THE ROLE OF NEK6 GENE EXPRESSION IN MULTIDRUG RESISTANCE
AND APOPTOSIS IN MCF-7 AND K-562 CELL LINES

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ABSTRACT

THE ROLE OF NEK6 GENE EXPRESSION IN MULTIDRUG RESISTANCE AND APOPTOSIS IN MCF-7 AND K-562 CELL LINES

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Drug resistance, either intrinsic or acquired, is the most important challenge in cancer treatment. Multidrug resistance (MDR) is a phenomenon in which tumor cells become resistant to structurally or functionally unrelated chemotherapeutic drugs. The increased drug efflux and decreased drug influx, elevated level of drug metabolism, impairment in apoptotic and DNA repair pathways are the basic reasons for MDR development.

Nek6 is a member of the NIMA-related kinase family. Nek6 is an important mitotic kinase for proper cell cycle progression. More specifically, Nek6 is crucial for metaphase to anaphase transition, mitotic spindle formation and chromosome segregation. Recent studies show that there is a connection between tumorigenesis and Nek6 activity. Nek6 is overexpressed in breast, lung, cervix, kidney, colorectal and laryngeal cancers. Overexpression of kinase-dead domain of Nek6 leads to decrease in tumor growth. The depletion of Nek6 cause mitotic arrest and induce apoptosis. Moreover, Nek6 has also role in DNA damage checkpoint. Although, the importance of Nek6 in cell cycle, tumorigenesis and cell cycle checkpoint has been studied, there is no study related to the role of Nek6 in multidrug resistance. For this

purpose, we aimed to search the involvement of Nek6 in multidrug resistance and apoptosis.

We screened the sensitive and resistant subtypes of breast and chronic myeloid leukemia cell lines for the expression of Nek6. Regarding Nek6 expression there is no significant difference between sensitive and chemo resistant breast cancer cell lines. On the other hand, Nek6 expression is significantly reduced in chemo resistant chronic myeloid leukemia cells, implying a possible role of Nek6 in drug resistance. Previous studies show that the impairments in apoptotic pathway are one of the major causes in imatinib resistance in chronic myeloid leukemia. Therefore, we investigated the expression profiles of apoptotic genes, such as Bax, Bcl-2, Survivin in leukemia cells. After silencing of the Nek6 gene by siRNA in sensitive leukemia cells, the expression profile of apoptotic genes showed a similar pattern with drug resistant cells.

Our results implied that Nek6 may have potential role in imatinib resistance through apoptotic pathway in chronic myeloid leukemia.

Keywords: Cancer, multidrug resistance, Nek6, apoptosis

ÖZ

NEK6'NIN K-562 VE MCF-7 HÜCRE HATLARINDA ÇOKLU İLAÇ DİRENÇLİLİĞİ VE APOPTOZDAKİ İŞLEVLERİ

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Kemoterapi kanser tedavisinde en etkili ve en çok başvurulan yöntemdir. Ancak tümör hücreleri yapısal özellikleri ve etki mekanizmaları farklı olan kemoterapötik ilaçlara karşı direnç gösterebilmektedir. Çoklu ilaç dirençliliği etkili kanser tedavisinin önündeki en büyük engeldir. Bu dirençlilik kemoterapötik tedavi öncesinde ya da tedavi sırasında ortaya çıkabilir. İlaç dirençliliğinin meydana gelmesinde bir çok farklı mekanizma vardır. İlacın hücre içine alımının azalması ya da hücreden atılımının artması, ilacın DNA molekülü üzerinde yarattığı hasarın hızlı bir şekilde onarılması, ilaç detoksifikasyonun artması veya hücrenin apoptoza eğiliminin azalması çoklu ilaç dirençliliğinin başlıca nedenleridir.

Nek6, NIMA-ilişkili serin-treonin kinaz ailesinin bir üyesidir. Bu kinaz mitotic hücre döngüsünün hatasız bir şekilde başlaması ve devam etmesinde önemli bir role sahiptir. Son zamanlarda yapılan çalışmalarda Nek6 kinaz aktivitesi ve tümorgenez arasında önemli bir ilişki bulunmuştur. Nek6 gen ifadesinin meme, rahim, akciğer, böbrek, gırtlak ve kalın bağırsak kanserlerinde arttığı gözlemlenmiştir. Nek6 aktivitesinin susturulması hücrenin mitotic döngüsünün durmasına ve apoptoza neden olmaktadır. Ayrıca, Nek6 DNA hasarı kontrol noktasında önemli bir proteindir. Nek6'nın çoklu ilaç dirençliliğindeki rolü ile ilgili bir çalışma

bulunmamaktır. Bu nedenle, amacımız Nek6'nın çoklu ilaç dirençliliği ve apoptoz ile ilişkisinin araştırılmasıdır.

Labarotuvarımızda geliştirilen ilaca dirençli ve duyarlı meme kanseri ve kronik miyeloid lösemi hücre hatlarında Nek6 gen ifadesi taranmıştır. Nek6 gen ifadesi ilaca karşı duyarlı ve dirençli olan meme kanseri hücre hatlarında önemli bir değişiklik göstermemiştir. Ancak ilaca karşı dirençli kronik miyeloid lösemi hücrelerinde, Nek6 ifade düzeyi duyarlı hücrelerle karşılaştırıldığında önemli bir azalma göstermiştir. Gen ifadesindeki bu farklılık Nek6'nın ilaç dirençliliğinde önemli bir rolü olabileceğini göstermektedir. Kronik miyeloid lösemide imatinibe karşı geliştirilen dirençlilikte apoptozda rol aldığı bilinen genlerin ifadesindeki değişikliklerin önemli bir rolü olduğu bilinmektedir. Bu nedenle Nek6'nın ilaç dirençliliğindeki olası rolünün bulunması için Bax, Bcl-2, Survivin gibi apoptoz ile ilişkilendirilmiş gen ifadeleri incelenmiştir. İlaca karşı duyarlı hücre hattında siRNA kullanılarak Nek6 geninin susturulması gerçekleştirilmiş ve bu hücrelerde Bax, Bcl-2, Survivin gibi apoptoz ile ilgili gen ifadelerinin dirençli hücre hatlarındaki gen ifade profillerine benzediği gözlemlenmiştir. Nek6'nın ilaç dirençliliğinin gelişmesine neden olması apoptoz yolağındaki bazı genlerin ifade düzeylerindeki farklılıklar ile ilişkilendirilmiştir. Elde ettiğimiz sonuçlar Nek6'nın kronik miyeloid lösemide çoklu ilaç dirençliliğinde potansiyel bir rolü olabileceğini göstermiştir.

Anahtar Sözcükler: Kanser, çoklu ilaç dirençliliği, Nek6, apoptoz

To my precious family…

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ABC   ATP-binding cassette

ABL   (Ableson Leukemia Virus Oncogene)

BCR   Breakpoint Cluster Region

BCRP   Breast cancer resistance protein

CDK   Cyclin dependent kinases

CML   Chronic Myelogenous Lekumia

DMSO   Dimethyl sufoxide

Dox   Doxorubicin

FADD   Fas-associated death domain

FBS   Fetal bovine serum

GST   Glutathione s transferase

HEPES   4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid

Ima   Imatinib

K562/DOX   1000 nM Doxorubicin-resistant K562 cell line

K562/IMA   1000 nM Imatinib-resistant K562 cell line

MCF7/DOC   1000 nM Docataxel-resistant MCF7 cell line

MCF7/DOX   1000 nM Doxorubicin-resistant MCF7 cell line

MCF7/ZOL   8 μM Zoledronic acid-resistant MCF7 cell line

MOMP	Mitochondrial outer membrane permeabilization
MRP	Multidrug resistance protein
MTX	Methotrexate
NIMA	Never-in-mitosis A
NMD	Nucleotide-binding Domain
OGT1	Organic Cation Transporters
P53	p-53 upregulated modulator of apoptosis
PARP	Poly [ADP-ribose] polymerase 1
PBS	Phosphate-buffered saline
P-gp	P-glycoprotein
PI	Propidium Iodide
PS	Phosphatidylserine
RISK	RNA-induced Silencing Complex
SAC	Spindle Assembly Checkpoint
Smac	Second Mitochondria-derived Activator
TMD	Transmembrane Domain
TNF	Tumor necrosis factor
TRADD	TNF receptor-associated death domain
UTR	Untranslated Region

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CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer is a general name for the diseases characterized by uncontrolled cell growth and invasion of these abnormal cells to other sides of body. Tumors that are unable to invade and remain encapsulated are called benign tumors. On the other hand, malignant tumors can migrate from primary site to other parts of body which is called metastasis. (Lauren Pecorino, 2008)

According to World Health Organization cancer is the most common cause of death around world, accounting for 8.2 million deaths and 14.1 million new cancer cases in 2012 (World Health Organization, 2015). Lung, prostate, colorectum, stomach, and liver cancer are the most encountered cancer types among men. Most common diagnosed cancers among woman are breast, colorectum, lung, cervix, and stomach cancer.

There are six characteristics shared by many cancers (Hanahan & Weinberg, 2000). Growth signal autonomy refers to cancer cells not requiring growth factor signalling to divide unlikely normal cells. Tumor cells can produce growth signal themselves and show attenuated dependence on growth stimulation from environment. A second characteristic of tumor cells is evasion from apoptosis. Impairments in the apoptotic pathway, in which dysregulation of anti- and pro-apoptotic gene expression is observed, is a common characteristic of cancer cells. Another hallmark is inresponsiveness to growth inhibitory signals; cancer cells do not respond to antiproliferative signals, resulting uncontrolled cell growth. Unlimited replicative potential as a result of maintaining telomere length, sustained angiogenesis, activating invasion and metastasis are additional characterists of tumor cells. There are also new emerging hallmarks such as, avoiding the immune system,

reprogramming cellular energetics, tumor promoting inflammation and genomic instabilty (Hanahan & Weinberg, 2011).

1.2   Breast Cancer

Breast cancer is the most prevalent cancer type among woman and it is a heterogeneous disease which each type has different etiology. The woman's breast comprises three basic parts: lobules that have milk producing glands, ducts responsible for carrying milk from lobules to nipples and stroma (connective and fatty tissue) (Breastcancer.org, 2013). Types of breast cancer can be categorized in three according to their origin. In Ductal carcinoma in situ (DCIS) abnormal cells observed in the lining the ducts and these cells have not grown into surrounding tissues such as lymph nodes yet. Invasive ductal carcinoma is the most common type and it starts in the milk duct of breast like DCIS but these cells have ability to invade in surrounding tissues. Invasive lobular carcinoma starts in lobules and able to metastasize. Moreover, breast cancer could be categorized according to its hormone receptor and HER2 status. If cancer cells contain estrogen or progesterone receptor, the case is called hormone receptor positive and they can be treated with hormone therapy. If they do not contain neither estrogen or progesterone receptor, then this type of breast cancer is named as hormone receptor negative. Cancer cells that have excessive HER2 protein or extra copies of the HER2 gene are named as HER2 positive. If HER2 protein level is quite low, they are called as HER2 negative. Triple negative type of breast cancer cells have neither of the two receptors and also do not have excessive HER2 protein. Triple positive breast cancer cells have both hormone receptors and also express high levels of HER2 protein.

5-10% of breast cancer cases are because of the inherited mutations, such as BRCA1, and BRCA2. The rest of them are due to random mutations. Gender, age, menstrual periods, usage of hormone therapy, certain kinds of birth control methods, tobacco smoke and alcohol abuse, not having children or having children at older age are the main risk factors for breast cancer (Macmillan, Chambers, & Tuck, 2013).

1.2.1   Treatment Strategies for Breast Cancer

1.2.1.1   Surgery

Surgery is the most applied treatment strategy among breast cancer patients. The principal aim of surgery is to take out as much of cancer cells as possible. The extent of surgery changes according to localization of tumor and what stage it is.

1.2.1.2   Radiation Therapy

Radiation therapy is treatment applied by using high-energy rays or particles to kill cancer cells. It is used to kill cancer cells remaining in the breast or surrounding tissues in order to prevent recurrence of cancer after surgery. External and internal beam radiations are two main ways for radiation therapy. In the external case, radiation is given by a machine and it focuses on the tumor. In internal beam radiation, radioactive pellets are delivered into breast tissue.

1.2.1.3   Hormone Therapy

This type of treatment is applied in hormone receptor positive breast cancers. The main aim is to either reduce hormone levels or block their actions on cancer cells. Drugs prevent hormone production or inhibit hormone-receptor interaction. Hormone therapy is also used to prevent cancer recurrence. Aromatase inhibitors, selective estrogen receptor modulators and estrogen receptor down regulators are the most commonly used drugs in hormone therapy.

1.2.1.4   Chemotherapy

Chemotherapy is the usage of drugs which can kill tumor cells. Treatment aims to shrink tumor before the surgery and kill any remaining tumor cells after the surgery. Chemotherapeutic drugs target the dividing cells in the body regardless of it is healthy or cancer cell. This explains some side effects of chemotherapy such as hair loss. Docetaxel, paclitaxel, doxorubicin, epirubicin are the commonly used drugs during breast cancer treatment (Breastcaner.org, 2013). Depending on the stage and type of cancer, drugs could be used as combinational. Especially, in early breast

cancer many drugs are used together, on the other hand, in advanced tumors targeted therapy with specific drug is preferred.

1.3   Chronic Myelogenous Lekumia (CML)

CML is a disease which starts in bone marrow. The term chronic states the disease has a tendency to progress slowly. The term myelogenous represents the affected blood cell in this disease. There are three phases in CML which are chronic, accelerated and blast crisis. In chronic phase precursor and mature form of myeloid cells accumulate in bone marrow and disease may last for many years whereas in accelerated phase precursor or progenitor myeloid cells accumulate rather than mature cells. In blast crisis the differentiation of myeloid and lymphoid cells is arrested and rapid progression of disease is observed (Calabretta & Perrotti, 2015). Bone marrow is responsible for production of mature blood cells from blood stem cells. Blood stem cells differentiate into either myeloid or lymphoid stem cells. Lymphoid stem cells further differentiate into white blood cells and myeloid stem cells further differentiate into either mature red blood cells, platelets or granulocytes (Figure 1.1). Granulocytes are responsible for fighting infections. There are three types of granulocytes which are neutrophils, eosinophils, and basophils. In CML patients, granulocytes are abnormal which causes inability to fight with infections and much more in number, as a result there is less space in bone marrow for healthy red blood cells and other white blood cells (Clarkson, Strife, Wisniewski, Lambek, & Liu, 2003).
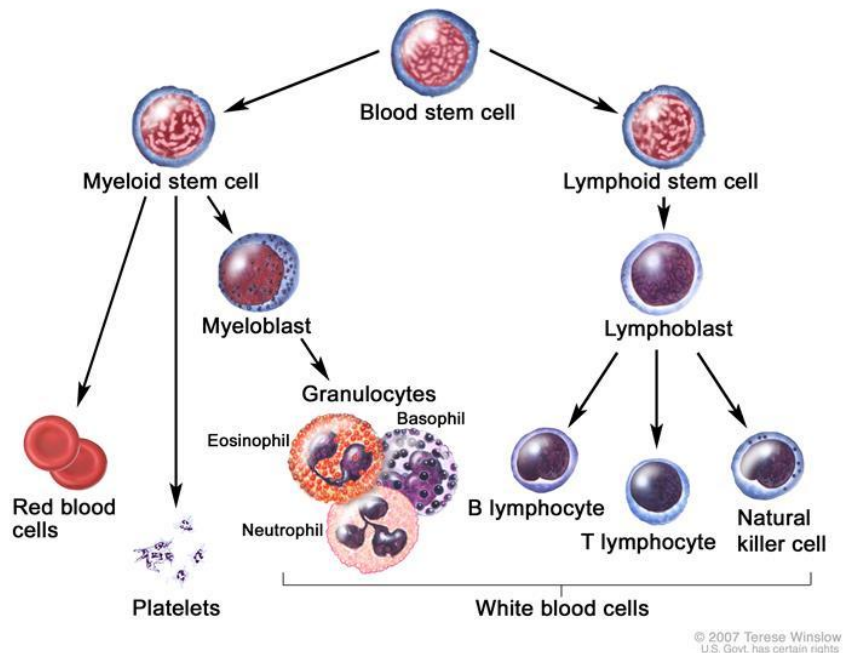
Figure 1.1 Development of blood cells. Retrieved from http://www.cancer.gov/types/leukemia/patient/cml-treatment-pdq#section/_1

In 90% of CML patients there is a translocation between chromosome 9 and 22 (t(9:22) (q34:q11.2)) creating an abnormal chromosome named as *Philadelphia chromosome* (Figure 1.2). The translocation causes fusion of BCR (Breakpoint Cluster Region) and ABL (Ableson Leukemia Virus Oncogene) genes. The hybrid oncogene (p210BCR/ABL) is responsible for expression of a hybrid p210 BCR/ABL protein. Studies show that expression of p210 BCR/ABL is sufficient condition for induction of CML (Danisz & Blasiak, 2013). BCR/ABL tyrosine kinase binds and transfers phosphate from ATP to own tyrosine residues and activates various signaling cascades. The BCR-ABL gene encodes a constitutively active form of tyrosine kinase, which activates downstream proteins those have roles in growth factor independent proliferation, evasion from apoptosis, altered adhesion and being resistant to DNA repair (Zhen & Wang, 2013). Eventually, granulocytes grow out of control and crowd out for healthy white blood cells.

