Alterations of Genetic Variants and Transcriptomic Features of Response to Tamoxifen in the Breast Cancer Cell Line

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Abstract

Background

Breast cancer is one of the most important causes of mortality in the world, and Tamoxifen therapy is known as a medication strategy for estrogen receptor-positive breast cancer. In current study, two hypotheses of Tamoxifen consumption in breast cancer cell line (*MCF7*) were investigated. First, the effect of Tamoxifen on genes expression profile at transcriptome level was evaluated between the control and treated samples. Second, due to the fact that Tamoxifen is known as a mutagenic factor, there may be an association between the alterations of genetic variants and Tamoxifen treatment, which can impact on the drug response.

Methods

In current study, the whole-transcriptome (RNA-seq) dataset of four investigations (19 samples) were derived from European Bioinformatics Institute (EBI). At transcriptome level, the effect of Tamoxifen was investigated on gene expression profile between control and treatment samples. Moreover, Tamoxifen is known as a mutagenic factor, therefore, its contribution to alterations of genetic variants and drug response were examined.

Results

Results achieved from RNA-seq analysis indicated the contribution of several candidate genes to tumor suppression process and consequently, the achievement of an effective treatment. For instance, *XIAP*-associated factor 1 (*XAF1*) was reported as an up-regulated gene under Tamoxifen therapy. *XAF1* is a tumor suppressor that contributes to the apoptosis induction and tumor growth inhibition along with *TP53*. Results of gene ontology enrichment analysis of differential gene expressions indicated that most of them could considerably lead to the cell death, apoptosis, and negative regulation of proteolysis process. Findings achieved from evaluating Tamoxifen mutagenicity effect on drug response was not confirmed perfectly. The most reported candidate genes, which were related to differential genetic variants between control and treated samples, played the oncogene and tumor suppressor dual roles and also their exact roles in breast cancer were not investigated precisely.

Conclusion

At transcriptome level, Tamoxifen consumption in MCF7 cell line could be associated with candidate genes and biological pathways that contribute to the apoptosis, proteolysis, and tumor suppression. The mutagenicity effect of Tamoxifen and its contribution to drug response was not confirmed perfectly.

Background

Cancer is one of the most important causes of mortality in the world and breast cancer is a common disease among women. Hormonal therapy is a medical strategy for breast cancer treatment (Akram et al. 2017). Tamoxifen is considered as the main non-steroidal drug in breast cancer treatment for postmenopausal women (Abo-Touk et al. 2010), which inhibits the estrogen activity through binding to the estrogen receptor competitively (Geisler, 2011).

There are several investigations carried out with the purpose of illustrating the hormonal therapy effects that provide a better understanding of the drug response mechanism and select an effective strategy for the therapeutic period (AlFakeeh and Brezden-Masley, 2018; Szostakowska et al. 2019; Fan et al. 2015; Moiseenko et al. 2017). The appropriate drug response is a complex interdependent procedure that is highly dependent upon several factors including the genetic variants background, lifestyle, climate, smoking, and alcohol consumption (Levy and Polatsek, 2002; Bachtiar and Lee. 2013).

Genetic variants refer to the genetic differences between individuals of a population (Kharrati et al., 2012). DNA is a vulnerable molecule against various mutagens including ultraviolet, toxins, chemical agent, and free radicals (Koopae and Esmailizadeh, 2014). Recently, high-throughput sequencing platforms have been applied as powerful tools in order to investigate the association between a massive number of genetic variants and drug response (Madian et al. 2012; Esfandiari et al., 2020).

It is shown that Tamoxifen has a mutagenic effect on the endometrium cells and increases the incidence of endometrial tumors (White and Smith, 1996). Results of evaluating the rat hepatic tissue showed that activated Tamoxifen could bind to guanine N2-position of DNA and consequently, produce pro-mutagenic lesion (Brown, 2009). More importantly, it was found that Tamoxifen mutagenicity effect induced DNA damages in human endometrial cells (Liapis et al., 2008). Considering the in vitro conditions, Tamoxifen would lead to the gene mutations and increased incidence of abnormal chromosomal structures in rat liver tissues (Davies et al., 1997). All of the above-mentioned literature reviews indicated that Tamoxifen could play a critical role in the alterations of genetic variants background. There are several examples about the role of genetic variants in drug response. To achieve a therapeutic effect, there has to be an interaction between the drug and its target. DNA variations can both increase and decrease the binding affinity of a drug to its target. In addition, genetic variations can transform the antagonist role of drug into an agonist one; therefore, the most common problem of treatment procedures is resistant mutations in drug targets. Tamoxifen blackens Estrogen (ER-positive cancer) in the breast cancer treatment procedure and consequently, decreases the risk of cancer recurrence. Tamoxifen is an anti-estrogen hormone that inhibits the Estrogen receptors; however, its efficiency would be decreased as a result of the occurrence of mutations in estrogen receptors and it leads to the conversion of RE-positive into progesterone-positive cancer. Consequently, it causes the drug resistance development and nonresponse to treatment (Wardell et al. 2019).

It is noteworthy that genetic variations may contribute to drug metabolism and influence the drug response. For instance, if the drug is rapidly metabolized, its concentration will decrease due to the

weaker drug action or side effects. Considering slower metabolism procedures, higher drug levels would result in the stronger or longer actions and side effects (Levy and Polatsek, 2002).

Gene expression analysis at transcriptome level is considered as another considerable tool that can show the effects of hormone therapy on genes expression profiles. It has been shown through several studies that there is a close association between transcriptome response and therapeutic drug consumption (Sjöström et al., 2020; Fekete et al., 2019; Men et al., 2018; Kumari et al., 2017 and Selli et al., 2016). It was found from one of the main investigations that carried out a comprehensive transcriptomic analysis on Tamoxifen resistance that ncRNAs profiling breast cancer cells would provide a new light on the identification of novel endocrine resistance biomarkers (Gao et al., 2020). Lanceta et al. (2020) carried out RNA sequencing (RNA-seq) and pathway analysis in ER + MCF7, and reported 2183 up-regulated and 1548 down-regulated transcripts that contributed to cell cycle, DNA replication, and DNA repair and autophagy.

