



Original article

Transcriptome analysis of neratinib treated HER2 positive cancer model vs untreated cancer unravels the molecular mechanism of action of neratinib



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ARTICLE INFO

Article history:

Received 1 April 2020

Accepted 25 June 2020

Available online 30 June 2020

Keywords:

Cancer

Expression profiling

Mouse array

Neratinib

Transcriptome

ABSTRACT

Human estrogen receptor positive cancer cells have mutations and make an excess of the HER2 protein and are far more aggressive than others cancers. Neratinib, an irreversible tyrosine kinase inhibitor is used to treat HER2 positive cancers. Neratinib targets HER2 and blocks its signal transduction resulting in inhibition of cell proliferation and induction of apoptosis without any information about the molecular mechanism involved. To understand the underlying molecular mechanism transcriptome analysis was carried out in normal vs cancer induced SWR/J nude mice. Cancer was induced in SWR/J nude mice with intraperitoneal injection of 5×10^6 SKBR3 cells for 14 days. Histopathology confirmed the induction of cancer in liver and kidney after the tumor size was at least 0.5 cm. Genome wide Mouse U133 Array was used to analyze the effect of neratinib treatment on cancer. Validation of expression was done by qPCR and ELISA. Microscopic examination revealed that neratinib treatment has potential effects on cancerous liver. Transcriptome expression profiling showed 1481 transcripts differentially expressed by neratinib treatment. Transcriptome Analysis Console (TAC) showed that 532 upregulated transcripts were exclusively belonging to cell cycle, inflammation, olfaction, oxidative stress, HER, and EGFR1 while 949 downregulated transcripts were involved in immunology, drug resistance such as histocompatibility, T cell receptors, and immunoglobulins. The differentially expressed genes were considered significant under the criteria of an adjusted p-value < 0.02 and \log_2 ratios ≥ 1.0 and/or \log_2 ratios ≤ -1.0 means two Fold change. qPCR assay and ELISA analysis was used to validate few genes involved in apoptosis and proliferation. This study provides new insights into the neratinib's mode of action by cyclin-dependent kinase inhibitor-3 and calcium-activated chloride channel 3 as markers for treatment progress.

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1. Introduction

Cancer is among the second most common causes of death accounting for 8.8 million deaths in 2015 according to World Health Organization (WHO) (Collaboration, 2019). Recently, cancer

treatment has been switched from cytotoxic chemotherapeutic agents to targeted therapies. Drugs designed to target mutated or overexpressed molecules within the cancer cells show more efficiency and less toxicity compared with the traditional chemotherapeutic agents due to their high selectivity (Natoli et al., 2010; Wani et al., 2018). Tyrosine kinase inhibitors (TKIs) are classified as small pharmaceutical molecules that target tyrosine kinases and one of these inhibitors, neratinib, has been recently approved by the United States Food and Drug Administration (USFDA) to treat HER2 + breast cancer (Ito et al., 2012; Martin et al., 2013).

Neratinib (Nerlynx) irreversibly inhibits tyrosine kinase targeting HER2 and preventing signal transduction by blocking the phosphorylation of the tyrosine domain, leading to inhibition of cell growth, migration, and induction of apoptosis (Schwab et al., 2015; Wani et al., 2020, 2015). The most common reported side

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Peer review under responsibility of King Saud University.



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effects associated with neratinib treatment are gastrointestinal, dermatological, ocular and hepatic symptoms (Davis, 2016; Huillard et al., 2014; Tanaka et al., 2014; Wong et al., 2009). Several reports have shown that neratinib treatment altered the expression of a variety of genes that are associated with cell cycle (O'Neill et al., 2013; Vogel and Marcotte, 2012; Wang et al., 2015) but to validate these results additional studies to explore other genes needs to be carried out. In this study, mechanism of action of neratinib at molecular level by elucidating the differential expression of genes on cancer induced mice. Studying cellular responses in the development of drugs is a useful approach for assessment of the safety and efficacy of these drugs. Several studies have reported some changes in genes expression after neratinib treatment, such as heat shock protein 90 (*Hsp90*) and genes associated with Akt pathway, including RB1 inducible coiled-coil 1 (*Rb1cc1*), forkhead box O3 (*Foxo3a*), and cyclin D1 (*Ccnd1*) (O'Neill et al., 2013; Schwab et al., 2015). Calcium-activated chloride channel 3 (CLC3) is a member of the CLC voltage-gated chloride (Cl⁻) channel superfamily. Previous studies have shown that CLC3 is associated with many diseases like several types of cancers and cardiovascular abnormalities as well as regulation of cell cycle (Duan, 2011). The CLC3 (Cl⁻ channel) has been associated to cell volume regulation, proliferation and apoptosis in vascular smooth muscle cells (Fan et al., 2006).

