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NEK6 gene silencing using siRNA for overcome multidrug resistance in chronic myeloid leukemia cells

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Abstract: Tumor cells become resistant to structurally or functionally unrelated chemotherapeutics which is called multidrug resistance (MDR). There are several mechanisms including the impairment of apoptotic pathway resulting in MDR development. *NEK6* is a member of NIMA-related kinase family and it is an important mitotic kinase for proper cell cycle progression. Recent studies showed that *NEK6* gene expression, protein level, and its kinase activity are increased in variety of cancer cells. We aimed to search the involvement of *NEK6* in multidrug resistance and apoptosis in chronic myeloid leukemia. The expression levels of *NEK6* and some of the apoptotic pathway genes such as *BAX*, *BCL-2* and *SURVIVIN* were determined in sensitive and drug resistant subtypes of K652 chronic myeloid leukemia cell lines by RT-PCR method. siRNA silencing studies were performed to examine the effect of expression of *NEK6* on apoptotic behavior in parental K-562 cell line. Cell viability assay was performed by XTT method in order to investigate whether *NEK6* silencing leads to resistance in parental K-562 cells. *NEK6* expression is significantly reduced in imatinib resistant K562 cells. After *NEK6* gene is silenced by specific siRNA in parental K562 cell line, the expression levels of some apoptotic genes, such as *BAX* and *SURVIVIN* were found similar to drug resistant K562 cells. *NEK6* may have potential role in imatinib resistance which may be through apoptotic pathway in chronic myeloid leukemia.

Keywords: Chronic myeloid leukemia, K562 cell line, multidrug resistance, Nek6, apoptosis, siRNA

1 Introduction

NEK6 is essential mitotic kinase for proper cell cycle progression. After G2-M phase transition its activity becomes important and during M phase *NEK6* activity increases. Studies showed that *NEK6* depletion results in mitotic arrest at metaphase, demonstrating that *NEK6* is essential for metaphase to anaphase transition (Fry et al. 2012; Lee et al. 2008; Moniz et al. 2011; Yin et al. 2003). After *NEK9* phosphorylates *NEK6* and *NEK7*, they phosphorylate *EG5*, microtubules, and *γ-TURC* which are important for microtubule dynamics. Depletion of either of these kinases results fragile spindles or mitotic arrest (Fry et al. 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 checkpoint is inhibited, cells delayed in late mitosis implying role of *NEK6* also in cytokinesis (O'Regan and Fry 2009).

The *NEK6* gene is situated on chromosome 9q33-34 that 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).

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. Decreased drug-influx and increased drug efflux, impairments in apoptotic pathways, problems in drug conversion to its active form, alteration in cell cycle checkpoints and ceramide metabolism and increased drug

metabolism are the major reasons for the development of MDR (Baran et al. 2011; Kars et al. 2006).

Chronic myeloid leukemia (CML) is a form of leukemia characterized by the increased and unregulated growth of predominantly myeloid cells in the bone marrow and the accumulation of these cells in the blood (Calabretta and Perrotti 2004). The aim of current study is to investigate the role of *NEK6* in drug resistance and apoptosis in both sensitive and drug resistant variants of K562 chronic myeloid leukemia cell lines.

2 Materials and Method

2.1 Cell Culture

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) resistant K-562 sublines were previously developed in our lab (Baran et al. 2011). The 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.

2.2 RNA Isolation and cDNA Synthesis

RNA isolation was performed using TriPure reagent (Roche, Germany) according to the manufacturer's instructions. RNA quality and quantity was determined by NanoDrop Spectrometer (Thermo Scientific, USA). RNA integrity was checked by 1% agarose gel electrophoresis. cDNA was synthesized from 2 mg DNase I-treated RNA by using RevertAid reverse transcriptase (Thermo Scientific, USA).

2.3 Gene Expression Analyses

Expression analysis of *NEK6* gene was performed by TaqMan Gene Expression Assay (Roche, Germany). β-actin gene was used as internal control. Expression analyses of apoptotic genes were performed by qRT-PCR experiments by using FastStart SYBR Green (Roche, Germany). Primer sequences and amplicon sizes of apoptotic genes is shown in Table 1.