Current study investigates two hypotheses of Tamoxifen consumption in breast cancer cell line (MCF7). First, there may be an association between genetic variants alterations and Tamoxifen treatment due to the fact that Tamoxifen is a mutagenic factor that impacts on the drug response. Second, how does Tamoxifen affect the gene expression profile at transcriptome level? Investigating Tamoxifen therapy-changed genetic variants and genes expression could be useful to understand the drug response mechanism; also, it would provide a new insight to the increase of the chance of survival, decrease the side effects, and select an appropriate strategy for the therapy period.

2. Materials

The summary of data collections, genetic variant, and RNA-seq analyses are represented in Figure 1.

2.1 Data collection

In current study, the whole-transcriptome (RNA-seq) dataset of four investigations were derived from EBI (<https://www.ebi.ac.uk/>). More details of collected datasets were provided in Table1.

Table1. More details of RNA-seq datasets to discover the genetic variants and gene expression analysis

Accession number	Control samples	Treatment samples	Drug type	Cell line
E-MTAB-822	1	2	Tamoxifen	MCF7
E-GEOD-59536	1	1	Tamoxifen	MCF7
E-GEOD-62613	1	1	Tamoxifen	MCF7
E-GEOD-78199	6	6	Tamoxifen	MCF7
Total	9	10	—	—

2.2 Quality control and trimming

It was observed that various parameters led to the appropriate quality control functions in CLC Genomic Workbench (12) for each sample including length distribution, GC content, ambiguous base content, Phred score, nucleotide contribution, enrich 5 mers, and duplicate sequences (Kharrati-Koopaei et al., 2019). Due to the fact that the adaptor sequences were cleaned in the achieved datasets, the adaptor trimming was not achieved.

2.3 Genetic variants analysis

2.3.1 Reference genome and alignments analysis

The reference genome (hg38) and all annotations were downloaded from Ensembl database (www.ensembl.org). Mapping short reads was carried out against reference genome through CLC Genomics Workbench 12 based on the following parameters: masking track= mRNA sequence, mismatch cost = 2, cost of insertions and deletions = linear gap cost, insertion cost = 3, deletion cost = 3, length fraction = 0.7, and similarity fraction = 0.8 (Mortazavi et al., 2008).

2.3.2 Variant calling and statistical analysis

CLC genomics workbench 12 applied the variant detections algorithm; also, there no constant ploidy level was observed within cancer cell lines. Therefore, the variant calling procedure was carried out using the low frequency algorithm on the basis of the following parameters: required variant probability (%) = 95.0 ignore broken pairs = yes, minimum coverage = 10, minimum count = 2, minimum frequency (%) = 30, base quality filter = Yes, neighborhood radius = 15, minimum central quality = 30, and minimum neighborhood quality = 25 (Doan et al., 2012). Chi-square test was performed with the purpose of explaining the differences of genetic variants distribution between control and treated samples.

2.3.3 Comparing the variants and gene ontology (GO) enrichment analysis

After performing the variants calling process, genetic variants of treatment samples were compared with the reads of control samples in order to remove the common genetic variants among treated and control samples. The file of gene ontology association, which included the gene names and associated gene ontology terms, was downloaded from the gene ontology consortium (<http://geneontology.org/>) and imported to CLC Genomic Workbench 12. Moreover, differential genetic variants were applied to perform GO enrichment analysis at levels of biological process, molecular function, and cellular component. The significance of the level of GO analysis was determined to be 0.01.

2.4 RNA-seq analysis

RNA-seq analysis was carried out based on the annotated genome reference with transcripts using various parameters including mismatch cost=2, insertion cost=2, deletion cost=2, length fraction=0.8, and similarity fraction= 0.8; also, the expression value was calculated through RPKM implementation. Reads per kilobase of transcript, per million mapped reads (RPKM) is a normalized unit of transcript expression. Results achieved from RNA-seq analysis of treated and control samples were compared in order to

identify the differential genes expression (DGE). The significant level ($P \leq 0.01$) was considered as a threshold of DGEs detection. Heat map and volcano plot were implemented to visualize the RNA-seq analysis results; also, the process of RNA-seq analysis was carried out using CLC genomic workbench 12.

3. Results

3.1 Genetic variants detection

Results of quality control indicated that there was not any necessary special trimming strategy for RNA-seq datasets. To minimize the alignment errors, 5% of the short reads with the lowest Phred scores were removed. Results of alignments of short reads against reference genome (hg 38) are provided in Table2. Furthermore, 66%-89% was reported for the mapping percentage.

Table2. The mapping summary of short reads against reference genome

Accession number	Samples	Total reads	Mapped reads%
E-GEOD-59536	T1	89713168	68.80
E-GEOD-62613	T2	112247072	85.91
E-GEOD-78199	T3	34981408	81.22
	T4	36012214	81.20
	T5	40160428	82.10
	T6	41384146	82.05
	T7	39870210	81.72
	T8	41063128	81.74
E-MTAB-822	T9	10069398	87.85
	T10	12018685	83.10
E-GEOD-59536	C1	97511228	66.10
E-GEOD-62613	C2	103822108	87.37
E-GEOD-78199	C3	39172180	82.47
	C4	40347538	82.44
	C5	44328838	80.16
	C6	45695050	80.15
	C7	36382948	82.53
	C8	37484422	82.50
E-MTAB-822	C9	8569125	89.25

T: treated samples, C: control samples

There were almost 5.8 million genetic variants identified in current study including single nucleotide variations (SNVs), multi nucleotide variations (MNVs), insertion, deletion, and replacement. The highest and lowest frequencies among detected genetic variants were respectively related to SNVs and replacement. More details of genetic variants frequencies are provided in Figure 2.