Cyclin-dependent kinase inhibitor 3 (CDKN3) is a member of protein phosphatase family that dephosphorylates CDK1 and CDK2 and has a potential role in regulating cell cycle progression. The CDKN3 has contradictory roles in different types of cancers. CDKN3 has been found downregulated in glioblastoma and hepatocellular carcinoma. In contrast, CDKN3 is found highly expressed in breast and prostate cancers (Nalepa et al., 2013; Rozovskaia et al., 2003; Yeh et al., 2003). Prior studies have reported the correlation of CDKN3 expression with reduced survival in several types of cancers, including breast, prostate, colorectal (Cress et al., 2017), hepatic (Wang et al., 2011) and head and neck cancers by controlling mitosis (Wang et al., 2017).

Expression profiling studies of tyrosine kinase inhibitors are focus of present studies on elucidating the changes in gene expression triggered by drug treatment which can be potentially linked to the drug's mode of action. This study can give new insight into mechanism of treatment by tyrosine kinase inhibitors and their acquired resistance that refers to the role of protein tyrosine kinases (PTKs) and the examination of their inhibitors as anti-cancer drugs (Jiao et al., 2018). In this study the effect of neratinib was studied on genome coding transcripts of cancer mouse model to elucidate the drug's mode of action on mouse transcriptome.

2. Materials and methods

2.1. Animals

Adult male SWR/J nude mice weighing 27–39 g were obtained from the Animal House Facility of King Saud University (SA) and caged in four groups with four in each cage: Normal group, normal group treated with neratinib, cancer induced group and neratinib treated cancer group. All the factors like microarray platform, assay protocol, data pre-processing were equal hence fewer animals were needed than human subjects (Jiao et al., 2018). All the samples were run in replicates. The experiments were conducted under the guidelines approved by King Saud University Ethical Review Board (IRB# no. KSU-SE-19-07). The mice were provided with fresh drinking water daily and fed rodent chow (Purina, CA) ad libitum and kept between 23 °C, 55–60% ambient humidity, under 12 h light and 12 h darkness until the experiments were

done. Human SKBR3 HER2+ breast cancer cells (ACTT, US) were used to induce cancer in mice models according to previous literature (Wang et al., 2015). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (Gibco, US) and cultured in 75 cm³ tissue culture flasks at 37 °C under a 5% CO₂ humidified environment. The mice were induced with intraperitoneal (i.p) injection of 5x10⁶ SKBR3 cells for 14 days to generate i.p carcinomatosis (Fischer et al., 2008). The treated group was treated orally with 40 mg/kg neratinib (Sigma-Aldrich, US) dissolved in 99.9% DMSO (Sigma-Aldrich, US) for two days and were sacrificed after 24 h of treatments (Tanaka et al., 2014).

2.2. Visual characterization and Histopathology of mouse model

After visual characterization, mice were sacrificed by CO₂ asphyxiation. After RNA extraction, livers were washed with PBS, fixed in 10% formalin, embedded in paraffin blocks, sectioned to 4–5 mm thick slides and stained with hematoxylin and eosin and then examined by an expert pathologist who was not informed about the sample assignment to the experimental groups using light microscopy (Leica DM 3000, DE) (Fischer et al., 2008).

2.3. RNA extraction and characterization

RNA was extracted by RNA Kit (Qiagen, CH) according to the manufacturers' instructions and RNA samples were characterized by measuring the concentration and absorbance using Agilent 2100 Bioanalyser (Agilent Technologies, US).

2.4. Affymetrix GeneChip Mouse Genome U133 Arrays

RNA samples that yielded RNA Integrity Number values equal to or greater than 8 were considered for global gene expression profiling performed in two duplicates for each group using Affymetrix GeneChip Mouse Genome U133 Arrays (Affymetrix, USA). Briefly, 500 ng of RNA samples were reverse transcribed to synthesize first strand of cDNA as well as *in vitro* transcription step reaction to synthesize the second strand. After purification, fragmentation, and washing, the hybridized microarrays were scanned and the intensity (cel) files with the acquisition and initial quantification of array images were generated using Transcriptome Analysis Console (TAC) for logarithmic ratio of difference in expression. Significantly modulated genes were defined as those with absolute fold change (FC) >2 and adjusted p-value < 0.05.