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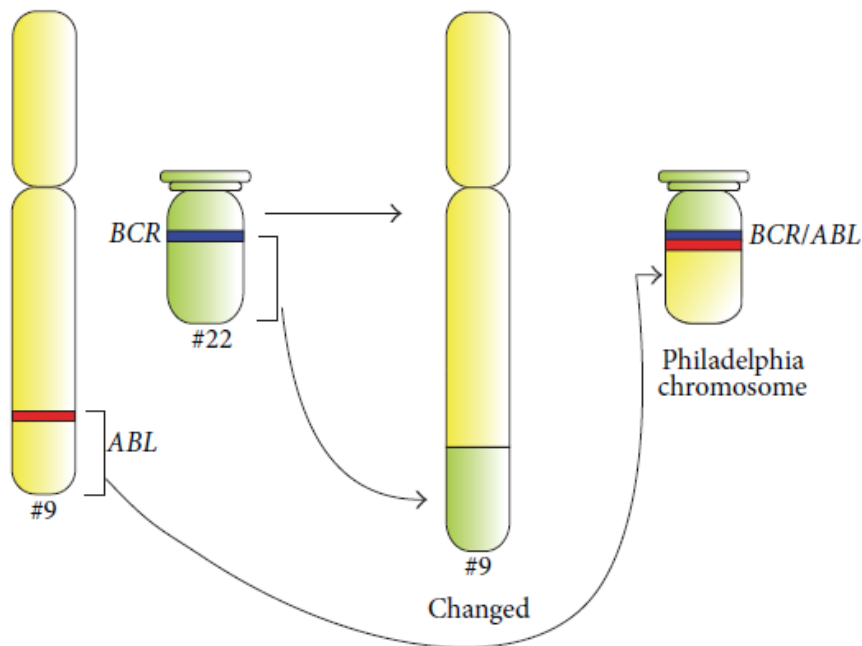
Figure 1.2 Philadelphia chromosome, translocation between chromosome 9 and 22, resulting p210 BCR/ABL fusion protein. Retrieved from Trela *et. al*., 2013.

A small percentage of CML patients, do not have Philadelphia chromosome even though they have BCR-ABL fusion protein. Moreover, some patients have neither Philadelphia chromosome nor BCR-ABL fusion proteins. In Ph-negative patients, the rearrangements in breakpoint of BCR gene is observed but their clinical characteristics and disease process is different from Ph-positive patients (Onida et al., 2002).

1.3.1 Options for CML Treatment

1.3.1.1 Bone Marrow Transplantation

Bone marrow transplantation is the definitive treatment for CML but the high risk of operation makes it latter option. For transplantation, high dose of chemotherapeutic drugs or radiation are given to patient in order to kill blood forming cells in bone

marrow of patient. Then, stem cells from a donor are transplanted into patient. Transplanted cells produce new healthy blood cells.

1.3.1.2 Targeted Therapy and Chemotherapy

Targeted therapy is a treatment option that targets specific cancer cells. In CML, tyrosine kinase inhibitors which attack cancer cells that produce excessive tyrosine kinase are used for targeted therapy. Imatinib that targets directly constitutively active form of tyrosine kinase, dasatinib, nilotinib are the most commonly used drugs for CML. (Kantarjian, Cortes, La Rosée, & Hochhaus, 2010). Some chemotherapeutic drugs such as hydroxycarmamide are combined with targeted drugs.

1.3.1.3 Biological Therapy

Biological therapy is applied when other treatments are not efficient enough or side effects of other drugs are too many. Interferon alpha is the commonly used drug in biologic therapy and it helps inhibiting tumor growth and proliferation. (Talpaz, Hehlmann, Quintás-Cardama, Mercer, & Cortes, 2012)

1.4 Multidrug Resistance (MDR)

Multidrug resistance is a complex phenomenon that cancer cells develop resistance to structurally or functionally unrelated drugs. MDR is the biggest obstacle in the treatment of cancer. There are various mechanisms behind MDR in cancer. Tumor cells develop resistance to cytotoxic drug either before the administration of any drug which is called intrinsic resistance, or they become resistance after exposed to drugs several times which is called acquired resistance. In acquired resistance, tumor cells not only develop resistance against administered drugs, but also they become cross resistant to functionally different drugs. Decreased drug influx and increased drug efflux, impair in apoptotic pathways, problems in drug conversion to its active form, alteration in cell cycle checkpoints and increased drug metabolism are the major reasons for the development of MDR (Baguley, 2010; Kars et al., 2006).

1.4.1 Mechanisms for MDR Development

Drugs can enter cells in two ways which are *(i)* passive diffusion (lipid or aqueous) and *(ii)* active transport (via carrier proteins). Lipophilic drugs such as doxorubicin, etoposide and paclitaxel enter cells via diffusion through cell membrane and hydrophilic drugs such as nucleoside analog, antifolates and cisplatin enter via transporters (carriers). In the case of hydrophilic drugs, drug resistance develops due to mutations in carrier proteins leading to inadequate drug accumulation in cell (Ambudkar, Kimchi-Sarfaty, Sauna, & Gottesman, 2003). Decreased drug influx is one of the reasons behind MDR development. For example, antifolate drugs enter cells via reduced folate carriers (RFC) and in children with CML disease RFCs are down regulated and as a result dihydrofolate reductase inhibitor methotrexate (MTX) could not reach critical threshold value in cell. In these patients, majority of drugs are found in the plasma (Longley & Johnston, 2005).

Another reason of drug resistance is the increased drug efflux from cells. Dysregulation of ATP-binding cassette (ABC) transporter proteins is the most important cause of alteration in drug efflux. The members of this family pump cytotoxic drugs out of cells and consequently drugs no longer take their effects in cells. ABC proteins are divided into seven classes (A-G) and they all have transmembrane (TMD) and nucleotide-binding (NBD) domains. The transmembrane domain is responsible for recognition and then intervention of chemical compounds. They generate energy from ATP via their nucleotide-binding domains and use this energy for active transport of various compounds. NBDs are evolutionally conserved due to same role among all organisms. On the other hand, TMDs show differences probably because of compound specificity (Sharom, 2008).

Three members of ABC transporter family are important for drug resistance development in cancer cells. ABCB1 (P-glycoprotein), ABCC1 (Multidrug Resistance Protein1), and ABCG2 (MXR, BCRP) are generally overexpressed in many types of cancer, including CML, causing inhibition of drug accumulation in tumor cells (Baguley, 2010).
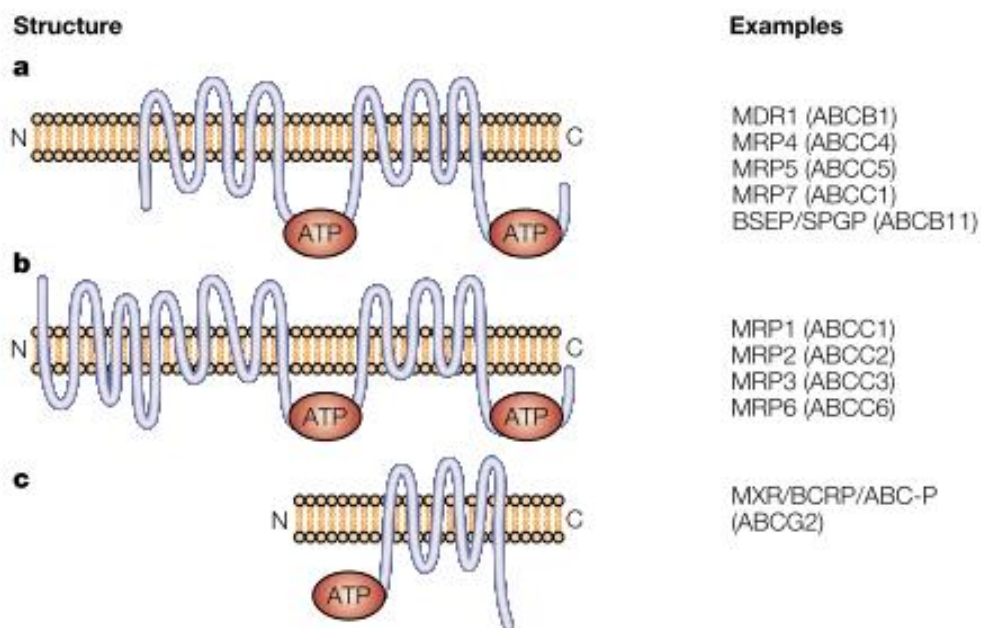
Figure 1.3 Structures of ABC transporter proteins link to drug resistance. Retrieved from Gottesman *et. al.,* 2002(Gottesman, Fojo, & Bates, 2002).

P-glycoprotein (P-gp), expressed by MDR1 gene, is the firstly identified ABC transporter protein and it consists of two transmembrane domains (TMD) that containing six transmembrane helices and one nucleotide binding domain (NBD), containing 4, 5 and 6 helices in the N-terminal half and 10, 11 and 12 helices in the C-terminal half (Wu, Calcagno, & Ambudkar, 2008). P-gp is expressed in epithelia cells of the liver, intestine kidney, and capillary endothelial cells in brain, ovary, and testis (Ambudkar et al., 2003). P-gp protects these tissues from xenobiotics but it has a role in development of drug resistance by increasing efflux of many chemotherapeutic drugs such as taxanes and anthracyclines and drug conjugates out of cells in tumor cells.

Multidrug resistance protein (MRP1) has similar structure to MDR1 with exceptionally an amino-terminal extension that contains five membrane- spanning domains. MDR1 and MRP1 also resemble each other in terms of transport specificity. Anionic and neutral hydrophobic drugs generally conjugated with

glutathione are transported by MRP1. It is overexpressed in various cancer types, such as leukemias and non-small-cell lung cancers.

ABCG2 (MXR and BCRP) composed of homodimer of two half-transporters, each containing an ATP-binding domain and six transmembrane segments. These proteins cause resistance to a broad spectrum of chemotherapeutic drugs similar to MDR1 and MRP1 such as doxorubicin, daunorubicin, and methotrexate (Gottesman et al., 2002).

Inactivation of drugs is another reason behind MDR development. Many chemotherapeutic drugs need to be activated in cell to implement their cytotoxic effects. Mutation and downregulation of drug-metabolizing proteins reduce the efficacy of drug. For example, cytarabine which is used in the treatment of leukemia, exert its effect after phosphorylated by deoxcytidine kinase. In drug resistant leukemia, down regulation of kinase is observed (Zahreddine & Borden, 2013). Alteration in drug detoxification pathways is another way of drug inactivation. Specifically, overexpression of GST (Glutathione s transferase) is commonly observed in many types of cancers. Overexpression of drug detoxifying protein leads to decrease in cytotoxic effects of drugs in cells by which inactivating drugs (Housman et al., 2014).

Alterations of drug targets are another mechanism for MDR development. Mutations in targets inhibit the interaction with drugs or gene amplification and overexpression of drug targets cause inefficacy of drugs even if they properly bind to target. For example, subsequent mutation in BCR/ABL gene in CML patients cause conformational change in which imatinib can no longer bind kinase (Zahreddine & Borden, 2013).

1.4.2   Imatinib Resistance in CML

Imatinib mesylate (IM) is a selector inhibitor of BCR/ABL. It directly binds the ATP-binding site and prevents auto-phosphorylation of tyrosine kinase. As a consequence of this inhibition, downstream pathways of tyrosine kinase such as PI3K/Akt or JAK/STAT could not be activated. Constitutive activation of PI3K/Akt

and JAK/STAT pathways leads to gain advantage to escape from apoptosis. Therefore, imatinib takes action by inhibiting proliferation and inducing apoptosis. Although imatinib is an effective drug for the treatment of CML, 15% of patients develop resistance (Trela, Glowacki, & Błasiak, 2014). IM resistance could develop either primary in which drug is ineffective from the beginning of treatment, or acquired in which drug loses its effect after treatment. There are various reasons for development of resistance against IM in the concept of BCR/ABL dependent and BCR/ABL independent mechanisms. Mutations in BCR/ABL gene that hinder interaction of kinase with imatinib, amplification and overexpression of gene resulting inefficacy of imatinib, decreased drug influx and elevated drug efflux in which overexpression of P-gp protein, epigenetic changes such as overexpression of DNA methyltransferases (DNMTs) and alterations in apoptotic and DNA repair pathways are the basic reasons for imatinib resistance in CML (Danisz & Blasiak, 2013; Trela et al., 2014). Moreover, in BCR/ABL independent mechanism additional mutations in patients and abnormal ceramide metabolism lead the fact that cancer cells do not require BCR/ABL fusion gene to proliferate anymore (Baran, Bielawski, Gunduz, & Ogretmen, 2011).

1.5   Multidrug Resistance and Apoptosis

Recent studies show that apoptosis is correlated with tumor development and resistance to therapy. Chemotherapy and other cancer treatment strategies activate apoptotic pathways in order to kill tumor cells. For this reason, impairment in apoptotic process cause cancer cells to gain resistance against applied therapy. Moreover, some cancer cells are intrinsically resistant to chemotherapeutic drugs due to their defective apoptotic pathways. Understanding of molecular mechanism of how tumor cells escape from apoptosis is important in order to develop more efficient treatment strategies (S Fulda & Debatin, 2006). Cells undergo apoptosis either through extracellular signals, or internal stimuli. In extrinsic pathway, extracellular signals, which are named as death signals, trigger apoptosis by the activation of death receptors. Specifically, Fas ligand or tumor necrosis factor (TNF) stimulate their receptor, Fas receptor and TNF receptor respectively, and cause conformational change and oligomerization of receptor. As a consequence of this

change cytoplasmic tail of the receptor which has the death domain, binds to adaptor proteins such as Fas associated death domain protein (FADD) or TNF receptor associated death domain protein (TRADD). When adaptor proteins are stimulated, initiator caspase-8 is activated; in turn it activates other downstream caspases. The caspase cascade induces the cleavage of specific proteins and eventuate apoptosis (Lauren Pecorino, 2008). In intrinsic pathway, an internal stimulus such as DNA damage, triggers apoptotic pathway through Bcl-2 protein family. This protein family has nearly 20 members all of which have Bcl-2 homology domain (BH) that intervene protein-protein interaction. Bcl-2 family members with pro-apoptotic characteristics promote apoptosis whereas the ones with anti-apoptotic characteristics protect cells from cell death. Figure 1.4 shows Bcl-2 family proteins and their roles in apoptosis. Bcl-2, Bcl-$_{xl}$, Mcl-1 are some of anti-apoptotic members of Bcl-2 family. They locate at mitochondrial membrane and prevent release of cytochrome c to prevent apoptosis. Moreover, they bind pro-apoptotic counterparts and prevent their activation by inhibiting undergo a conformational change or oligomerisation of these proteins (van Loo et al., 2002). Among pro-apoptotic proteins there is a subgroup called BH3-only proteins. They share only BH3 domain and they promote apoptosis either inducing other pro-apoptotic proteins or inhibiting the activity of anti-apoptotic proteins. Upon an internal apoptotic signal, alterations in mitochondrial membrane occur, such as loss of trans-membrane potential and mitochondrial outer membrane permeabilization (MOMP) (Elmore, 2007). Then, cytochrome c, Smac (Second Mitochondria-derived Activator)/DIABLO complex, and the serine protease HtrA2/Omi are released from mitochondria. Cytochrome-c binds and activates Apaf-1 and procaspase-9, forming *"apoptosome"*. Smac/DIABLO and HtrA2/Omi hinder inhibitors of apoptosis protein (IAPs) by competing with caspase-9 to bind them (van Loo et al., 2002).
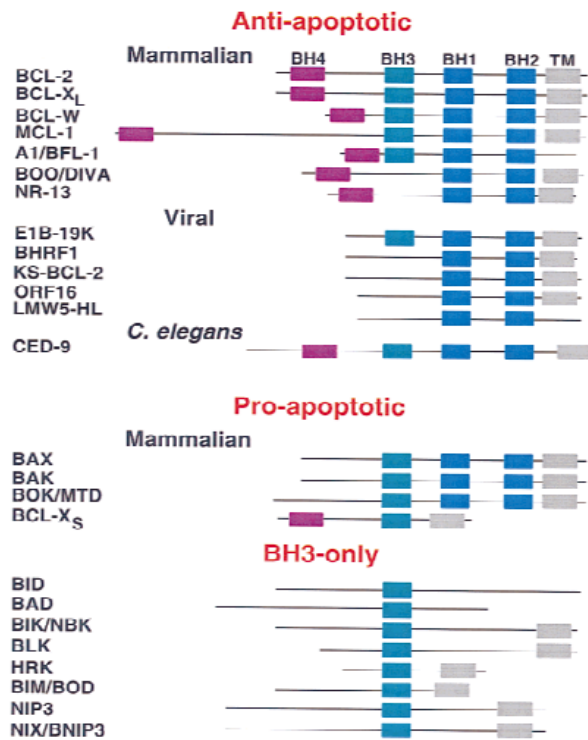
Figure 1.4 Members of Bcl-2 family proteins. Retrieved from Gross et. al., 1999.