To investigate the effect of Tamoxifen on genetic variants distribution within control and treated samples, a statistical analysis was separately carried out for each genetic variant on the basis of chi-square test for total genetic variants in the control and treatment samples. Results showed that genetic variants distribution within control and treated samples was significant ($P \leq 0.05$). Also, it was found that all of the genetic variants distributions in control and treatment samples were significant (Table 3), which indicated the possible effects of Tamoxifen on the genetic variants frequency.

Table3. Results of statistical analysis of genetic variants distribution in control and treatment samples

Genomic variants	P-value
SNV ^{***}	0.00006
MNV ^{***}	0.0001
Insertion ^{***}	0.0003
Deletion ^{***}	0.0012
Replacement ^{***}	0.001

Results of the comparison between genetic variants of control and treatment samples indicated that there were 117 differential genetic variants. Among all of the differential variants, 13 genetic variants were located in the coding regions and 12 variants led to the amino acid sequence transformation within the protein structure. Table 4 shows more details of differential genetic variants.

Table 4. The classification of differential genetic variants within control and treatment samples

Genetic variants	Differential variants	Coding region	Non-coding regions	Amino acid changes
SNV	93	12	81	11
MNV	9	1	8	1
Insertion	2	0	2	0
Deletion	13	0	13	0
Replacement	0	0	0	0
Total	117	13	104	12

The process of gene ontology enrichment analysis of differential genetic variants was carried out at three levels of biological process, cellular component, and molecular function; therefore, a total number of 77 significant GO terms were reported (Table 5). At the biological process level, the most repetitive of reported overlapping gene names were *GEN1*, *HSPA5*, *NSMCE2*, *AURKA*, and *DDX11* candidate genes. *GEN1* (Flap endonuclease GEN homolog 1) encoded a member of Rad2/xeroderma pigmentosum group G nuclease family. As it was observed for *BRCA1* and *BRCA2*, findings showed that *GEN1* contributed to resolve the Holliday junction in the homologous recombination. It is noteworthy that Holliday junction can play a vital role in the cancer chemo-sensitivity (Wu et al., 2016). Somatic truncating *GEN1* mutations have been reported in breast cancers; therefore, it would indicate the fact that *GEN1* may be a predisposition gene in breast cancer. However, it was shown that although it plays a critical role in the double-strand DNA break repair, *GEN1* would not make any appreciable contribution to breast cancer susceptibility through acting as a high- or intermediate-penetrance breast cancer predisposition gene

such as *BRCA1*, *BRCA2*, *CHEK2*, *ATM*, *BRIP1*, and *PALB2* (Turnbull et al., 2010). Sun et al. (2014) suggested that *GEN1* would play a vital role in DNA damage response; therefore, its alteration could lead to the breast cancer. *HSPA5* (Heat-shock protein 5) is considered as a marker of poor prognosis in breast cancer patients, and plays a critical role in promoting the drug resistance and metastasis (Chang et al., 2014). A close association was observed between the cancer behaviors of heat shock proteins family; however, all members of HSP family have not been studied completely (Zoppino et al., 2018). *NSMCE2* is an E3 SUMO ligase and a subunit of SMC5/6 complex that could be associated with DNA repair (Pond et al., 2019). Although SMC5/6 complex functions were not described precisely, it was reported that it could act as a tumor suppressor in mice (Jacome et al., 2015). *AURKA* (Aurora Kinase A) is a serine/threonine kinase that contributes to the regulation of cell cycle progression; therefore, it could be a potential cancer susceptibility gene (Cox et al., 2006). Furthermore, it is considered as a promising target in the treatment processes of patients with cancer (Staff et al., 2010). *DDX11* is a DNA helicase that plays a role in DNA replication, sister chromatid cohesion establishment, and general chromosome structure. The effects of DNA helicases among patients with cancer are dependent upon their genetic background and tumor type; however, it has not been illustrated precisely and there are various reports of their activities. For example, it was suggested that DNA helicase may have a tumor suppressor function, and the expression level of several DNA helicases at pre-cancerous stages would be increased significantly (Mahtab et al., 2021).

Results achieved from molecular function analysis indicated that the most frequent enriched candidate genes in significant GO term were *IL6ST*, *COX15*, and *FNTA*.

Interleukin-6 (IL-6) is a cytokine released by various cells such as cancerous cells, and plays a vital role in the expansion and differentiation of tumor cells (Masjedi et al., 2018). It was also shown that *IL6ST* may respectively act as a main factor and a tumor suppressor gene in triple-negative breast (TBC) progression, and diagnosis and treatment procedures (Jia et al., 2021). Additionally, *IL6ST* was reported as a specific candidate gene for TBC. Therefore, the expression of *IL6ST* and other three genes (*ANKRD30A*, *ANP32E*, and *DSC2*) classified TBC from non-TBC (Mathe et al., 2015). *COX15* encodes cytochrome C Oxidase subunit 15 and contributes to mitochondrial respiratory chain (UniProtKB: Q7KZN9). Gao et al. (2017) reported that the high-level expression of *COX5B* candidate gene was associated with a poor prognosis in breast cancer. It was suggested that the level of *COX5B* protein may be related to the tumor size; also, its up-regulated form showed a worse disease free-survival. However, there was not enough evidence to explain the clinical implications of *COX5B* in breast cancer. *FNTA* is located on chromosome 8 and encodes the subunit alpha of protein farnesyltransferase (FTase) enzyme (UniProtKB: P49354). It was found that *FNTA* could be a key gene for tumor progression; moreover, its abnormal copy numbers were associated with pathological transformations of breast cancer. Therefore, it could be considered as a main target of developing drugs (Tian et al., 2020). The cellular component analysis showed that nucleus and nucleoplasm were the most important cellular parts that may contribute to the hormone therapy.