2.5. qPCR

The relative expression of CLC3 and CDKN3 was measured by qPCR assay using the ABI 7500 Sequence Detection System (ABI, US). For this purpose, 100 ng total RNA extracted were reverse transcribed into cDNA using Sensiscript Kit (Qiagen, US) according to manufacturer instructions. The PCR assay was performed using Quantitect SyBR GreenKit (Qiagen, US), employing GAPDH as the endogenous control All reactions were conducted in triplicates and the data were analyzed using the delta delta CT method. The sequence of primers is listed here:

Gene	Primer sequence	Amplicon size (bp)
<i>Gapdh</i>	F GCCTGCTTACCACCTTCT	84
	R CCCCAATGTGTCCGTCGTG	
<i>Clc3</i>	F ACAGAAAAGCTCGGCGGATG	126
	R CGTCAGCCACCACTTAGGG	
<i>Cdkn3</i>	F CCTGTCTTGCTGCTGTCTC	85
	R CCTCTGACATCTCGAAGGCT	

2.6. ELISA

The levels of CLC3 and CDKN3 were determined by ELISA Kits according to the manufacturer's instructions (Bioassay Technology Laboratory, CN). Several dilutions of BSA were prepared ranging from 150 to 1800 ng/L and the samples were diluted to 10 ng/L. After that, the standards and the samples were added to the ELISA plate with streptavidin-HRP and incubated for 1 h at 37 °C. Then, the plates were washed 5 times by washing buffer and the substrates were added and incubated for 10 min at 37 °C. Finally, the stop solution was added to all wells and then the absorbance was read at 450 nm.

3. Results

Cancer was confirmed after the tumor size on hind limbs was at least 0.5 cm. Histopathological analysis of liver revealed neoplasma revealed by increased cell numbers and morphology of cells. The neratinib treated cancerous group, however, showed changes associated with the anticancer effect of neratinib. The Liver histology of mice groups showed normal hepatocytes with well-distinguished nucleus and cytoplasm in control and neratinib treated normal groups. Abnormal growth with dense cells and constriction in the hepatic vein were seen in cancerous group depicting the induction of cancer and soft organ metastasis. The hepatic vein in neratinib treated cancerous group showed less constriction with less cells

as well as the appearance of necrotic cells. Normal hepatocytes with well-distinguished nucleus and cytoplasm can be seen in neratinib treated control with no difference noticed than normal control (Fig. 1). Also cancerous groups developed eye pathologies within two days of treatment with neratinib while the normal groups didn't show any such pathologies.

Samples that yielded RIN equal to or greater than 8 were considered for microarray analysis and two duplicates of each sample were run on Affymetrix GeneChip Genome U133 Arrays (Affymetrix, USA). The chromosome maps imported from TAC (Fig. 2a, b) showed the locations of the differentially expressed genes on the chromosomes. Fold change cut-offs of >2 and p-values of < 0.02 were chosen to select the genes that were significantly variable in expression between the groups. The differentially expressed genes after treatment were 1481 in which 532 were up-regulated and 949 were down-regulated. Volcano plots of differentially expressed upregulated transcripts were shown in red color and down regulated in green color are shown in Fig. 2c). Volcano plots provide global view of expression levels compared to heat-maps. In volcano plot, the log transformed adjusted p-values are plotted on the y-axis and log₂ fold change values on the x-axis. The volcano plot can be easily drawn by using ggplot2. In volcano plot, results shown clearly indicates whether or not the gene is considered differentially expressed based on p-adjusted and log₂ fold change values.

Fig. 3 shows differential expression of some common genes and their links with pathways associated with apoptotic and prolifera-