As mentioned earlier evasion from apoptosis is one of the hallmarks of cancer. Expression levels of many apoptotic genes change in cancer cells. Expressions of many death receptors are downregulated in different types of cancer. For example, one of death receptors; CD95 expression is significantly decreased in resistant types of leukemia suggesting the role of death receptor in drug resistance. Increased expression of *Bcl-2* gene is common characteristics in lymphoma and leukemia. Chromosomal translocation of Bcl-2 gene leads to overexpression of this gene. Pro-apoptotic gene, *bax* expression is downregulated in hematological malignancies and many solid tumors (Simone Fulda, 2009). *Survivin*, which is an anti-apoptotic gene that inhibits the caspase-9, is overexpressed in many cancers such as, gastric, breast, colorectal and hematologic malignancies (Mita, Mita, Nawrocki, & Giles, 2008). P-53 upregulated modulator of apoptosis (PUMA) and Noxa are members of pro-apoptotic Bcl-2 family. Both genes are induced by tumor suppressor p53 gene. Expression profiles of these two genes also are found dysregulated in many hematologic malignancies.

1.6 Never in Mitosis A (NIMA) Related Kinases (Neks)

Dysregulation in cell cycle is one of the hallmarks of cancer as mentioned earlier. Cyclin dependent kinases (CDKs), Aura and Polo kinase families are important regulator of cell cycle. Recent studies showed that Nima related kinase family proteins also have important roles in cell cycle progression. Never-in-mitosis A (NIMA) protein of *Aspergillus nidulans* is the founding member of NEK family. Early studies showed that NIMA is important in chromatin condensation, assembly of mitotic spindle, cytokinesis and most importantly NIMA degradation is necessary for mitotic exit in yeast. NIMA consists of three important domains which are N-terminal catalytic domain that shares same motifs with other serine-threonine kinases, coiled-coiled domains which are essential for oligomerization of kinase and PEST-sequence that have role in ubiquitin dependent proteolysis (Moniz, Dutt, Haider, & Stambolic, 2011). There are eleven genes that encode Nek, Nek1 to Nek11, in human. Figure 1.5 shows the structural characteristics of all Neks.
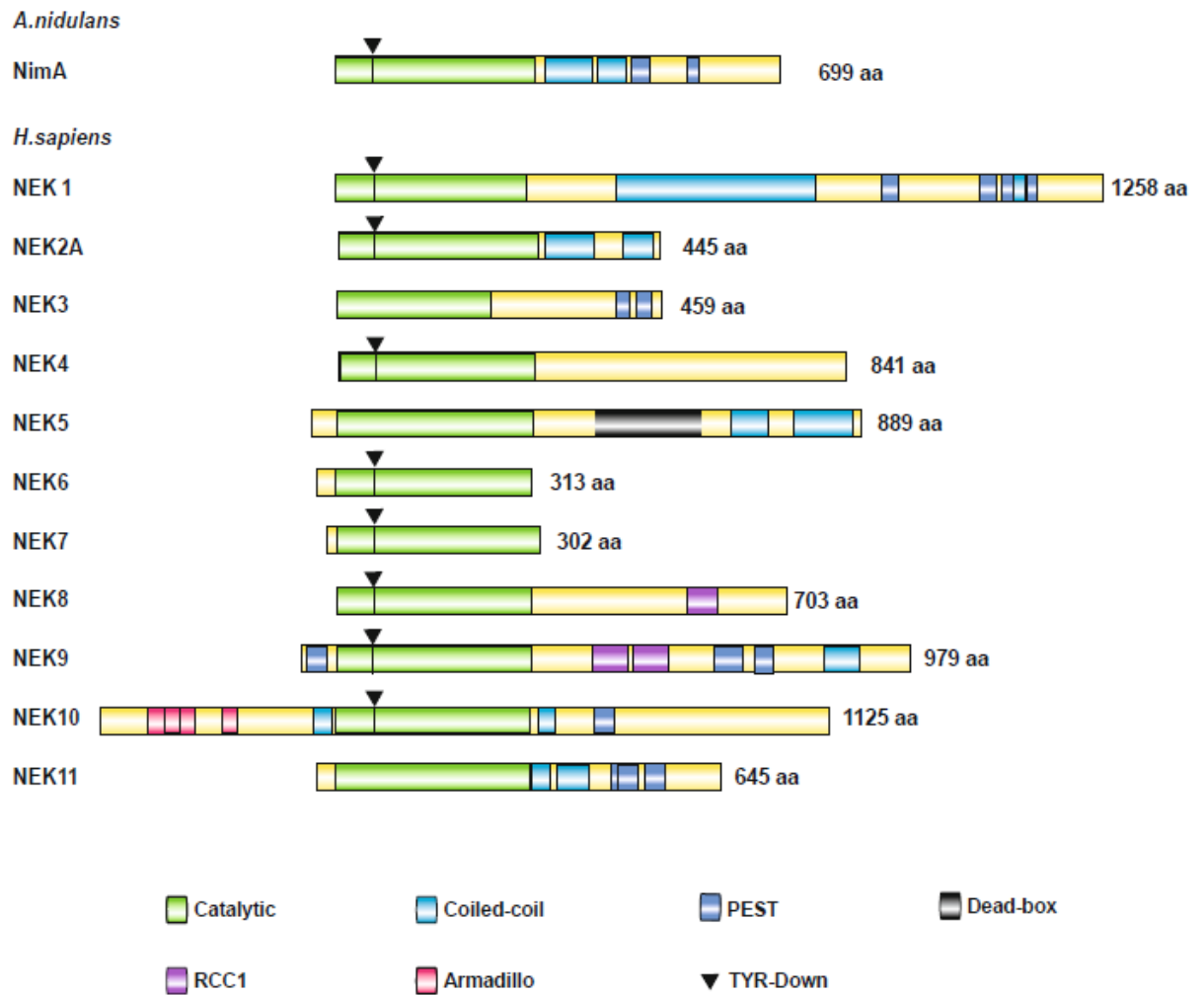
Figure 1.5 Structural characteristics of Nek family. Retrieved from Moniz et. al., 2011.

As mentioned earlier Nek family kinases have several roles in cell cycle progression. Nek2 is required for proper chromosome separation. Studies showed that Nek2 inhibition causes failure in chromosome separation and spindle assembly (Fry, Meraldi, & Nigg, 1998; Maruvada, Dmitrieva, East-Palmer, & Yen, 2004). Nek2 is also important for maturation of chromosome and disjunction. Nek1 has a role in the repair of DNA strand breaks in response to ultraviolet light and ionizing radiation at G1 to S and G2 to M transitions. Microtubule deacetylation is regulated by Nek3 in neurons (Moniz et al., 2011). Nek10 and Nek11 are important for DNA damage response checkpoint in G2 to M transition. Nek6, Nek7, and Nek9 have roles in appropriate spindle pole formation. Belham et al. showed that Nek9 phosphorylates

Nek6 and Nek7 during mitosis and depletion of Nek6 and Nek7 leads to mitotic arrest in prometaphase to metaphase and apoptosis (Belham et al., 2003; O'Regan & Fry, 2009).

Nek2, Nek6, Nek8 and Nek10 expression level is increased in different types of cancer. Elevated Nek2 level in breast and colorectal cancers, mutations in Nek10 gene in ovarian, lung and brain cancers, overexpression of Nek8 in primary breast tumors show the possible role of Nek family in cancer progression (Moniz et al., 2011).

1.6.1 Nek6 and Cancer

Nek6 protein comprises only catalytic domain with a short N-terminus unlikely other members of Nek family proteins. It is composed of roughly 34% a-helices, 12% β-strands and 54% coils (Meirelles et al., 2011).
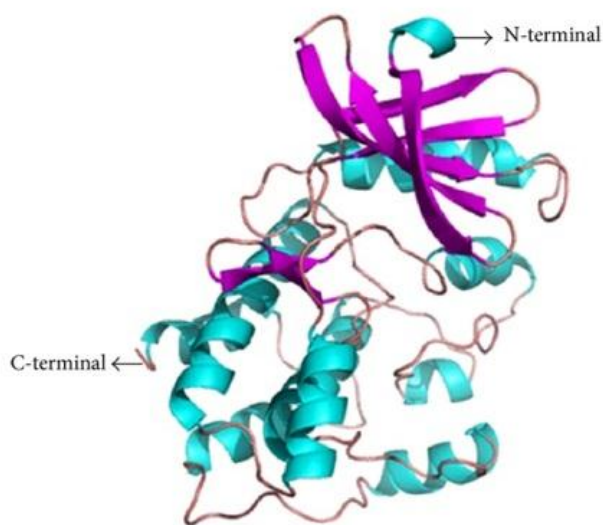
Figure 1.6 Three dimensional homology model for Nek6 protein. Retrieved from Srinivasan *et. al.,* 2014.

Nek6 is essential mitotic kinase for proper cell cycle progression. After G2-M phase transition its activity becomes important and during M phases Nek6 localizes at spindle microtubules and its activity increases. Studies showed that Nek6 depletion results in mitotic arrest at metaphase, demonstrating that Nek6 is essential for

metaphase to anaphase transition (Yin, Shao, Voehringer, Smeal, & Jallal, 2003). Figure 1.7 shows the involvement of Nek6 in cell cycle progression. After Nek9 phosphorylates Nek6 and Nek7, they phosphorylate kinesin and some microtubule associated proteins which are important for microtubule dynamics. Depletion of either of these kinases results fragile spindles or mitotic arrest (Fry, O'Regan, Sabir, & Bayliss, 2012). Moreover, depletion of Nek6 and Nek7 leads to spindle assembly checkpoint (SAC) activation and inhibits metaphase to anaphase transition (Moniz et al., 2011). Overexpression of kinase dead domain Nek6 results in M phase arrest and subsequently apoptosis (Yin et al., 2003). During cytokinesis Nek6 localizes at midbody and Nek6 depletion studies showed that when SAC inhibitor is used in order to inhibit checkpoint, cells delayed in late mitosis implying role of Nek6 also in cytokinesis (O'Regan & Fry, 2009).
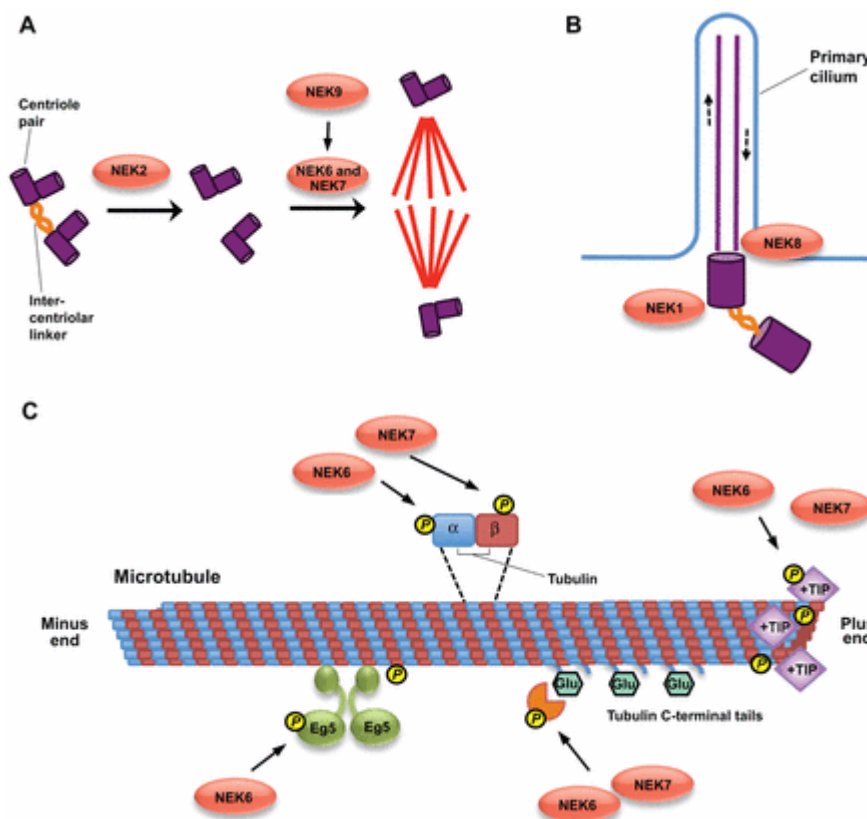
Figure 1.7 The roles of Nek6 in cell cycle progression. Retrieved from Fry et. al., 2012.

The Nek6 gene is situated on chromosome 9q33-34, which is a locus associated with various human cancers, such as neuroblastoma, bladder cancer, and renal cell carcinoma (Jee et al., 2010). Tissue microarray studies show that Nek6 is overexpressed in breast, colorectal, lung , and laryngeal cancers (Capra et al., 2006). Nassirpour et al, showed that Nek6 gene transcript, protein level and kinase activity is significantly upregulated in colon, lung, kidney, and cervix cancers (Nassirpour et al., 2010). Furthermore, Nek6 is also important in anchorage-independent growth in breast (MDA-MB-231) and cervical (HeLa) cancers. Elevated level of Nek6 kinase activity causes cancer cell transformation and Nek6 depletion results inhibition of tumor growth *in vivo* in HeLa cells. Additionally, Nek6 knockdown induce apoptosis in these cancer cell lines (Nassirpour et al., 2010). Nek6 expression decreases in premature and replicative senescence. Overexpression of Nek6 gene inhibits p-53 induced premature senescence in a variety of cancer cells, and its kinase activity is essential for this inhibition implying non-mitotic role of Nek6 (Jee et al., 2010).

Aim of Study

Nek6 gene is significantly upregulated in many types of cancer. Nek6 is essential for metaphase to anaphase transition, and its depletion causes mitotic arrest. Moreover, the inhibition of endogenous Nek6 gene or exogenous overexpression of kinase-dead domain form of Nek6 gene causes repression of cancer cell transformation, and induction of apoptosis. Multidrug resistance is correlated with impairment in apoptotic pathway. However, the role of Nek6 gene in multidrug resistance is still unknown. The aim of the current project is to investigate the role of Nek6 in drug resistance and apoptosis in CML cell lines. Our principal goals are:

- To screen expression level of Nek6 in drug sensitive and drug resistant forms of breast and chronic myeloid leukemia cancer cell lines.
- To determine the role of Nek6 in MDR by screening expression levels of some important genes involved in apoptotic pathway after silencing Nek6 with siRNA.
- To investigate the effect of transient Nek6 gene silencing in apoptosis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell lines and culture conditions

MCF-7 cell line was purchased from ŞAP Institute, Ankara. 1000 nM doxorubicin resistant (MCF-7/Dox), 120 nM docataxel resistant (MCF-7/Doc), and 8000 nM zoledronic acid resistant (MCF-7/Zol) MCF-7 variants were previously developed in our laboratory (Kars *et.al.*, 2006; Kars *et.al.,* 2007). Parental Ph+ K-562 (K562/S) cell line was purchased from German Collection of Microorganisms and Cell Culture, Germany. 1000 nM doxorubicin (K-562/Dox) and 1000 nM imatinib (K-562/Ima) K-562 variants were previously developed in our lab.

All cell lines were cultured in RPMI 1640 medium (Thermo Scientific, USA) with 10% fetal bovine serum (FBS) (Biochrome, Germany) and 0.1% gentamycin (Biological Industries, Israel). Drug resistant sublines were grown with their reported drug concentrations. The cells were incubated in a Heraeus incubator (Hanau, Germany) at 37°C, in the presence of humidified atmosphere with 5% CO_2.