Table5. Results of gene ontology enrichment analysis of differential genetic variants between control and treated samples

GO type	GO term	Description	Overlapping gene name
Biological process	0000722	Telomere maintenance via recombination	<i>NSMCE2, SMC6</i>
	0007052	Mitotic spindle organization	<i>AURKA, RAN, TTK</i>
	0007051	Spindle organization	<i>AURKA, TTK</i>
	0007041	Lysosomal transport	<i>IGF2R, VPS54</i>
	0090398	Cellular senescence	<i>NSMCE2, SMC6</i>
	0000724	Double-strand break repair via homologous recombination	<i>GEN1, NSMCE2, YY1</i>
	0034612	Response to tumor necrosis factor	<i>ADAM10, GGT1</i>
	0001315	Age-dependent response to reactive oxygen species	<i>SOD2</i>
	0003069	Vasodilation by acetylcholine involved in regulation of systemic arterial blood pressure	<i>SOD2</i>
	0033316	Meiotic spindle assembly checkpoint	<i>TTK</i>
	0036316	SREBP-SCAP complex retention in endoplasmic reticulum	<i>INSIG1</i>
	0051089	Constitutive protein ectodomain proteolysis	<i>ADAM10</i>
	0060904	Regulation of protein folding in endoplasmic reticulum	<i>HSPA5</i>
	0061015	snRNA import into nucleus	<i>RAN</i>
	0070862	Negative regulation of protein exit from endoplasmic reticulum	<i>INSIG1</i>
	0071139	Resolution of recombination intermediates	<i>GEN1</i>
	0090044	Positive regulation of tubulin deacetylation	<i>FNTA</i>
	1901303	Negative regulation of cargo loading into COPII-coated vesicle	<i>INSIG1</i>
	1901668	Regulation of superoxide dismutase activity	<i>SZT2</i>
	1903891	Regulation of ATF6-mediated unfolded protein response	<i>HSPA5</i>
	1903897	Regulation of PERK-mediated unfolded protein response	<i>HSPA5</i>
	1904426	positive regulation of GTP binding	<i>CLN5</i>
	0060271	cilium assembly	<i>IFT80, INTU, SEPT7, WDR19</i>

0031297	Replication fork processing	<i>DDX11, GEN1</i>
0018279	Protein N-linked glycosylation via asparagine	<i>MAGT1, MCFD2</i>
0000303	Response to superoxide	<i>SOD2</i>
0000768	Syncytium formation by plasma membrane fusion	<i>ERVW-1</i>
0006784	Heme a biosynthetic process	<i>COX15</i>
0006949	Syncytium formation	<i>ERVW-1</i>
0031179	Peptide modification	<i>GGT1</i>
0034085	Establishment of sister chromatid cohesion	<i>DDX11</i>
0035281	Pre-miRNA export from nucleus	<i>RAN</i>
0035437	Maintenance of protein localization in endoplasmic reticulum	<i>HSPA5</i>
0070085	Glycosylation	<i>CLN5</i>
0071140	Resolution of mitotic recombination intermediates	<i>GEN1</i>
0072369	Regulation of lipid transport by positive regulation of transcription from RNA polymerase II promoter	<i>HNRNPK</i>
0090045	Positive regulation of deacetylase activity	<i>FNTA</i>
0097421	Liver regeneration	<i>AURKA</i>

Table5. Continued

GO type	GO term	Description	Overlapping gene name
Biological process	0035437	Maintenance of protein localization in endoplasmic reticulum	<i>HSPA5</i>
	0070085	Glycosylation	<i>CLN5</i>
	0071140	Resolution of mitotic recombination intermediates	<i>GEN1</i>
	0072369	Regulation of lipid transport by positive regulation of transcription from RNA polymerase II promoter	<i>HNRNPK</i>
	0090045	Positive regulation of deacetylase activity	<i>FNTA</i>
	0097421	Liver regeneration	<i>AURKA</i>
	1901980	Positive regulation of inward rectifier potassium channel activity	<i>ALG10B</i>
	1990700	Nucleolar chromatin organization	<i>DDX11</i>
	2001076	Positive regulation of metanephric ureteric bud development	<i>BASP1</i>
	0006631	Fatty acid metabolic process	<i>GGT1, MSMO1</i>
Molecular function	0016740	transferase activity	<i>ALG10B, UHMK1</i>
	0004712	protein serine/threonine/tyrosine kinase activity	<i>AURKA, TTK</i>
	0000400	four-way junction DNA binding	<i>GEN1, YY1</i>
	0005537	mannose binding	<i>CLN5, IGF2R</i>
	0004569	glycoprotein endo-alpha-1,2-mannosidase activity	<i>MANEA</i>
	0016627	oxidoreductase activity, acting on the CH-CH group of donors	<i>COX15</i>
	0033781	cholesterol 24-hydroxylase activity	<i>CYP46A1</i>
	1902945	metalloendopeptidase activity involved in amyloid precursor protein catabolic process	<i>ADAM10</i>
	0003865	3-oxo-5-alpha-steroid 4-dehydrogenase activity	<i>SRD5A1</i>
	0004661	protein geranylgeranyltransferase activity	<i>FNTA</i>
	0004662	CAAX-protein geranylgeranyltransferase activity	<i>FNTA</i>
	0004915	interleukin-6 receptor activity	<i>IL6ST</i>
	0004923	leukemia inhibitory factor receptor activity	<i>IL6ST</i>
	0016653	oxidoreductase activity, acting on NAD(P)H, heme protein	<i>COX15</i>

as acceptor

0019981	interleukin-6 binding	<i>IL6ST</i>
0045509	interleukin-27 receptor activity	<i>IL6ST</i>