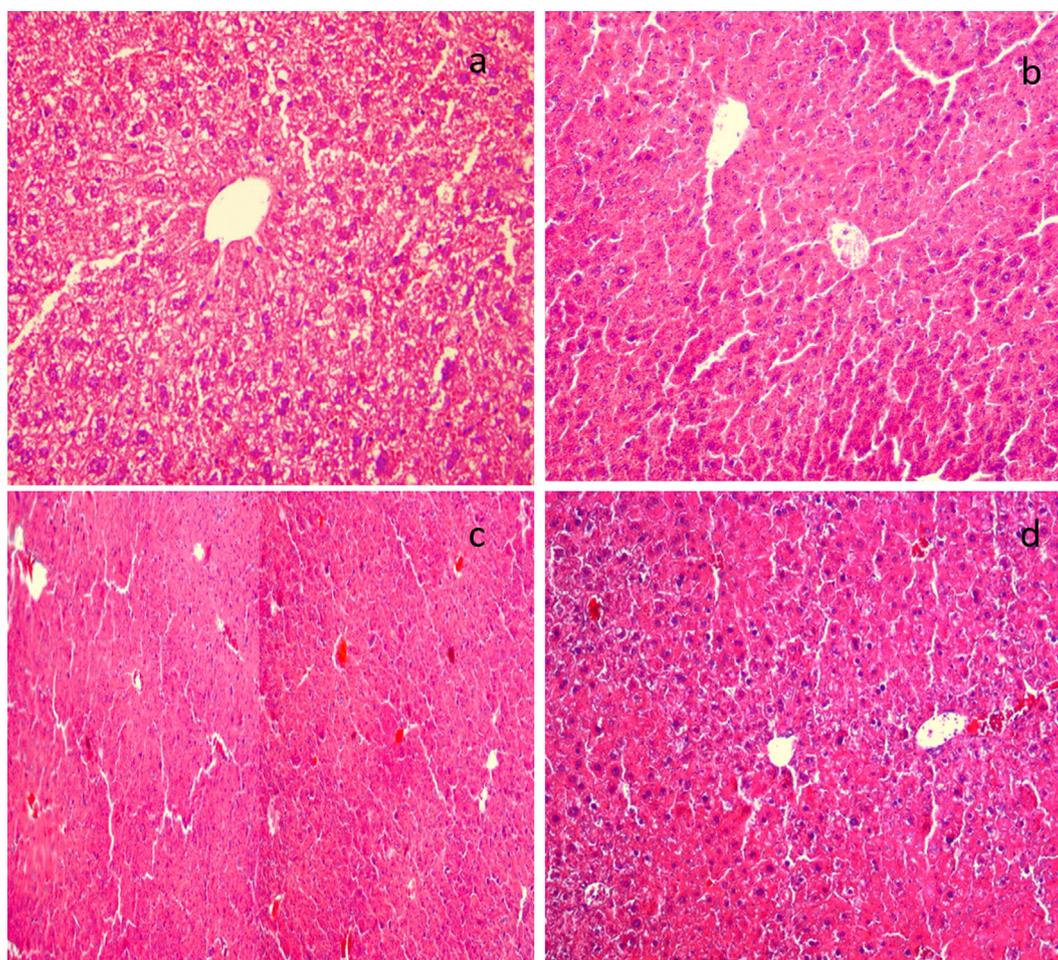


Fig. 1. Liver histology for the mice groups. (a) Normal liver, (b) neratinib treated normal, (c) cancerous mouse model liver, and (d) neratinib treated cancerous mouse model liver. Normal hepatocytes with well-distinguished nucleus and cytoplasm can be seen in (a and b) Abnormal growth with dense cells and constriction in the hepatic vein are seen in (c) reflecting the induction of cancer.

