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Gene expression study on similarity effect of Thymoquinone on epithelial breast cancer and colorectal cancer cell lines

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Abstract: Adenocarcinoma is known as a common type of cancer which includes 85% of breast carcinoma and 95% of colorectal carcinoma. Up until now, the cytotoxic effect of Thymoquinone on different types of tumor cells has been reported. It was hypothesized that Thymoquinone has similar effect on cancer arising from epithelial cells through gene expression analysis in MCF7 breast cancer and HT-29 colon cancer cell lines. The quantity and quality of RNA samples were identified using RNeasyPlus Mini kit and RNA 6000 Nano LabChip kit, respectively. The purified RNA samples of MCF7 cells were used in two-color 8×60K cDNA array platform with SurePrint Agilent technology. The hybridized cRNA/cDNA probes were identified due to labeling with red and green cyanine dyes using GE_X hybridization buffer HI-RPM. LOWESS normalization reduced the dye bias on the array slide using feature extraction software. Gene ontology analysis was done after performing different steps of filtering to reduce not satisfied genes using Gene Spring software. Two-step RT-qPCR assay using Taq Man fast advanced master mix analyzed the most up and down regulated genes in MCF7 compared to HT-29 cancer cell lines. The 10 different cancer cell lines in a form of universal reference RNA was used as standard data set comparison and as a positive control RNA in cDNA array and RT-qPCR assay, respectively. The T-test statistical analysis of independent samples showed that there is no significant difference between two types of cell lines due to Thymoquinone treatment with *p*-value 0.844. Among the selected genes; *CARD16*, *UGT1A8*, *SLC7A11*, *IFIT1*, *IF16* and *IFIT3* were expressed significantly (0.009, 0.0001, 0.037, 0.098, 0.0001 and 0.033, respectively) in breast cancer compare to colon cancer cells. The findings indicated similar effect of Thymoquinone on cancer arising from epithelial cells.

Keywords: Thymoquinone, Epithelial Cancer Cells, cDNA Array, RT-qPCR Assay

1. Introduction

Carcinoma is the common type of cancer which origin from epithelial cells. Adenocarcinoma is known as a type of epithelial cells which emerges in the body's mucus-secreting glands [1]. Colorectal carcinoma is known as the third common cancer in the world between men and women, and breast cancer is the common cause of death in women worldwide [1]. Thymoquinone is an active compound of *Nigella Sativa* seed which its anti-cancer effect has been reported on various types of tumors. Till now, alteration of some genes has been stated due to the exposure of cancer cells to Thymoquinone. Previous study on pancreatic cancer cells showed that the treatment with Thymoquinone 50 µM concentration for 24 hours is able to produce down-regulated genes involved in tumor growth and metastasis through doing microarray gene expression analysis. The down-regulated genes are *TRIM24*, *SI00A4*, *MMP7*, *RORA* and *MMP13*. Besides, *STC2*, *IL24*, *TRIB3*, *ERRFI1*, *GADD45A*, *CYP27B1* and *RND3* are reported as up-regulated genes. The function of some of these genes such as *TRIB3* and *GADD45A0* is reported to be involved in the activation of MAPK pathway, including JNK and p38-MAPK [2]. In addition, the down-regulation of anti-apoptotic *Bcl2*, *Bcl-xL* and PPAR-γ-related genes due to the Thymoquinone treatment was reported in MCF7 cell line [3]. Furthermore, increase in the expression level of *TP53* gene in colorectal carcinoma cells [4], androgen-positive prostate cancer cells [5] and

human osteosarcoma p53-mutant cells [6] were reported due to the Thymoquinone effect. Down-regulation of *p65, XIAP, Bcl2, COX-2, VEGF* and *p-Akt* genes were seen after Thymoqionone treatment in cholangio-carcinoma (CCA) cell line [7]. Hence, this study designed to find the genes involved in the therapeutic effect of Thymoquinone on carcinoma cells of two different cell lines of breast and colon.