Table5. Continued

GO type	GO term	Description	Overlapping gene name
Cellular component	0000803	Sex chromosome	<i>SMC6</i>
	0005634	Nucleus	<i>ADAM10, ANP32A, AURKA, BASP1, COA7, COX15, CUTC, DDX11, DECR1, FBXL3, FGF13, GJB4, GTF2H3, HNRNPK, HSPA5, NSMCE2, PSMD8, RAN, RPAP2, SEPT7, SMC6, SNRPD3, TMPO, TTK, UHMK1, XAF1, YY1, ZNF37A, ZNF471, ZNF480, ZNF550</i>
	0005654	Nucleoplasm	<i>ANP32A, ARPP19, AURKA, CUTC, DDX11, DECR1, GEN1, GTF2H3, HNRNPK, NSMCE2, PSMD8, RAN, RPAP2, RXYLT1, SMC6, SNRPD3, UBXN8, UHMK1, VPS54, WDR19, YY1, ZNF480</i>
	0005783	Endoplasmic reticulum	<i>ALG10B, ANP32A, CLN5, CYP46A1, HSPA5, INSIG1, LCLAT1, MAGT1, MSMO1, RCN1, TECRL, UBXN8</i>
	0005789	Endoplasmic reticulum membrane	<i>CYP46A1, HSPA5, INSIG1, LCLAT1, MCFD2, MSMO1, PIGC, SRD5A1</i>
	0005900	Oncostatin-M receptor complex	<i>IL6ST</i>
	0005953	CAAX-protein geranylgeranyltransferase complex	<i>FNTA</i>
	0008180	COP9 signalosome	<i>BASP1, HSPA5</i>
	0030496	midbody	<i>AURKA, DDX11, HSPA5, RAN</i>
	0030915	Smc5-Smc6 complex	<i>NSMCE2, SMC6</i>
	0042565	RNA nuclear export complex	<i>RAN</i>
	0070069	Cytochrome complex	<i>COX15</i>
	0071598	Neuronal ribonucleoprotein granule	<i>UHMK1</i>

3.2 RNA-seq analysis

PCA results derived from the findings of performing RNA-seq analysis for all samples indicated that control and treated samples were clustered separately (Fig. 3). Therefore, it could be said that Tamoxifen

therapy may impact on gene expression profiles at the transcriptome level among treated and control samples.

The visualization of RNA-seq analysis results derived from control and treated samples were shown in a heat map (Fig 4). It was also shown that mitochondrial respiratory genes were expressed at lower levels within treated samples compared to control ones. In current study, it was reported that *MT-CO1*, *MT-CO3*, *MT-ND2*, *MT-ND4*, *MT-ND5*, *MT-ND6*, and *MT-ATP6* were the mitochondrion respiratory genes. *MT-ND* genes provide NADH dehydrogenase. This protein is a part of a large enzyme complex encoded by the mitochondrial genome. Moreover, the dysfunction of MT-ND proteins would lead to the electron transport chain disruption and ATP production. *MT-CO* genes encode Cytochrome C Oxidase subunits within mitochondria. It is found that they were the last enzyme in the mitochondrial electron transport chain for ATP synthesis (Kharrati-Koopae et al., 2019). Findings derived from heat map analysis suggested that several candidate genes including *ALDOA*, *RPL13*, *HSPB1*, *GATA3*, *KRT18*, *IGFBP4*, and *SULF2* were associated with the lowest gene expression level in treated samples. Aldolase (*ALDOA*) is known as an oncogene, which is a glycolytic enzyme that promotes the metastatic progression of cancers (Chang et al., 2019; Ji et al., 2016). It was shown that there was an association between *ALDOA* knock down and proliferation reduction of breast cancer cells (Zhang et al., 2017). *RPL13* encodes a ribosomal protein, which is a component of 60S subunit. Ribosomal proteins (RP) expression patterns were implemented as a diagnostic strategy in human cancers. Reports of several cancers indicated the dysregulation of RP expression (e.g. *RPL13*) (Dolezal et al., 2018); therefore, it could be said that *RPL13* would be expressed at significantly higher levels in benign breast lesions compared to that of breast carcinomas (GeneCards: GC16P089674). *HSPB1* is a member of heat shock proteins, which are considered as a large family of proteins with breast cancer behavior (Zoppino et al., 2018). It was reported that the down-regulation of *HSPB1* protein may induce the expression of phosphatase and tensin homologue (PTEN) as a tumor suppressor gene. In other words, PTEN stabilization depends upon *HSPB1* low-level expression (Cayado-Gutiérrez et al., 2013).

GATA binding protein 3 (*GATA3*) is a highly conserved transcription factor that belongs to *GATA* family and leads to the expression of a large number of important genes (Voduc et al., 2008). Furthermore, it contributes to the human growth and differentiation cells including the mammary tissue. Lower levels of *GATA3* expression in breast tumors are associated with larger tumors. Therefore, *GATA3* is considered as an important gene in breast cancer development; however, its exact role as an oncogene or tumor suppressor is unclear (Afzaljavan et al., 2021; Takaku et al., 2015). Keratin 18 (*KRT18*) is a member of the intermediate filament family of cytoskeletal protein that is involved in the tissue integrity, and its over-expression has been reported in many cancers (Zhang et al., 2019). It was also reported that *KT18* was over-expressed in breast cancer and played a vital role in the breast tumorigenesis and tumor dedifferentiation (HA et al., 2011). Insulin-like growth factor binding proteins (*IGFBPs*) would regulate many cellular processes such as cell proliferation. *IGFBPs* act as binding proteins for insulin-like growth factor (IGF); furthermore, it is evidenced that they play a critical role in the cancer progression, especially in breast cancer (Hermani et al., 2013). However, there are various reports regarding their activities as oncogenes or tumor suppressors. *IGFBP5* may be considered as an oncogene due to its contribution to

metastasis, proliferation, and limited responses to endocrine treatment; also, it acts as a tumor suppressor because of its apoptotic role, anti-metastatic function, and anti-migratory effects (Akkiprik et al., 2015)

Sulfatase family, which includes sulfatase1 (*SULF1*) and sulfatase 2 (*SULF2*), plays an important role in the multiple biological pathways through regulating the sulfation status (Jiang.et.al, 2020). It was confirmed that *SULF2* would promote the breast cancer progression and regulate the tumor-related genes expression in breast cancer (Zhu.et.al, 2016).