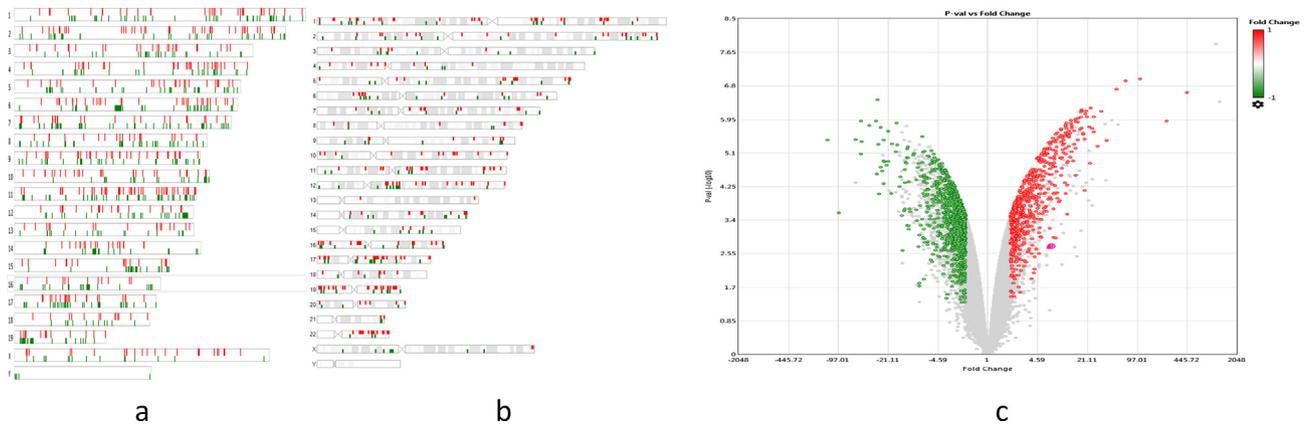


Fig. 2. Chromosome summary of microarray analysis after neratinib treatment and Volcano plots showing fold change against the p-value of differentially expressed genes in experimental groups. (a) Chromosome map of cancerous group. (b) Chromosome map of neratinib treated cancerous group. (c) Volcano plot of differentially expressed transcripts (p-values < 0.02) with red spots representing up-regulated and the green ones representing the down-regulated genes with fold change in X-axis and P value on Y axis. The grey colored region represents the non-significantly differentially expressed gene.

Pathway associated with the differentially expressed genes

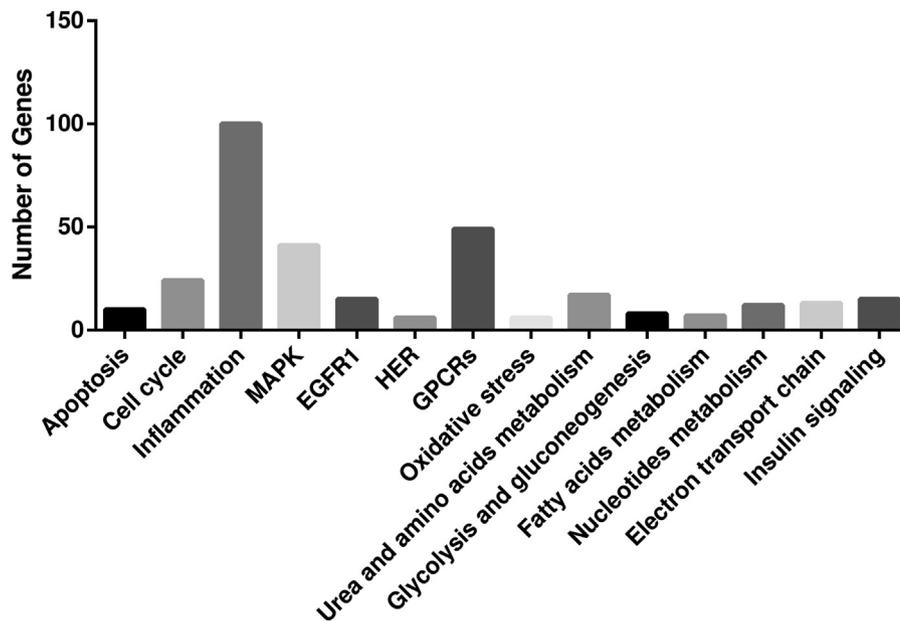


Fig. 3. The Pathway analysis of differentially expressed genes and their associated pathways. The number of differentially regulated genes were plotted against each pathway. This analysis showed the involvement the differentially regulated genes in many metabolic pathways, such as fatty acids, glycolysis, nucleotides, and amino acids metabolism, with maximum involvement of genes of cell cycle, inflammation, MAPK and GPCRs.

tion. The functional pathway analysis showed the differential expression of maximum genes of four main pathways namely cell cycle, Inflammation, G-protein coupled receptors (GPCRs) and Map kinases (MAPK). As depicted in Fig. 4 almost 25 genes of cell cycle and 100 genes of inflammation were downregulated while as 40 genes of Map kinase pathway and 50 G-protein coupled receptors were upregulated with neratinib treatment in cancer groups than without treatments. The differentially expressed genes were considered significant under the criteria of an adjusted p-value < 0.02 and \log_2 ratios ≥ 1.0 and/or \log_2 ratios ≤ -1.0 means two Fold change. Table 1 highlights highly significant of the differentially regulated genes associated with many biological pathways, including cell cycle, oxidative stress, inflammatory response, and voltage-gated channels. The functional annotations were taken from UniProt database.

Fig. 4 shows gene expression of Ccl3 and Cdkn3, the proliferation markers. The increased levels in cancer groups were significantly decreased after neratinib treatment showing the involvement of these two genes in drug's mechanism of action. Ccl3 gene expression correlated with CCL3 protein expression while as CDKN3 protein levels increased after treatment showing protein stability increases after neratinib treatment than mRNA stability.