2. Materials and Methods

2.1. Cell Culture and Treatment

RPMI-1640 medium containing L-glutamine (Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen/Gibco) and 1 unit penicillin/streptomycin (HyClone, USA) was used to culture the MCF7 breast cancer cell line (ATCC® HTB22™) and HT-29 colon cancer cell line (ATCC® HTB38™). The cells were seeded at $3\text{-}4\times10^6$ cells/well and subcultures were prepared according to the ATCC protocol. Four biological replicates from each sample were prepared in separate culture flasks. The cells were treated with Thymoquinone 50 µM (Sigma-Aldrich, France) for 24 h. The control cells were treated with 0.05% dimethyl sulfoxide (Sigma-Aldrich, France).

2.2. RNA Preparation

The manufacturer's instructions of RNeasy Plus Mini kit (Qiagen, USA) and RNA 6000 Nano LabChip kit (Agilent Technologies, USA) were followed to identify the quantity and quality of RNA samples, respectively. Spectrophotometer (NanoDrop 2000c; Thermo Scientific) measured the purity and concentration of RNA and Agilent 2100 Bioanalyser determined the RNA integrity number (RIN). The samples with the yield of ≥ 2.0 ng/µl and RIN > 9 were selected for gene expression analysis.

2.3. Gene Expression Assay

The samples of MCF7 breast cancer cell line was used in cDNA microarray experiment and the results were compared with HT-29 colon cancer cell line doing RT-qPCR analysis.

2.4. cRNA Labelling and Hybridization

The isolated RNA samples (200 ng for two-color) from MCF7 breast cancer cells were used to generate the labeled cRNA samples. The Agilent Low Input Quick Amp Labeling kit (USA) containing two cyanine-3 and -5 was used to label samples of Thymoqionone-treated, -untreated and internal control, respectively. According to the reference design protocol for using internal control, the universal human reference RNA (Agilent Technologies) normalized the signal intensity from two-color microarray and reduced the dye bias. The labeled cRNA samples were hybridized with the cDNA on the slide array using GE_X hybridization buffer HI-RPM (Agilent Technologies, USA) in a brochette oven rotated at 10 rpm with temperature 65˚C for 17 h. The slide washing and image processing were followed according to the SureHyb Technology and SureScan High-Resolution Technology, respectively.

2.5. Analysis of cDNA Microarray Findings

The hybridized cRNA/cDNA probes were qualified using Feature Extraction software v10.7.3.1 through removing the outlier pixels and reduction of inliers pixels of features and backgrounds which were produced by cyanine dyes. The probe sets were filtered according to the signal intensity values, detected or not detected flags, data file of breast cancer and coefficient variation (%CV < 50%) using GeneSpring software v12.1. The filtered probe sets were scored using 3 dimensional scatter plot to qualify through principal component analysis. Statistically, T-test unpaired compared treated samples (T) versus untreated samples (C). Multiple testing correction was Benjamini-Hochberg (FDR). The fold change wizard of GeneSpring software identified the up- and down-fold genes. The molecular functions, bio-logical processes and cellular components of gene products yielded from hybridization were identified through Gene Ontology analysis.

2.6. Two-Step RT-qPCR Assay

The 10 most up-regulated and 10 most down-regulated genes resulted from microarray analysis were compared in MCF7 and HT-29 cell lines doing two-step RT-qPCR assay. The high capacity RNA-to-cDNA Kit (Applied BioSystems, USA) reverse transcribed the total RNA samples with 2000 ng/µl concentration to cDNA strand. The thermal cycler (Master cycler gradient, Eppendrof, Germany) amplified cDNA strands. The C_T value of each target gene was identified using TaqMan fast advanced master mix (Life Technologies Corp, Carlsbad, CA, USA) which contained TaqMan probe and primer of the target genes. The Universal Human Reference RNA was used as positive control to determine the amplification efficiency of target genes based on the C_T value of each target gene. The *GAPDH* was used as internal control. The ΔC_T value of treated and untreated samples obtained from the StepOnePlus Real Time PCR 96-well system (Applied Biosystem, USA) was used in the Livak method to perform the relative quantification assay ($2^{-\Delta\Delta C_T}$).