2.4 siRNA Transfection

Small interfering RNA 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) according to manufactor's instructions.

2.5 Annexin V/PI Staining

In order to determine apoptotic status of cells, Annexin-V-FLUOS Staining Kit (Roche, Germany) was used according to manufactor's intructions. Cells were analyzed with Acuri Flow Cytometer in FL-1 and FL-3 channels.

Table 1 Primer sequences and amplicon sizes of apoptotic genes

Gene	Sequence 5'-3'	Amplicon size (bp)
BAX	F-TCTGACGGCAACTTCAACTG	188
	R-TTGAGGAGTCTCACCCAACC	
BCL-2	F-CCCGCGACTCCTGATTCATT	166
	R-AGTCTACTTCCTCTGTGATGTTGT	
SURVIVIN	F-AGCCAGATGACGACCCCATAGAGG	60
	R-AAAGGAAAGCGCAACCGGACGA	
B-ACTIN	F-CCAACCGCGAGAAGATGA	97
	R- CCAGAGGCGTACAGGGATAG	

2.6. Tryphan Blue Exclusion Assay

Cell viability was determined by tryphan blue exclusion assay (Biological Industries, Israel). In order to analyze cell viability of siRNA treated K562 cells, $3x10^4$ cells were seeded on 6-well plates with a total volume of 1 ml. Cells were remained untreated or treated with 50 nM either mock or Nek6 siRNA. After 48 and 72 hours incubation alive cells that were not stained by tryphan blue were counted on 16 squares of hematocytometer and total cell number in 1 ml was calculated.

2.7 Statistical analysis

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

3 Results

3.1 *NEK6* expression profiles in K-562 cell line and its drug resistant sublines

The expression profiles of *NEK6* gene in K-562 cell line and its drug resistant sublines were examined. According to the results, *NEK6* gene expression was significantly decreased in doxorubicin and imatinib resistant K-562 cell lines compared to its sensitive subline. Nek6 expression level decreased approximately 3 fold in both resistant K562 cell lines (Figure 1).

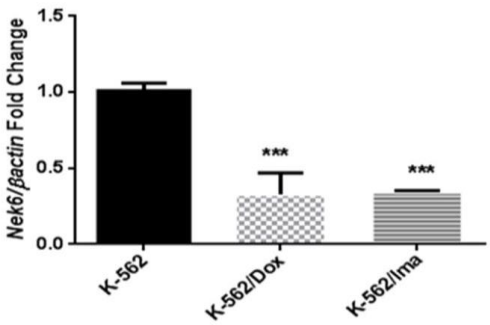

Fig. 1 Expression levels of *NEK6* gene in drug sensitive and drug resistant K562 cell lines. ***$p<0.05$

3.2 Expression profile of apoptotic genes in parental K-562 cells and its resistant sublines

Screening apoptotic gene expression levels in sensitive K-562 and its resistant sublines were performed to understand the potential role of *NEK6* in MDR. According to the results, *BAX*, and *BCL-2* expression levels were significantly decreased in imatinib and doxorobucin resistant K562 cells whereas *SURVIVIN* expression was significantly downregulated and upregulated in doxorubicin and imatinib resistant K-562 cells, respectively compared to parental cells (Figure 2).

The expression level of *BAX* and *BCL-2* were significantly decreased in both doxorubicin and imatinib resistant cells. *BAX* to *BCL-2* ratio in the imatinib resistant subline was approximately 3 which may imply 3 fold increased tendency to apoptosis (Figure 2a and 2b). Interestingly, *SURVIVIN* expression was only increased in imatinib resistant subline (Figure 2c).

3.3 *NEK6* silencing by specific siRNA

siRNA silencing studies were performed to examine the effect of expression of *NEK6* on apoptotic behavior in parental K-562 cell line. *NEK6* expression analysis by qRT-PCR after transfection of parental K562 cells with *NEK6* specific siRNA was given in Figure 3.