4. Discussion

Neratinib is an irreversible pan-HER inhibitor that has promising activity in treating solid tumors, however, more studies are needed to elucidate its mechanism of action and side effects.

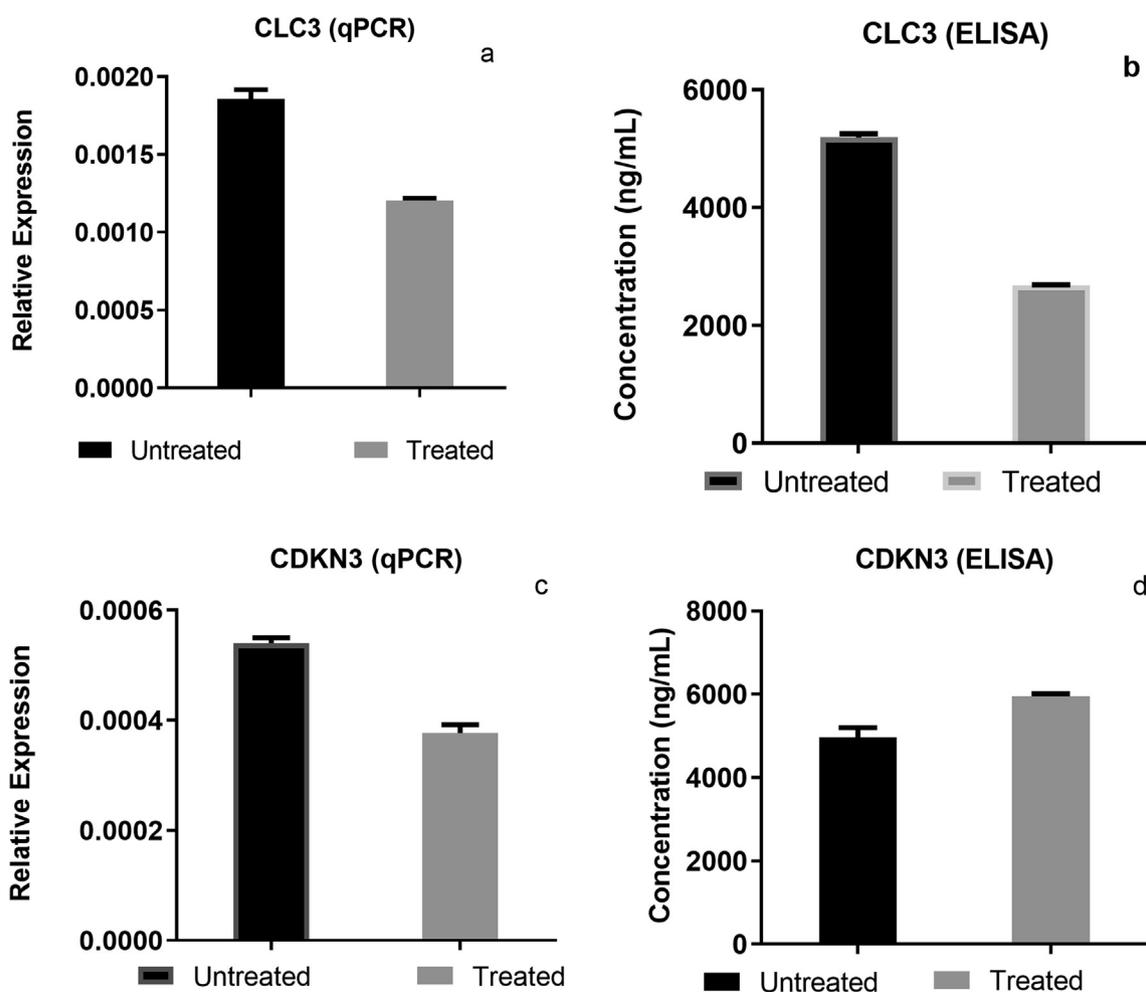


Fig. 4. Gene expression and protein expression levels of Clc3 and Cdkn3. The increased mRNA expression levels in cancer induced group decreased after neratinib treatment. The data represent the mean \pm standard error ($n = 3$, **** $p < 0.0001$). CLC3 Protein levels showed significant decrease after treatment. The data represent the mean \pm standard deviation (* $p < 0.05$, ns: non-significant). CDKN3 protein levels increased after treatment. The data represent the mean \pm standard deviation (ns: non-significant).