2.1.1 Maintanence of MCF-7 and K-562 Cell Culture

All cell types are subcultured at various ratios when they reached 80% confluence. For MCF-7 sensitive and resistant subtypes, the medium was removed and the surface of flask to which cells adhered was washed with 4 ml phosphate buffered saline (pH 7.2). In order to detach cells from surface 0.25% Trypsin-EDTA solution (Biological Industries, Israel) was added and incubated for five minutes at 37°C. After incubation, 3 ml RPMI 1640 medium was added into flask and cells were homogenized by pipetting. Then, cell suspension was taken into 15 ml falcon (Greiner), and was centrifuged at 1000 rpm for 5 minutes. Finally, the supernatant is removed and cells were resuspended in appropriate fresh medium and required amount was taken into flask with total volume of 12 ml. For resistant subtypes proper

amount of drug was added into flask to achieve previously indicated drug concentrations.

For K562 sensitive and resistant cell lines, the medium was taken into 15 ml falcon and was centrifuged at 1000 rpm for 5 min. Then, the supernatant was discharged and cells were washed with 4 ml PBS. Cells in PBS were centrifuged one more time at 1000 rpm for 5 min. Finally, PBS is discharged and cells were resuspended in appropriate fresh medium. Required amount was taken into flask and fresh medium was added to get total 12 ml volume. For resistant subtypes appropriate amount of drug was added into flask to get previously indicated drug concentrations.

2.1.2 Freezing and thawing cells

Cells were frozen regularly if the need arises for further studies. For this purpose, MCF-7 cells were washed with PBS, trypsinized and resuspended with 3 ml fresh medium. Then, cell suspension was taken into 15 ml falcon tubes and centrifuged at 1000 rpm for 5 min. After centrifuge step, the supernatant was discharged and cells were suspended in 1 mL of cold freezing medium prepared by mixing 90% complete growth medium with 10% DMSO (Sigma-Aldrich, USA). Finally, cells were taken into cryovials. K562 cells were taken into 15 ml falcon and were centrifuged at 1000 rpm for 5 min. Supernatant was removed and cells were washed in 4 ml PBS and centrifuged again. Then, supernatant was discharged and cells were suspended in 1 ml cold freezing medium. Finally, this cell suspension was taken into cryovials. The cells in cryogenic vials were kept in -20 C for overnight. Finally, they were stored at liquid nitrogen, -196º C.

For thawing process, the cryogenic vials were incubated at water bath at 37°C in order to get the highest amount of viable cells. Then, suspension was transferred into 15 ml falcon and centrifuged at 1000 rpm for 5 min. After the supernatant was removed, cells were resuspended in 3 ml growth medium and cultured in flask with total volume of 12 ml.

2.1.3 Cell counting

Viable cell count was implemented by using hemacytometer and tyrphan blue (Biological Industries, Israel). Tryphan blue is dye which could not enter into viable cells due to their lipid bilayer in their membrane. Because the membranes of dead cells are corrupted, the dye easily enters into dead cells and cause to be seen as blue. For counting process, 90 µl cell suspension was mixed with 10 µl tryphan blue in microfuge tube. Meanwhile, coverslip was cling on hemacytometer. Next, 10 µl of stained cell suspension was placed the spaces between chamber and coverslip. There are 16 squares were composed of 256 small squares in cytometer. Under the light microscope the viable cells, not stained as blue, were counted in these 16 squares and total cell number in 1 ml was calculated according to formula below:

$$\frac{\text{Cell number}}{\text{mL}} = \frac{\text{Number of cells counted on 16 squares}}{256} \times 4 \times 10^6$$

In order to analyze cell viability of siRNA treated K-562 cells by tryphan exclusion test, $3\text{x}10^4$ cells were seeded on 6-well plates with a total volume 1000 mL. Cells were remained untreated or treated with 50 nM either mock or Nek6 siRNA. After 48 and 72 hours incubation, cells were stained with tryphan blue and counted live cells as mentioned earlier.

2.2 Gene Expression Analysis

2.2.1 RNA Isolation

Total RNA isolation was performed by using TriPure (Roche, Germany). Pipette tips and microfuge tubes were treated with DEPC before the experiment. The first step of RNA isolation is to harvest cells from culture flasks. MCF-7 cells were trypsinized, taken into 15 ml falcon and centrifuged at 1000 rpm for 5 min. Then, they were washed with 4 ml PBS and centrifuged again. After discharging PBS, cells were resuspended in 1 ml TriPure reagent and mixed by pipetting to homogenize and then, transferred into 2 ml microfuge tubes. K562 cells were taken into 15 ml falcon and

centrifuged at 1000 rpm for 5 min. After supernatant was removed, cells were washed with 4 ml PBS and centrifuged again. Then, PBS was discharged and cells were resuspended in 1 ml TriPure reagent, mixed by pipetting and were taken into 2 ml microfuge tubes.

The cells in Tri-Pure were incubated on ice for 5 min. Next, 0.2 ml chloroform (Sigma Aldrich, Germany) was added into tubes, mixed by inversion for 30 seconds and incubated on ice for 5 minutes. Tubes were centrifuged at 12000 g for 15 min at 4 C. Aqueous phase which was at the top, was transferred into 1.5 ml microfuge tubes and 0.5 ml ice-cold isopropanol was added. After mixing the aqueous phase with isopropanol by pipetting, tubes were incubated at room temperature for 5 minutes. Then, they were centrifuged at 12000 g for 10 minutes at 4º C. Supernatant was removed; pellet was washed with 1 ml 75% ethanol and centrifuged at 12000 g for 15 minutes at 4ºC. After removing supernatant pellets were placed for air-drying for 10-15 minutes. When air-drying step finished, pellet was resuspended with proper amount of nuclease-free water (HyClone, USA) and incubated at 55º C for 10 minutes in order to break secondary structures. Isolated RNAs were kept at -80ºC.

2.2.2 Quantitation of RNA

NanoDrop Spectrometer (Thermo Scientific, USA) was used in order to obtain purity and quantity of RNA. A260/280 ratio of Nanodrop shows whether any protein contamination exists or not. Higher than 1.8 means there is no or little DNA contamination, in turn, RNA can be used for further analysis. A260/230 ratio indicates organic solvent contamination if the value is not near 2.0.

RNA integrity was controled by agarose gel electrophoresis. 1% agarose gel was prepared by adding 0.5 g agarose powder in 50 ml TAE. To dissolve powder the mixture was heated for 5 minutes in microwave. Agarose solution was cooled and stained with 0.5 μg/mL ethidium bromide (Sigma Aldrich, Germany). Then, solution was poured on tray, placed with comb and wait for cooling. After gel freeze, comb was removed. RNA solution was mixed with 2X RNA loading buffer (Thermo Scientific, USA) for 1:1 ratio. Then, the mixture of RNA and RNA loading dye was loaded on agarose gel and run at 75-80 V for 55-60 minutes. Image was taken in Vilber Lourmat UV imager. The expected RNA bands are 28S rRNA, 18S rRNA, and 5S rRNA. If RNA is degraded, the smear image between 28S and 18S rRNA

bands is observed. If there is a band below the wells, it indicates genomic DNA contamination.

2.2.3 DNase I Treatment

DNase I treatment is performed in order to prevent amplification of genomic DNA during gene expression analysis. Prior to cDNA synthesis, RNA solutions were treated with DNA removal kit (Thermo Scientific, USA) to get rid of any gDNA. Initially, 2 μg RNA was mixed with 2 μL 10X DNase buffer and 2 μl DNase I. Total volume was completed to 20 mL with nuclease free water. The mixture was incubated at 37°C for 30 minutes. Then, 2 μl EDTA was added into solution and the mixture was incubated at 65 C for 10 minutes.

2.2.4 cDNA Synthesis

After DNase I treatment, cDNA was synthesized by using 2μg RNA by using RevertAid reverse transcriptase (Thermo Scientific, USA). The mixture was mixed with 2 μl Random Hexamer and 1 μl nuclease free water. The solution was incubated at 65ºC for 5 minutes. Then, master mix which contains 8 μl reverse transcriptase buffer, 4 μl 10 mM dNTPs, 40U/mL 1 μl Riboblock RNase inhibitor and 2 μl RevertAid reverse transcriptase was prepared. 15 μl of master mix was added into each tube and they were incubated at 25°C for 10 minutes, 42ºC for 1 hour and 70ºC for 10 minutes. Finally cDNAs were diluted 1:5 ratio and stored at − 20 º C.

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

Expression analysis of Nek6 gene was performed by TaqMan Gene Expression Assay (Roche, Germany). β-actin gene was used as internal control. The master mix was prepared by using 2x LightCycler 480 Probe Master, 20x RealTime Ready Assay and nuclease free water. The reaction was performed on LightCycler 480 instrument in METU Central Laboratory. The amount of reaction components and PCR conditions were shown in Table 2.1.

Table 2.1 The amounts of reaction components and PCR conditions for Nek6 Gene.

Component	Concentration	Volume	Final Concentration
LightCycler 480 Probes Master	2X	10 μL	1X
Real Time Ready Assay	20X	1 μL	Primers 8 pmol each, 4 pmol UPL probe
PCR grade water	--	4 μL	--
Total Volume		15 μL	--

Pre-incubation	95°C	10 min	1 cycle
Amplification	95°C	10 sec	
	60°C	30 sec	45 cycles
	72°C	1 sec	
Cooling	40°C	30 sec	1 cycle

Expression analysis of apoptotic genes were performed by qRT-PCR experiments. The master mix was prepared by adding 10 mL of 2x FastStart SYBR Green Master Mix (Roche, Germany) which contains reaction buffer, hot-start *Taq* polymerase, dNTP mix, $MgCl_2$ and SYBR Green, preestablished amount of forward and reverse primers and nuclease free water which makes total volume to 20 μl. The reactions were performed on Rotor Gene Q real-time thermal cycler (Qiagen, Germany). The amounts of reaction components and PCR conditions were shown in Table 2.2.

Table 2.2 The amounts of reaction components and PCR conditions for apoptotic genes.

	Bax	Bcl-2	Survivin	β-actin
2x FastStart SYBR Green Master Mix	10 µl	10 µl	10 µl	10 µl
Forward Primer 10 µM	0.2 µl	0.2 µl	0.2 µl	0.2 µl
Reverse Primer 10 µM	0.2 µl	0.2 µl	0.2 µl	0.2 µl
Nuclease-free water	5.6 µl	5.6 µl	5.6 µl	5.6 µl

	Bax	Bcl-2	Survivin	β-actin
Initial Denaturation	95˚C, 10 min	95˚C, 10 min	95˚C,10 min	95˚C, 10 min
Denaturation	95 ˚C, 15 sec	95 ˚C, 15 sec	95 ˚C, 15 sec	95 ˚C, 15 sec
Annealing	60 ˚C, 60 sec	60 ˚C, 60 sec	60 ˚C, 60 sec	60 ˚C, 45 sec
Extension				72 ˚C, 45 sec
Cycle Number	35	40	35	30

Table 2.3 List of primers

	Sequence 5'-3'	Amplicon Size (bp)	Reference
Bax	TCTGACGGCAACTTCAACTG TTGAGGAGTCTCACCCAACC	188	İseri, 2009
Bcl-2	CCCGCGACTCCTGATTCATT AGTCTACTTCCTCTGTGATGTTGT	166	
Survivin	AGCCAGATGACGACCCCATAGAGG AAAGGAAAGCGCAACCGGACGA	60	
β-actin	CCAACCGCGAGAAGATGA CCAGAGGCGTACAGGGATAG	97	Kaplan, 2012

2.2.6 Quantification of qRT-PCR data

qRT-PCR data was quantitated according to delta delta Ct ($2^{\Delta\Delta Ct}$) method (Livak & Schmittgen, 2001). The treated and untreated groups were normalized to the internal control, beta actin gene.

$$\Delta\Delta C_t = \Delta C_{t"Treated"} - \Delta C_{t"Untreated"}$$

$$\Delta C_{t"Treated"} = (C_{tT})_{Treated} - (C_{ti})_{Treated}$$

$$\Delta C_{t"Untreated"} = (C_{tT})_{Untreated} - (C_{ti})_{Untreated}$$

The fold change in gene expression was calculated with the formula $2^{-\Delta\Delta Ct}$.

2.3 siRNA Transfection

Small interfering RNA targeting specifically the Nek6 encoding mRNA was purchased from Qiagen, Germany and used to transiently silence *Nek6* gene. siRNA

that did not have a target in human transcriptome was used as control (Santa Cruz, USA). siRNA delivery was performed by HiPerfect Transfection Reagent (Qiagen, Germany).

2.3.1 Optimization of Transfection Efficiency

In order to optimize required siRNA amount and transfection time, 10 μM Alexa Fluor® 488 conjugated negative siRNA (Qiagen, Germany) was used. 2.0 x 10^5 cells were seeded in 2300 μl into each well of 6-well plates before transfection. Various amounts of siRNAs were prepared to reach final concentrations of 1nM, 2nM, 5nM, 10nM, 20nM, 50 nM, 100 nM and 200 nM by diluting them in serum-free culture medium. The solution was mixed with 12 μl HiPerfect transfection reagent (Qiagen, Germany). In order to allow the formation of tranfection complex, prepared solutions were vortexed and incubated at room temperature for 15 minutes. Then, 100 μl of siRNA transfection mix was added into each well by dropwise. Cells were incubated at 37 ℃ in the presence of humidified atmosphere with 5% CO_2. After 24 hours of transfection, cells were harvested, washed in 2 ml PBS and resuspended in 1 ml PBS. They were analyzed by using flow cytomety (Accuri, BD, USA) at FL-1 channel (for green fluorescence).

2.3.2 Transfection at 6-well plates and validation of knockdown

K-562 sensitive cells were harvested and 3.0 x 10^5 cells were seeded in 1100 ml medium in 6-well plates. Control and Nek6 siRNAs were diluted with serum-free medium to get 50 nM final concentrations in separate eppendorf tubes. 6 μl transfection reagents were added in each eppendorf tubes and the complexes were vortexed and incubated for 15 minutes. 100 μl either control or Nek6 siRNA complex was added onto cells. One well was remained as untransfected. Cells were incubated for 48 and 72 hours at 37 ℃ in the presence of humidified atmosphere with 5% CO_2. At specific time points, cells were harvested, washed with PBS and lysed in TriPure reagent for RNA isolation.

2.3.3   Transfection at 96-well plate and XTT

50 nM siRNA was mixed with 0.75 µl HiPerfect transfection reagent and total volume completed to 25 µl with serum free medium. The mixture remained incubation for 15 minutes at room temperature. Meanwhile, highest drug concentration of 1000 nM imatinib mixed with 150 µl medium and applied on third column of plate while other columns contain 150 µl medium. Then, 100 µl of highest drug containing well removed and subsequently added into next column. Serial dilution (2/3) was completed in this way until twelfth column. Cells were harvested and washed with PBS. After cell counting, $3x10^4$ cells in 25 µl were seeded in each well from second column. First column remained for medium control. Next, 25 µl of siRNA mixture was added each well beginning from third column. After 72 hours, 5 ml XTT reagent was mixed with 0.1 ml activator reagent and 50 µl of mixture was added into each well. XTT is a tetrazolium salt which converted to formazan crystals that are soluable by mitochondrial enzymes of alive cells. The more intensity represent higher viability. After 4 hour incubation at cell culture incubator, absorbance analysis was performed at 492 nm by 96-well plate reader.

2.4   Determination of Apoptosis by Annexin V/PI Staining

K-562 sensitive cells were harvested and 3.0×10^5 cells were seeded in 1100 ml medium in 6-well plates. For negative control, cells in first plate left untreated. For positive control, cells were treated with 4 µl etoposide. For siRNA treatment, one plate was treated with transfection reagent only. The second and third plates were treated with mock and Nek6 specific siRNA, respectively.

In order to determine apoptotic status of cells Annexin-V-FLUOS Staining Kit (Roche, Germany) was used. The kit contains three compounds; incubation buffer (HEPES), propidium iodide (PI) and Annexin V solutions.

Annexins are a family of calcium-dependent phospholipid-binding proteins. In viable cells, phosphatidylserine (PS) is located on the cytoplasmic side of the cell membrane. When cells undergo apoptosis, phosphatidylserine loses its asymmetric dispersion in lipid bilayer and translocates from inner part of plasma membrane to extracellular membrane. Therefore, PS residues are exposed to external cellular

environment and detected by fluorescently labeled Annexin V protein that has high affinity for PS. Propidium iodide (PI) is not able to pass through viable cell membrane so the dye is used to detect dead cells. It binds to double stranded DNA by intercalating between basepairs with little or no sequence predilection. Unlike Annexin V, PI stains only late apoptotic cells because membrane of early apoptotic cells excludes PI. Therefore cells stained with only Annexin V show early apoptotic cells; on the other hand cells stained with Annexin V/PI are late apoptotic cells. Annexin V is excited at 488 nm and has a peak emission at 518 nm and PI can be excited at 488-540 nm and its emission wavelength is at 617 nm.