The analysis shows that *NEK6* gene expression level was decreased two folds after 48 hour *NEK6* specific siRNA treatment, compared to untransfected cells (Figure 3). In siRNA transfection efficiency studies by flow cytometry, we showed that 60% of cells transfected by fluorescein conjugated siRNA (data not shown). Figure 3 shows that transfection of K562 cells with 50 nM specific siRNA leads two folds decrease in mRNA levels of *NEK6*. *NEK6* expression level did not change in cells transfected with control siRNA. Since the expression level of *NEK6* was recovered after 72 hours, further studies performed by treating cells with *NEK6* siRNA for 48 hours.

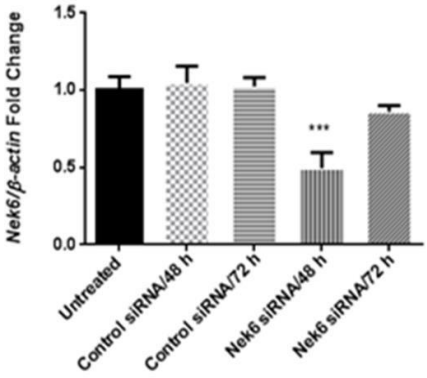

Fig 3. *NEK6* expression in parental K562 cell line following 48 hours and 72 hours post-transfection. *** p<0.00

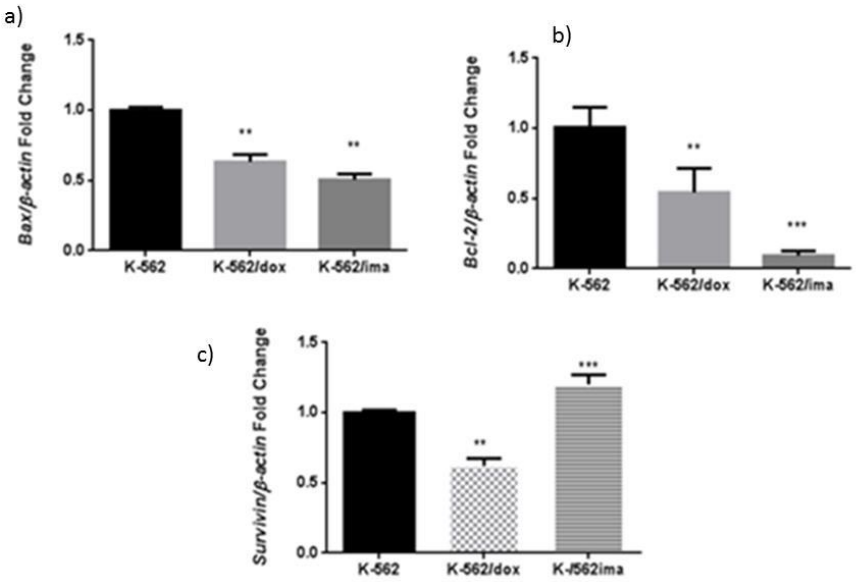

Fig. 2 Expression levels of A. *BAX*, B. *BCL-2* and C. *SURVIVIN* genes in K562 cell lines, when p<0.05.