Microscopic examination of liver tissues revealed that neratinib treatment has potential effects on cancerous tissues whereas no changes were seen in the normal treated tissues (Fig. 1). Hematoxylin and eosin staining of liver tissue showed abnormal growth and constriction in the hepatic vein after cancer induction as well as appearance of necrotic cells that was reversed after neratinib treatment with less cells and less constriction of the hepatic vein. Similar observations of hepatic cells necrosis as well as cell shrinkage were seen in a patient after imatinib treatment which is a TKI and has a similar action as neratinib (Foringer et al., 2005). These results are consistent with earlier studies (Foringer et al., 2005; Pou et al., 2003) where the patients developed renal and hepatic failure after imatinib treatment. Neratinib treatment within two days showed ocular side effects on cancerous mice group. Four out of eight mice treated with neratinib (4 normal and 4 cancer treated) exhibited ocular toxicity. Similarly, in the previous studies, one out of nine mice showed atrophy in the corneal epithelium after neratinib treatment (25 mg/kg) (Davis, 2016; Yao et al., 2015). These symptoms are consistent with the ocular side effects reported for TKIs since HER1 is highly expressed in the eyes (Chang et al., 2011; Rajala et al., 2017). Microarray analysis showed differences in gene expression after neratinib treatment in the cancerous group. Approximately, 1481 transcripts were differentially expressed after treatment among which 532 were up-regulated and 949 genes were down-regulated (Fig. 3). Transcriptome analy-

sis showed down-regulation of major transcripts associated with immunity, such as histocompatibility, T cell receptors, and immunoglobulins. These results are in line with those previous studies on patients treated with TKIs and experienced lower immunity with neutropenia and thrombocytopenia (Rajala et al., 2017). Additionally, ATP-binding cassette sub-family E member 1 (*Abce1*) was down-regulated after treatment which may be associated with drug resistance reversal action by neratinib when treated with other anticancer drugs. Moreover, genes associated with cell cycle regulation were up-regulated after treatment, including retinoblastoma 1 (*Rb1*), f-box protein 7 (*Fbxo7*), forkhead box N3 (*Foxn3*), and mitogen-activated protein kinase kinase 3 (*Map2k3*) which are involved in cell cycle arrest and apoptosis induction as well as genes associated with oxidative stress like glutathione-disulfide reductase (*Gsr*), cytochrome B-245 (*Cyba*), catalase (*Cat*), and NAD(P)H quinone dehydrogenase 1 (*Nqoa*) (Fig. 4). Interestingly, neratinib may have a stress induction effect on the cell since it up-regulated the genes involved with stress response activation and down-regulated the genes that inhibit ROS production, resulting in stress response activation and higher production of ROS. These findings are consistent with previous studies of some TKIs that induced ROS production which eventually causes the increasing of oxidative stress enzymes. Chang et al (2011) reported an increased ROS production in melanoma cell lines treated with imatinib (Chang et al., 2011). Likewise, Okon et al (2015) studied the

Table 1

Differently expressed genes compared between cancer and after neratinib treatment with highly significant *P* values according to Benjamini-Hochberg method. All the genes were significantly chosen by making comparison with both normal control and neratinib treated control groups.

