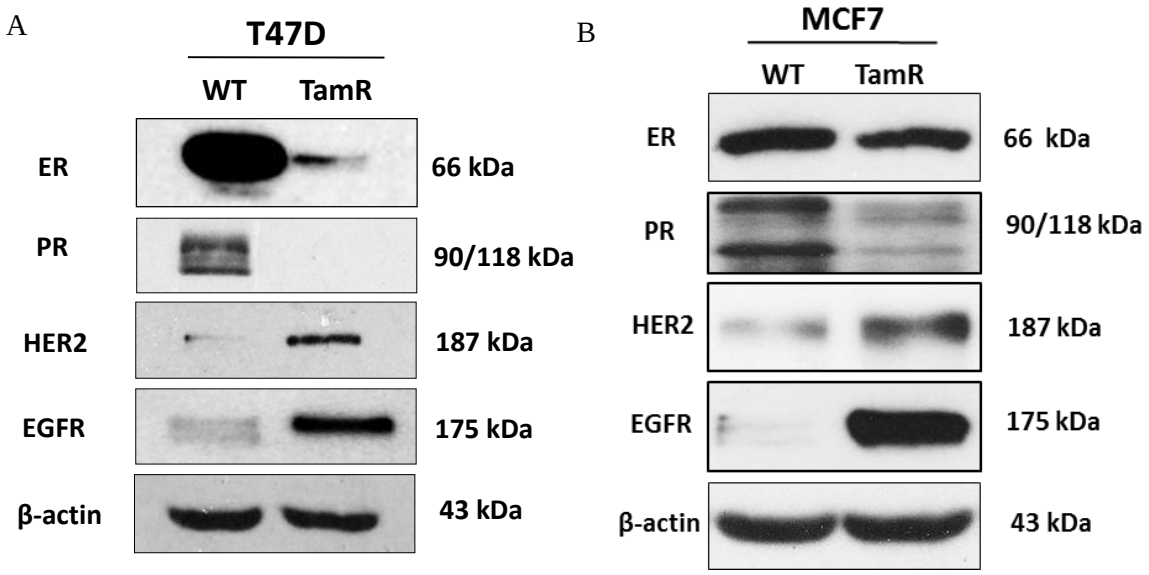


Figure 9: Western Blot results of tamoxifen resistance models with characteristic alterations in hormone and growth factor receptors. T47D-TamR cells manifested low ER and PR levels and elevated HER2 and EGFR expression compared to sensitive counterparts (A). The profile seen in T47D model was in line with previously developed MCF7 model (B). Actin was used as a loading control.

Acquired tamoxifen resistance is frequently associated with overactivation of the PI3K/AKT axis. As can be seen in **Figure 10**A and 10B, AKT1/2, main executer of the cell survival, was observed to be more activated at both phosphorylation sites in tamoxifen resistant TamR cells than their WT counterpart. In terms of cell proliferation, ERK activation was observed in both TamR models. As a consequence, *in vitro* acquired tamoxifen resistant cells (T47D-TamR and MCF7-TamR) showed increased cell survival and cell proliferation, further confirming unsuppressed viability under tamoxifen treatment (***Figure 10***)

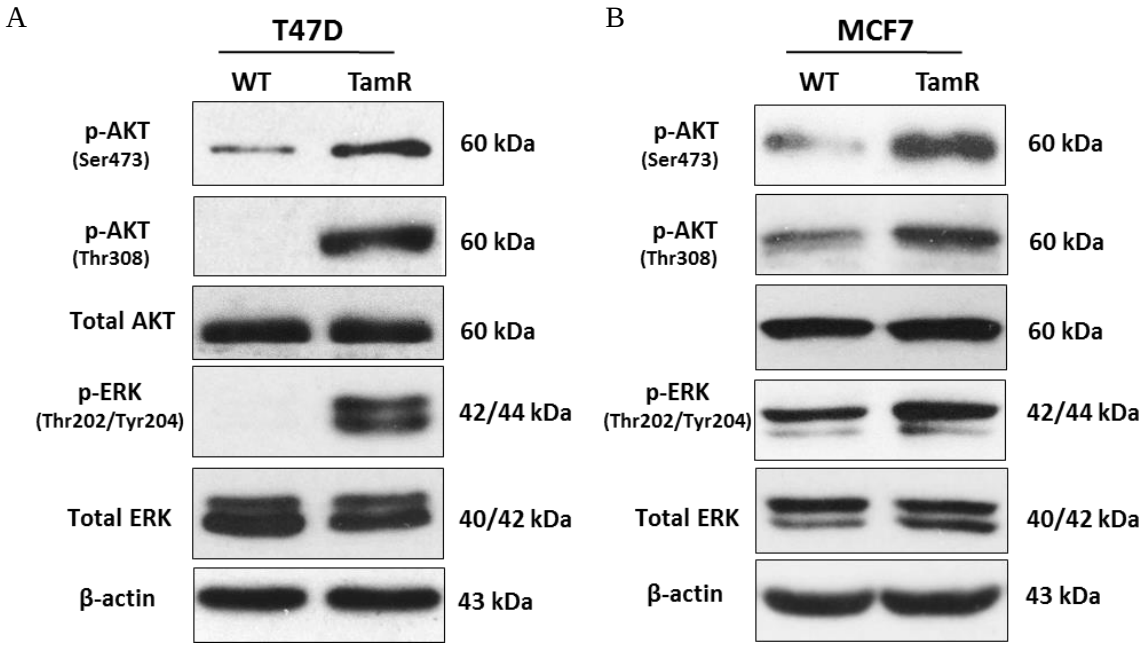


Figure 10: Western Blot results of tamoxifen resistant models for survival and proliferation markers. In both T47D-TamR (A) and MCF7-TamR (B) cells both survival and proliferation pathways were activated upon resistance development. Actin was used as a loading control

Tamoxifen resistant cells were shown to have more mesenchymal phenotype, leading to cancer more aggressive state. For this reason, I also examined the expression levels of EMT markers in both T47D and MCF7-TamR and WT cells. The expression levels of epithelial markers (CDH1, KRT18 and ZO1) were decreased while those of mesenchymal markers (CDH2, ZEB1, ZEB2, MMP9 and SNAIL2) were elevated in T47D-TamR cell lines compared to sensitive counterparts (***Figure 12***). On the other hand, expression levels of epithelial markers did not change while those of mesenchymal markers increased substantially in MCF7-TamR cells compared to WT cells. These results could be an indicative of partial EMT where cells still keep their contact with neighbor cells while cells at the edge become more migratory [83] [84] [85] [86]. Overall, these results support the notion that when cells get more resistant to tamoxifen, they also acquire more mesenchymal phenotype by inducing EMT or partial EMT programs.

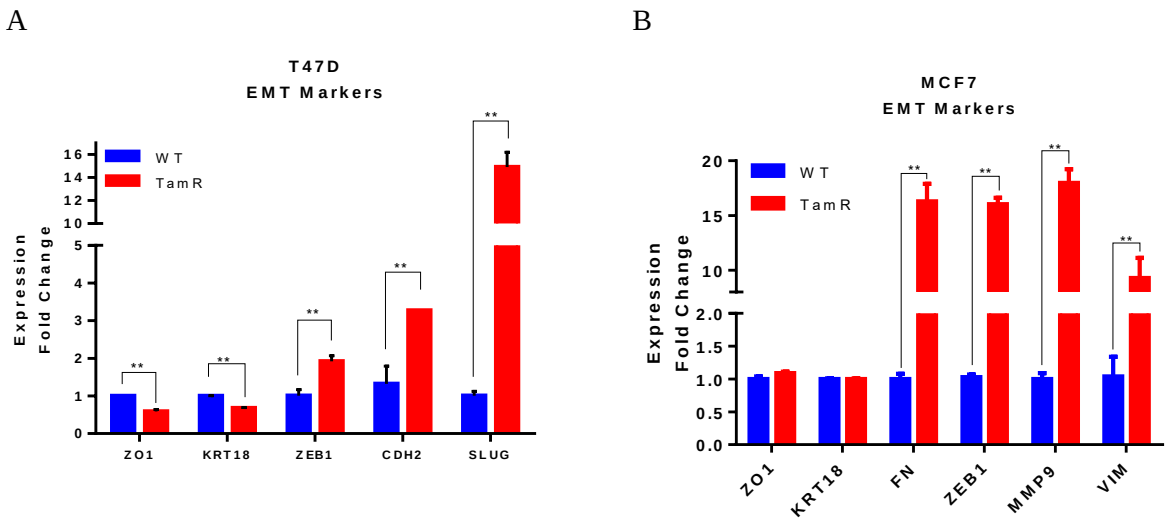


Figure 11: Expression of epithelial and mesenchymal markers in both tamoxifen resistant models. Both T47D (A) and MCF7 (B) models manifested EMT-like properties by qRT-PCR analysis. ZO1 and KRT18 are epithelial markers and ZEB1, CDH2, FN, MMP9, VIM and SLUG are mesenchymal markers. HPRT and GAPDH were used as housekeeping controls. All genes were performed in triplicates for qRT-PCR. (**p<0.01)

3.2 Whole Transcriptome Characterization of the Tamoxifen Resistance

Next generation RNA-sequencing is an invaluable tool to reveal transcriptome profiles of the any given samples as well as genetic and genomic aberrations, such as mutations and translocations [87]. To identify long non-coding RNAs (lncRNAs) potentially contributing to the development of tamoxifen resistance, the total RNAs isolated from MCF7-WT and MCF7-TamR cells were shipped to McGill University, Canada and were subjected to RNA-sequencing following the preparation of ribosomal RNA (rRNA)-depleted libraries. Since the transcriptome is a snapshot of the gene expression at any given time, the total RNA samples were isolated from 3 different passages (biological replicates) of both MCF7-WT and TamR cells to eliminate passage-specific effects and to have a more precise picture of the expression alterations between the groups. Around 65 millions paired-end reads (2x100 bp) were obtained from each replicate following RNA-sequencing and were analyzed *via* Tuxedo protocol [81]. Schematic representation of the whole procedure is given in ***Figure 12***.

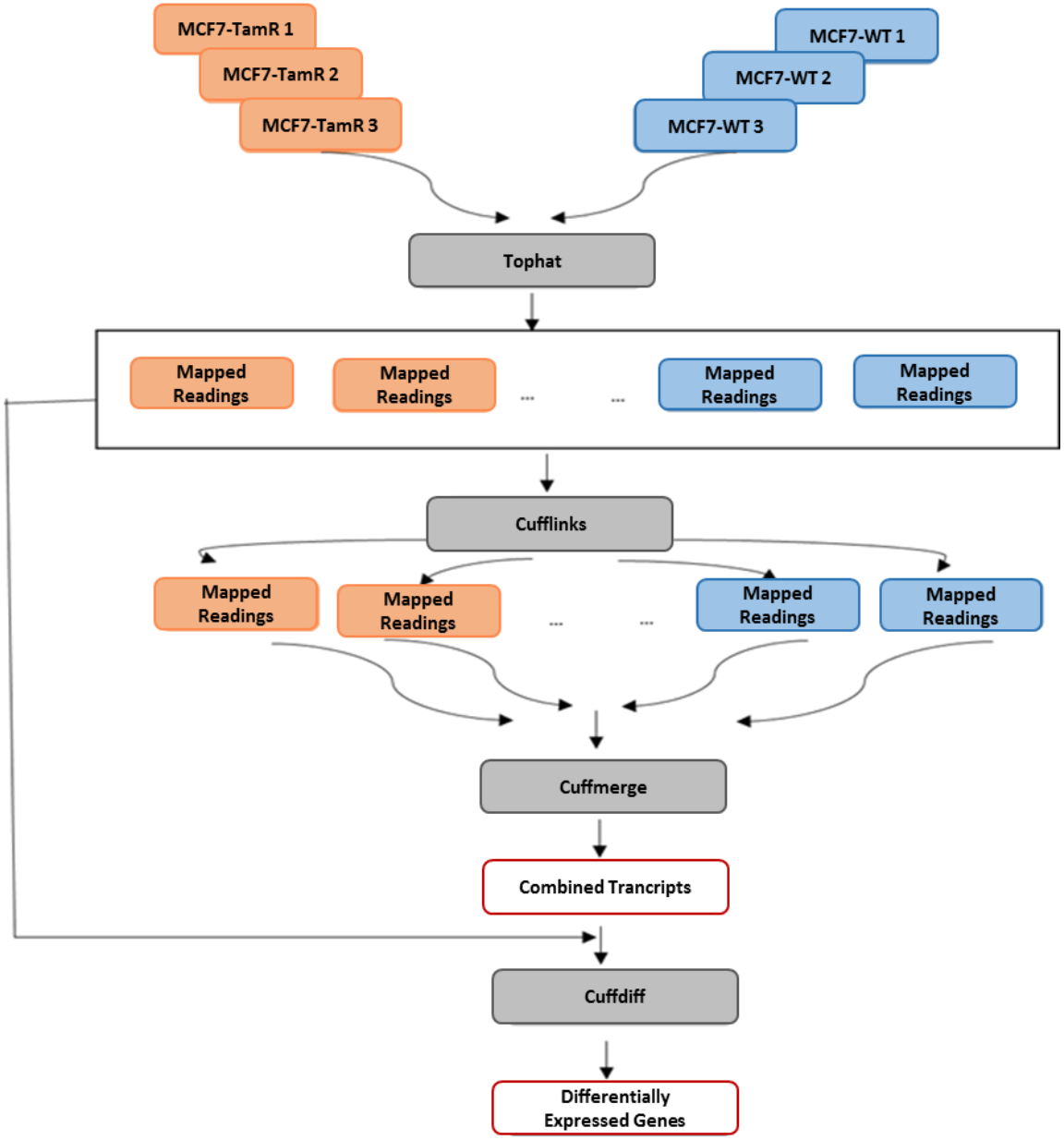


Figure 12*:* RNA-Seq analysis flow chart. Analysis was done according to Tuxedo protocol. CuffDiff was the final step where differentially expressed transcripts between tamoxifen sensitive and resistant cell lines obtained.

The quality of raw sequencing data was examined in terms of several quality control parameters, such as per base sequence quality and sequence length distribution by FASTQC v0.11.4 [88]. I first obtained the gene type information for each gene with an annotation from BioMart tool of ENSEMBL [89]. As I was interested in studying long non-coding RNAs in the context of tamoxifen resistance, I just kept the genes classified as lncRNAs according to VEGA Genome Browser's gene and transcript type classification [90], and did not include the

others in the downstream analysis. The lncRNAs genes that did not display a logarithmic fold change of ≥+1 or ≤-1 (log2FC ≥ +1 or log2FC ≤ -1) or did not have an adjusted p value less than 0.05 ($p < 0.05$) were filtered out from the list, leaving 191 significantly upregulated and 139 significantly downregulated lncRNAs. Unsupervised clustering of the samples by these 330 lncRNAs separated MCF7-TamR and WT cells into clear-cut 2 groups, implying the potential roles of lncRNAs in tamoxifen resistance (**Figure 13*A***). The majority of these lncRNAs was annotated as lincRNAs followed by antisense RNAs that represent 2 largest classes of lncRNAs (**Figure 13*B***) [91] .