After 48 hour treatment, cells were harvested and washed with PBS twice. Then, cells were resuspended in 400 μl of PBS and transferred into eppendorf tubes. 100 μl incubation buffer (HEPES) was added into eppendorfs and 2 μl Annexin or/both 2 μl PI was added each setups. Cells were analyzed with Acuri Flow Cytometer in FL-1 and FL-3 channels.

2.5   Statistical analysis

Results obtained from two experiments were analyzed by GraphPad Prism Version 5 with one-way ANOVA followed by Tukey's Test as $p < 0.05$.

CHAPTER 3

RESULTS AND DISCUSSION

3.1   Total RNA Isolation

Total RNA was isolated by using TriPure. RNA quality was determined by agarose gel electrophoresis in order to see whether it can be used for cDNA synthesis. According to Figure 3.1, the main RNA bands; 28S, 18S and 5S rRNA, were observed successfully on gel results. The intensity ratio of 28S rRNA band to 18S rRNA band should be approximately 2 for high quality of RNA. The band at the top of gel represents genomic DNA. For this reason, DNase treatment was applied before Cdna synthesis.
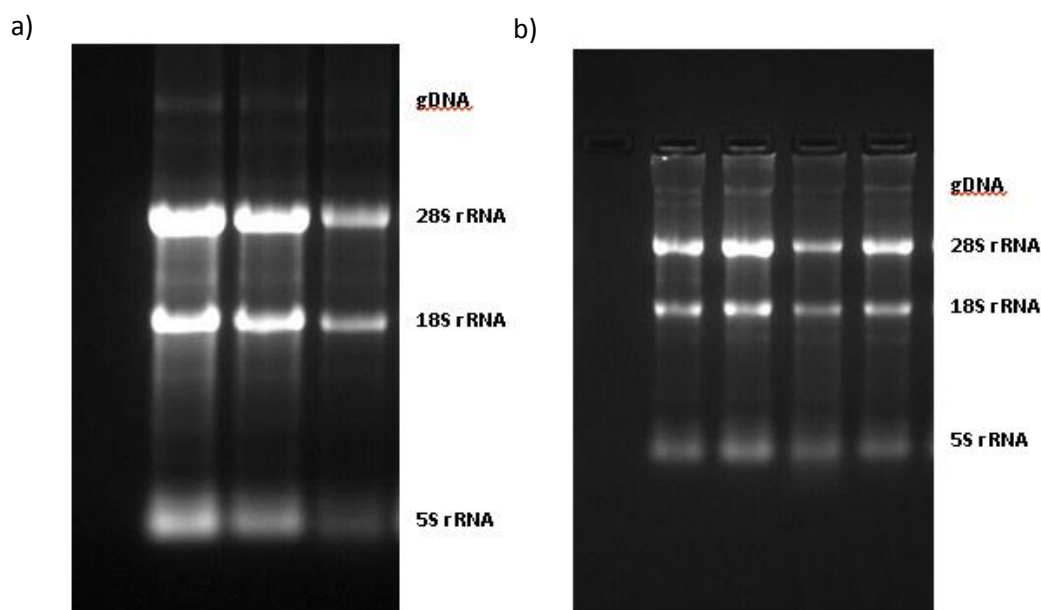

Figure 3.1 RNA gel image (1% agarose) showing main RNA bands. a) K-562 sensitive, doxorubicin, and imatinib resistant subtypes, b) MCF-7 sensitive and docataxel, doxorubicin and zoledronicacid resistant subtypes.

The quantity and purity of RNA was determined by spectrophotometric analysis. Absorbance wavelengths at 280 nm, 260 nm, and 230 nm were measured. 280/260

ratio and 260/230 ratio are important for RNA purity. A260/280 ratio of Nanodrop shows whether any protein contamination exists or not. A value higher than 1.8 means there is little or no DNA or protein contamination. A260/230 ratio indicates organic solvent contamination if the value is different from 2.0. Table 3.1 shows the concentration and both 280/260 and 260/230 ratios of isolated RNAs. According to results, RNA isolates could be used for further analysis.

Table 3.1 The concentration and absorbance results of isolated RNAs.

Cell Line	N.A Conc.	Unit	260/280	260/230
K-562	2389.9	ng/µl	1.98	1.75
K-562/Dox	2153.1	ng/µl	1.96	1.63
K-562/Ima	690.3	ng/µl	2.06	1.91
MCF-7	1505.8	ng/µl	1.9	1.81
MCF-7/Dox	1824.1	ng/µl	1.98	1.72
MCF-7/Doc	2446.8	ng/µl	2.0	1.57
MCF-7/Zol	1976.3	ng/µl	1.87	1.54

3.2 Nek6 Gene Expression Profiles in MCF-7 and K-562 Cell Lines and Drug Resistant Subtypes

According to literature, Nek6 mRNA and protein level and its kinase activity are significantly increased in breast, colon uterus, stomach, rectum, ovary, lung, kidney, pancreas, thyroid, cervix, and prostate cancers. Moreover, cancer cell transformation is promoted when Nek6 activity is increased (Nassirpour et al., 2010). However, the expression pattern of Nek6 gene in drug resistant subtypes has been unknown.

The expression profiles of Nek6 gene in MCF-7 and K-562 cell lines and their drug resistant subtypes were examined. According to results represented in Figure 3.2, Nek6 gene expression is significantly decreased in doxorubicin and imatinib resistant K-562 cell lines compared to sensitive one. On the other hand, there is no significant difference in Nek6 gene expression level between sensitive MCF-7 and its resistant subtypes.
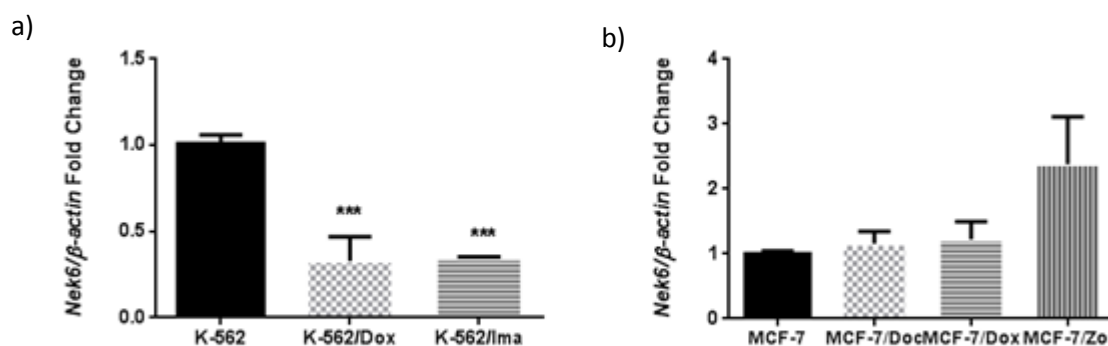

Figure 3.2 a) Expression levels of *Nek6* gene in K-562 and its resistant subtypes, b) Expression levels of *Nek6* gene in MCF-7 and its resistant subtypes, when $p < 0.05$ Experiment was performed twice in triplicate.

Figure 3.3 shows cycling profile of *Nek6* gene in K-562 and MCF-7 cells. Amplification curves are exponential representing each amplicon is ideal.
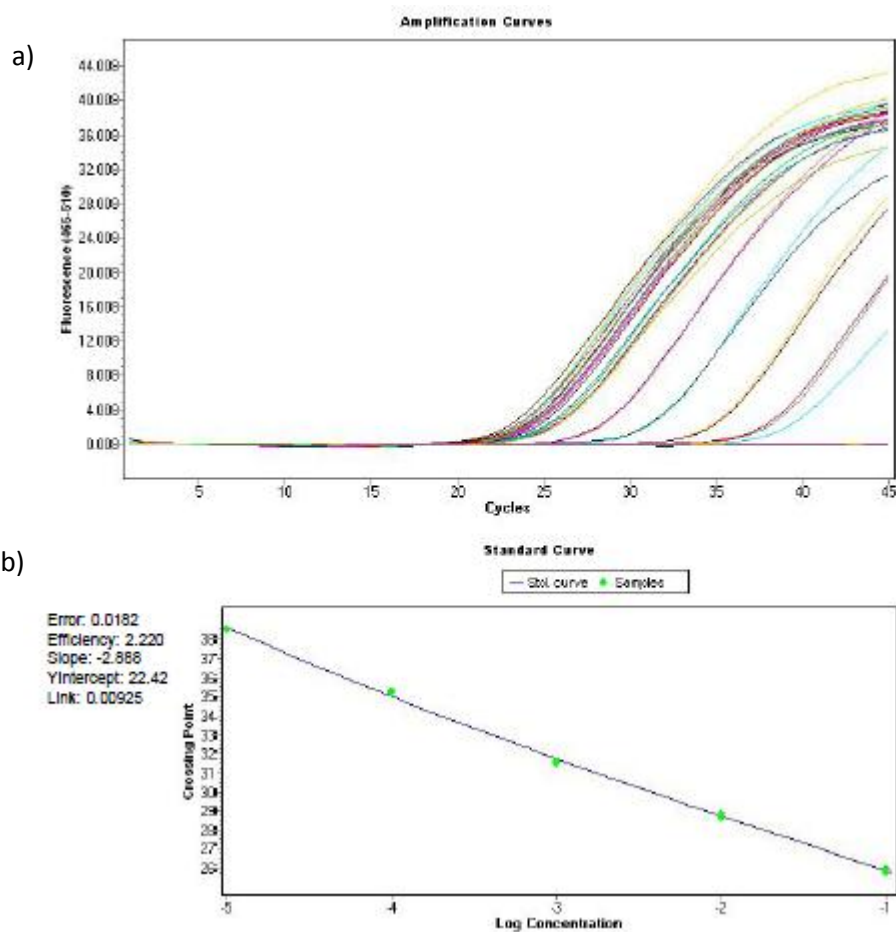
Figure 3.3 a) Cycling profile, and b) standard curve for *Nek6* amplicon.

Impairment in apoptotic pathways is one of the main reasons for MDR development. As mentioned earlier, some pro-apoptotic genes, such as *Bax* and *Bak*, are down regulated and the expression levels of some anti-apoptotic genes, such as *Bcl-2* and *Survivin*, are increased in various types of hematological malignancies (Kaufmann & Vaux, 2003). Furthermore, alterations in apoptotic gene expression profile is one of the underlying causes in resistance against imatinib (Trela et al., 2014). Moreover, previous studies show that depletion on Nek6 triggers apoptosis in many types of cancer (Nassirpour et al., 2010). Surprisingly, the attenuated expression of Nek6 gene in resistant K-562 subtypes may imply a role of Nek6 in drug resistance by modulating apoptotic pathway. Because there is no significant change between MCF-

7 and its resistant subtypes, further experiments were performed with K-562 cell lines.

3.3 Apoptotic Gene Expression Levels in K-562 Cell lines

Screening apoptotic gene expression levels in sensitive K-562 and its resistant subtypes were performed to see potential role of Nek6 in MDR. As mentioned earlier, impairments in apoptotic gene regulation is one of the major reason for MDR development. In order to overcome programmed cell death, the expression profile of many apoptotic genes change while gaining resistance to chemotherapeutic drugs. Aberrant expressions of inhibitor of apoptosis proteins, anti-apoptotic, pro-apoptotic proteins account for drug resistance in tumors (Simone Fulda, 2009). In CML, two mechanisms contribute to drug resistance: BCR/ABL dependent and BCR/ABL independent mechanisms. In the BCR/ABL dependent mechanism, point mutations in Bcr-abl fusion gene causes failure in imatinib tyrosine kinase interaction by changing conformation of the kinase. The deterioration in kinase drug interaction causes decreased efficacy of imatinib (Nestal de Moraes et al., 2012). BCR/ABL also leads to drug resistance by activating PI3K/Akt signaling pathway which in turn resulting degradation or inactivation of pro-apoptotic genes. Moreover, activation of Akt by BCR/ABL hinders mitochondrial apoptotic pathway in different ways, such as over expression of Bcl-2 or Bcl-x_L by phosphorylation of STAT5. BCR/ABL independent mechanisms for imatinib resistance are quite complex. Transcriptional regulation in DNA repair, drug metabolism, drug transporters and apoptotic genes are the basic reason for resistance to occur (Danisz & Blasiak, 2013). Studies show that imatinib resistance in CML cells arises from over expression of anti-apoptotic Bcl-2 family proteins and inhibitor of apoptotic proteins (IAPs) (Trela et al., 2014). Parallel with these findings, the apoptotic genes expression profiles were examined in sensitive and resistant CML cell lines in the study.

As shown in Figure 3.4, the expression level of *Bax* is significantly decreased in imatinib resistant cells. Whereas, *Survivin* expression is increased in same resistant subtype. The increase in anti-apoptotic gene expression levels and reduced expression of pro-apoptotic gene is expected because cancer cells can become resistant to drugs, by escaping apoptosis for survival. Our findings are as expected

that demonstrate several alterations in apoptosis signaling pathway. Decreased expression level of *Survivin* in doxorubicin resistant K-562 cell line could be clarified by the fact that apoptotic pathway is a complex process which many proteins take part in. Moreover, in one of studies showed that Survivin gene expression level did not significantly change in vincristine resistant K-562 cells. However, Survivin locates in the cytoplasm instead of translocating to the nucleus (Rumjanek, Vidal, & Maia, 2013). Hence, stability and location of Survivin has an effect on apoptosis and also other inhibitor apoptotic proteins may compensate the attenuated level of Survivin. Surprisingly, *Bcl-2* expression level is significantly decreased in both doxorubicin and imatinib resistant cell types. This unexpected result could be explained by BCR-ABL dependent drug resistance in CML. Overexpression of drug efflux protein such as P-gp, decreased expression of drug influx pump for imatinib which is OCT1 (Organic cation transporters) or amplification and overexpression of BCR/ABL also contribute resistance (Nestal de Moraes et al., 2012). Moreover, STAT5 activation by BCR/ABL leads to increased expression of either Bcl-2 or Bcl-$_{xl}$ implying that high level of Bcl-x_L could compensate the decreased expression of Bcl-2 to maintain resistance. Besides, previous studies show that Bax is upregulated in breast cancer through feedback loop of increased Bcl-2 expression (Kaufmann & Vaux, 2003). Conceivably, decreased Bax expression may be responsible for decrease expression of Bcl-2.
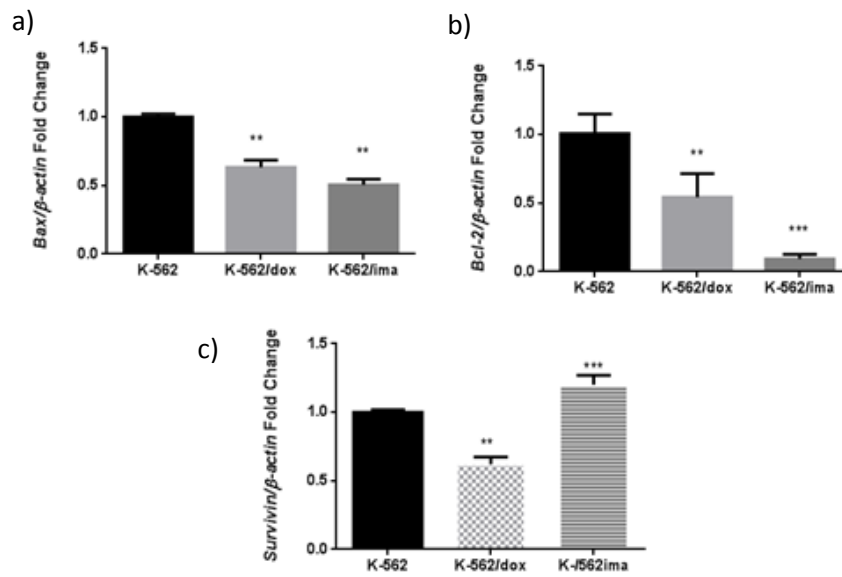
Figure 3.4 Expression levels of a) *Bax* **b)** *Bcl-2* **and c)** *Survivin* **genes in K562 cell lines, when p<0.05. Experiment was performed in duplicate.**