Results of the whole transcriptome analysis showed that there were 21515 DGEs among control and treated samples, while findings of Volcano plot indicated that most of DGEs were classified as the down-regulated genes. At significant levels ($P < 0.01$, $-\text{Log}_{10}(\text{P-value}) > 2$), there were 910 and 3 candidate genes reported as the significant down- and up-regulated ones (Fig.5).

Results of Volcano plot showed that three candidate genes including *GREB1*, *EGR3*, and *XAF1* were clustered as the up-regulated genes. It was also found that the estrogen-based growth regulation in breast cancer 1 (*GREB1*) was an early estrogen-responsive gene, and there was a close association between *GREB1* expression and estrogen levels in breast cancer patients. In fact, *GREB1* was an ESR1 (estrogen receptor 1) that could mediate the estrogen action. It was reported that the optimal level of *GREB1* expression was required for breast cancer cells proliferation (Cheng.et al, 2018). However, *GREB1* knockdown could prevent the breast cancer cell lines proliferation; therefore, it was found that targeting *GREB1* could provide a possible treatment strategy through inhibiting the tumor-promoting pathways (Hodgkinson.et al., 2018). Early growth response (*EGR*) is a family of transcription factors that contributes to various biological pathways (Pio.et al., 2013). It was reported that *EGR3* could be induced by estrogen in breast cancer *MCF-7* cells and consequently, become involved in the estrogen-signaling pathway (Inoue.et al., 2004). Moreover, *EGR3* levels were significantly higher within tissue samples derived from patients with recurrent breast cancer compared to those with primary tumors (Knudsen.et al., 2020). XIAP-associated factor 1 (*XAF1*) is a tumor suppressor observed in the multiple human neoplasms (Shin et al., 2017). It was shown that *XAF1* loss expression would be resulted from tumor staging and its dysfunction was associated with tumor progression. Moreover, its appropriate expression could play a critical role in the apoptosis inductions and tumor growth inhibition in the gastric cancer (Tu.et al., 2009). Pinto.et al. (2020) reported that *XAF1* may be considered as a *TP53* function modifier through increasing the transcriptional activity of hypomorphic *TP53* variants. *TP53* is one of the most significant tumor suppressor genes, which is commonly mutated in various cancers such as breast cancer (Børresen-Dale, 2003).

It is noteworthy that *PROM1*, *FBN2*, and *KLHL14* were highly down-regulated (Fig.5). Prominin 1 (*PROM1* or *CD133*) is known as a biomarker of cancer stem cells; however, its biological role is not illustrated perfectly (Saha et al., 2020). Findings showed that there was an association between *PROM1* levels and malignancy properties stages including initiation, progression, and metastasis. Moreover, it was reported that *PROM1* would contribute to the cell motility and invasion, and may affect the

malignancy of breast tumors. Also, *PROM1* genes were highly expressed in TNBC cell lines (Brugnoli et al., 2019; Guo et al., 2017). Fibrillin-2 (*FBN2*) is an extracellular calcium-binding microfibril that contributes to several biological pathways including the bone mineralization, osteoblast maturation, and calcium binding (UniProtKB: P35556). *FBN2* is considered as a biomarker of cancers early diagnosis. For example, Promotor hypermethylation of *FBN2* is associated with colorectal cancer as an early event. In fact, Methylation may lead to *FBN2* down-regulation in primary tumors (Yi.et al., 2012), while Kelch-like protein 14 (*KLHL 14*) belongs to Kelch family genes and interacts with torsin-1A (UniProtKB: Q9P2G3). It was shown that *KLHL 14* was significantly overexpressed in breast cancer compared to normal breast tissues, and had a positive relationship with tumor aggressiveness (Fritzsche.et al, 2006). Moreover, findings indicated the vital role of *KLHL 14* in the development of various cancers including ovarian cancer (Chen.et.al, 2020).

Results derived from GO enrichment analysis of *DGEs* showed that most of *DGEs* were enriched in the regulations of apoptosis and cell death pathways (Table 6). Cell cycle damage is considered as the main cause of cancer incidence; therefore, the balance between proliferation and cell death is disrupted in cancers (Robert and David, 2005). It was shown that apoptosis inactivation would play a vital role in the process of cancer development (Brown and Attardi, 2005). Therefore, it could be said that significant GO term of apoptosis pathways could contribute to cancerous cell death under Tamoxifen therapy. Proteolysis is a hydrolysis reaction that occurs when peptide bonds and proteins are broken down into smaller polypeptides or amino acids. There is an association between the metastasis of malignancy tumor and overexpression of proteolytic enzyme. More importantly, proteolysis inactivation in cancerous tissue plays a critical role in the inhibition of tumor invasion, angiogenesis, and migration (Wyganowska-Świątkowska et al., 2019). Interestingly, our findings suggested that negative proteolysis regulation and consequent regulations of proteolytic pathways could be regarded as considerable GO terms that control the cancer under Tamoxifen treatment (Table 6).