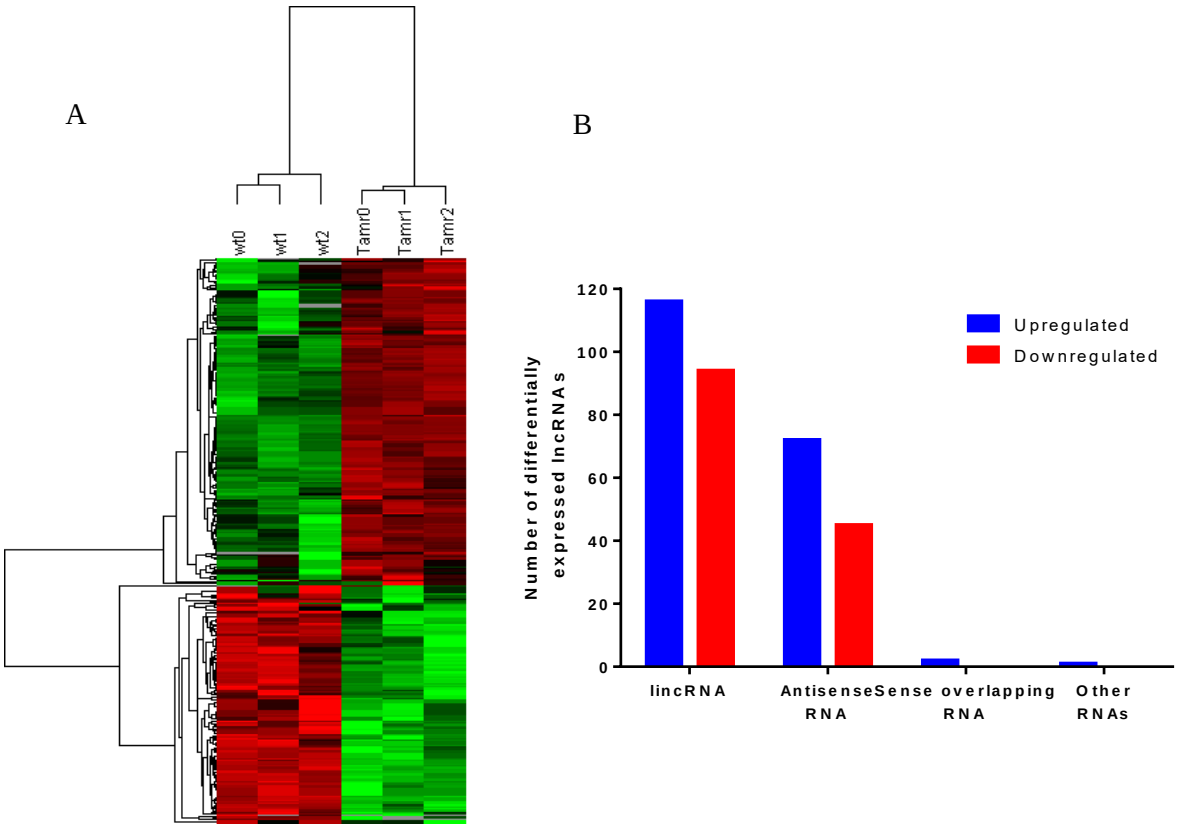


Figure 13: Preliminary *in silico* analysis and representation of RNA-Seq results. (A) Unsupervised clustering heatmap of all differentially expressed lncRNAs where lncRNAs associated with resistance and sensitiveness to tamoxifen were clearly separated. (B) Abundance of lncRNAs according to their subclasses. lincRNAs were the most abundant class in the RNA-Seq result, followed by Antisense RNAs. All statistically significant transcripts were included in both analysis (**p*<0.05).

The *CuffDiff* output file was processed through a funnel approach aiming to narrow down the list of differentially expressed lncRNAs that will be studied further. To avoid overwhelming characterization process of novel lncRNAs and lncRNAs that can be potentially discontinued/withdrawn in the future, I examined the RefSeq status of each lncRNA gene and eliminated those with a status other than validated or reviewed [92]. I did a detailed literature search for the remaining 27 well-annotated and validated lncRNA genes in the context of cancer. Only 1 upregulated (LINC00152) and 1 downregulated (HIF1A-AS1) lncRNA was found to be associated with cancer. Since it is relatively easier to modulate the expression, thus activity of an upregulated lncRNA compared with a downregulated one, LINC00152 was selected as the top candidate lncRNA for functional analysis (**Figure 14**).

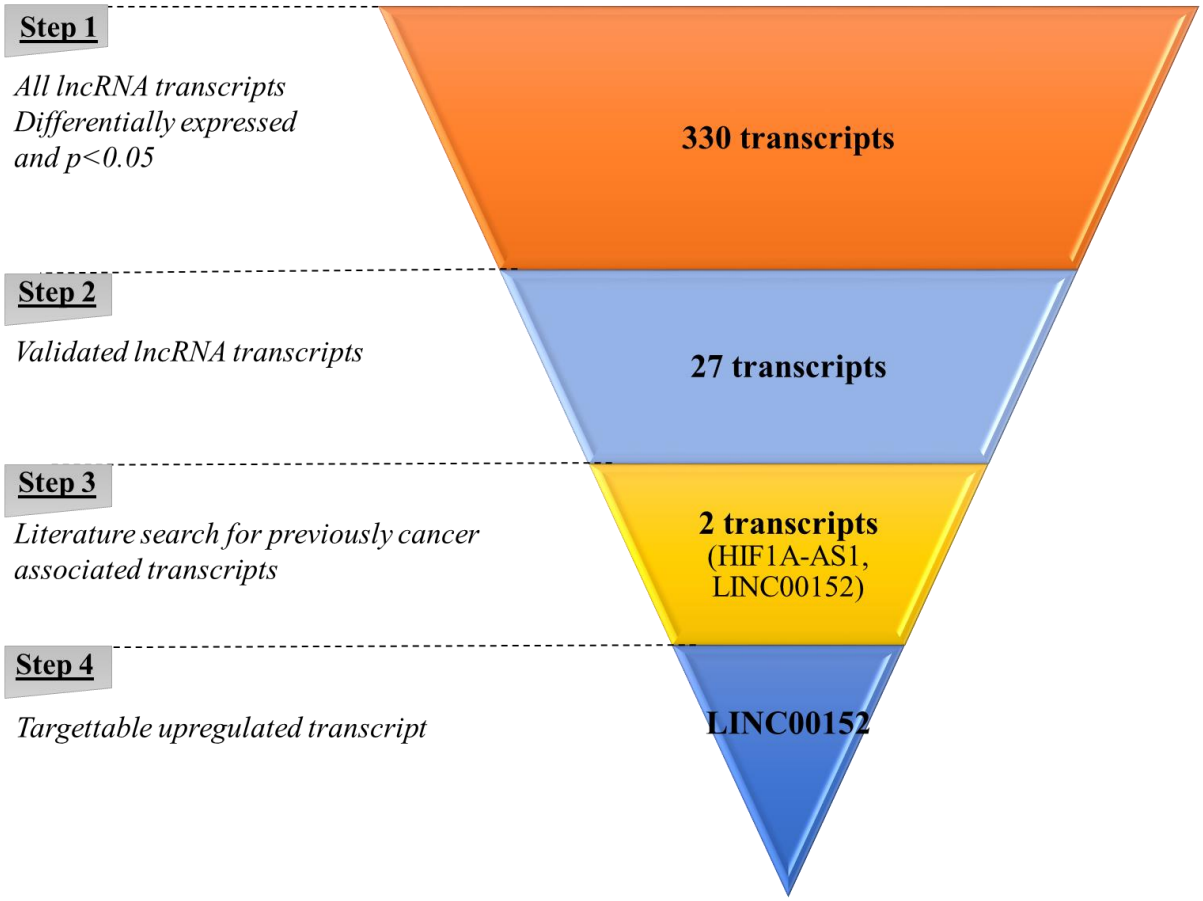


Figure 14: Funnel approach applied to identify candidate lncRNAs to be tested in functional studies. Among 330 possible candidate transcripts, LINC00152 was selected according to the criteria described in the text.

3.2.1 Validation of RNA-Seq Results

Although RNA-sequencing can capture the absolute state and nature of the transcriptome with a great detection rate and resolution, the tools/algorithms developed for RNA-seq analyses may introduce errors in every step including quantification and differential expression [86]. Therefore, to confirm both the presence and differential expression of the lncRNAs, qRT-PCR was performed using cDNA samples prepared with oligo-dT or random primers. While oligo-dT primers selectively synthesize cDNA from poly(A)-bearing RNAs, random primers convert any RNA to cDNA without a discrimination. As a result, I validated the upregulation of LINC00152 and downregulation of HIF1A-AS1 in the MCF7-TamR cells compared to MCF7-WT cells (***Figure 15***). The expression fold changes were also very similar between RNA-seq and qRT-PCR, especially for LINC00152. This experiment also provides a proof that both lncRNAs have a poly(A) tail.

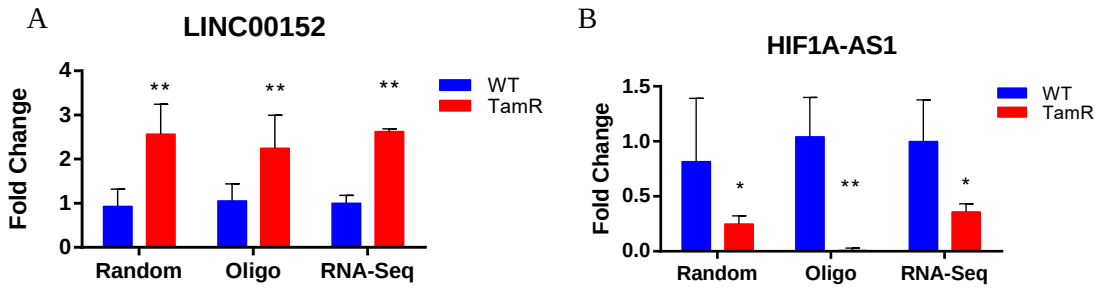


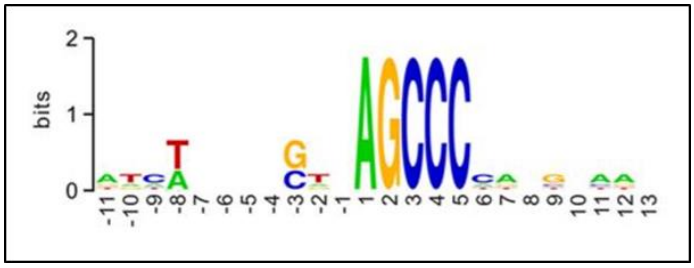
Figure 15*:* Validation of RNA-Seq results by qRT-PCR. Both random oligomer and oligo-dT primers were used for qRT-PCR. Both LINC00152 (A) and HIF1A-AS1 (B) expression were shown as fold change normalized to sensitive cell line counterpart (MCF7-WT). 2 different biological replicates of MCF7-WT and MCF7-TamR were used in triplicates. HPRT was used as housekeeping control (**p<0.01, *p<0.05).

3.2.2 Cellular Localization of the LINC00152

In order to predict the localization of LINC00152 *in silico*, I retrieved cDNA sequences of LINC00152 corresponding to each transcript from ENSEMBL and examined for the presence of a nuclear retention signal (NRS) provided in the reference [89]. This signal consists of a pentamer sequence AGCCC with nucleotide restrictions at position -3 (G or C) and at position -8 (A or T). A perfect NRS meeting above criteria will lead to a strict nuclear localization,

whereas the absence of compatible nucleotides at position -3 or -8 will result in partial nuclear and cytoplasmic distribution. There were 2 NRS sites in the representative transcript of the LINC00152 gene, which lack of the required A/T nucleotide at position -8 (**Figure *16***). As a result, LINC00152 was concluded to be a partially nuclear lncRNA.

A



B

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GTTCCAATGAGAATGAAGGCTGAGGTGTGCGCCTTTTTTTTTTTTTCCTTCTTAGTCGTGTGTACATCATTGGGAATGGAGGGAAATAAATGACTGGATG  < 100

                                          >Pentamer
                                      >-3 (G/C)
                                  >-8 (T/A)
                                  |    |    |
GTCGCTGCTTTTTAAGTTTCAAATTGACATTCCAGACAAGCGGTGCCTGAGCCCGTGCCTGTCTTCAGATCTTCACAGCACAGTTCCTGGGAAGGTGGAG  < 200

                                                                                  >Pentamer
                                                                              >-3 (G/C)
                                                                          >-8 (T/A)
                                                                          |    |    |
CCACCAGCCTCTCCTTGAATAACTGGGAGATGAAACAGGAAGCTCTATGACACACTTGATCGAATATGACAGACACCGAAAATCACGACTCAGCCCCCTC  < 300