Figure 3.5-8 show cycling profiles of *β-actin, Bax, Bcl-2* and *Survivin* genes. All amplification curves were exponential and reached platau showing ideal amplification. In melt curve analysis, one peak was observed, indicating only desired products were amplified.
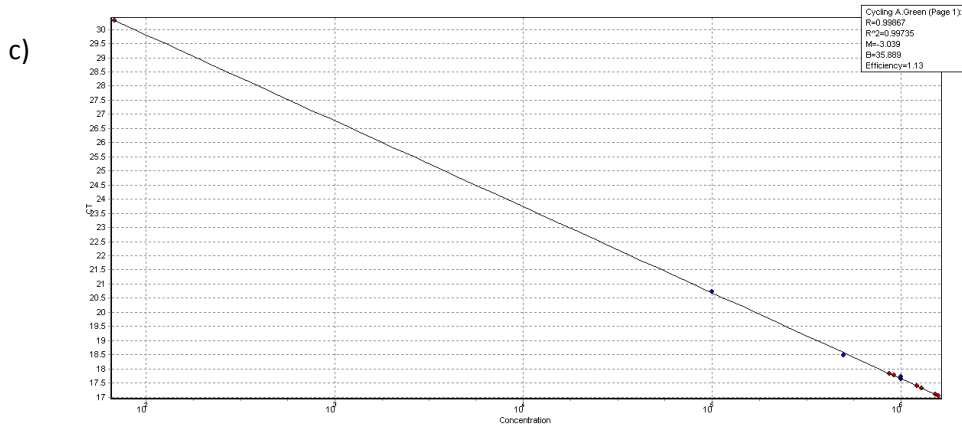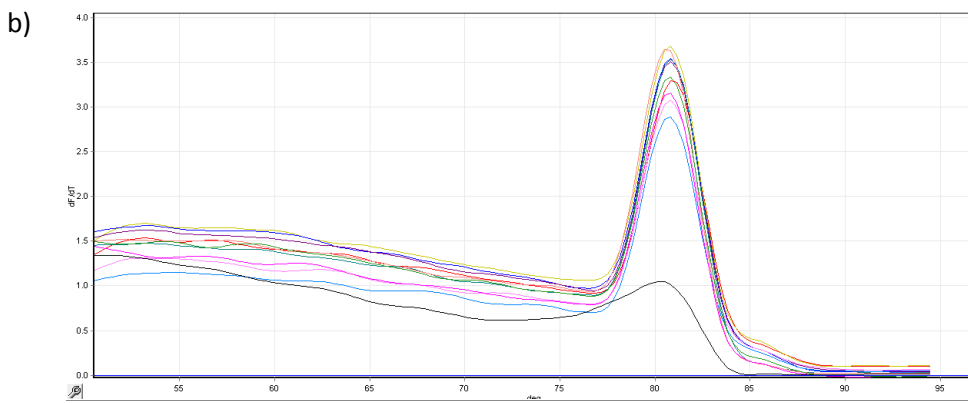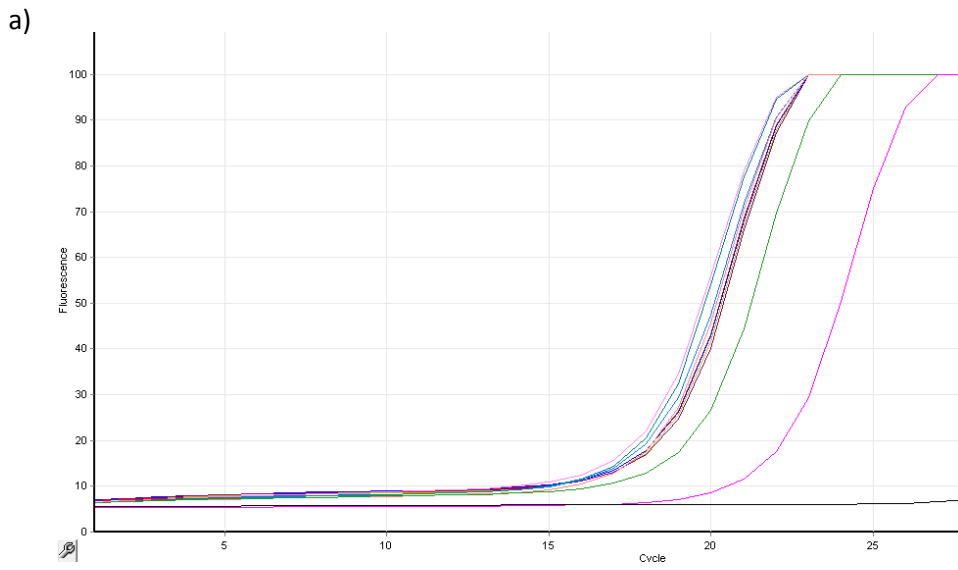
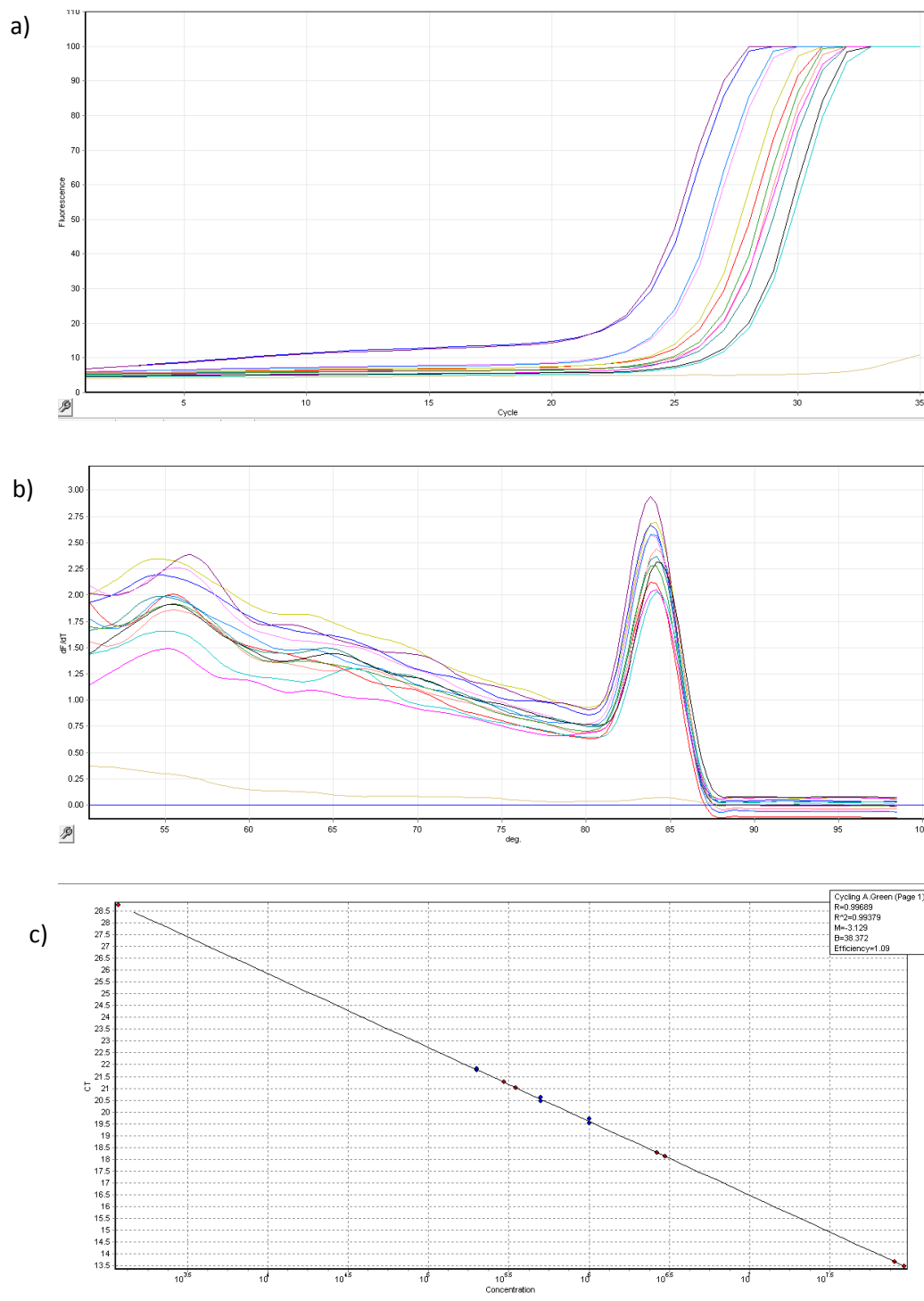.**Figure 3.5 a) Cycling profile, b) melt curve, and c) standard curve for *β-actin* amplicon**

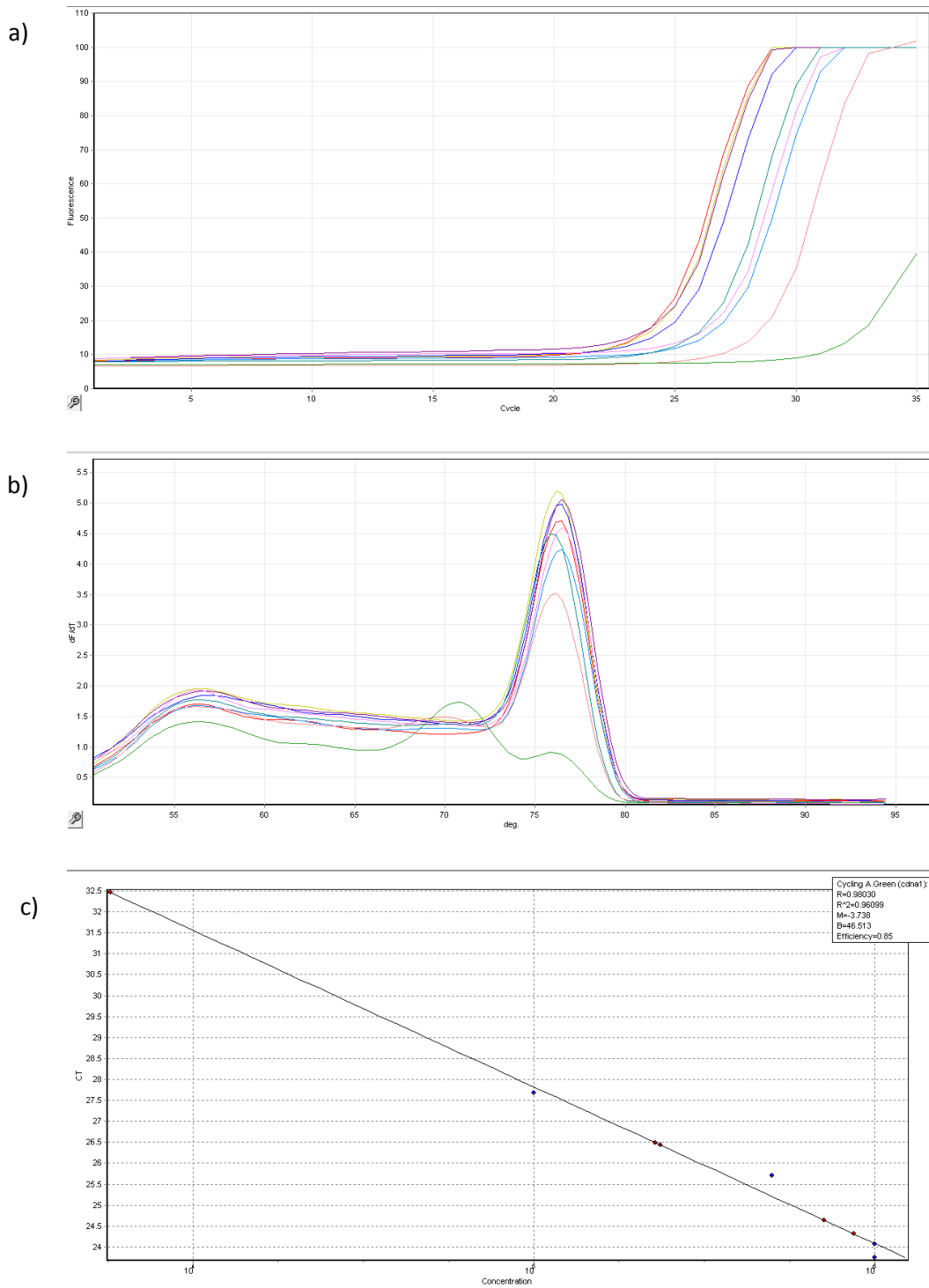**Figure 3.6 a) Cycling profile, b) melt curve, and c) standard curve for *Bax* amplicon.**

Figure 3.7 a) Cycling profile, b) melt curve, and c) standard curve for *Bcl-2* amplicon.
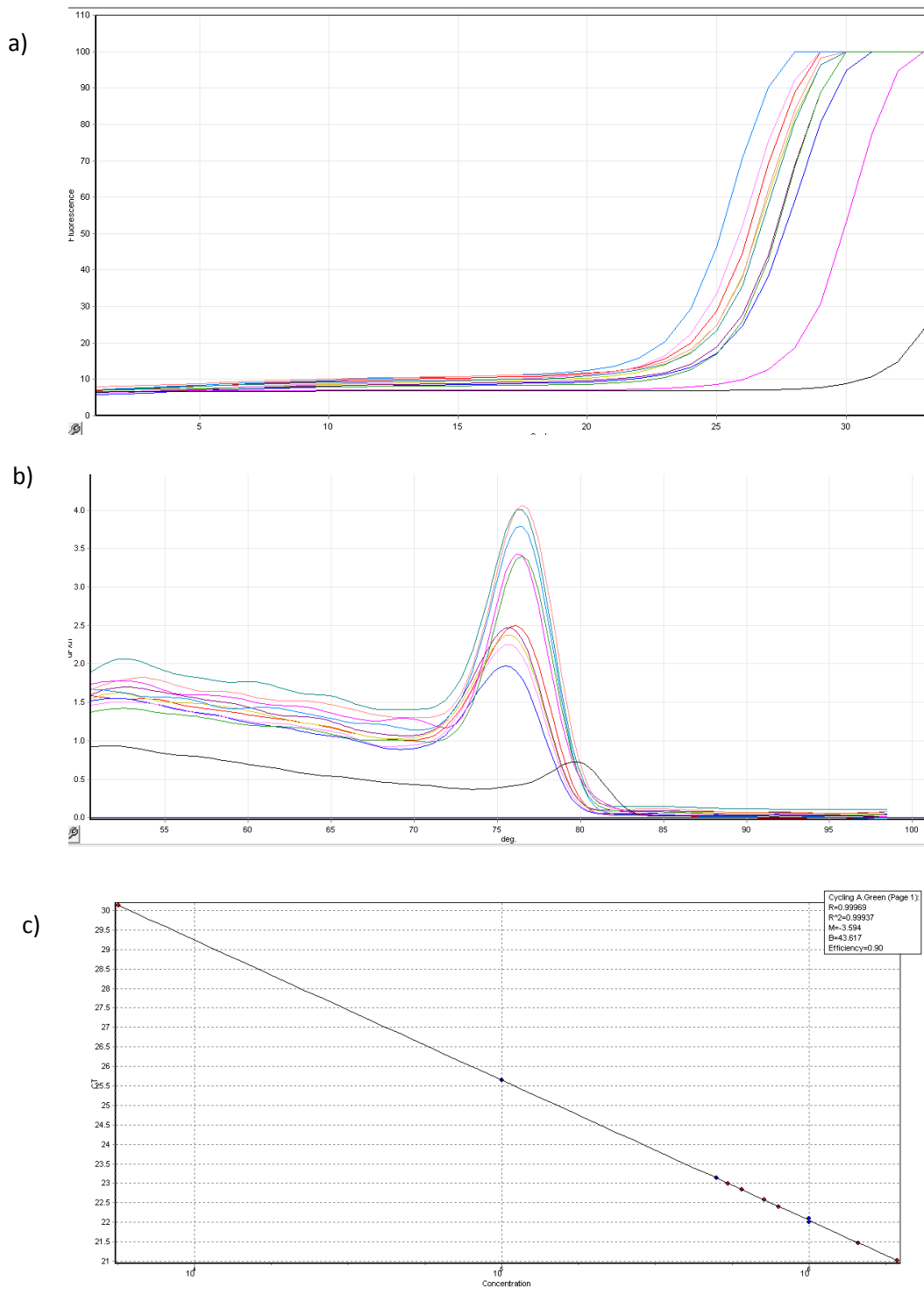
Figure 3.8 a) Cycling profile, b) melt curve, and c) standard curve for *Survivin* amplicon.

Next, siRNA silencing studies were performed in order to show whether decreased expression of Nek6 has an effect on apoptotic behavior in K-562 cell lines.