Table6. Results of GO enrichment analysis of DGEs

GO type	GO term	Reference genes in category	Description	DGEs count	Gene names
Biological process	0051248	20	Negative regulation of protein metabolic process	8	<i>CTSZ, GPX1, GSTP1, IGFBP3, ITM2C, MAGEA2, MAGEA3, MECOM</i>
	0010941	30	Regulation of cell death	10	<i>CTSZ, GPX1, GSTP1, IGFBP3, MAGEA3, MALT1, MECOM, MSX1, PAX8, TRIM2</i>
	0030162	6	Regulation of proteolysis	4	<i>CTSZ, GPX1, MAGEA3, MALT1</i>
	0032269	17	Negative regulation of cellular protein metabolic process	7	<i>CTSZ, GPX1, GSTP1, IGFBP3, MAGEA2, MAGEA3, MECOM</i>
	0043281	4	Regulation of cysteine-type endopeptidase activity involved in apoptotic process	7	<i>GPX1, MAGEA3, MALT1</i>
	0045861	4	Negative regulation of proteolysis	3	<i>CTSZ, GPX1, MAGEA3</i>
	0052548	4	Regulation of endopeptidase activity	3	<i>GPX1, MAGEA3, MALT1</i>
	2000116	4	Regulation of cysteine-type endopeptidase activity	3	<i>GPX1, MAGEA3, MALT1</i>
	0043067	29	Regulation of programmed cell death	9	<i>GPX1, GSTP1, IGFBP3, MAGEA3, MALT1, MECOM, MSX1, PAX8, TRIM2</i>
Molecular function	0019904	12	Protein domain specific binding	5	<i>CCN1, CXADR, GPX1, SCNN1A, TCEAL9</i>
	0004602	2	Glutathione peroxidase activity	2	<i>GPX1, GSTP1</i>
Cellular component	0070062	49	Extracellular exosome	14	<i>ACSL4, AKR1B1, CRISPLD1, CTSZ, FSTL1, GREB1, GSTP1, IGFBP7, ITM2C, LDHB, MSN, PSAT1, RAB34, SCNN1A</i>
	0043230	50	Extracellular organelle	14	<i>ACSL4, AKR1B1, CRISPLD1, CTSZ, FSTL1, GREB1, GSTP1, IGFBP7, ITM2C, LDHB, MSN, PSAT1, RAB34, SCNN1A</i>

1903561	50	Extracellular vesicle	14	<i>ACSL4, AKR1B1, CRISPLD1, CTSZ, FSTL1, GREB1, GSTP1, IGFBP7, ITM2C, LDHB, MSN, PSAT1, RAB34, SCNN1A</i>
0044432	28	Endoplasmic reticulum part	9	<i>ACSL4, COL9A2, CTSZ, FADS2, FSTL1, GJA1, IGFBP3, IGFBP7, RCN1</i>

Discussion

Generally, breast cancer tumors are hormone receptor-positive with highly estrogen- and progesterone-dependent growth rates. Tamoxifen is a type of hormonal therapy implemented with the purpose of treating the estrogen receptor-positive breast cancer; also, it can decrease the risk of invasive cancer development. However, it is able to affect the gene expression profile at transcriptome level (Taylor et al., 2010). Furthermore, the activated form of it could bind to DNA and act as a mutagen factor (Brown, 2009); therefore, it may impact on the drug response process through the alteration of genetic variants. To confirm this idea, Chan et al. (2016) showed that the genetic alteration of variants would contribute to drug response and provide a new insight about pharmacogenomics implication. Therefore, it could be said that Tamoxifen consumption may lead to several biological pathways that can increase or decrease the chances of survival and effective treatments. Results achieved from RNA-seq analysis in MCF7 cell line under Tamoxifen therapy revealed that there were several candidate genes and biological pathways that could result in the tumor suppression and consequently, effective treatments. XIAP-associated factor 1 (*XAF1*) can be up-regulated under Tamoxifen therapy; also, it is a tumor suppressor that plays a critical role in the apoptosis induction and tumor growth inhibition in gastric cancer (Tu et al., 2009). Interestingly, it was reported that the combination of *XAF1* with *TP53* would act as a modifier. *TP53* is a key tumor suppressor gene, which is generally mutated in various cancers such as breast cancer (Børresen-Dale, 2003; Pinto et al., 2020). It was found in current study that *PROM1* and *KLHL14* were down-regulated under hormone therapy; also, an association was observed between *PROM1* levels and malignancy features including initiation, progression, and metastasis. Results showed that *PROM1* led to the cell motility and invasion and may impact on the breast tumors malignancy (Brugnoli et al., 2019; Guo et al., 2017). It was observed that there was a high level of *KLHL14* expression in breast cancer compared to the normal breast tissues, which was associated with considerable tumor aggressiveness (Fritzsche et al., 2006). *KLHL14* also played a vital role in the development of various cancers such as ovarian cancer (Chen et al., 2020), and results derived from GO enrichment analysis of DGEs indicated that most of DGEs could considerably result in the cell death, apoptosis, and negative regulation of proteolysis process. Generally, apoptosis has a critical role in the inhibition of cancerous cell growth.

Proteolysis activation in cancerous tissues would result in the tumor invasion (Wyganowska-Świątkowska et al., 2019; Brown and Attardi, 2005). All of the above-mentioned findings were in accordance with those of Rouhimoghadam et al. (2018) who showed that tamoxifen treatment on the breast cancer MCF-7 cell line could lead to the apoptosis induction through activating the

apoptotic signaling pathways including *Tp53* and MAPKs. Comparable results reported that Tamoxifen induced the apoptosis through inhibiting the cancerous inhibitor of protein phosphatase 2A (CIP2A) and phospho-Akt (p-Akt) in ER-negative breast cancer cell lines (Liu et al., 2014). Frasar et al. (2006) also reported that Tamoxifen could regulate the gene expression in breast cancer cells and indicated several Tamoxifen-regulated candidate genes including *SOCS1* and *IEX-1*, which was in accordance with our findings. Cytokine Signaling 1 (*SOCS1*) suppressor is considered as a tumor suppressor that plays a negative regulatory role for cytokine action through JAK/STAT pathway and suppresses the growth of hepatocellular carcinomas. *IEX-1*, which is an immediate early response gene that can widely be expressed in various tissues, is overexpressed in breast cancer cells and has an inhibitory effect on breast cancer cell proliferation. Generally, it could be concluded that Tamoxifen implementation in the treatment procedures could be beneficial at the transcriptome level; however, it may induce the oncogene expression in rodent uterine (Nephew et al., 1996).