CAGCACCTCTACCTGTTGCCCGCCGATCACAGCCGGAATGCAGCTGAAAGATTCCCTGGGGCCTGGTTCCAACCGCCCACTGTGGACTCTGAGGCCTCTG  < 400

CATTTGCGGGTGGTCTGCCTGTGATATTTTGGTCATGGGCTGGTCTGGTCGGTTTCCCATTTGTCTGGCCAGTCTCTATGTGTCTTAATCCCTTGTCCTT  < 500

CATTAAAAGCAAAACTAAAGAAAACAGAA  < 529
```

Figure 16: Nuclear retention signal (NRS) in LINC00152 sequence. (A) NRS is composed of AGCCC sequence with nucleotide restrictions at position -3 (G or C) and at position -8 (A or T). (B) In the cDNA sequence of representative LINC00152 transcript, NRS is found with a mismatch in the -8 position where G/C is found instead of A/T.

To validate *in silico* findings and provide further support regarding partially nuclear localization of LINC00152, I did cell fractionation followed by isolation of the nuclear and cytoplasmic RNAs. qRT-PCR results presented as percentage and showed that 60% of the LINC00152 transcripts produced by the cells were located in the cytoplasm whereas the rest was found in the nucleus. Here, MALAT1 is used as a nuclear control while ACTB and GAPDH are cytoplasmic/nuclear controls (***Figure 17***).

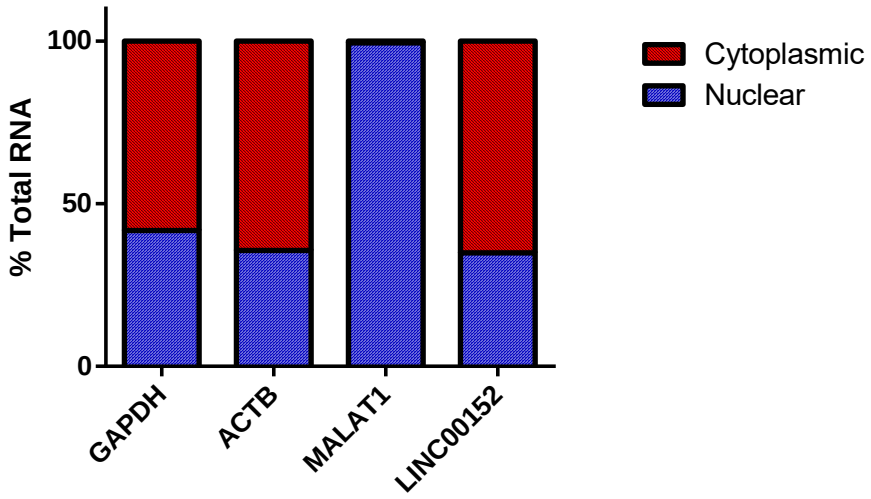

Figure 17: Percentage representation of the localization of LINC00152 RNA in the cell. MALAT1 is nuclear RNA while ACTB and GAPDH are primarily cytosolic RNAs.

3.3 ASO Transfection with LINC00152 and Tamoxifen Sensitization

Few studies have demonstrated that targeting nuclear-retained RNAs with small fragments of complementary DNAs called antisense oligonucleotides (ASOs) was more successful than siRNA-mediated silencing [93]. ASOs are about 20-nucleotide-long complementary DNA fragments that trigger the degradation of target RNA molecules. DNA-based structure allows them to freely move into nucleus and hybridize to target RNA. RNA-DNA heteroduplex is sensed by RNase H, which then cuts RNA molecule and release ASO as intact molecule. Therefore, it is expected that ASO-mediated knockdown will be more efficient and at greater level than siRNA-mediated knockdown. Accordingly, silencing of LINC00152 was carried out by ASOs in MCF7-TamR cells in order to test whether LINC00152 knockdown was able to sensitize TamR cells to tamoxifen and make them similar to their sensitive counterparts. Another goal was to check if LINC00152 upregulation is causally involved in tamoxifen resistance.

3.3.1 Targeted Non-Coding RNA Knockdown

ASOs targeting LINC00152 were designed against the most upregulated transcript of the gene according to RNA-Seq data (LINC00152 ASO: GTGTGTCATAGAGCTTCCTG and Control

ASO: GCGTATTATAGCCGATTAAC, all nucleotides are PS modified). Although ASOs can be directly taken up by the cells, cells were transfected *via* lipofectamine to ensure sufficient uptake of the oligonucleotides. To optimize ASO dose for knockdown, 10 nM, 20 nM, 25 nM, 50 nM and 100 nM doses were selected and compared for the knockdown efficiencies. Knockdown efficiency was more than 80% and was very similar for 25 nM, 50 nM and 100 nM doses after 72 hours of transfection. Later, optimum duration for knockdown was determined by the comparison of 3 different time points (24, 48, and 72 h) for 25 nM and 50 nM ASO concentrations. The knockdown was stable at 48 h and 72 h for both doses, while it was not sufficient at 24 h with 60% decrease. After the successful optimization of the amount and duration of ASO treatment needed for a good knockdown efficiency in MCF7-TamR cells, LINC00152 knockdown was also confirmed with the same experimental setup in T47D-TamR cells (***Figure 18***).

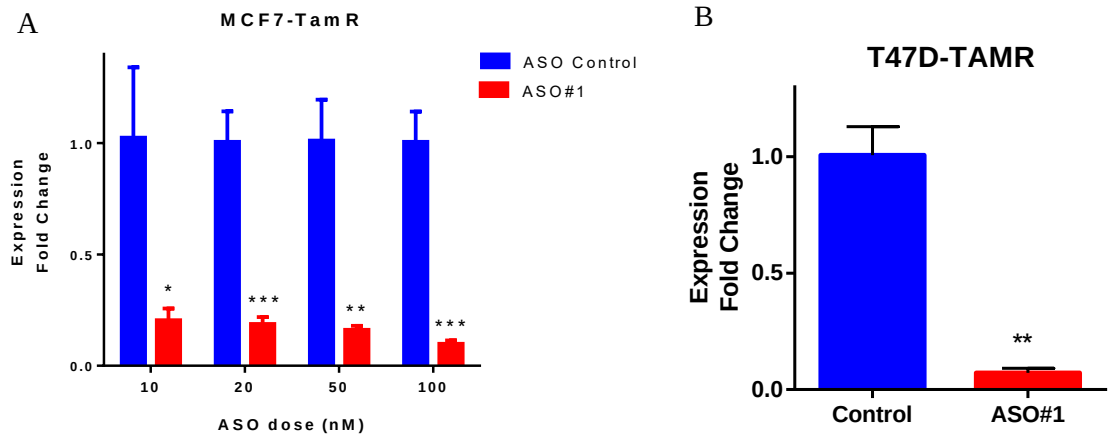

Figure 18*:* qRT-PCR results for ASO-mediated knockdown of LINC00152. Optimization study for ASO knockdown was performed with a dose range of 10 nM to 100 nM in MCF7-TamR (A) cell lines, and knockdown was confirmed by 25 nM of ASO in T47D-TamR cells (B). HPRT and GAPDH were used as housekeeping genes.

3.3.2 Tamoxifen Sensitization by LINC00152 Knockdown in Both TamR Cell Line Models

After successfully silencing LINC00152 expression, sensitization assays were carried out. LINC00152 silencing showed improved response to tamoxifen in both MCF7-TamR and T47D-TamR resistant models. While 25nM ASO treatment inhibits growth 30%, 5uM of

tamoxifen alone had an effect of 15%. The combination of same doses of ASO and tamoxifen inhibits growth at 45% in MCF7 cell model (**Figure *19***), which was an additive effect of LINC00152 on tamoxifen. Similarly, combination showed same profile of additive effect in T47D cell model as well (**Figure *19***). These result showed TamR cells require LINC00152 for their survival and growth, and its expression contributes to tamoxifen resistance in these cells.

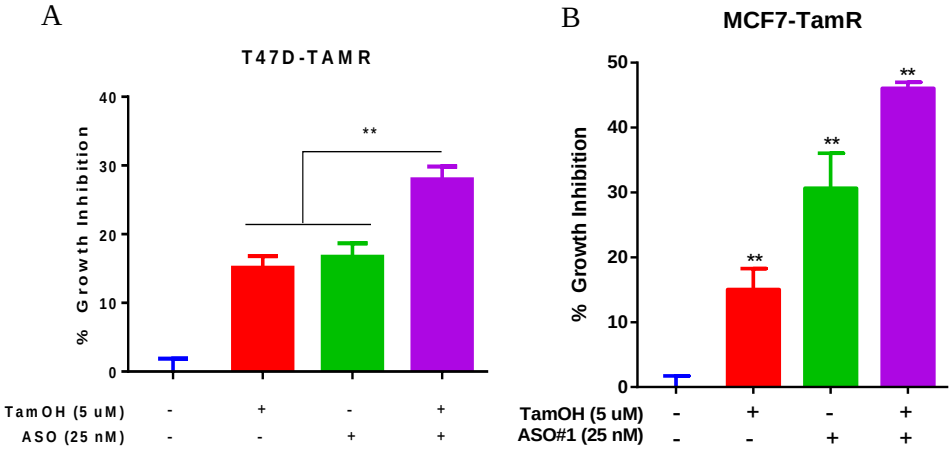

Figure 19: LINC00152 knockdown re-sensitizes tamoxifen resistant cell models to tamoxifen. Cells were seeded in quadruplets as 6,000 cell/well in 96-well plate. After 1 day of incubation, they received 25nM ASO, and next day they were treated with 5uM tamoxifen. Cell viability was assessed by Cell Titer Glo viability assay at 72 hours of treatment. Tamoxifen inhibited growth 15% in both MCF7-TamR (A) and T47D-TamR (B) cells while LINC00152 knockdown with ASO yielded 15% growth inhibition in T47D-TamR and 30% in MCF7-TamR. Combination had good additive effect in both cell models. (**p<0.01)

3.3.3 Silencing of LINC00152 Results in Enhanced Autophagy and Apoptosis in Tamoxifen Resistant Cell Models

After assessing tamoxifen sensitization capacity of LINC00152 knockdown, I continued with measuring the cell death levels. For this, protein levels of essential apoptosis pathway member were detected. In literature MCF7 cells was reported lacking caspase 3 expression, therefore I continued with only caspase 7 in my experiments [94]. Protein level of cleaved-caspase 7 was greatly induced in LINC00152 knockdown samples, most significantly when combined with tamoxifen. In line with our expectation, 72h of tamoxifen treatment in MCF7-TamR cell did not induce any detectable cleaved caspase 7 protein level.

Apoptosis can be induced upon autophagy which is a critical process for maintenance of the homeostasis [95] [96] [97] . Therefore, I examined autophagy induction levels to investigate autophagic effect of LINC00152 knockdown in the same setup where cells received 25nM of ASO and a day after, 5uM of tamoxifen. During autophagosome production, LC3 I (16 kDa) is converted into LC II (14 kDa). Measurement of LC II/LC3 I ratio is a highly reliable method for autophagy detection [98]. In our TamR MCF7 model, it was found that knockdown of LINC00152 greatly contributes to conversion of LCI to LCII (**Figure *20***).

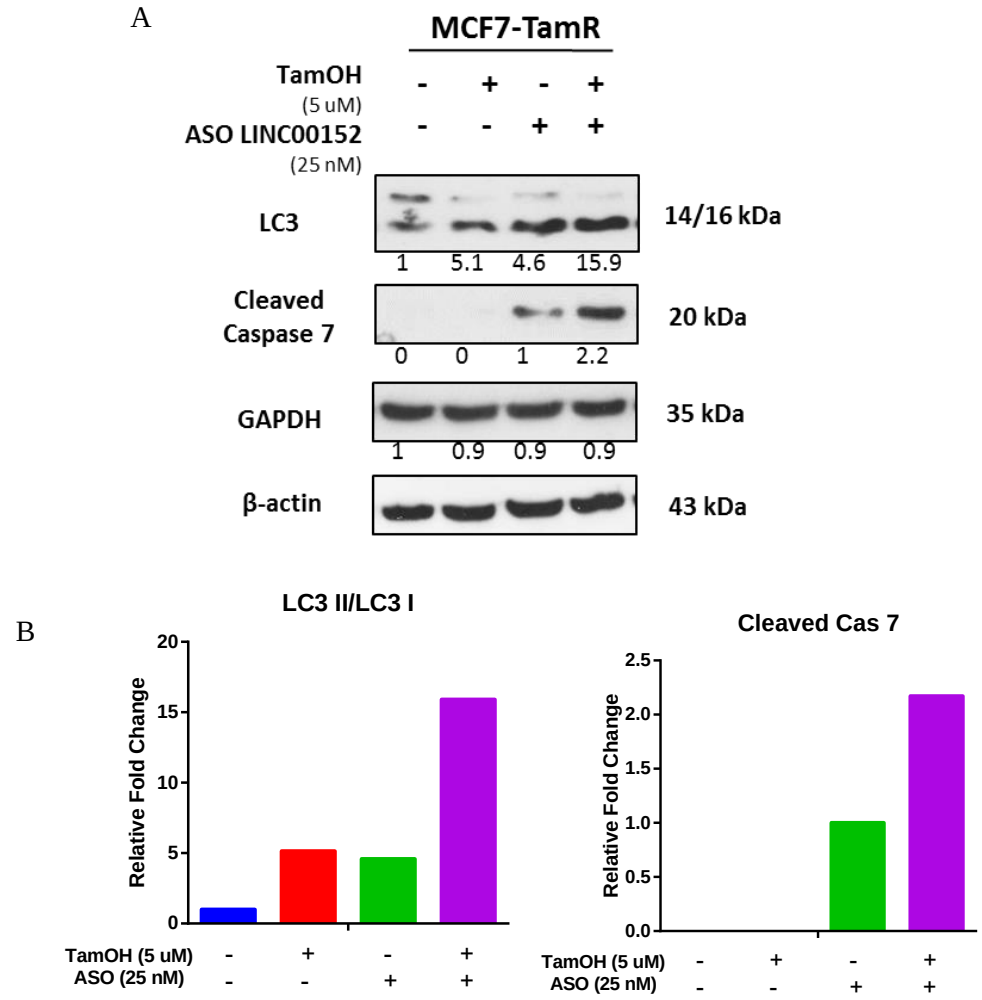

Figure 20: Main apoptosis and autophagy markers were checked by Western Blot after transfection and treatment with ASO and tamoxifen, respectively. (A) Western blot and (B) its quantification. Apoptotic bodies and protein samples were collected after 72h of 5uM tamoxifen treatment following one day of 25nM ASO transfection. LC3, major autophagy marker was induced with tamoxifen treatment and knockdown of LINC00152 enhanced this effect. Cleaved caspase 7 were only induced by LINC00152 knockdown. Actin and GAPDH were used as loading controls.

Increased ratio of LC3 II to LC3 I in tamoxifen and ASO combination indicate that autophagy might have a role in sensitization of MCF7-TamR cells to tamoxifen through LINC0052 knockdown.

3.4 Clinical Relevance of Findings

I search for several databases and examined the clinical relevance of LINC00152 expression in breast cancer patients in order to support and strengthen my hypothesis. Primary source of survival analysis was METABRIC data [99]. Two of 3 LINC00152 probes in METABRIC were targeting the transcripts (ILMN_1665515 and ILMN_2143795). In the discovery cohort, breast cancer patients representing all subtypes were stratified based on their LINC00152 expression and divided into two groups: Patients with low LINC00152 levels and patients with high LINC00152 levels. Higher expression of LINC00152 showed poorer overall survival compared to patients with lower expression (****p:*0.0007 n=996) (**Figure 21**).

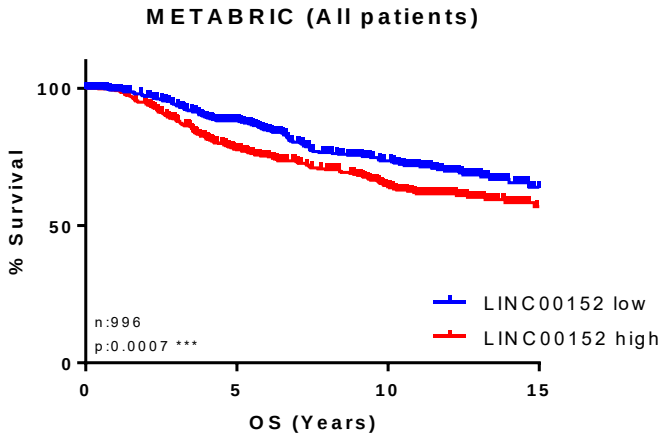

Figure 21: LINC00152 expression associates with lower survival in breast cancer patients in METABRIC data set (n=996, ****p*<0.01)

Further, to investigate the role of LINC00152 expression in clinical outcome with respect to breast cancer subtypes, I separated the patients as Luminal, Basal, HER2 and normal-like. The higher expression of LINC00152 was associated with poorer OS in Luminal A and B subtypes (**p*=0.0325) (***Figure 22A***). When patients were separated according to the

IHC results of ERα, a lower survival rate was again observed in patients with high LINC00152 expression than in patients with low expression (*p=0.0377) (**Figure *22B***). Moreover, survival analysis was performed with other subtypes of breast cancer or ERα-negative patients, and LINC001552 expression was failed to differentiate OS of the patients (***Figure 22C-E***).

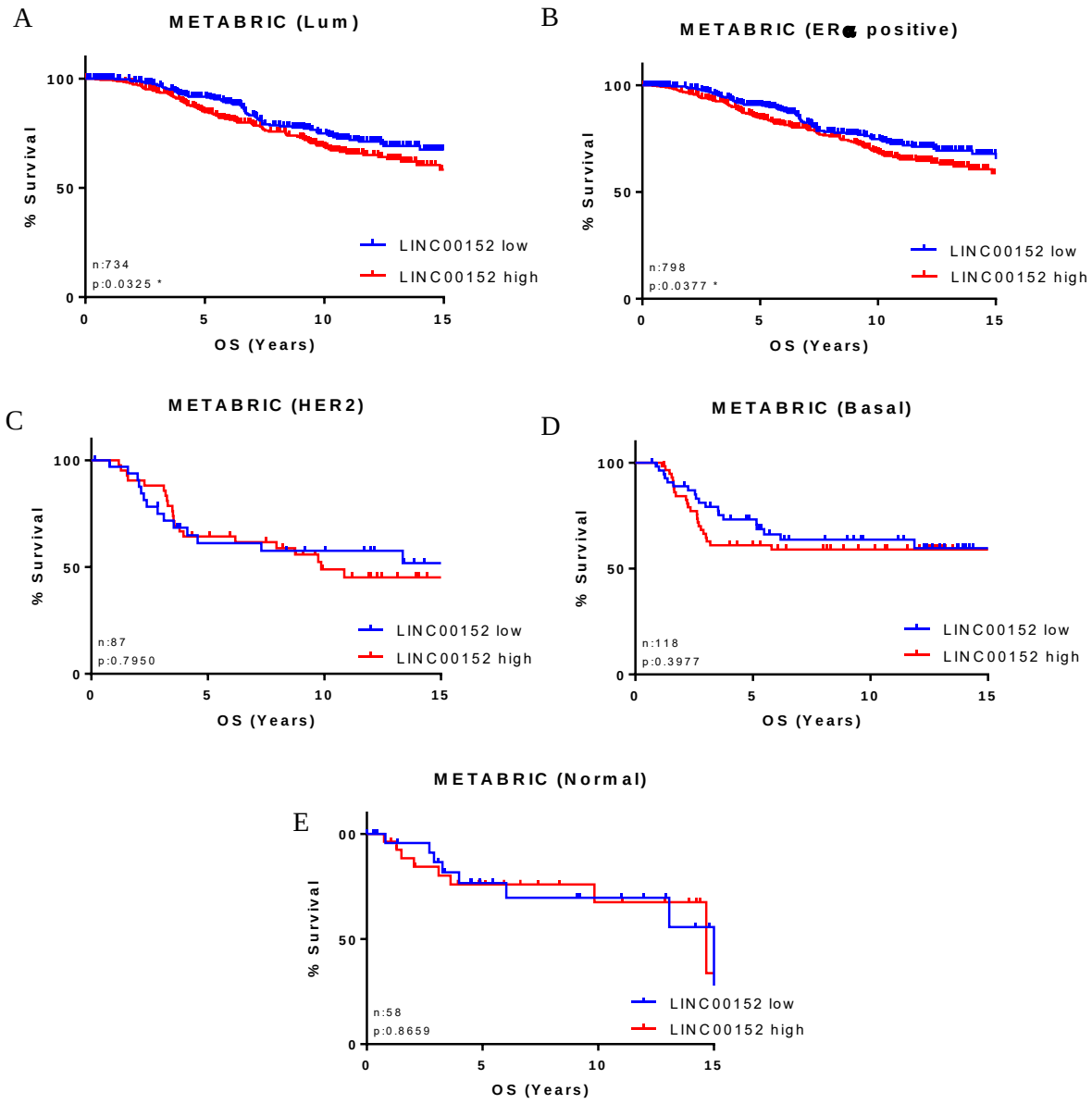

Figure 22: LINC00152 expression associates with poor prognosis is ER-positive or Luminal patients in METABRIC data set. Patients stratified according to clinical (A) and molecular classification (B) of the ERα-positive subtypes. High LINC00152 expression was associated with

shorter OS duration in both Luminal and ERα-positive subtypes (*p<0.05). LINC00152 cannot differentiate OS rate in non-ER-positive breast cancer patients; HER2 enriched (C), Basal (D) or Normal (E) subtypes of breast cancer (p>0.5).

Survival analysis with patient data also pointed out that, LINC00152 was more likely associated with ERα-positive patients, not ERα-negative patients. However, regarding the context of drug resistance, overall survival is not enough to support if LINC00152 might have critical role in tamoxifen response of ERα-positive breast cancer. Therefore, I analyzed different patient data sets for diseases-free survival (DFS), relapse-free survival (RFS) and distant metastasis-free survival (DMFS). For DMFS, appropriate suitable, tamoxifen-treated ERα-positive patient data with a statistically enough number of samples was not found. Yet, there were other datasets for RFS and DFS analysis. In both GSE9195 and GSE6532 datasets, which have all ERα-positive, tamoxifen- treated patients with RFS info, I demonstrated that higher LINC00152 expression is associated with shorter RFS (**p=0.0053 and **p=0.0088, respectively) (***Figure 23A-B***). Similarly, in GSE58644, which has the same clinical profile of patients with DFS info, I showed that higher LINC00152 expression is associated with shorter DFS (***Figure 23C***) (**p=0.0011).

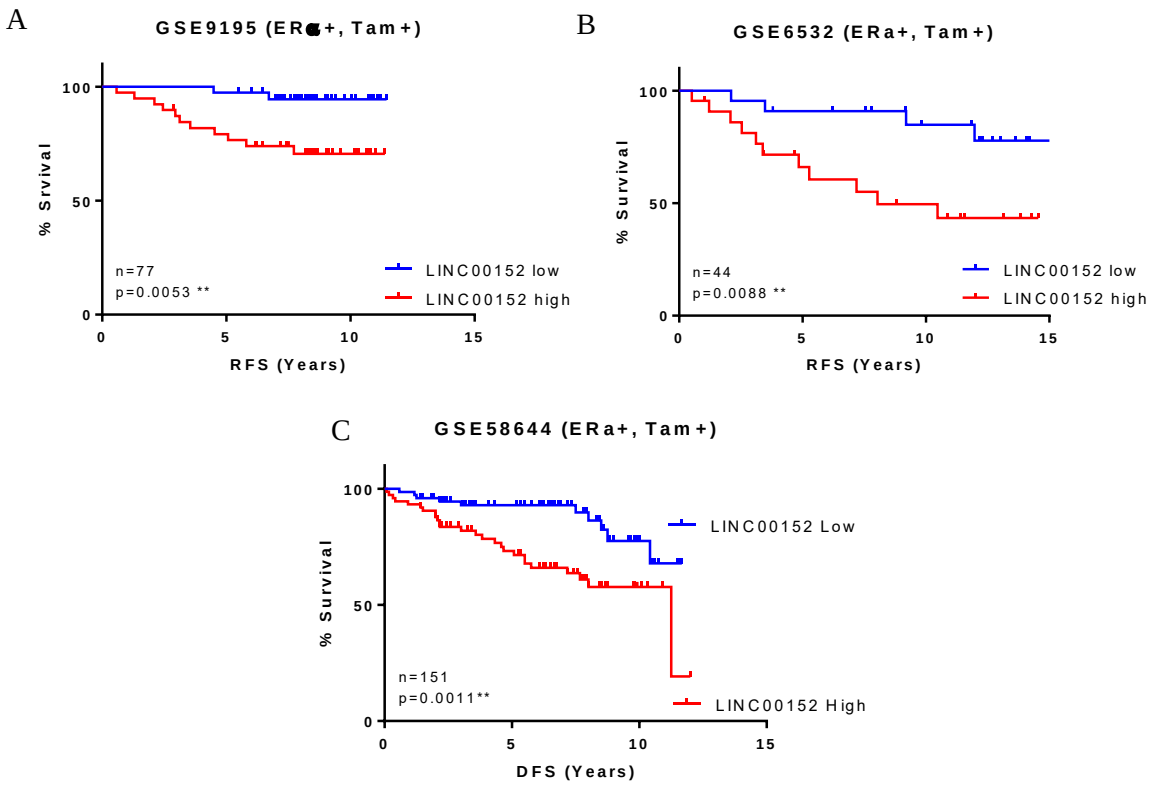

Figure 23: LINC00152 higher expression shortens relapse-free and disease-free survival time in ERα-positive breast cancer patients received tamoxifen treatment. In 3 independent patient datasets (GSE9195, GSE6532, GSE58644) LINC00152 high expression were associated with decreased relapse-free (A and B) and disease-free survival (C). All datasets were composed of ERα-positive, tamoxifen treated breast cancer patients. (**p<0.01)

In conclusion, the expression of LINC00152 is associated with RFS and DFS in ERα-positive patients treated with tamoxifen. These results suggest that patients with higher LINC00152 expression had increased risks of relapse and metastasis compared to patients with low expression.

CHAPTER 4

DISCUSSION

Tamoxifen, one of the most prescribed drugs for ERα-positive breast cancer patients in the world, has a good clinical outcome, where it drastically reduces mortality rate in the first 15 years after therapy. Unfortunately, almost half of the patients fail to response and suffer from relapse of the disease in the long-run. Some of the reasons giving rise to therapy failure and drug resistance are investigated and proposed. However, the suggested mechanisms were mostly dependent on the coding genes of the genome. Due to the recent advancements in sequencing technologies and downstream functional assays, it is known that non-coding part of the genome has a crucial role in various biological processes. Hence, in order to draw a more complete picture of tamoxifen resistance mechanisms, non-coding region of the genome, both small and long ncRNAs, should be studied in this context.