3.4   siRNA Transfection Efficiency

In order to find optimum siRNA concentration and treatment time, transfection efficiency studies were performed. Cells were transfected with various amounts of fluorescein-conjugated negative siRNA and fluorescence intensity coming from transfected cells was analyzed by flow cytometer. Figure 3.9 shows the transfected cell percentage according to their fluorescence intensity after various concentrations of siRNA treatment. The results show that approximately 60% cells were transfected when treated with 50 nm siRNA. Cells treated with 100 nm siRNA started to die because of cytotoxic effect of high concentration siRNA. Increasing concentration of siRNA causes a significant decrease in cell viability regardless of siRNA target (Fedorov et al., 2006). This unintended phenotypic effect of high siRNA concentration could be a result of stimulation of immune pathways and off-target effects, meaning undesirable gene silencing. There are two reasons for off-target effects. Seed complementarity of siRNA in 3'UTR to any miRNA and high amount of siRNA delivered into cell may occupy RISK (RNA-induced silencing complex), in turn prevent miRNAs to interact with RISK so miRNA-mediated gene regulation is deteriorated (Caffrey et al., 2011). Figure 3.9 panel b shows flow cytometer results where upper left quarter of each quadrands represent cells transfected with fluorescein-conjugated siRNA successfully. In subpanel G, 61% of cell population was successfully transfected with 50 nm siRNA concentration. When cells treated with 100 nm siRNA concentration, transfected cell amount decreased approximately to 40%. (Figure 3.9-b H) Moreover, 200 nm siRNA concentration decreased cell viability significantly (data not shown).

In order to prevent off-target effects, 50 nm siRNA concentration was chosen for further analysis.
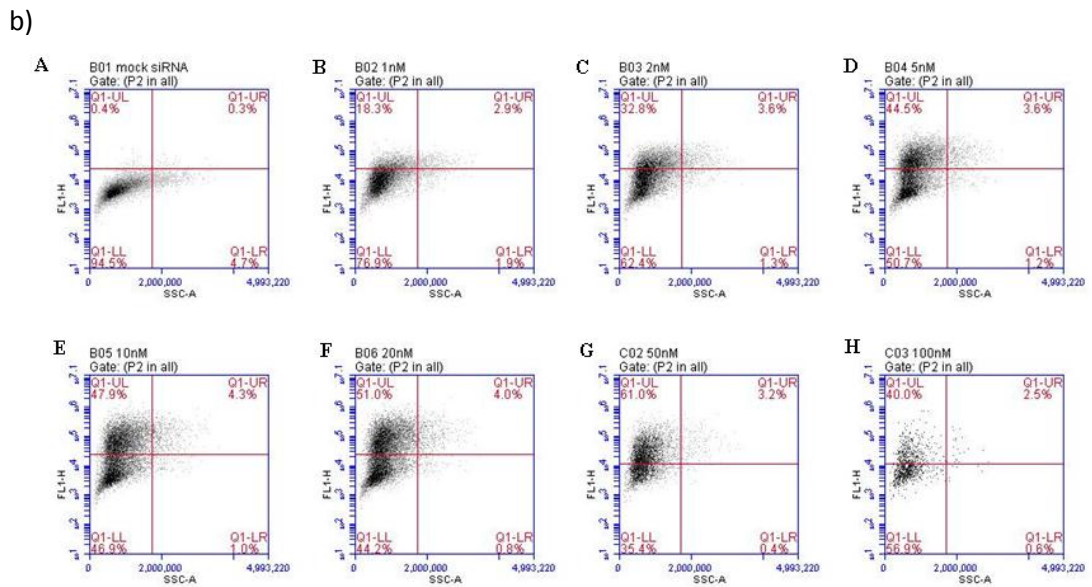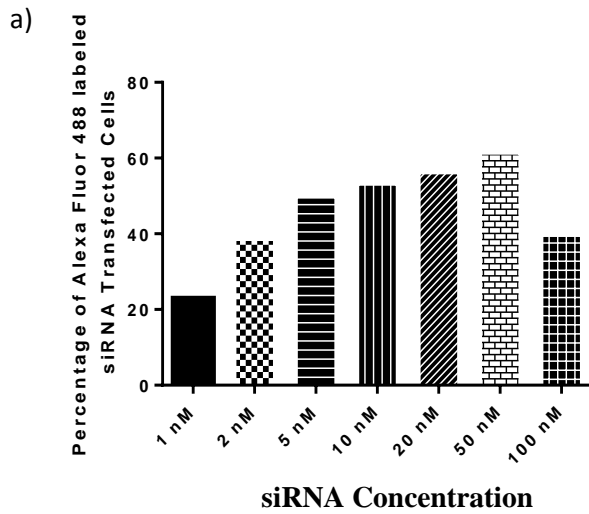
Figure 3.9 a) Graphical representation of Alexa Fluor 488 labeled siRNA transfected K-562 cells after 48 hour treatment. b) Optimization of transfection efficiency. K-562 sensitive cells were transfected with transfection reagent alone (A), 1 nM siRNA (B), 2 nM siRNA (C), 5 nM siRNA (D), 10nM siRNA (E), 20 nM siRNA (F), 50 nM siRNA (G), and 100 nM siRNA (H)

3.5 Validation of Nek6 Knockdown

Quantative real time PCR analysis shows that Nek6 gene expression level was decreased to 50% after 48 hour Nek6 specific siRNA treatment compared to untransfected one. In siRNA transfection efficiency studies, we showed that 60% of cells transfected fluorescein conjugated siRNA. Figure 3.10 shows that transfection of cells with 50 nm specific siRNA for Nek6 leads 50% decrease in mRNA level of Nek6. Moreover, Nek6 expression level did not change in cells transfected with control siRNA. Because the expression level of Nek6 was recovered after 72 hours, the following studies performed by treating cells with Nek6 siRNA for 48 hours. To sum up, Nek6 gene expression is successfully reduced to study potential role of Nek6 in apoptotic process and resistance.
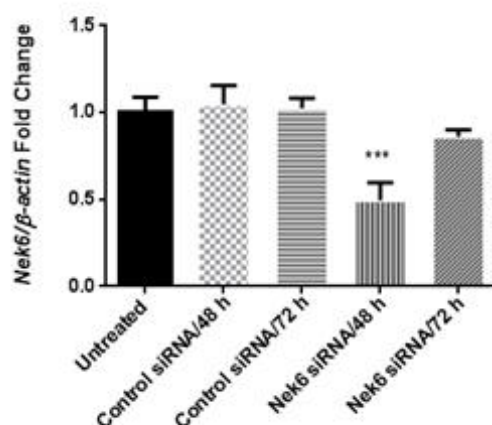
Figure 3.10 Nek6 expression following 48 hours and 72 hours post-transfection. Data analyzed by one-way ANOVA followed by Tukey's Multiple Comparison Test using GraphPad Prism and presented as mean ± SEM and. Experiment was performed twice in triplicate. (p<0.001)

3.6 Effect of Nek6 Silencing on Apoptosis

In order to figure out whether Nek6 gene has a role in MDR in CML through dysregulated apoptotic pathway, the expression levels of apoptotic genes after Nek6 gene silencing in sensitive K-562 cell line were investigated. According to results shown in Figure 3.11, Bax expression level is decreased 2 fold in sensitive cells.

After Nek6 silencing, the expression level of Bax gene in sensitive K-562 cells resembled the expression level of Bax in imatinib and doxorubicin resistant counterparts. Although, Bcl-2 expression level is down regulated in resistant subtypes of K-562, evaluated level of Bcl-2 gene after Nek6 silencing, represents gaining resistance to chemotherapeutic drugs. Increase in Bcl-2 level after 72 hours of transfection could be explained by indirect effect of Nek6 on expression of Bcl-2. Therefore, it could be concluded that Nek6 silencing make sensitive cells to exhibit similar gene expression pattern with resistant ones. Accordingly, Nek6 gene has a potential role in the development of drug resistance in CML.
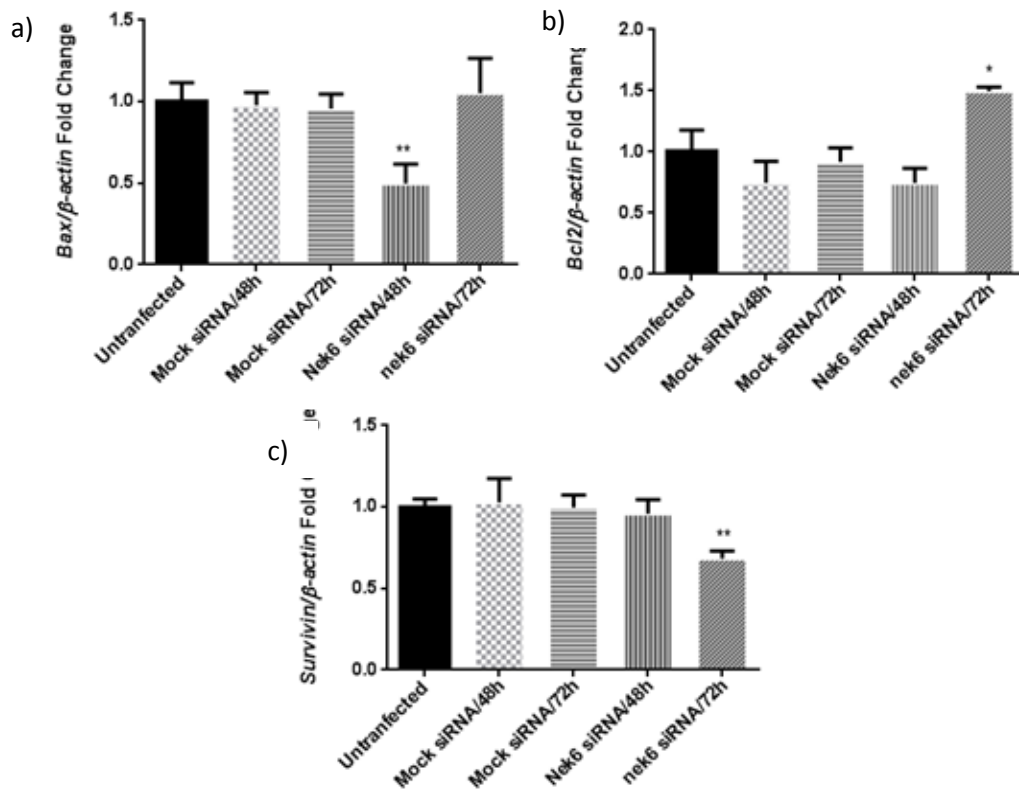

Figure 3.11 Expression levels of a) *Bax,* **b)** *Bcl-2,* **and c)** *Survivin* **genes in K562 cell lines, when p<0.05. Experiment was performed in duplicate.**

3.7 Annexin V/PI Staining

Annexin V/PI staining was performed in order to show apoptotic status of transfected cells. The lower right quadrant represents early apoptotic cells and the upper right quadrant shows late apoptotic and necrotic cells. Etoposide that is highly cytotoxic chemotherapeutic drug was used for positive control and for negative control cells were remained untreated. According to results shown in Figure 3.12-a, more than 97% of untreated cells were alive in both 48 and 72 hours after treatment. More than 50% and 70% of etoposide treated cells were apoptotic after 48 and 72 hours treatment, respectively (Figure 3.12-b). Cells treated with only lipofectamine were also living similar to untreated cells. After siRNA treatment, only 7% of cells underwent apoptosis after 48 hours. Apoptotic cell amount after 72 hour of silencing declined 3%. This result is consistent with apoptotic gene expression analysis in the context of escaping from apoptosis. Interestingly, more than 30% of cells transfected with mock siRNA appeared early apoptotic (Figure 3.12-c). The underlying reason could be off target effect of using high concentration of siRNA. The gene expression analysis did not underpin apoptotic behavior of cells transfected with mock siRNA. These cells could undergo cellular death via necrosis instead of apoptosis.

a)

b)

Figure 3.12 Flow cytometer results of Annexin V/PI staining. a) Cells with no treatment after 48 and 72 hours. b) Cells with etoposide treatment after 48 and 72 hours. After 48 and 72 hours treatment cells were stained with PI alone, Annexin V alone and both Annexin V/PI. Experiment was performed in duplicate (The rest of the figure on the next page).
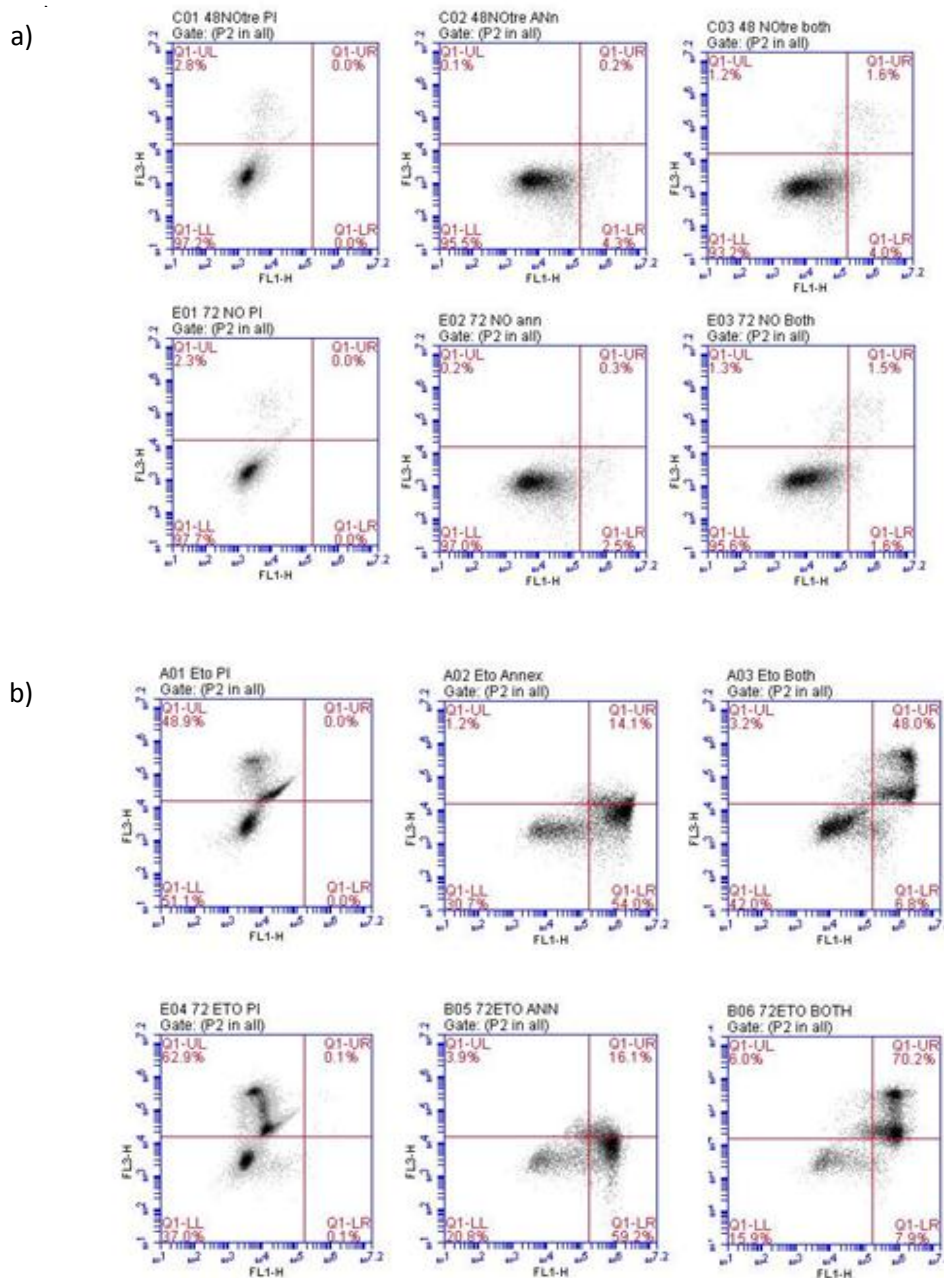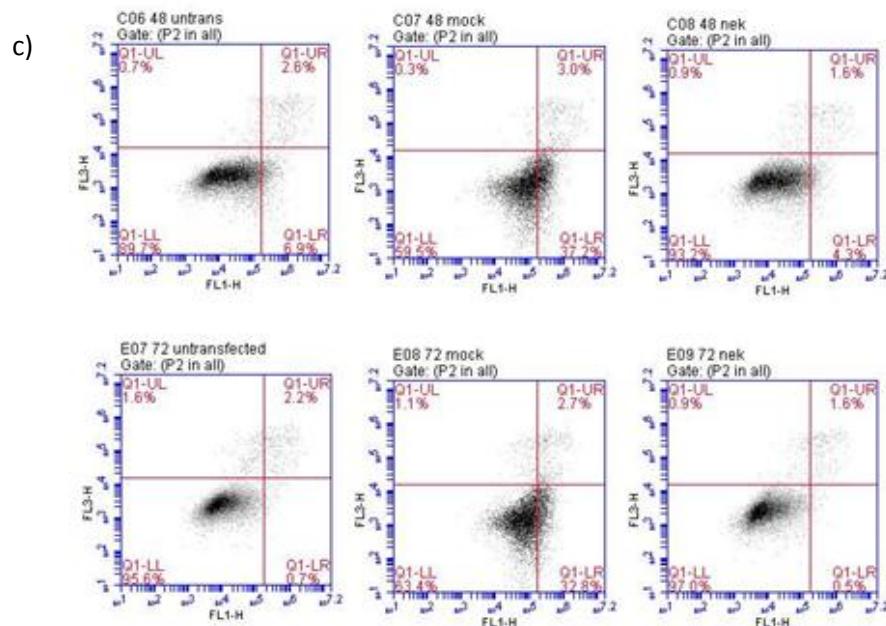
Figure 3.12 Flow cytometer results of Annexin V/PI staining. a) Cells with no treatment after 48 and 72 hours. b) Cells with etoposide treatment after 48 and 72 hours. c) Cells treated with lipofectamine alone, mock siRNA, and Nek6 siRNA after 48 and 72 hours. K-562 sensitive cells were transfected with 50 nm Nek6 specific siRNA. After 48 and 72 hours treatment cells were stained with PI alone, Annexin V alone and both Annexin V/PI. Experiment was performed in duplicate.