Our hypothesis regarding Tamoxifen mutagenicity effect and its role in the drug response was not approved appropriately. It was found that most of the candidate genes with differential genetic variants had dual roles as oncogenes or tumor suppressors; however, their exact contribution in breast cancer has not been investigated precisely.

For example, findings of the genetic variant analysis revealed that differential genetic variants between control and treated samples (under Tamoxifen therapy) were overlapped with *NSMCE2* and *DDX11* candidate genes. *NSMCE2* contributes to DNA repair pathway and acts as a cancer suppressor in mice (Jacome.et.al, 2015). *DDX11* is a member of helicase family, which involves in DNA replication pathways; however, its role in cancer has not been investigated precisely. DNA helicases may have a tumor suppressor function in cancers (Mahtab.et.al, 2021).

It was revealed by GO analysis that reported differential genetic variants were associated with *FNTA*, *HSPA5*, *IL-6*, and *AURKA* candidate genes. FTase enzyme (*FNTA*) could lead to the tumor progression and abnormal copy numbers of *FNTA* were associated with pathological changes of breast cancer (Tian.et.al, 2020). *HSPA5* (Heat-shock protein 5) promoted the drug resistance and metastasis (Chang et al., 2014), while Interleukin-6 (IL-6) contributed to the expansion and differentiation of tumor cells (Masjedi et al., 2018). *AURKA* gene was associated with potential cancer susceptibility and became involved in the cell cycle progression regulation (Cox et al., 2006).

Conclusion

Results of current study that was carried out at transcriptome level showed that Tamoxifen consumption in MCF7 cell line could be associated with candidate genes and biological pathways that contribute to the apoptosis, proteolysis, and tumor suppression. Moreover, Tamoxifen could decrease the expression of candidate genes involved in tumor progression, invasion, and metastasis. The mutagenicity effect of Tamoxifen and its contribution to alterations of genetic variants was not confirmed perfectly. Therefore, it

was suggested that Tamoxifen could not have any significant role in an effective treatment through changing the genetic variants profile.

Abbreviations

EBI

European Bioinformatics Institution

SNV

single nucleotide variation

MNV

multi nucleotide variation

MCF7

Michigan Cancer Foundation-7

DDX11

DEAD/H-Box Helicase 11

DGEs

differential gene expressions

MT-CO1

Mitochondrially Encoded Cytochrome C Oxidase I

MT-CO3

Mitochondrially Encoded Cytochrome C Oxidase III

MT-ND2

Mitochondrially Encoded NADH:Ubiquinone Oxidoreductase Core Subunit 2

MT-ND4

Mitochondrially Encoded NADH:Ubiquinone Oxidoreductase Core Subunit 4

MT-ND5

Mitochondrially Encoded NADH:Ubiquinone Oxidoreductase Core Subunit 5

MT-ND6

Mitochondrially Encoded NADH:Ubiquinone Oxidoreductase Core Subunit 6

MT-ATP6

Mitochondrially encoded ATP synthase membrane subunit 6

ALDOA

Aldolase

RPL13

Ribosomal Protein L13

HSPB1

Heat shock protein beta-1

PTEN

phosphatase and tensin homologue

KRT18

Keratin 18
GATA3
GATA binding protein 3
IGFBPs
Insulin-like growth factor binding proteins
IGF
Insulin-like growth factor
SULF1
Sulfatase1
SULF2
Sulfatase 2
GREB1
Growth regulation in breast cancer 1
ESR1
Estrogen receptor 1
EGR
Early growth response
XAF1
XIAP-associated factor 1
PROM1
XIAP-associated factor 1
TNBC
Triple-negative Breast Cancer
KLHL14
Kelch-like protein 14
FBN2
Fibrillin-2
FNTA
FTase enzyme
HSPA5
Heat-shock protein 5
IL-6
Interleukin-6
GEN1
Flap endonuclease GEN homolog 1
AURKA
Aurora Kinase A
BRCA1
Breast cancer type 1
BRCA1

Breast cancer type 2
COX15
cytochrome C Oxidase subunit 15

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets analyzed during the current study are available in the European Bioinformatics Institution (EBI) repository (Table1).

Conflict of interest

The authors declare no conflict of interest.

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Author's contributions

HKK, MNC and STH conceived the study. HKK contributed to data analysis and prepared the primary draft of manuscript. AD, MD, and KBL revised the manuscript. All authors read and approve the manuscript.

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Figures

Figure 1

The summary of genetic variants detection and RNA-seq analysis of breast cancer cell line under Tamoxifen therapy.

Figure 2

Frequencies of reported genetic variants in the control and treated samples. Parts A and B illustrate the frequencies of detected genetic variants within control and treated samples. A total number of 2,853,482, and 2,988,729 genetic variants were reported for control and treated samples.

Figure 3

The classification of control and treated samples using PCA based on RNA-seq analysis results. Blue and red points show the control and treated samples. Results indicated that hormone therapy may influence the gene expression profile among treated and control samples.

Figure 4

The heat map of RNA-seq analysis for control and treated samples

Figure 5

Results of Volcano plot that visualized the distribution of significant DGEs. Volcano plot shows the statistical significance ($-\log_{10}(\text{P-value})$) versus $\log_2$ (fold change) value. Also, the up- and down-regulated genes were respectively shown in blue and red, while the most statistically significant genes were towards the top of dash line.