Here, I investigated tamoxifen resistance in ERα-positive breast cancer patients with a specific focus on long non-coding transcriptome alterations. The results suggested that LINC00152 upregulation contributes to tamoxifen resistance. According to cell viability assays, it was confirmed that LINC00152 expression influences the cell survival rate and additionally, silencing of the lncRNA re-sensitizes tamoxifen resistant cell line models (MCF7-TamR and T47D-TamR) to tamoxifen. Furthermore, I showed that the effect of LINC00152 loss on the cell viability was a result of enhanced apoptosis evidenced by increased cleaved caspase 7 levels. Further experiments determining LC3 II/LC3 I ratio, a well-established marker of autophagy, showed that apoptosis in the LINC00152 knockdown samples was a result of the high autophagy induction. I also supported role of LINC00152 in the tamoxifen treatment with analysis of a very large patient cohort of breast cancer patients where higher expression of LINC00152 is associated with worse survival of tamoxifen-treated patients. Here, I propose that LINC00152 upregulation contributes to tamoxifen resistance *via* blockade of autophagy and apoptosis, and the expression of LINC00152 may be used as a biomarker of tamoxifen resistance.

4.1 Tamoxifen Resistance is Characterized by ERα Downregulation and Upregulation of Growth Factor Receptors Activating PI3K and MAPK Pathways

Cancer is a complex disease caused by mutations, epigenetic alterations and chromosomal aberrations leading to abnormal activations in genes regulating cell proliferation, cell cycle and cell survival. This is a multi-step process and occurs when multiple factors are causally involved. Cancer with its complex nature displays a great diversity and heterogeneity [100]. This heterogeneous profile is reflected at carcinogenesis, cancer progression, and drug response. Therefore, it is crucial to study high number of samples in clinical studies or multiple cell lines originating from different patient background *in vitro* to capture a more precise profile of common molecular alterations in the context of tumor heterogeneity. Accordingly, MCF7-TamR and T47D-TamR cell models were derived from their parental/wild-type counterparts and characterized at cellular and molecular levels to study the mechanisms involved in acquired tamoxifen resistance in breast cancer. Of these two resistance models, MCF7-TamR had been previously developed and successfully used [49] [48] while T47D-TamR has been generated during this thesis work. Resistant cells were developed by culturing parental cells (WT) in the presence of 5 μM 4-hydroxytamoxifen for ~1 year. WT cells were cultured in parallel to resistant ones without tamoxifen treatment. Understanding the molecular mechanisms of resistance against tamoxifen will help to both reverse resistance through identification of new targets and predict patients' response to therapy [101].

Estrogen receptor signaling pathway possesses an important role for breast tumor formation and progression. ERα expression level is, accordingly, a powerful biomarker to predict the response of patients to tamoxifen. One of the major mechanisms leading to tamoxifen resistance is the decrease in ERα expression followed by a decrease in progesterone receptor (PR) at protein level and activation of some compensatory proteins/pathways such as EGFR and HER2 [102].

EGFR and HER2 belong to same protein family (epidermal growth factor receptor family), and their functions are similar to each other. Both require dimerization for activation, and are able to form homodimer as well as heterodimers with other members of the family. In other words, they can dimerize with themselves or change partner. Due to this property, overexpressed EGFR or HER2 alone has effect on tumor progression [103] [104]. At this point, the expression of ER, EGFR and HER2 for in both MCF7 and T47D-TamR cell models was assessed at protein level. In line with the literature, resistant models that I have

developed as a part of this thesis had elevated expression of HER2 with decreased ER levels (**Figure 9**). I showed that EGFR was also upregulated in tamoxifen resistant T47D-TamR cells compared to their sensitive counterparts. Moreover, ERα level along with PR, a direct transcriptional target of ERα, was almost lost after continuous tamoxifen treatment in T47D-TamR cells. A similar phenotype was observed in MCF7-TamR cell line model. In conclusion, the resistant cells I developed to investigate acquired tamoxifen resistance mechanisms consistently represent the literature findings in terms of loss of ER and PR expression and gain of other growth factor receptors.

AKT (protein kinase B) is a protein kinase promoting cell viability and suppressing apoptotic signaling pathways, which may contribute to the resistance mechanisms. In addition to over-activated hormone and growth receptors, phosphorylation status of AKT at Serine 473 and Threonine 308 was elevated in both resistant models. Similarly, the resistant cells had dramatically higher levels of phosphorylated ERK1/2 at Threonine 202/Tyrosine 204 compared with WT counterparts (**Figure 10**). Hence, it suggests that cell survival and proliferation pathways were more active in tamoxifen-resistant cells in comparison to the WT cells, implying higher viability of TamR cells at basal level. This may lead to overrepresentation of the resistant cell population over time within a heterogeneous tumor, resulting in a more aggressive and non-responsive subpopulation. The overexpression of EGFR or HER2 in ER-positive breast cancers can confer resistance to tamoxifen [105] [106] [107]. In this line, AKT and ERK1/2 activation as downstream events of EGFR and HER2 can also contribute to resistance profile. Antiestrogen-resistant cell lines have been generated by several groups through long-term exposure of ER-positive breast cancer cells to either tamoxifen or fulvestrant. In these studies, acquired resistance is often accompanied by overexpression of the EGFR and activation of downstream molecules such as ERK1/2, PI3K, and AKT [108] [109] [110]. This way, the survival disadvantage imposed by decreased proliferative ER transcriptional repertoire, can be compensated by proteins mentioned above.

4.2 LINC00152 Contributes to Tamoxifen Resistance

Genome-wide mutation analyses have revealed a comprehensive catalog of functional mutations within the noncoding genome, exerting profound effects on the expression of long noncoding RNAs [111]. This was largely made possible by the next-generation sequencing, which is one of the major breakthroughs in recent years and accelerates lncRNA research

incredibly quickly. Owing to the discovery of their key roles in gene regulation and various aspects of cellular homeostasis including proliferation, survival, migration or genomic stability, lncRNA-disease association has become a hot topic in many fields [112]. As the number of cancer transcriptome sequenced and characterized is increasing dramatically, it is becoming evident that lncRNAs are causally involved in all cancer hallmark processes [82] [113]. For example, a conserved mammalian lncRNA called NORAD maintains genomic stability [114]. Another lncRNA, SOCS2-AS1, inhibits apoptosis in addition to promoting cell growth in prostate cancer [115]. Likewise, UCA1 both enhances cell proliferation and confers resistance to 5-fluorouracil in colorectal cancer [116]; MALAT1 promotes tumor angiogenesis by upregulating pro-angiogenic genes in neuroblastoma [117]; and HOTAIR fosters metastasis of primary breast tumors *via* chromatin reprogramming [118].

Breast cancer is not an exception, for which long noncoding RNA landscape has been repeatedly characterized in great detail by seminal studies [79] [119] [120] [121]. For example, BCAR4 enhances cell migration, thereby facilitating breast cancer metastasis [122]. Another lncRNA called APOC1P1-3 inhibits apoptosis of breast cancer cells by reducing α-tubulin acetylation [123]. One of these studies even showed that LINC00152, which they called CYTOR, regulates genes involved in EGFR and mTOR pathways, alters proliferative and migratory capacity of the breast cancer cells, and is involved in cytoskeletal organization. In addition to their role in cancer hallmarks in breast cancer, lncRNAs can also control breast cancer-specific alterations/outcomes and can be used to classify breast cancer patients into molecular subtypes [124] [125].

Here, to investigate the potential roles of lncRNAs specifically in the context of tamoxifen resistance in ERα-positive breast cancer, MCF7-WT and MCF7-TamR cells were sequenced at whole transcriptome level. Total RNA from 3 different biological replicates of both parental and resistant cells was used for sequencing to minimize the passage-related effects, thus obtaining a better transcriptomic profile regulating tamoxifen resistance. RNA-Seq libraries were prepared by rRNA-depletion to capture a higher number of lncRNAs, which could have been missed if they were prepared by poly(A)-enrichment. As a result of RNA-Seq data analysis, a total of 330 significantly differentially expressed lncRNAs were obtained.

As it is not possible to study this number of lncRNAs at molecular level in detail, I needed to systematically filter out these lncRNAs by a funnel approach. First, lncRNAs

should be annotated as genuine lncRNA in the Ref-Seq with a status of validated or reviewed, excluding proposed or model lncRNA species. Second, the change in their expression between WT and TamR groups should be statistically significant ($p<0.05$). Third, they should be differentially expressed at least 2-fold ($log2FC \geq +1$ or $log2FC \leq -1$). Finally, they should have been previously shown to be associated with any cancer type (**Figure 14**). Since the great majority of the lncRNAs are not conserved at sequence or even structure level (whose conservation is an indicator for functional importance), this allowed us to make sure the candidate lncRNAs will be functional. In addition, it is not possible to limit the number of lncRNAs by pathway enrichment analysis or using the domain information to predict function as lncRNAs do not possess any domains as proteins do. As a result of the approach I followed, I obtained 2 lncRNAs that were significantly differentially expressed between groups and associated with cancer. They were LINC00152 and HIF1A-AS1, of which the first is upregulated and the second is downregulated in MCF7-TamR cells in comparison to MCF7-WT cells. Strikingly, some lncRNAs previously linked to breast cancer such as HOTAIR, MALAT1 and BCAR4 did not meet all the criteria and omitted from the downstream analyses. Although I initially focused on both candidate lncRNAs, I failed to amplify downregulated transcripts due to their non-conserved nature, long transcript length and GC-rich content and abandoned downregulated lncRNA. Moreover, it is much easier to modulate the expression and activity of upregulated lncRNA. Therefore, all the experiments were carried out for only LINC00152.

Long intergenic non-coding RNA 152 (LINC00152), also known as cytoskeleton regulator RNA (CYTOR), was characterized as an oncogene in many cancer types, including renal cell carcinoma [126], gastric cancer [127], hepatocellular carcinoma [128], bladder cancer [129]. Furthermore, inhibition of LINC00152 was shown to negatively regulate cancer cell proliferation, migration and invasion as reported by many studies [130] [131]. Accordingly, LINC00152 may serve as an important biomarker for both diagnosis and prognosis, which has already been confirmed by large clinical samples. For instance, the findings that overexpression of LINC00152 is associated with poor prognosis have clearly been revealed in 133 colon and gastric cancers. Likewise, LINC00152 has been shown to function as an indicator for invasion and metastasis to lymph nodes and predicted poor survival in 97 gastric cancer patients. LINC00152 was shown to exert these critical effects on its target genes by forming competing endogenous RNA networks in cytoplasm and through epigenetic effector proteins such as PRC2. However, its potential role in fostering breast

cancer development and progression is not clear, and it has not been studied in the context of endocrine therapy resistance. Therefore, this study constitutes the first example of the involvement of LINC00152 in tamoxifen resistance.