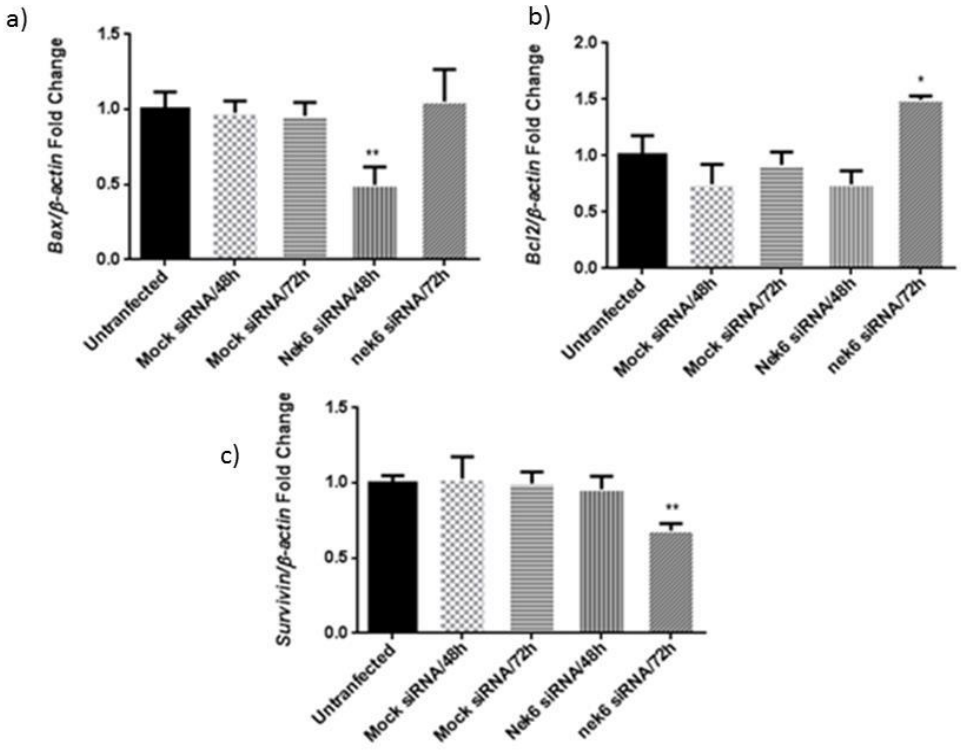

Fig. 4 Expression levels of A. *BAX*, B. *BCL-2* and C. *SURVIVIN* genes in parental K562 cell line after silencing of *NEK6* gene, when $p<0.05$.

3.4 The effect of *NEK6* silencing on apoptosis in K-562 cell line

In order to show whether *NEK6* gene has a role in MDR in CML through disregulated apoptotic pathway, the expression levels of apoptotic genes after *NEK6* gene silencing in parental K-562 cell line were investigated (Figure 4). According to results shown in Figure 4, *BAX* to *BCL-2* ratio decreased to approximately 0.7 (Figure 4a and 4b). This significant decrease (approximately 4 folds) in *BAX* to *BCL-2* ratio after *NEK6* silencing indicates escape from apoptosis. This may represents parental K562 cells gain resistance to imatinib through disregulating the apoptotic pathway after *NEK6* silencing. Interestingly, when *BAX* to *BCL-2* ratio decrease, *SURVIVIN* gene expression is also decrease in this cell line (Figure 4c).

3.5 Apoptosis studies by Annexin V/PI staining and tryphan blue exclusion assay

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 (Figure 5). According to Figure 5 more than 95% of cells treated with only lipofectamine were alive. 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 after *NEK6* silencing. Interestingly, more than 30% of cells transfected with mock siRNA appeared as early apoptotic. 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. Figure 6 shows the cell viability of 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 90% alive and it is statistically significant with respect to etoposide treated K562 cells (Figure 6).

3.6 Imatinib resistance in parental K562 cells after *NEK6* silencing

K562 parental cells were subjected to different concentration of imatinib and IC50 value was determined by XTT analysis after *NEK6* silencing. Cell viability assay was performed by XTT in order to investigate whether *NEK6* silencing leads to resistance in parental K-562 cells. IC50 value for untreated K-562 cells is 150 nM whereas after *NEK6* silencing IC50 increased to 1780 nM. More than 10 fold increase in IC50 after *NEK6* silencing, indicates that K562 cells show high resistance to imatinib. When parental K-562 cells treated with imatinib only we observed an intrinsic resistance up to 200 nM. This resistance may be contributed by *BCR/ABL* dependent or independent mechanisms as mentioned earlier. In *NEK6* silenced K-562 cells treated with imatinib, cell viability did not decrease more than 50% percent. This behaviour could be explained by *NEK6* recovery after 72 hours of transfection.