According to the literature, Nek6 depletion triggers apoptosis by increasing Bax and Bad expression level. Besides, cleaved caspase-3 and PARP levels also increase after functional knockdown of Nek6 in HeLa and MCF-7 cell lines (Nassirpour et al., 2010). However, Nek6 silencing by lipofectamine does not cause apoptosis. This contradictory result could be explained by the efficiency of Nek6 depletion. We were not able to silence Nek6 more than 50% by lipid-based transfection method. Stable transfection or overexpression of kinase dead domain as in the case of Nassirpour study may result inducing apoptosis. On the other hand, 50% decrease in Nek6 expression level could not be sufficient to trigger apoptosis. Moreover, studies showed that when K-562 cells were treated with imatinib Bcl-2 level decreases whereas some studies indicated that Bcl_{xl} level decreases. Clonal variation among cancer cells accounts for differences in gene expression profiles (Junya Kuroda et al.,

2006). In order to validate potential role of Nek6 in drug resistance elicited by alteration in apoptotic pathway, these studies should be repeated in stably transfected K-562 cells.

Figure 3.13 shows the cell viability K-562 cells after variety of treatment analyzed by tryphan blue exclusion test. Results were consistent with Annexin V/PI staining. Cells transfected with Nek6 siRNA were alive in the proportion of 90%.
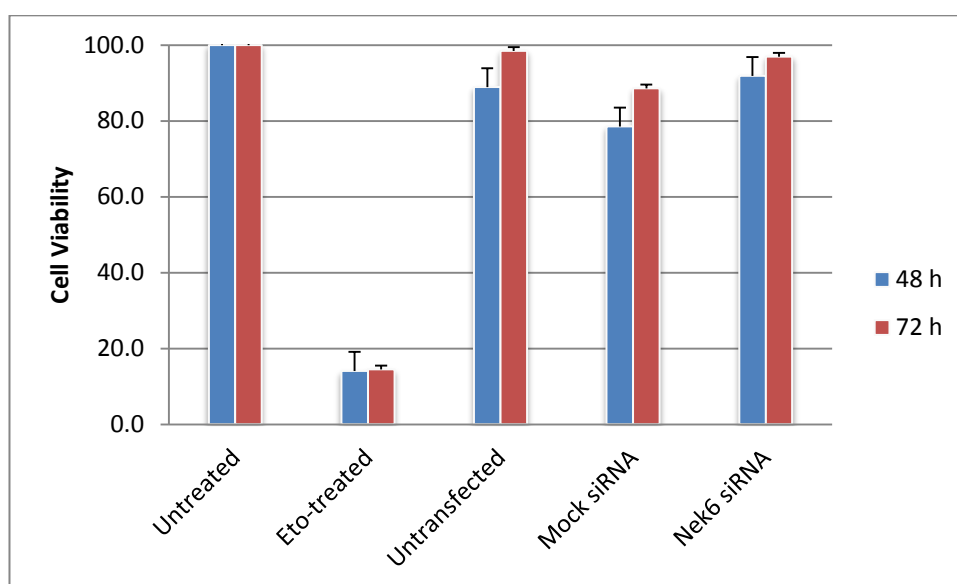
Figure 3.13 Tryphan Blue staining of K-562 cells under no treatment, etoposide treatment, lipofectamine treatment, mock siRNA and Nek6 siRNA treatment after 48 and 72 hours. Experiment was performed in duplicate.

3.8   Cell Viabilty

Cell viability assay was performed by XTT in order to investigate whether Nek6 silencing leads to gain of resistance in K-562 cells. Preliminary data showed that K-562 cells after Nek6 silencing become more resistant to imatinib (Figure 3.14). IC_{50} value for untreated K-562 cells is 150 nM whereas after Nek6 silencing IC_{50} increased to 1780 nM. IC_{50} values were calculated via formula determined in the Figure 3.14.

a)

y = -23.01ln(x) + 5.8035
R² = 0.8735

◆ K-562 Untreated

—— Log. (K-562 Untreated)

b)

y = -13.61ln(x) + 57.817
R² = 0.8401

◆ K-562 Nek6 siRNA

—— Log. (K-562 Nek6 siRNA)
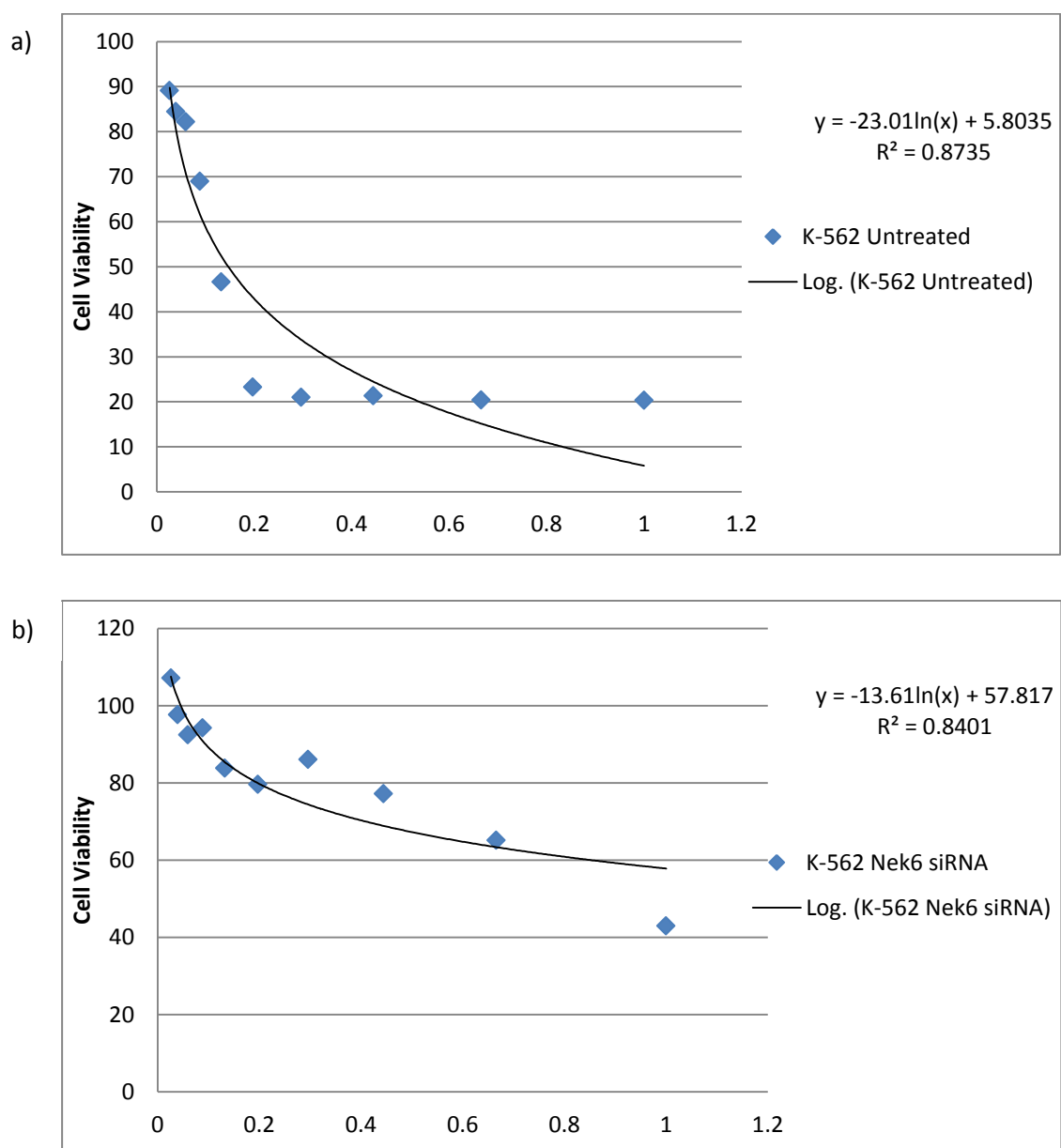
Figure 3.14 Cell proliferation analysis by XTT. a) Imatinib treated K-562 cells after 72 hour, b) Imatinib treated Nek6 silenced K-562 cells after 72 hour.

Gene expression analyses and cell viability assays showed that Nek6 is important for developing drug resistance in CML. After Nek6 silencing, the pro-apoptotic gene expression level, Bax, was 2 fold decreased and anti-apoptotic gene expression level, Bcl-2, was 1.5 fold increased in K-562 cell lines (Figure 3.11). Annexin V/PI staining and tryphan blue exclusion assay confirmed that cells transfected with Nek6 did not go apoptosis (Figure 3.12 and 3.13). Their viability did not significantly

change compared to untransfected ones. Moreover, cell viability assay demonstrated that IC_{50} value against imatinib was significantly increased in Nek6 transfected cells, 1.78 nM compared to untreated, 0.15 nM (Figure 3.14).

Although depletion studies cause mitotic arrest followed by apoptosis in solid tumors, Nek6 may have different role in hematological malignancies (Nassirpour et al., 2010). There are different kinase families that have role in mitotic progression, such as Polo-like kinases, and Aurora kinases, and the expression level of these kinases differ with respect to type of cancer (Degenhardt & Lampkin, 2010; Malumbres, 2011). They have either oncogenic or tumor suppressor roles in cancer cells. For example, point mutations identified in Plk1 gene cause protein instability and Plk1[(+/-)] mice show higher susceptibility for cancer development (Malumbres, 2011). On the other hand, overexpresion of Plk1 in fibroblast cells induce cancer transformation and depletion studies result mitotic arrest and apoptosis. Silencing Plk1 in CML cell lines cause an increase in arrested cell number and decrease viability of cells (Degenhardt & Lampkin, 2010; Gleixner et al., 2010). Studies showed that Plk2 was significantly downregulated in resistant ovarian cancer cells and decrease in expression pattern was mediated by methylation of CpG island of Plk2 promoter (Syed et al., 2011). Plk3 expression level is increased in ovarian and breast cancer whereas it is downregulated in lung and neck cancers but the underlying reason behind these expression differences is still unknown (Degenhardt & Lampkin, 2010). To sum up, cell cycle kinases display different expression patterns in various cancer cells and their roles in proliferation and drug resistance depend on cancer type, epigenetic status and their interaction with other proteins.

Parallel with these findings, Nek6 may also display different characteristics depending on cancer cell type. The increased expression level in solid tumors and apoptotic behaviour after silencing of Nek6 in these tumors could refer its oncogenic-like role whereas the decreased expression level in resistant cells imply that Nek6 may have an anti-proliferative role or epigenetic status of Nek6 could be related to resistance in hematological malignancies.

3.9 Future Prospects

In order to validate role of Nek6 in drug resistance, different approaches should be examined. Firstly, Nek6 expression pattern in healthy blood cells should be investigated. If the expression pattern of Nek6 is higher compared to CML cells, this may imply its anti-proliferative role in hematological malignancies. Silencing studies must be repeated by stable transfection or overexpression of kinase dead domain in order to show whether stable silencing also leads to gain of resistance characteristics in sensitive cells.

In order to confirm role of Nek6 in drug resistance, it could be overexpressed in resistant subtypes of K-562 cells which normally exhibits decreased expression pattern and investigate forced overexpression of Nek6 cause apoptosis or not.

The mechanism of Nek6 in drug resistance could be analyzed by studying its interaction with transcription factors, specifically those have role in BCR/ABL independent resistance, or its epigenetic status in sensitive and resistant subtypes.

CHAPTER 4

CONCLUSION

1) Real time PCR studies showed that Nek6 gene expression is significantly reduced in K-562 doxorubicin and imatinib resistant subtypes compared to sensitive parental cells, whereas there was no significant change between sensitive MCF-7 and its resistant sublines.

2) Gene expression analyses demonstrated that expression levels of anti-apoptotic genes, Survivin, was increased and pro-apoptotic gene expression level, Bax was decreased in resistant K-562 cell lines.

3) Fluorescein-conjugated siRNA transfection studies in K-562 sensitive cells indicated that with 50 nm siRNA concentration, one can successfully transfect more than 60% of cells after 48 hour.

4) Nek6 mRNA level was reduced 50% after treatment with Nek6 specific siRNA for 48 hour.

5) After Nek6 silencing in drug sensitive K-562 cells, the expression profiles of apoptotic genes showed similarity with imatinib resistant cells.

6) Apoptotic assay indicated that cells transfected with Nek6 siRNA did not undergo apoptosis. Instead, they exhibited characteristics of resistant cells.

Our study demonstrated that Nek6 gene could have potential role in MDR in CML. This role may be related with evasion from apoptosis which drug resistant cancer cells developed.

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APPENDIX A

BUFFERS AND SOLUTIONS

Freezing medium

9ml FBS (Biochrome, Germany)

1ml DMSO (Applichem, Germany)

Mixed and stored at +4°C

Phosphate buffered saline (pH 7.2)

1 PBS tablet (Sigma, Germany) in 200ml distilled water.

After the tablet dissolved PBS is autoclaved at 121°C for 20 minutes

Tris acetate buffer (pH 8.0)

20 ml 50X TAE

980 ml distilled water

Mixed and stored at room temperature

AGAROSE GEL IMAGES OF AMPLICONS

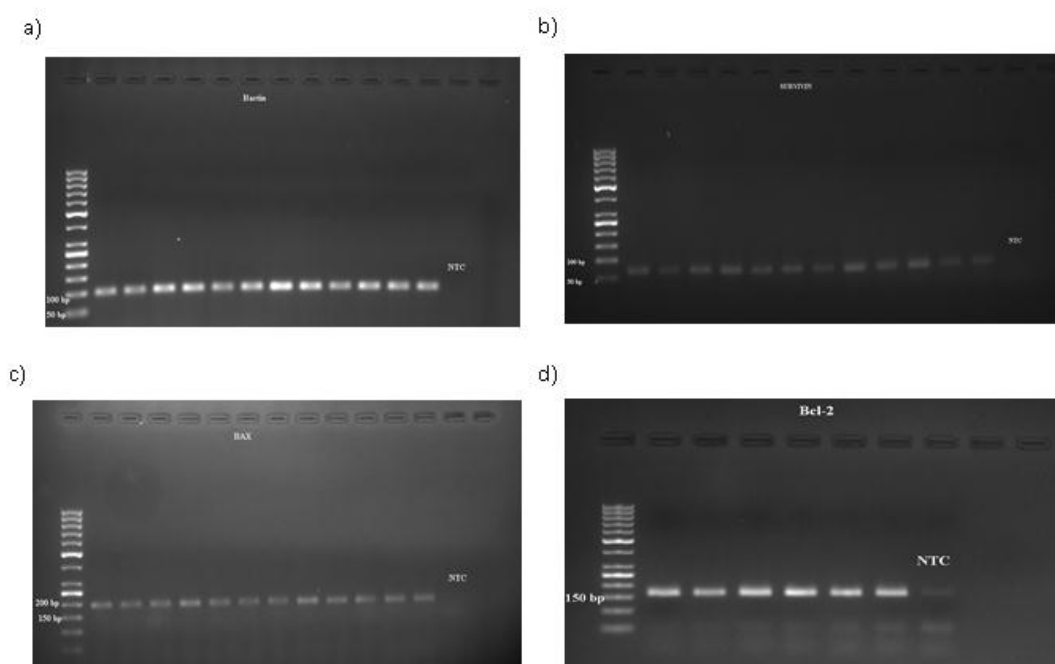**B.1. Representative agarose gel images for each amplicon**

Figure B.1 Representative gel images of a) * β-actin*, b) *Survivin*, c) *Bax*. d) *Bcl-2*. Each amplicon was load on 2% agarose gel and run at 90 V.