Initially, the upregulation of LINC00152 in the tamoxifen-resistant MCF7 cells predicted by RNA-Seq data analysis was confirmed in both resistant cell line models by qRT-PCR analysis. This led us to explore the effect of LINC00152 on tamoxifen resistance, which requires the modulation of LINC00152 expression or activity. Similar to many lncRNAs, LINC00152 also exhibits both nuclear and cytoplasmic distribution [132]. As antisense oligonucleotides (ASOs) are capable of targeting RNA transcripts efficiently in both nucleus and cytoplasm unlike siRNAs. LncRNA-knockdown by ASOs was shown to be more effective than RNAi-based molecules [133]. Consequently, I decided to use ASO to target LINC00152. The designed ASO at selected dose of 25nM was able to knockdown LINC00152 by 80% (***Figure 18***). As sensitization assays are key to the study of drug resistance, I examined the viability of TamR cells following tamoxifen, ASO alone or tamoxifen plus ASO treatment. Expression level of LINC00152 has been significantly reduced upon knockdown in both resistant models. While ASO or tamoxifen (5uM) alone was showed around 30% or 15% growth inhibition, respectively; the combination of tamoxifen with ASO resulted in 45% inhibition in tamoxifen-resistant MCF7 cells. The same experiment was repeated for T47D-TamR cells, yielding a similar outcome: LINC00152 knockdown or tamoxifen treatment alone resulted in around 15% growth inhibition, whereas the combination led to about 30% proliferation inhibition. When those inhibition rates analyzed, it was shown that 25nM of ASO has an additive effect on 5uM of tamoxifen in both resistance MCF7 and T47D cell lines (**Figure *19***). The sensitization assay, thus, demonstrated that LINC00152 was causally involved in the acquired tamoxifen resistance. In consistent with our results, many lncRNAs has been linked to drug resistance in breast cancer through diverse mechanisms. For instance, the lncRNA ATB is positively correlated with trastuzumab resistance of breast cancer patients [134]. Mechanistically, it functions as a ceRNA of ZEB1 and ZNF-217 against miR-200c, leading to their upregulation followed by enhanced epithelial-mesenchymal transition [134]. In another study, downregulation of lncRNA GAS5 in HER2-positive breast cancer cells was reported to enhance cell proliferation and confer trastuzumab resistance *via* decreased expression of PTEN by miR-21 [135]. Similarly, overexpression of HOTAIR by estrogen receptor-modulated mechanisms in breast cancer cells caused enhanced cell proliferation and tamoxifen resistance, which was lost or reversed upon its depletion.

In summary, it is apparent that LINC00152 could be an important mediator of tamoxifen resistance in both MCF7 and T47D-TamR models. However, all my conclusions were obtained from *in vitro* experiments merely. To overcome weak point of my data that was lacking *in vivo* confirmation, I wanted to examine LINC00152 status in breast cancer patients, and whether its expression is associated with tamoxifen resistance in patients. One of the largest breast cancer cohorts, METABRIC, along with 3 independent patient data were utilized for bioinformatics analysis. I first started with METABRIC, a reputable dataset about 2,000 breast cancer patients covered with follow-up data for up to 20 years, and high expression of LINC00152 showed lower survival in all patients (**Figure 21**). Then I divided patients according to their subtypes and ERα status. Luminal A and B patients showed lower overall survival with high LINC00152 expression; same profile applies to ERα-positive patients as well. Later, I wanted to test whether that relation only account for ERα levels, thus I tested HER2 enriched, Basal, and Normal-like subtypes. Analysis of these subtypes showed that LINC00152 expression is not associated with their survival (**Figure *22***). Overall, Kaplan-Meier survival analysis performed with METABRIC patients showed that LINC00152 is more likely related with ERα-positive and Luminal subtypes rather than other subtypes. However, the effect on overall survival cannot directly help the hypothesis claiming LINC00152 contributes tamoxifen resistance, due to the fact that METABRIC patients were indicated to be treated with general hormone therapy, not particularly tamoxifen. Moreover, it was necessary to have more independent datasets with tamoxifen therapy information. To investigate the association of LINC00152 and tamoxifen resistance, I have benefited from three independent cohorts consisting of ERα-positive, tamoxifen- treated patients. GSE9195 and GSE6532 datasets had all ERα-positive, tamoxifen- treated breast cancer patients with RFS information. In addition to those, GSE5944 was used for DFS analysis. My analyses revealed that higher levels of LINC00152 were associated with shorter relapse free and disease free survival (**Figure *23***). Hence, the data confirmed that LINC00152 may strongly be associated with tamoxifen resistance. This outstanding entire clinical findings about LINC00152 supporting the hypothesis that it associates with tamoxifen resistance in ERα-positive breast cancer.

4.3 LINC00152 Confers Tamoxifen Resistance *via* Regulating Autophagy and Apoptosis

LINC00152, a long non-coding spanning about 700 nucleotides, was found to be differentially hypomethylated in progression of liver carcinoma [136] in a computational study on hepatocellular carcinoma which combines methylome analysis and integrative genomic and transcriptomic profiling analysis. Global hypomethylation of the genome, which generally allows gene activation in general sense, is frequently seen in tumors and has been recognized as a contributing factor favoring oncogenesis [137] [138] [139]. In addition, hypomethylation is reported to be most common methylation alteration in solid tumors (i.e. hepatocellular carcinoma). After this computational study, several groups confirmed that, indeed, LINC00152 was upregulated in gastric cancer compared to normal tissue [140] [141] and elevated LINC00152 level induces EMT, proliferation, migration and invasion [127] [142] [143]. Despite the fact that most of the LINC00152-related research was carried out in the context of gastric cancer, in several other cancer types, this RNA was also shown to have a tumor promoting role and suggested to be a prognostic biomarker in hepatocellular carcinoma [128] [144] [145], renal cell carcinoma [146] [126], gallbladder cancer [147], lung adenocarcinoma [131], and last of all squamous cell carcinoma [148]. Literature search clearly shows that LINC00152 might have a general oncogenic effect. However, studies on LINC00152 in the context of breast cancer are limited, such that, there is no research in literature examining LINC00152 effect in tamoxifen resistance.

I studied role of potential non-coding RNAs modulating tamoxifen resistance in the ERα-positive breast cancer. After analysis of the RNA-Seq results, LINC00152 was found as the top candidate. Indeed, I also showed LINC00152 upregulation was contributing tamoxifen resistance in the both cell models (Fig. 18). In order to find out the main mechanism which had a role in cell survival in high expression of LINC00152, a series of cell based assays were carried out. In literature, it was reported that acquired tamoxifen resistance may result majorly in alterations in 2 biological pathways; estrogen receptor pathway or growth factor mediated cellular signaling pathways that work as compensatory to the ER pathway. These alterations modulate anti-proliferative or pro-apoptotic influence of the tamoxifen [31]. Considering LINC00152 knockdown leads to proliferation inhibition, it was worth inspecting LINC00152 effect on apoptosis. Additionally, in the previous studies of the Sahin and his colleagues, apoptosis suppression was shown to be one of the resistance mechanisms of the tamoxifen resistant MCF7 cell model [49]. Western Blot analysis was conducted in order to examine apoptosis at protein levels in the tamoxifen resistant MCF7-TamR cells after LINC00152 knockdown and tamoxifen treatment. The main executer of apoptosis is generally considered

to be caspase 3 and 7. However, one of the cell lines I used, MCF7, is caspase 3 negative. In **Figure *20***, I showed in the MCF7 cells, caspase 7 was cleaved when cells were transfected with LINC00152 ASO. The cleaved caspase 7 protein level was 2 folds in combination sample when compared to solely ASO-transfected sample. Cleaved caspase 7 levels in tamoxifen resistant MCF7 cell line treated with tamoxifen was stated to be weak in the literature [149]. Similarly, in my experiment, tamoxifen given MCF7-TamR samples did not result in caspase cleavage confirming resistant phenotype of my cell line. Hence, the results confirmed that the apoptosis was induced in the LINC00152 silenced samples, implying LINC00152 upregulation was causing apoptosis suppression in the resistant cells. In the literature, different studies stated that LINC00152 upregulation was associated with inhibition of apoptosis in gastric cancer [127], renal cell carcinoma [146] and HeLa cells [150]. Yet, none of these studies explained how LINC00152 regulating apoptosis.

In order to further investigate the pathways participating in apoptosis inhibition, I looked for the upstream regulators of the apoptosis. Here, as tamoxifen is a known autophagy inducer, I proposed that tamoxifen resistance in the cells may partially be originating from autophagy suppression, resulting in anti-apoptosis phenotype [78]. Thus, I also checked autophagy levels in the same setup as apoptosis. During autophagosome production, LC3 I (16 kDa) is converted into LC3 II (14 kDa) ensuring the last and key step of the autophagy initiation. Therefore, measurement of LC II/LC3 I ratio is a highly reliable method for autophagy detection [98]. Similar to apoptotic marker cleaved caspase 7, LC3 II/LC3 I ratio was at its greatest level in the combination samples (15.9 folds of conversion compared to control). These results imply LINC00152 expression greatly inhibits apoptosis in the tamoxifen resistant cell model *via* suppression of the autophagy within the cell (**Figure *20***).

Autophagy has dual role in cancer development. In the early stages of the tumorigenesis it is believed that autophagy induction is disfavored in cancer progression and act as tumor-suppressor. On the other hand, in the more advance stages, autophagy is considered giving an advantage to tumor development. Moreover, there is no info in literature linking LINC00152 with autophagy neither directly nor indirectly. Here, I have proposed, long non-coding RNA LINC00152 is upregulated in tamoxifen resistant ERα-positive breast cancer cell models, and its elevated expression contributes to tamoxifen resistance by inhibiting apoptosis *via* regulating autophagy in the cells. Considering the still

unresolved, dichotomous role of autophagy in drug resistance, this study contributes to the sensitizer role of autophagy in drug resistance field.

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

LINC00152 is a frequently dysregulated lncRNA in various cancer types including, but not limited to, lung cancer, glioma and hepatocellular carcinoma. It has been implicated in a wide range of cellular processes such as cell proliferation, epithelial-mesenchymal transition and chemotherapy resistance, leading to tumorigenesis or tumor progression. Accordingly, in this thesis, I investigated its role in tamoxifen resistance in ERα-positive breast cancer. Although I demonstrated that LINC00152 contributes to the development and/or maintenance of tamoxifen resistance in the cell line models, the molecular mechanisms upon which it acts are still unclear. Recent literature suggests that lncRNAs can function as transcriptional regulators, epigenetic modulators, miRNA sponges and even encode functional small peptides, adding to their complexity. Therefore, LINC00152 may be potentially exerting its effect on tamoxifen resistance at multiple levels simultaneously.

In my studies, I have used just one ASO against LINC00152, which seems to be specific to LINC00152 in computational analyses though it may act on different RNA targets or cause non-specific effects *via* its toxicity *in vitro*. To rule out this possibility and provide a stronger support for the causal role of LINC00152 in tamoxifen resistance, new ASOs with the same modification backbone and with a less toxic modification backbone should be tested under the same experimental conditions.

LINC00152 is a promising lncRNA that can be used as a prognostic marker in ERα-positive breast cancer cells. Its expression level not only affects the patient survival rate but also predicts clinical outcome, such as relapse and disease free survival. Therefore, high LINC00152 level can serve as an indication for tamoxifen resistance in clinics. Fluorescent in situ hybridization (ISH) against LINC00152 can be performed in breast cancer patient samples to demonstrate its clinical relevance and predictive power even further. Accordingly, an antisense oligonucleotide or a similar molecule targeting LINC00152 may be tested in clinical trials together with current drug regimens, and/or it may be conjugated to targeted or chemotherapeutic agents commonly utilized in ERα-positive breast cancer patients. There are