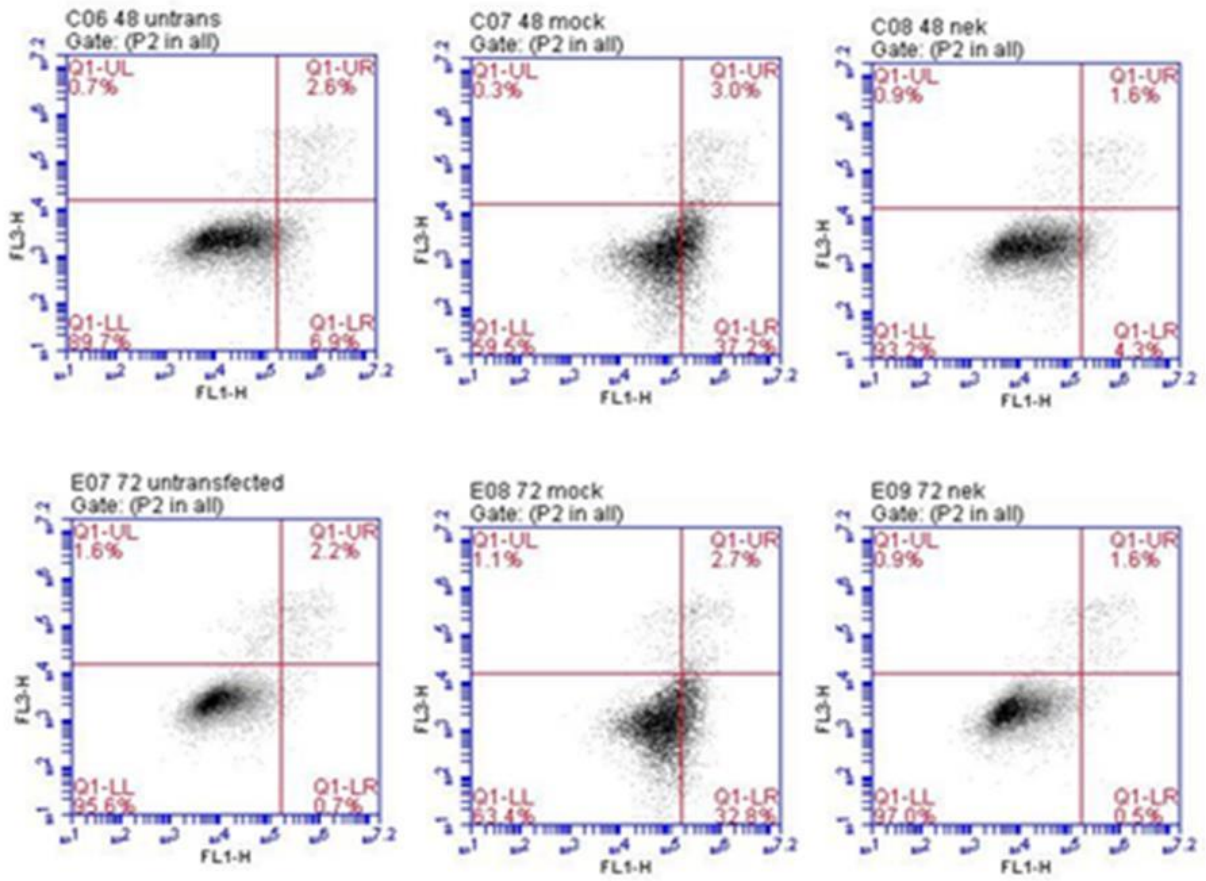

Fig. 5 Flow cytometer results of Annexin V/PI staining. Cells treated with **A.** lipofectamine alone, **B.** mock siRNA and **C.** *NEK6* siRNA after 48 hours. Cells treated with **D.** lipofectamine alone, **E.** mock siRNA and **F.** *NEK6* siRNA after 72 hours. K-562 sensitive cells were transfected with 50 nm *NEK6* specific siRNA. After 48 and 72 hours treatment cells were stained with Annexin V/PI.