Gene symbol	Chromosome	Fold change	Differential expression between treated and cancerous	Functional annotation*	P-value of differential expression	Pathway
Apol10a	15: 77,477,047–77,491,069	75.95	Upregulated in cancer vs control and downregulated in cancer vs treated	Lipid exchange and cholesterol transport	<0.0001	Endocytic lysosomal degradation Pathway
Cdkn3	14: 54,396,867–54,420,217	20.12	Upregulated in cancer vs control and downregulated in cancer vs treated	Cell cycle	<0.01	Cell proliferation and invasion Pathway
Hagh	17: 24840143–24864450	13.62	Upregulated in cancer vs control and downregulated in cancer vs treated	Oxidative stress	<0.0001	Oxidative stress
Chil3	3: 106147554–106167564	22.99	Upregulated in cancer vs control and downregulated in cancer vs treated	Inflammation	<0.0001	Inflammation
Clc3	8: 60910389–60983317	15.04	Upregulated in cancer vs control and downregulated in cancer vs treated	Exchange of ions against protons and regulation of neural cell function	<0.0001	Neutralization of the electron flow generated by Nox1
Map2k3	11: 60932054–60952811	8.06	Upregulated in cancer vs control and downregulated in cancer vs treated	Stress induced activation and induction of inflammation and apoptosis	<0.0001	Inflammation and apoptosis
Olf1055	2: 86,346,624–86,350,284	8.82	Downregulated in cancer vs control and upregulated in cancerous with treatment	Olfaction	<0.001	G protein-coupled receptor signaling pathway
Abce1	8: 79683442–79711740	5.02	Downregulated in cancer vs control and upregulated in cancerous with treatment	Multidrug resistance	<0.0001	Type I interferon-mediated signaling pathway
P2ry13	3: 59207892–59210882	6.51	Downregulated in cancer vs control and upregulated in cancerous with treatment	Vision	<0.0001	G protein-coupled receptor signaling pathway
Mycbp2	14: 103113411–103346840	2.62	Downregulated in cancer vs control and upregulated in cancerous with treatment	Synapse and vision	<0.0001	Breast Cancer and ubiquitination Pathway
Ccnd2	6: 127125162–127152188	5.07	Downregulated vs control and upregulated with treatment	Cell cycle	<0.0001	ubiquitin-mediated proteolysis and cell cycle pathway
Lbh	1: 72918305–72941946	5.61	Downregulated vs control and upregulated with treatment	Modulation of cardiogenesis	<0.0001	WNT Signaling Pathway
Tubb5	6: 30,720,351–30,725,421	4.09	Downregulated vs control and upregulated with treatment	Drug binding, microtubules function	<0.0001	Phagocytosis pathway

* The functional annotations were taken from UniProt ([Consortium, 2014](#)).

effect of gefitinib treatment on lung cancer cell lines and found that gefitinib increased ROS production (Okon et al., 2015). Furthermore, changes in glycolytic genes expression were observed, including glucose carrier family 2 (*Slc2a1* and *Slc2a3*), and enolase 3 (*Eno3*), and various genes associated with amino acids metabolism, including arginase 2 (*Arg2*) and arginosuccinate synthetase 1 (*Ass1*), as well as genes involved in tricarboxylic acid cycle (TCA), such as oxoglutarate dehydrogenase (*Ogdh*), fumarate dehydratase (*Fh1*), and isocitrate dehydrogenase (*Ish3b*). Similar effects were reported after TKIs treatment on glycolysis, amino acids, and energy metabolism genes expression (Anderson et al., 2018; Hong et al., 2015). These changes in expression may contribute to the apoptotic effect of TKIs, including neratinib, since cancerous cells rely heavily on glycolysis and TCA pathways as fuel sources. Because of their role in apoptosis and proliferation that is main mechanism behind neratinib's action, we further validated CLC3 and CDKN3 genes by qPCR and ELISA.

ELISA analysis showed that CLC3 levels significantly decreased after neratinib treatment and directly correlated with the gene expression. This may be because CLC3 contributes to cancer development since the proliferation process requires an increase in cell volume that is important for proliferation mechanism. Previous reports about neratinib's potential action on CLC3 to prevent its proliferative role is consistent with present study (Hong et al., 2015). In this study, CDKN3 protein levels increased after treatment which is reverse of gene expression where the increased gene expression in hepatocellular cancer significantly decreased with neratinib treatment. This may be due to the apoptotic effect of the protein by inactivating cyclin-dependent kinase 2 via dephosphorylation resulting in the inhibition of cell cycle progression. Further, mRNA may be modified by methylation on which the translational efficiency is dependent e.g. m6a methylation can allow cap independent translation under stressed conditions. Another factor for increased protein levels may be more stability of protein formed in stressed conditions. Some protein have a long half life while others have to be immediately destroyed for proper function (Yu et al., 2017). Further, in previous studies CDKN3 expression was negatively-associated with the pathological stage of the tumor. Inhibition of CKDN3 promoted the clonogenic capacity and chemotherapeutic tolerance and hence cell survival (Dai et al., 2016). Also, CDKN3 is an important contributor to cellular senescence and can interact with Mdm2 and form a complex with p53 and Mdm2 (Huda et al.), hence showing contradictory results between protein and gene expression. In conclusion, these results have expanded our knowledge about the molecular mechanisms of action of neratinib on HER⁺ cancers, which will provide potential directions and valuable resources for further research on HER⁺ cancer response to neratinib. In future, other differentially expressed genes can be validated by qPCR and ELISA. Also, additional studies similar to this study can be performed on other cancer types and other tissues, to confirm CDKN3 and CLC3 as efficient biomarkers. Further studies with larger sample sizes can be used to validate the current findings.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Deanship of Scientific Research, King Saud University; Research group No. RG-1435-073

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