phase I/II clinical studies using ASO as treatment for various diseases [104] [105] including EGFR antisense for neck carcinoma (ClinicalTrials.gov identifier: NCT01592721), XIAP antisense for hepatocellular carcinoma (ClinicalTrials.gov identifier: NCT00882869) and for AML (ClinicalTrials.gov identifier: NCT00363974) as well as safety study of antisense products for breast and bladder carcinoma (ClinicalTrials.gov identifier: NCT00487786). Hence, LINC00152 knockdown *in vivo* by ASO can be important as a pre-clinical study. Although patient gene expression and survival data were in compliance with *in vitro* results, this thesis lacks *in vivo* studies that would back up the causal involvement of LINC00152 in mediating tamoxifen resistance. Therefore, mice xenograft studies should be carried out to further validate LINC00152 contribution to the tamoxifen response. Inducible LINC00152 knockdown system using CRISPR/Cas9 can be developed from both MCF7-TamR and T47D-TamR cells followed by mammary tumor development in a mouse model. In this way, the effect of LINC00152 expression alone and in combination with tamoxifen treatment could be examined in *in vivo* settings.

Autophagy is a natural intracellular degradation system to avoid accumulation of non-functional organelles and balance energy and nutrient use. It occurs in a step-wise manner and is interconnected to endolysosomal pathway. LC3 I to LC3 II conversion is a crucial step and marker for autophagosome formation, which strictly drives the completion of autophagy. Upstream events controlling LC3 status, such as Beclin1 and p62, therefore, exert their effect at earlier time points. Consequently, events upstream of LC3 must be investigated in a time-dependent manner to uncover the mechanisms *via* which LINC00152 regulates autophagy process. In another project from our lab, it was shown that endoplasmic reticulum stress was associated with tamoxifen resistance (data not published). As ER stress is one of the upstream events of autophagy and both contributes to the tamoxifen resistance, their crosstalk through LINC00152-mediated mechanism is worth to be investigated.

Another modulator of the autophagy is mTOR signaling, which may be connected *via* nutrient sensing. In a study conducted in hepatocellular carcinoma, LINC00152 was suggested to be associated with mTOR pathway [68]. mTOR is a known autophagy regulator, and there are several studies demonstrating autophagy can be induced by inhibiting mTOR [106] [107]. In addition, mTOR and its signaling components are stated to be overexpressed, overactive or mutated in many cancer types, including breast cancer [16] [108]. Inducing autophagy *via* targeting mTOR signaling pathway elements is shown to decrease viability in breast cancer cell lines [83] [82]. In a clinical study conducted with Everolimus, it was

revealed that direct mTOR inhibitors can improve progression-free survival as second-line therapy when combined with endocrine therapy in patients with advanced ERα-positive breast cancer after failure with first-line therapies [16], such as tamoxifen treatment. In addition; AKT, ERK and mTOR pathways will be monitored after LINC00152 knockdown in tamoxifen resistant cell lines.

Apoptosis, as extent result of induced autophagy, was found to be enhanced when LINC00152 was silenced in the tamoxifen resistant cell model. There are two main MAPK pathways controlling apoptosis initiation; p38 and JNK MAPK pathways [151] [152] [153]. JNK-triggered apoptosis is suggested to be influenced by wide range of stimuli within the cell, same accounts for the other mitogen-activated kinase, p38 [153]. For this reason, investigating the role of LINC00152 in apoptosis initiation requires highly detailed series of experiments. Luckily enough, our lab's (unpublished) data and few studies in literature examined p38/JNK induction in the context of tamoxifen, providing insights into the upstream mechanism [154] [155] [156] [157].

Overall, I developed and characterized tamoxifen resistance *in vitro* in Luminal A breast cancer cell model T47D, and identified a lncRNA, LINC00152, that is partially responsible for the resistance *via* inhibiting autophagy. Further studies need to be undertaken to elucidate the detailed mechanisms of LINC00152-induced tamoxifen resistance.

APPENDIX

List of 330 statistically significant and differentially expressed non-coding RNAs in tamoxifen resistant MCF7-TamR cell line compared to tamoxifen sensitive MCF7-WT cell line. ncRNAs are sorted according to their log2FC values in descending order.

Gene ID	log2FC	q value
CTD-2307P3.1	7,07	0,0007
RP11-266N13.2	6,16	0,0029
RP11-129M6.1	5,80	0,0007
LINC00473	5,53	0,0007
RP11-619A14.2	4,97	0,0007
CTD-3096P4.1	4,71	0,0007
TM4SF1-AS1	4,56	0,0007
RP11-353N4.6	4,51	0,0007
CTA-833B7.2	4,46	0,0092
CTD-2298J14.2	4,43	0,0007
AF240627.2	4,38	0,0019
RP5-1010E17.1	4,33	0,0007
RP11-206M11.7	4,23	0,0007
CTB-140J7.2	4,20	0,0007
RP11-818F20.4	4,01	0,0007
LINC00623	3,93	0,0007
RP11-838N2.3	3,84	0,0007
RP11-576D8.4	3,73	0,0007
RP11-369C8.1	3,68	0,0007
RP11-32K4.1	3,51	0,0007
GS1-179L18.1(LINC01446)	3,50	0,0007
RP11-809H16.5	3,41	0,0007
LINC00277 (EWSAT1)	3,41	0,0007
RP11-269F21.2	3,34	0,0013
RP11-269F21.3	3,34	0,0013
RP11-483P21.2	3,31	0,0007
PGM5-AS1	3,25	0,0284
AC104801.1	3,18	0,0007
MIR181A1HG	3,10	0,0007
AC116609.2	3,07	0,0007
AC116609.3	3,07	0,0007
RP11-897M7.1	3,00	0,0019
AL132709.5	2,96	0,0007
AL132709.8	2,96	0,0007
MEG8	2,96	0,0007

RP11-909M7.3	2,96	0,0007
AC002451.3	2,96	0,0341
RP11-1299A16.3	2,93	0,0007
LDLRAD4-AS1	2,86	0,0191
RP11-227F19.5	2,84	0,0029
LINC00636	2,82	0,0007
RP11-317P15.4	2,81	0,0007
RP1-124C6.1	2,79	0,0245
RP11-53B2.3	2,77	0,0007
RP11-691H4.4	2,73	0,0007
LINC00665	2,73	0,0007
RP4-735C1.6	2,71	0,0007
CTD-2223O18.1	2,68	0,0080
RP11-675F6.3	2,66	0,0007
RP11-675F6.4	2,66	0,0007
RP11-776H12.1	2,57	0,0007
RP1-35C21.2	2,57	0,0007
BFSP2-AS1	2,54	0,0007
RP1-230L10.1	2,51	0,0007
RP11-483P21.3	2,50	0,0344
AC004066.3	2,49	0,0007
AC005592.1	2,48	0,0007
AC005592.2	2,48	0,0007
AC005592.3	2,48	0,0007
RP11-1096D5.2	2,38	0,0019
LINC00869	2,37	0,0039
RP4-773N10.6	2,36	0,0187
RP4-536B24.2	2,35	0,0007
RP11-638F5.1	2,34	0,0007
AP001059.5	2,31	0,0007
RP11-273G15.2	2,30	0,0323
AC005013.5	2,30	0,0007
AC005162.5	2,30	0,0007
CTB-113D17.1	2,30	0,0007
ZBED3-AS1	2,28	0,0007
AC018359.1	2,26	0,0007
AC123023.1	2,26	0,0007
RP11-148O21.2	2,25	0,0019
RP11-148O21.3	2,25	0,0019
RP11-148O21.4	2,25	0,0019
RP11-148O21.6	2,25	0,0019
RP11-127O4.3	2,22	0,0007
CTB-147C13.1	2,20	0,0007

CTC-265N9.1	2,20	0,0007
RP11-1399P15.1	2,18	0,0475
RP1-140K8.5	2,14	0,0007
AC142293.3	2,14	0,0007
RP11-114F10.2	2,08	0,0044
RP4-536B24.3	2,07	0,0132
RP11-90D11.1	2,06	0,0044
RP11-349I1.2	2,05	0,0013
RP11-185E8.1	2,05	0,0235
RP11-266K4.9	2,05	0,0024
AQP4-AS1	2,02	0,0024
RP11-17A19.2	2,02	0,0024
CTD-2013M15.1	2,01	0,0067
CTD-2215E18.1	2,00	0,0007
AC011747.3	1,99	0,0007
AC011747.4	1,99	0,0007
LINC00298	1,99	0,0007
LINC00299	1,99	0,0007
RP11-20B7.1	1,98	0,0007
RP11-38L15.3	1,96	0,0007
CTC-321K16.1	1,95	0,0218
RP11-293M10.6	1,93	0,0024
RP11-661P17.1	1,93	0,0007
RP11-692C24.1	1,93	0,0007
RP11-486M23.1	1,93	0,0007
RP11-486M23.2	1,93	0,0007
RP11-321G12.1	1,92	0,0407
AC007319.1	1,92	0,0007
RP11-705O24.1	1,92	0,0007
RP11-92C4.3	1,90	0,0007
RP4-704D21.2	1,90	0,0007
RP11-355F16.1	1,89	0,0024
RP1-60O19.1	1,89	0,0007
AC129492.6	1,87	0,0007
AP001057.1	1,86	0,0019
RP5-1065P14.2	1,85	0,0007
CTC-340D7.1	1,82	0,0013
CTC-537E7.2	1,82	0,0013
CTC-537E7.3	1,82	0,0013
LINC00882	1,81	0,0048
RP11-109P11.1	1,80	0,0007
RP11-18F14.1	1,80	0,0007
RP11-525K10.3	1,80	0,0007

LINC00111	1,74	0,0281
AC009502.4	1,72	0,0007
AC067956.1	1,72	0,0007
AC093843.1	1,72	0,0007
AC114765.1	1,72	0,0007
AC114765.2	1,72	0,0007
RP11-77P16.4	1,71	0,0007
RP13-726E6.1	1,71	0,0007
RP11-435O5.4	1,68	0,0480
VIM-AS1	1,68	0,0344
CTD-2503I6.1	1,66	0,0437
CASC19	1,62	0,0007
CCAT1	1,62	0,0007
PCAT2	1,62	0,0007
RP11-168K11.3	1,57	0,0177
RP11-18B16.2	1,57	0,0177
RP11-37N22.1	1,56	0,0007
AC017002.1	1,55	0,0029
CTD-3064M3.4	1,54	0,0019
RP11-224O19.2	1,53	0,0105
RP11-549B18.1	1,51	0,0148
RP11-409I10.2	1,50	0,0053
STARD4-AS1	1,48	0,0007
RP11-265N7.1	1,47	0,0019
RP11-624L4.1	1,47	0,0019
RP5-1050E16.2	1,47	0,0007
RP11-128L5.1	1,46	0,0136
ST8SIA6-AS1	1,42	0,0007
AP000475.2	1,42	0,0287
LINC00152	1,39	0,0007
RP11-734K21.5	1,38	0,0113
RP11-445F12.1	1,36	0,0350
AP001619.3	1,35	0,0125
RP11-983P16.4	1,34	0,0338
RP11-133L19.1	1,33	0,0228
RP11-31L22.3	1,32	0,0281
RP11-265N7.2	1,31	0,0092
C2orf48	1,31	0,0007
KRTAP5-AS1	1,30	0,0076
RP11-573G6.6	1,27	0,0007
CTD-2175A23.1	1,27	0,0007
RP11-210M15.2	1,26	0,0007
AP001043.1	1,24	0,0207

RP11-342K6.4	1,24	0,0181
CTD-2523D13.2	1,21	0,0007
KB-1836B5.1	1,21	0,0007
RP11-181E10.3	1,20	0,0007
RP11-803D5.4	1,20	0,0007
CTD-2147F2.1	1,20	0,0221
RP11-611D20.2	1,20	0,0284
RP13-467H17.1	1,19	0,0007
RP11-736K20.6	1,18	0,0007
RP11-582J16.4	1,16	0,0007
TMEM254-AS1	1,16	0,0007
AF064858.6	1,16	0,0390
AP001044.2	1,16	0,0390
ZNF503-AS1	1,15	0,0287
LINC00885	1,12	0,0488
CTC-480C2.1	1,11	0,0007
CTD-2651B20.3	1,10	0,0341
RP4-813D12.3	1,10	0,0029
RP11-666A20.4	1,09	0,0007
AC009410.1	1,09	0,0423
RP11-64B16.4	1,08	0,0410
AC061961.2	1,08	0,0166
RP11-173M1.4	1,07	0,0194
RP11-123O22.1	1,06	0,0053
RP11-156L14.1	1,03	0,0488
CTD-2516F10.4	1,02	0,0088
LINC00984 (INAFM2)	1,00	0,0013
JHDM1D-AS1	-1,00	0,0490
AC013463.2	-1,01	0,0044
AC019181.2	-1,01	0,0044
RP4-792G4.2	-1,05	0,0335
RP11-430B1.2	-1,12	0,0419
AF127577.11	-1,13	0,0304
AC011526.1	-1,14	0,0007
LINC00589	-1,17	0,0007
RP11-94H18.1	-1,17	0,0007
RP11-473E2.2	-1,21	0,0007
RP11-473E2.4	-1,21	0,0007
LINC00578	-1,23	0,0297
RP11-114M1.1	-1,23	0,0297
RP11-2L8.1	-1,23	0,0297
RP11-91K9.1	-1,23	0,0297
RP3-510L9.1	-1,25	0,0007

RP11-65J21.3	-1,27	0,0007
RP11-384C4.7	-1,28	0,0048
CASC15	-1,28	0,0356
RP11-524C21.2	-1,28	0,0356
RP11-73M7.6	-1,30	0,0423
RP11-73M7.9	-1,30	0,0423
CTD-3179P9.1	-1,31	0,0062
RP11-146I2.1	-1,35	0,0007
RP11-330A16.1	-1,35	0,0007
RP11-131L23.1	-1,36	0,0024
RP11-131L23.2	-1,36	0,0024
RP11-197K6.1	-1,39	0,0071
RP5-1027G4.3	-1,43	0,0007
CTD-2555A7.2	-1,46	0,0007
AP004372.1	-1,47	0,0084
HIF1A-AS1	-1,48	0,0044
HIF1A-AS2	-1,48	0,0044
TMCC1-AS1	-1,48	0,0053
RP11-548P2.2	-1,52	0,0201
RP11-502M1.2	-1,55	0,0024
AC019117.1	-1,57	0,0248
RP11-97N19.2	-1,60	0,0395
AC017060.1	-1,61	0,0109
AC019117.2	-1,61	0,0109
H19 (LINC00008)	-1,63	0,0007
RP11-21C17.1	-1,63	0,0007
RP11-865I6.2	-1,63	0,0007
RP11-963H4.3	-1,66	0,0013
RP3-399L15.3	-1,67	0,0007
LINC00222	-1,70	0,0007
RP11-465B22.8	-1,70	0,0034
RP11-434D2.2	-1,71	0,0284
LA16c-325D7.1	-1,73	0,0007
LINC00887	-1,80	0,0007
CPEB2-AS1	-1,81	0,0007
LINC00504	-1,81	0,0007
ADAMTS9-AS1	-1,82	0,0092
ARHGEF19-AS1	-1,84	0,0048
RP11-47I22.2	-1,84	0,0105
CTC-806A22.1	-1,89	0,0187
LINC00908	-1,93	0,0278
RP11-624M8.1	-1,97	0,0088
RP4-710M3.2	-2,02	0,0136

LINC00865	-2,03	0,0007
RP11-419C23.1	-2,04	0,0007
RP11-65J21.1	-2,05	0,0338
RP11-97O12.2	-2,05	0,0287
TET2-AS1	-2,10	0,0024
RP11-310E22.4	-2,11	0,0007
RP11-310E22.5	-2,11	0,0007
CTD-3010D24.3	-2,12	0,0464
LINC00536	-2,12	0,0007
RP11-536K17.1	-2,12	0,0007
RP11-284F21.10	-2,16	0,0007
RP11-284F21.7	-2,16	0,0007
RP11-284F21.9	-2,16	0,0007
RP11-499O7.7	-2,16	0,0248
LINC00632	-2,16	0,0390
ARHGAP26-AS1	-2,18	0,0125
RP11-234K24.3	-2,19	0,0007
RP11-290F20.3	-2,32	0,0007
RP4-550H1.4	-2,33	0,0310
AC006262.10	-2,34	0,0092
AC006262.5	-2,34	0,0092
AC073316.1	-2,37	0,0007
AC073316.2	-2,37	0,0007
C20orf197	-2,38	0,0044
RP11-347E10.1	-2,39	0,0007
RP1-80N2.2	-2,45	0,0007
CTD-2555I5.1	-2,45	0,0007
RP11-179A16.1	-2,45	0,0007
RP11-179A16.2	-2,45	0,0007
RP11-686G23.2	-2,45	0,0007
RP11-356C4.3	-2,46	0,0024
RP11-655G22.1	-2,48	0,0071
CTC-518B2.10	-2,49	0,0013
RP11-179A7.2	-2,57	0,0019
RP11-37L2.1	-2,57	0,0019
RP11-93K22.6	-2,57	0,0007
LOXL1-AS1	-2,57	0,0007
RP11-734I18.1	-2,59	0,0007
AC068580.7	-2,66	0,0024
AC073325.1	-2,67	0,0007
LINC01021	-2,67	0,0007
ELOVL2-AS1	-2,71	0,0053
RP11-556I14.1	-2,73	0,0007

CTD-2023M8.1	-2,90	0,0007
LINC00327	-2,93	0,0007
RP5-1029K10.2	-2,95	0,0007
RP5-1029K10.4	-2,95	0,0007
DPYD-AS1	-2,99	0,0007
RP11-438N16.1	-3,02	0,0029
RP11-225N10.1	-3,04	0,0062
AC004053.1	-3,15	0,0034
AC004063.1	-3,15	0,0034
CTD-2005H7.1	-3,17	0,0007
CTD-2005H7.2	-3,17	0,0007
RP11-245G13.2	-3,20	0,0191
RP11-400K9.4	-3,23	0,0007
AC007743.1	-3,32	0,0007
RP11-553A10.1	-3,35	0,0029
LINC01016	-3,38	0,0007
AC012594.1	-3,91	0,0007
AF038458.5	-3,91	0,0007
RP11-57A1.1	-3,96	0,0007
LINC00683	-4,06	0,0007
RP11-317J10.2	-4,10	0,0007
CTB-164N12.1	-4,16	0,0007
CTB-32H22.1	-4,16	0,0007
CTB-33O18.1	-4,16	0,0007
CTB-33O18.2	-4,16	0,0007
CTB-33O18.3	-4,16	0,0007
RP11-47P18.1	-4,18	0,0007
RP11-1081M5.1	-4,25	0,0007
RP11-1081M5.2	-4,25	0,0007
AC005301.8	-4,27	0,0007
LINC00511	-4,27	0,0007
PDZRN3-AS1	-4,52	0,0132
RP11-395B7.4	-4,57	0,0088
RP11-152C17.1	-4,76	0,0007
RP11-713M6.2	-5,13	0,0007
RP11-809M12.1	-5,31	0,0007