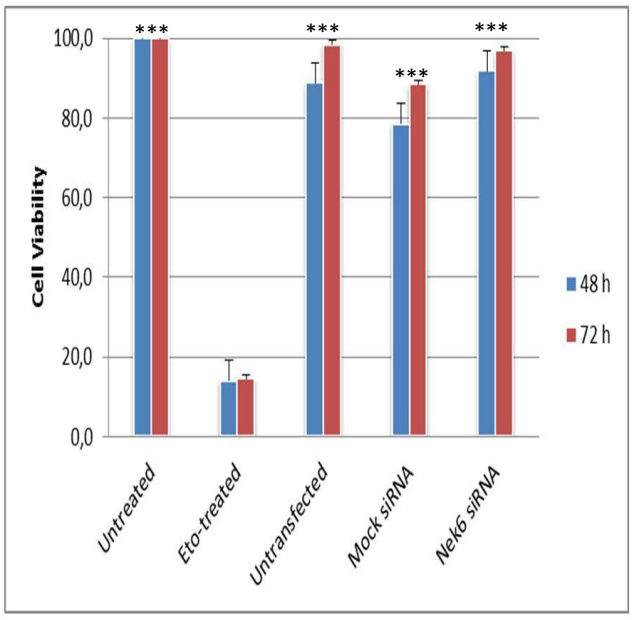

Fig. 6 Tryphan Blue staining of K-562 cells under without treatment, with etoposide treatment, lipofectamine treatment, mock siRNA and *NEK6* siRNA treatment after 48 and 72 hours. ***p<0.05

4 Discussion

In this study, we aimed to search the possible involvement of *NEK6* in drug resistance in chronic myeloid leukemia. According to our results, *NEK6* gene expression was significantly decreased in doxorubicin and imatinib resistant K-562 cell lines compared to its sensitive subline. However, *BAX* to *BCL-2* ratio in the imatinib resistant subline was approximately 3 which may imply 3 fold increased tendency to apoptosis. This ratio is not as significant (approximately 1.4 fold) in doxorubicin resistant subline. Impairment in apoptotic pathways is one of the main reasons for MDR development. 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 hematological malignancies (Kauffmann and Vaux 2003). In recent years, several studies have investigated the significance of *BAX/BCL-2* ratio and its correlation with several diseases including cancer. In many organisms, *BCL-2* family members modulate apoptosis via *BAX/BCL-2* ratio serving to as a rheostat to determine cell susceptibility to apoptosis. On the other hand, *SURVIVIN* expression was slightly upregulated in imatinib resistant K562 cells compared to parental cells whereas it decreased in doxorubicin resistant K-562 cell line. The different

expression patterns of *BAX* to *BCL-2* ratio and *SURVIVIN* apoptotic pathway genes in doxorubicin and imatinib resistant K562 cells may indicate that these two drugs activate different pathways during drug resistance development.

NEK6 gene expression was transienly silenced by specific siRNA in order to show whether *NEK6* gene has a role in MDR through disregulated apoptotic pathway in CML cells. The expression levels of some apoptotic genes and *BAX/BCL-2* ratios were investigated in parental K562 cells after *NEK6* silencing. According to our results, *BAX* to *BCL-2* ratio decreased to 4 folds indicates escape from apoptosis after *NEK6* silencing. This may represent parental K562 cells gain resistance to imatinib through disregulating the apoptotic pathways. Therefore, *NEK6* gene may have a potential role in the development of drug resistance in CML.

According to literature, in HeLa and MCF-7 cells *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* (Nassirpour et al. 2010). *NEK6* depletion cause mitotic arrest followed by apoptosis in solid tumors. The results obtained with K562 cells seem to be contradictory to these results. This may indicate possible different roles of *NEK6* on different types of cells which either adherent or in suspension. On the other hand, in this study, *NEK6* siRNA transfection was transient and this treatment only cause two folds decrease in mRNA level of *NEK6* within 48 hours while the expression level was recovered after 72 hours. Stable transfection with siRNA or overexpression of kinase dead domain as in the case of Nassirpour study may result inducing apoptosis (Nassirpour et al. 2010). 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.

5 Conclusion

As a conclusion, in this study we observed that *NEK6* expression is significantly reduced in imatinib resistant K562 cells. After *NEK6* gene is silenced by specific siRNA in parental K562 cell line, the expression levels of some apoptotic genes, such as *BAX* and *SURVIVIN* were found similar to drug resistant K562 cells. These results may indicated that *NEK6* can have potential role in imatinib resistance which may be through apoptotic pathway in chronic myeloid leukemia.

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Formal Analysis: Ayça Nabioğlu, Pelin Mutlu, Ufuk Gündüz

Data curation: Ayça Nabioğlu, Pelin Mutlu

Original draft preperation: Ayça Nabioğlu, Pelin Mutlu

Review and editing: Pelin Mutlu, Serap Yalçın-Azarkan, Ufuk Gündüz

Conflict of interest disclosure:

The authors of this study declare that they have no conflict of interest.

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