RP13-895J2.7	-10,74	0,0007

BIBLIOGRAPHY

1. Perou, C.M., et al., *Molecular portraits of human breast tumours.* Nature, 2000. **406**(6797): p. 747-52.
2. Colombo, P.E., et al., *Microarrays in the 2010s: the contribution of microarray-based gene expression profiling to breast cancer classification, prognostication and prediction.* Breast Cancer Res, 2011. **13**(3): p. 212.
3. Sorlie, T., et al., *Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.* Proc Natl Acad Sci U S A, 2001. **98**(19): p. 10869-74.
4. Carroll, J.S., et al., *Deciphering the divergent roles of progestogens in breast cancer.* Nat Rev Cancer, 2017. **17**(1): p. 54-64.
5. Klinge, C.M., *Estrogen receptor interaction with estrogen response elements.* Nucleic Acids Res, 2001. **29**(14): p. 2905-19.
6. Heldring, N., et al., *Estrogen receptors: how do they signal and what are their targets.* Physiol Rev, 2007. **87**(3): p. 905-31.
7. Wang, G.S., H. Zhu, and S.J. Bi, *Pathological features and prognosis of different molecular subtypes of breast cancer.* Mol Med Rep, 2012. **6**(4): p. 779-82.
8. Cuzick, J., et al., *Prognostic value of a combined estrogen receptor, progesterone receptor, Ki-67, and human epidermal growth factor receptor 2 immunohistochemical score and comparison with the Genomic Health recurrence score in early breast cancer.* J Clin Oncol, 2011. **29**(32): p. 4273-8.
9. Mohammed, H., et al., *Progesterone receptor modulates ERalpha action in breast cancer.* Nature, 2015. **523**(7560): p. 313-7.
10. Braun, L., et al., *Intrinsic breast cancer subtypes defined by estrogen receptor signalling-prognostic relevance of progesterone receptor loss.* Mod Pathol, 2013. **26**(9): p. 1161-71.
11. Ahn, H.J., et al., *Differences in Clinical Outcomes between Luminal A and B Type Breast Cancers according to the St. Gallen Consensus 2013.* J Breast Cancer, 2015. **18**(2): p. 149-59.
12. Cuzick, J., et al., *Selective oestrogen receptor modulators in prevention of breast cancer: an updated meta-analysis of individual participant data.* Lancet, 2013. **381**(9880): p. 1827-34.
13. Moyer, V.A. and U.S.P.S.T. Force, *Medications to decrease the risk for breast cancer in women: recommendations from the U.S. Preventive Services Task Force recommendation statement.* Ann Intern Med, 2013. **159**(10): p. 698-708.
14. Tran, B. and P.L. Bedard, *Luminal-B breast cancer and novel therapeutic targets.* Breast Cancer Res, 2011. **13**(6): p. 221.
15. Wong, Z.W. and M.J. Ellis, *First-line endocrine treatment of breast cancer: aromatase inhibitor or antioestrogen?* Br J Cancer, 2004. **90**(1): p. 20-5.
16. Zhang, M.H., et al., *Estrogen receptor-positive breast cancer molecular signatures and therapeutic potentials (Review).* Biomed Rep, 2014. **2**(1): p. 41-52.
17. Baselga, J., et al., *Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer.* N Engl J Med, 2012. **366**(6): p. 520-9.

18. Mauri, D., et al., *Survival with aromatase inhibitors and inactivators versus standard hormonal therapy in advanced breast cancer: meta-analysis.* J Natl Cancer Inst, 2006. **98**(18): p. 1285-91.
19. Hackshaw, A., et al., *Long-term benefits of 5 years of tamoxifen: 10-year follow-up of a large randomized trial in women at least 50 years of age with early breast cancer.* J Clin Oncol, 2011. **29**(13): p. 1657-63.
20. Smith, G.L., *The Long and Short of Tamoxifen Therapy: A Review of the ATLAS Trial.* J Adv Pract Oncol, 2014. **5**(1): p. 57-60.
21. Cole, M.P., C.T. Jones, and I.D. Todd, *A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474.* Br J Cancer, 1971. **25**(2): p. 270-5.
22. Muyan, M., et al., *Modulation of Estrogen Response Element-Driven Gene Expressions and Cellular Proliferation with Polar Directions by Designer Transcription Regulators.* PLoS One, 2015. **10**(8): p. e0136423.
23. Hurtado, A., et al., *FOXA1 is a key determinant of estrogen receptor function and endocrine response.* Nat Genet, 2011. **43**(1): p. 27-33.
24. Ring, A. and M. Dowsett, *Mechanisms of tamoxifen resistance.* Endocr Relat Cancer, 2004. **11**(4): p. 643-58.
25. Schiff, R., et al., *Advanced concepts in estrogen receptor biology and breast cancer endocrine resistance: implicated role of growth factor signaling and estrogen receptor coregulators.* Cancer Chemother Pharmacol, 2005. **56 Suppl 1**: p. 10-20.
26. Dowsett, M. and A. Howell, *Breast cancer: aromatase inhibitors take on tamoxifen.* Nat Med, 2002. **8**(12): p. 1341-4.
27. Housman, G., et al., *Drug resistance in cancer: an overview.* Cancers (Basel), 2014. **6**(3): p. 1769-92.
28. Gottesman, M.M., *Mechanisms of cancer drug resistance.* Annu Rev Med, 2002. **53**: p. 615-27.
29. Early Breast Cancer Trialists' Collaborative, G., et al., *Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials.* Lancet, 2011. **378**(9793): p. 771-84.
30. Kennecke, H., et al., *Metastatic behavior of breast cancer subtypes.* J Clin Oncol, 2010. **28**(20): p. 3271-7.
31. Musgrove, E.A. and R.L. Sutherland, *Biological determinants of endocrine resistance in breast cancer.* Nat Rev Cancer, 2009. **9**(9): p. 631-43.
32. Osborne, C.K. and R. Schiff, *Mechanisms of endocrine resistance in breast cancer.* Annu Rev Med, 2011. **62**: p. 233-47.
33. Hart, C.D., et al., *Challenges in the management of advanced, ER-positive, HER2-negative breast cancer.* Nat Rev Clin Oncol, 2015. **12**(9): p. 541-52.
34. Miller, K., et al., *Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer.* N Engl J Med, 2007. **357**(26): p. 2666-76.
35. Johnston, S., et al., *Lapatinib combined with letrozole versus letrozole and placebo as first-line therapy for postmenopausal hormone receptor-positive metastatic breast cancer.* J Clin Oncol, 2009. **27**(33): p. 5538-46.
36. Finn, R.S., et al., *Estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2 (HER2), and epidermal growth factor receptor expression and*

benefit from lapatinib in a randomized trial of paclitaxel with lapatinib or placebo as first-line treatment in HER2-negative or unknown metastatic breast cancer. J Clin Oncol, 2009. **27**(24): p. 3908-15.

37. Cristofanilli, M., et al., *Phase II, randomized trial to compare anastrozole combined with gefitinib or placebo in postmenopausal women with hormone receptor-positive metastatic breast cancer.* Clin Cancer Res, 2010. **16**(6): p. 1904-14.
38. Osborne, C.K., et al., *Gefitinib or placebo in combination with tamoxifen in patients with hormone receptor-positive metastatic breast cancer: a randomized phase II study.* Clin Cancer Res, 2011. **17**(5): p. 1147-59.
39. Thomas, C. and J.A. Gustafsson, *A CUE hints at tumor resistance.* Nat Med, 2011. **17**(6): p. 658-60.
40. Riggins, R.B., et al., *Pathways to tamoxifen resistance.* Cancer Lett, 2007. **256**(1): p. 1-24.
41. Widschwendter, M., et al., *Association of breast cancer DNA methylation profiles with hormone receptor status and response to tamoxifen.* Cancer Res, 2004. **64**(11): p. 3807-13.
42. Ottaviano, Y.L., et al., *Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells.* Cancer Res, 1994. **54**(10): p. 2552-5.
43. Lapidus, R.G., et al., *Methylation of estrogen and progesterone receptor gene 5' CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors.* Clin Cancer Res, 1996. **2**(5): p. 805-10.
44. Fan, M., et al., *Diverse gene expression and DNA methylation profiles correlate with differential adaptation of breast cancer cells to the antiestrogens tamoxifen and fulvestrant.* Cancer Res, 2006. **66**(24): p. 11954-66.
45. Holloway, J.N., S. Murthy, and D. El-Ashry, *A cytoplasmic substrate of mitogen-activated protein kinase is responsible for estrogen receptor-alpha down-regulation in breast cancer cells: the role of nuclear factor-kappaB.* Mol Endocrinol, 2004. **18**(6): p. 1396-410.
46. Shou, J., et al., *Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer.* J Natl Cancer Inst, 2004. **96**(12): p. 926-35.
47. Geisler, S. and J. Coller, *RNA in unexpected places: long non-coding RNA functions in diverse cellular contexts.* Nat Rev Mol Cell Biol, 2013. **14**(11): p. 699-712.
48. Ward, A., et al., *Re-expression of microRNA-375 reverses both tamoxifen resistance and accompanying EMT-like properties in breast cancer.* Oncogene, 2013. **32**(9): p. 1173-82.
49. Ward, A., et al., *MicroRNA-519a is a novel oncomir conferring tamoxifen resistance by targeting a network of tumour-suppressor genes in ER+ breast cancer.* J Pathol, 2014. **233**(4): p. 368-79.
50. Batista, P.J. and H.Y. Chang, *Long noncoding RNAs: cellular address codes in development and disease.* Cell, 2013. **152**(6): p. 1298-307.
51. Wapinski, O. and H.Y. Chang, *Long noncoding RNAs and human disease.* Trends Cell Biol, 2011. **21**(6): p. 354-61.

52. Jin, P., R.S. Alisch, and S.T. Warren, *RNA and microRNAs in fragile X mental retardation.* Nat Cell Biol, 2004. **6**(11): p. 1048-53.
53. Faghihi, M.A., et al., *Expression of a noncoding RNA is elevated in Alzheimer's disease and drives rapid feed-forward regulation of beta-secretase.* Nat Med, 2008. **14**(7): p. 723-30.
54. Visel, A., et al., *Targeted deletion of the 9p21 non-coding coronary artery disease risk interval in mice.* Nature, 2010. **464**(7287): p. 409-12.
55. El Azzouzi, H., P.A. Doevendans, and J.P. Sluijter, *Long non-coding RNAs in heart failure: an obvious lnc.* Ann Transl Med, 2016. **4**(9): p. 182.
56. Beltrán-Anaya, F.O., et al., *Insights into the Regulatory Role of Non-coding RNAs in Cancer Metabolism.* Frontiers in Physiology, 2016. **7**.
57. Fabbri, M., 2014.
58. Huarte, M., *RNA. A lncRNA links genomic variation with celiac disease.* Science, 2016. **352**(6281): p. 43-4.
59. Hajjari, M. and A. Salavaty, *HOTAIR: an oncogenic long non-coding RNA in different cancers.* Cancer Biol Med, 2015. **12**(1): p. 1-9.
60. Taft, R.J., et al., *Non-coding RNAs: regulators of disease.* J Pathol, 2010. **220**(2): p. 126-39.
61. Kumar, V., et al., *Human disease-associated genetic variation impacts large intergenic non-coding RNA expression.* PLoS Genet, 2013. **9**(1): p. e1003201.
62. Ji, P., et al., *MALAT-1, a novel noncoding RNA, and thymosin beta4 predict metastasis and survival in early-stage non-small cell lung cancer.* Oncogene, 2003. **22**(39): p. 8031-41.
63. Ding, X., et al., *Long non-coding RNAs may serve as biomarkers in breast cancer combined with primary lung cancer.* Oncotarget, 2017.
64. Lo, P.K., et al., *Dysregulation of the BRCA1/long non-coding RNA NEAT1 signaling axis contributes to breast tumorigenesis.* Oncotarget, 2016. **7**(40): p. 65067-65089.
65. Zhang, M., et al., *lncRNA NEAT1 is closely related with progression of breast cancer via promoting proliferation and EMT.* Eur Rev Med Pharmacol Sci, 2017. **21**(5): p. 1020-1026.
66. Meijer, D., et al., *Functional screen for genes responsible for tamoxifen resistance in human breast cancer cells.* Mol Cancer Res, 2006. **4**(6): p. 379-86.
67. Godinho, M.F., et al., *Relevance of BCAR4 in tamoxifen resistance and tumour aggressiveness of human breast cancer.* Br J Cancer, 2010. **103**(8): p. 1284-91.
68. Godinho, M., et al., *Characterization of BCAR4, a novel oncogene causing endocrine resistance in human breast cancer cells.* J Cell Physiol, 2011. **226**(7): p. 1741-9.
69. Xing, Z., et al., *LncRNA BCAR4 wires up signaling transduction in breast cancer.* RNA Biol, 2015. **12**(7): p. 681-9.
70. Mathew, R., V. Karantza-Wadsworth, and E. White, *Role of autophagy in cancer.* Nat Rev Cancer, 2007. **7**(12): p. 961-7.
71. Kisen, G.O., et al., *Reduced autophagic activity in primary rat hepatocellular carcinoma and ascites hepatoma cells.* Carcinogenesis, 1993. **14**(12): p. 2501-5.
72. Kondo, Y., et al., *The role of autophagy in cancer development and response to therapy.* Nat Rev Cancer, 2005. **5**(9): p. 726-34.

73. Yang, Z.J., et al., *The role of autophagy in cancer: therapeutic implications.* Mol Cancer Ther, 2011. **10**(9): p. 1533-41.
74. White, E., J.M. Mehnert, and C.S. Chan, *Autophagy, Metabolism, and Cancer.* Clin Cancer Res, 2015. **21**(22): p. 5037-46.
75. Liang, X.H., et al., *Induction of autophagy and inhibition of tumorigenesis by beclin 1.* Nature, 1999. **402**(6762): p. 672-6.
76. Scarlatti, F., et al., *Role of non-canonical Beclin 1-independent autophagy in cell death induced by resveratrol in human breast cancer cells.* Cell Death Differ, 2008. **15**(8): p. 1318-29.
77. Kuo, P.L., Y.L. Hsu, and C.Y. Cho, *Plumbagin induces G2-M arrest and autophagy by inhibiting the AKT/mammalian target of rapamycin pathway in breast cancer cells.* Mol Cancer Ther, 2006. **5**(12): p. 3209-21.
78. Samaddar, J.S., et al., *A role for macroautophagy in protection against 4-hydroxytamoxifen-induced cell death and the development of antiestrogen resistance.* Mol Cancer Ther, 2008. **7**(9): p. 2977-87.
79. Van Grembergen, O., et al., *Portraying breast cancers with long noncoding RNAs.* Sci Adv, 2016. **2**(9): p. e1600220.
80. Zhang, B., et al., *A novel RNA motif mediates the strict nuclear localization of a long noncoding RNA.* Mol Cell Biol, 2014. **34**(12): p. 2318-29.
81. Trapnell, C., et al., *Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks.* Nat Protoc, 2012. **7**(3): p. 562-78.
82. Iyer, M.K., et al., *The landscape of long noncoding RNAs in the human transcriptome.* Nat Genet, 2015. **47**(3): p. 199-208.
83. Ovadya, Y. and V. Krizhanovsky, *A new Twist in kidney fibrosis.* Nat Med, 2015. **21**(9): p. 975-7.
84. Lovisa, S., et al., *Epithelial-to-mesenchymal transition induces cell cycle arrest and parenchymal damage in renal fibrosis.* Nat Med, 2015. **21**(9): p. 998-1009.
85. Lamouille, S., J. Xu, and R. Derynck, *Molecular mechanisms of epithelial-mesenchymal transition.* Nat Rev Mol Cell Biol, 2014. **15**(3): p. 178-96.
86. Jolly, M.K., et al., *Implications of the Hybrid Epithelial/Mesenchymal Phenotype in Metastasis.* Front Oncol, 2015. **5**: p. 155.
87. Wang, Z., M. Gerstein, and M. Snyder, *RNA-Seq: a revolutionary tool for transcriptomics.* Nat Rev Genet, 2009. **10**(1): p. 57-63.
88. Brown, J., M. Pirrung, and L.A. McCue, *FQC Dashboard: integrates FastQC results into a web-based, interactive, and extensible FASTQ quality control tool.* Bioinformatics, 2017.
89. Aken, B.L., et al., *The Ensembl gene annotation system.* Database (Oxford), 2016. **2016**.
90. Wilming, L.G., et al., *The vertebrate genome annotation (Vega) database.* Nucleic Acids Res, 2008. **36**(Database issue): p. D753-60.
91. Harrow, J., et al., *GENCODE: the reference human genome annotation for The ENCODE Project.* Genome Res, 2012. **22**(9): p. 1760-74.

92. O'Leary, N.A., et al., *Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation.* Nucleic Acids Res, 2016. **44**(D1): p. D733-45.
93. Watts, J.K. and D.R. Corey, *Silencing disease genes in the laboratory and the clinic.* J Pathol, 2012. **226**(2): p. 365-79.
94. Liang, Y., C. Yan, and N.F. Schor, *Apoptosis in the absence of caspase 3.* Oncogene, 2001. **20**(45): p. 6570-8.
95. Ryter, S.W., S.M. Cloonan, and A.M. Choi, *Autophagy: a critical regulator of cellular metabolism and homeostasis.* Mol Cells, 2013. **36**(1): p. 7-16.
96. Jing, K. and K. Lim, *Why is autophagy important in human diseases?* Exp Mol Med, 2012. **44**(2): p. 69-72.
97. Das, G., B.V. Shravage, and E.H. Baehrecke, *Regulation and function of autophagy during cell survival and cell death.* Cold Spring Harb Perspect Biol, 2012. **4**(6).
98. Nath, S., et al., *Lipidation of the LC3/GABARAP family of autophagy proteins relies on a membrane-curvature-sensing domain in Atg3.* Nat Cell Biol, 2014. **16**(5): p. 415-24.
99. Curtis, C., et al., *The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups.* Nature, 2012. **486**(7403): p. 346-52.
100. Abraham, D.J., D.P. Rotella, and A. Burger, *Burger's medicinal chemistry, drug discovery and development*. 7th ed. 2010, Hoboken, N.J.: Wiley.
101. Ali, S., et al., *Molecular mechanisms and mode of tamoxifen resistance in breast cancer.* Bioinformation, 2016. **12**(3): p. 135-139.
102. Uhlmann, S., et al., *Global microRNA level regulation of EGFR-driven cell-cycle protein network in breast cancer.* Mol Syst Biol, 2012. **8**: p. 570.
103. Moasser, M.M., *The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis.* Oncogene, 2007. **26**(45): p. 6469-87.
104. Wieduwilt, M.J. and M.M. Moasser, *The epidermal growth factor receptor family: biology driving targeted therapeutics.* Cell Mol Life Sci, 2008. **65**(10): p. 1566-84.
105. Kurokawa, H., et al., *Inhibition of HER2/neu (erbB-2) and mitogen-activated protein kinases enhances tamoxifen action against HER2-overexpressing, tamoxifen-resistant breast cancer cells.* Cancer Res, 2000. **60**(20): p. 5887-94.
106. Kurokawa, H. and C.L. Arteaga, *Inhibition of erbB receptor (HER) tyrosine kinases as a strategy to abrogate antiestrogen resistance in human breast cancer.* Clin Cancer Res, 2001. **7**(12 Suppl): p. 4436s-4442s; discussion 4411s-4412s.
107. Kurokawa, H. and C.L. Arteaga, *ErbB (HER) receptors can abrogate antiestrogen action in human breast cancer by multiple signaling mechanisms.* Clin Cancer Res, 2003. **9**(1 Pt 2): p. 511S-5S.
108. Sommer, A., et al., *Studies on the development of resistance to the pure antiestrogen Faslodex in three human breast cancer cell lines.* J Steroid Biochem Mol Biol, 2003. **85**(1): p. 33-47.
109. Jensen, B.L., et al., *Differential regulation of specific genes in MCF-7 and the ICI 182780-resistant cell line MCF-7/182R-6.* Br J Cancer, 1999. **79**(3-4): p. 386-92.

110. McClelland, R.A., et al., *Enhanced epidermal growth factor receptor signaling in MCF7 breast cancer cells after long-term culture in the presence of the pure antiestrogen ICI 182,780 (Faslodex).* Endocrinology, 2001. **142**(7): p. 2776-88.
111. Schmitt, A.M. and H.Y. Chang, *Long Noncoding RNAs in Cancer Pathways.* Cancer Cell, 2016. **29**(4): p. 452-63.
112. Huarte, M., *The emerging role of lncRNAs in cancer.* Nat Med, 2015. **21**(11): p. 1253-61.
113. Gutschner, T. and S. Diederichs, *The hallmarks of cancer: a long non-coding RNA point of view.* RNA Biol, 2012. **9**(6): p. 703-19.
114. Lee, S., et al., *Noncoding RNA NORAD Regulates Genomic Stability by Sequestering PUMILIO Proteins.* Cell, 2016. **164**(1-2): p. 69-80.
115. Misawa, A., et al., *Androgen-induced Long Noncoding RNA (lncRNA) SOCS2-AS1 Promotes Cell Growth and Inhibits Apoptosis in Prostate Cancer Cells.* J Biol Chem, 2016. **291**(34): p. 17861-80.
116. Bian, Z., et al., *LncRNA-UCA1 enhances cell proliferation and 5-fluorouracil resistance in colorectal cancer by inhibiting miR-204-5p.* Sci Rep, 2016. **6**: p. 23892.
117. Tee, A.E., et al., *The long noncoding RNA MALAT1 promotes tumor-driven angiogenesis by up-regulating pro-angiogenic gene expression.* Oncotarget, 2016. **7**(8): p. 8663-75.
118. Gupta, R.A., et al., *Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis.* Nature, 2010. **464**(7291): p. 1071-6.
119. Niknafs, Y.S., et al., *The lncRNA landscape of breast cancer reveals a role for DSCAM-AS1 in breast cancer progression.* Nat Commun, 2016. **7**: p. 12791.
120. Xu, S., et al., *Oncogenic long noncoding RNA landscape in breast cancer.* Mol Cancer, 2017. **16**(1): p. 129.
121. Yan, X., et al., *Comprehensive Genomic Characterization of Long Non-coding RNAs across Human Cancers.* Cancer Cell, 2015. **28**(4): p. 529-40.
122. Xing, Z., et al., *lncRNA directs cooperative epigenetic regulation downstream of chemokine signals.* Cell, 2014. **159**(5): p. 1110-1125.
123. Liao, X.H., et al., *Long intergenic non-coding RNA APOC1P1-3 inhibits apoptosis by decreasing alpha-tubulin acetylation in breast cancer.* Cell Death Dis, 2016. **7**: p. e2236.
124. Meng, J., et al., *A four-long non-coding RNA signature in predicting breast cancer survival.* J Exp Clin Cancer Res, 2014. **33**: p. 84.
125. Su, X., et al., *Comprehensive analysis of long non-coding RNAs in human breast cancer clinical subtypes.* Oncotarget, 2014. **5**(20): p. 9864-76.
126. Wang, Y., et al., *Long intergenic non-coding RNA 00152 promotes renal cell carcinoma progression by epigenetically suppressing P16 and negatively regulates miR-205.* Am J Cancer Res, 2017. **7**(2): p. 312-322.
127. Zhao, J., et al., *Long non-coding RNA Linc00152 is involved in cell cycle arrest, apoptosis, epithelial to mesenchymal transition, cell migration and invasion in gastric cancer.* Cell Cycle, 2015. **14**(19): p. 3112-23.

128. Ji, J., et al., *LINC00152 promotes proliferation in hepatocellular carcinoma by targeting EpCAM via the mTOR signaling pathway.* Oncotarget, 2015. **6**(40): p. 42813-24.
129. Cai, Q., et al., *Long non-coding RNA LINC00152 promotes gallbladder cancer metastasis and epithelial-mesenchymal transition by regulating HIF-1alpha via miR-138.* Open Biol, 2017. **7**(1).
130. Yu, Y., et al., *LINC00152: A pivotal oncogenic long non-coding RNA in human cancers.* Cell Prolif, 2017. **50**(4).
131. Chen, Q.N., et al., *Long intergenic non-coding RNA 00152 promotes lung adenocarcinoma proliferation via interacting with EZH2 and repressing IL24 expression.* Mol Cancer, 2017. **16**(1): p. 17.
132. van Heesch, S., et al., *Extensive localization of long noncoding RNAs to the cytosol and mono- and polyribosomal complexes.* Genome Biol, 2014. **15**(1): p. R6.
133. Lennox, K.A. and M.A. Behlke, *Cellular localization of long non-coding RNAs affects silencing by RNAi more than by antisense oligonucleotides.* Nucleic Acids Res, 2016. **44**(2): p. 863-77.
134. Shi, S.J., et al., *LncRNA-ATB promotes trastuzumab resistance and invasion-metastasis cascade in breast cancer.* Oncotarget, 2015. **6**(13): p. 11652-63.
135. Li, W., et al., *Downregulation of LncRNA GAS5 causes trastuzumab resistance in breast cancer.* Oncotarget, 2016. **7**(19): p. 27778-86.
136. Neumann, O., et al., *Methylome analysis and integrative profiling of human HCCs identify novel protumorigenic factors.* Hepatology, 2012. **56**(5): p. 1817-27.
137. Das, P.M. and R. Singal, *DNA methylation and cancer.* J Clin Oncol, 2004. **22**(22): p. 4632-42.
138. Shibata, T. and H. Aburatani, *Exploration of liver cancer genomes.* Nat Rev Gastroenterol Hepatol, 2014. **11**(6): p. 340-9.
139. Ehrlich, M., *DNA methylation in cancer: too much, but also too little.* Oncogene, 2002. **21**(35): p. 5400-13.
140. Cao, W.J., et al., *Analysis of long non-coding RNA expression profiles in gastric cancer.* World J Gastroenterol, 2013. **19**(23): p. 3658-64.
141. Pang, Q., et al., *Increased expression of long intergenic non-coding RNA LINC00152 in gastric cancer and its clinical significance.* Tumour Biol, 2014. **35**(6): p. 5441-7.
142. Zhou, J., et al., *Linc00152 promotes proliferation in gastric cancer through the EGFR-dependent pathway.* J Exp Clin Cancer Res, 2015. **34**: p. 135.
143. Chen, W.M., et al., *Long intergenic non-coding RNA 00152 promotes tumor cell cycle progression by binding to EZH2 and repressing p15 and p21 in gastric cancer.* Oncotarget, 2016. **7**(9): p. 9773-87.
144. Deng, X., et al., *Linc00152 promotes cancer progression in hepatitis B virus-associated hepatocellular carcinoma.* Biomed Pharmacother, 2017. **90**: p. 100-108.
145. Li, J., et al., *HULC and Linc00152 Act as Novel Biomarkers in Predicting Diagnosis of Hepatocellular Carcinoma.* Cell Physiol Biochem, 2015. **37**(2): p. 687-96.
146. Wu, Y., et al., *Long non-coding RNA Linc00152 is a positive prognostic factor for and demonstrates malignant biological behavior in clear cell renal cell carcinoma.* Am J Cancer Res, 2016. **6**(2): p. 285-99.

147. Cai, Q., et al., *Upregulation of long non-coding RNA LINC00152 by SP1 contributes to gallbladder cancer cell growth and tumor metastasis via PI3K/AKT pathway.* Am J Transl Res, 2016. **8**(10): p. 4068-4081.
148. Yu, J., et al., *Upregulated long non-coding RNA LINC00152 expression is associated with progression and poor prognosis of tongue squamous cell carcinoma.* J Cancer, 2017. **8**(4): p. 523-530.
149. Miller, T.E., et al., *MicroRNA-221/222 confers tamoxifen resistance in breast cancer by targeting p27Kip1.* J Biol Chem, 2008. **283**(44): p. 29897-903.
150. Notzold, L., et al., *The long non-coding RNA LINC00152 is essential for cell cycle progression through mitosis in HeLa cells.* Sci Rep, 2017. **7**(1): p. 2265.
151. Sui, X., et al., *p38 and JNK MAPK pathways control the balance of apoptosis and autophagy in response to chemotherapeutic agents.* Cancer Lett, 2014. **344**(2): p. 174-9.
152. Cai, B., et al., *p38 MAP kinase mediates apoptosis through phosphorylation of BimEL at Ser-65.* J Biol Chem, 2006. **281**(35): p. 25215-22.
153. Wada, T. and J.M. Penninger, *Mitogen-activated protein kinases in apoptosis regulation.* Oncogene, 2004. **23**(16): p. 2838-49.
154. Feng, Y., et al., *Tamoxifen-induced apoptosis of rat C6 glioma cells via PI3K/Akt, JNK and ERK activation.* Oncol Rep, 2010. **24**(6): p. 1561-7.
155. Moodbidri, M.S. and N.V. Shirsat, *Activated JNK brings about accelerated apoptosis of Bcl-2-overexpressing C6 glioma cells on treatment with tamoxifen.* J Neurochem, 2005. **92**(1): p. 1-9.
156. Mandlekar, S. and A.N. Kong, *Mechanisms of tamoxifen-induced apoptosis.* Apoptosis, 2001. **6**(6): p. 469-77.
157. Zhang, C.C. and D.J. Shapiro, *Activation of the p38 mitogen-activated protein kinase pathway by estrogen or by 4-hydroxytamoxifen is coupled to estrogen receptor-induced apoptosis.* J Biol Chem, 2000. **275**(1): p. 479-86.

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