3. Results

Filter by signal intensity, flag value, data files and CV% caused the reduction of 17574 entities from the total 42545 entities. The volcano plot revealed the significant expression of 9 entities out of 24971 at *p*-value ≤ 0.05 “Fig. 1”, which were listed in Table 1. The expression level of 1127 entities out of 24971 was more than 2.0 fold which contained 519 up- and 608 down-regulated genes. The Gene ontology analysis showed 109 GO terms satisfying corrected *p*-value cut-off 0.05 when the entity list chosen from the filter on Error-CV < 50.0%. The GO terms included molecular function (31.46%), cellular component (32.83) and biological process (35.72%). In addition, when the entity list chosen from T-test unpaired (T)

versus (C), 14 GO terms included molecular function (32.26%) and biological process (67.74%) satisfying corrected *p*-value cut-off 0.05 were displayed as listed in Table 2. Furthermore, when the entity list chosen from fold change ≥ 2.0, 8 GO terms included biological process (83.87%) and molecular function (16.13%) satisfying corrected *p*-value cut-off 0.05 were displayed as listed in Table 3. The cellular response to chemical stimulus was the important biological process which included *EBI3, HMOX1, IFI6, CYP1B1, IFIT1, UGT1A6* and *UGT1A8* genes. The C_T value of each targeted primer revealed their sufficient amount and strength of positive reactions by using human universal reference RNA. The RT-qPCR analysis showed that the alteration of relative quantification (RQ value) for the selected genes in MCF7 breast cancer and HT-29 colon cancer cell lines. The statistical analysis of T-test was done for independent samples of breast and colon cancers which indicated there is no significant difference between two types of cell lines due to Thymoquinone treatment with *p*-value 0.844. According to the EST profile of each selected gene sources in NCBI, the transcript of the selected genes had very low/no expression in the breast cancer profile except for the genes *CARD16, MTR* and *MOBKL2B* between the up-regulated ones and the genes *SLC7A11, IFI6, IFIT1, IFIT2, IFIT3, UGT1A6* and *HMOX1* between the down-regulated ones. The EST profile of HT-29 colon cancer cell line showed that the transcript of the *CARD16, MTR, MOBKL2B, SLC7A11, IFIT1, IFIT2, IFIT3, IFI6, UGT1A6* and *HMOX1* genes are less than 300 bp indicated low expression of these genes in colon cancer. The T-test analysis of genes showed that expression of *CARD16, UGT1A8, SLC7A11, IFIT1, IF16* and *IFIT3* genes were significant (0.009, 0.0001, 0.037, 0.098, 0.0001, and 0.033, respectively), but *IFIT2* and *UGT1A6* were not significant (0.613 and 0.592, respectively) at *p*-value 0.05 (Table 4, 5).

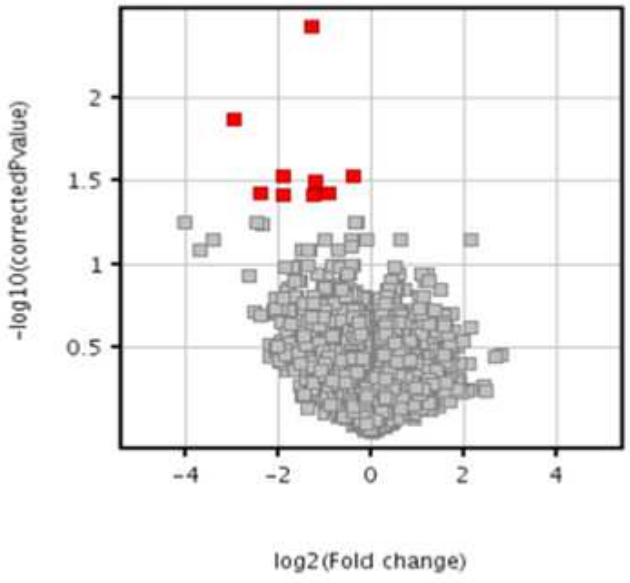

Figure 1. *Volcano plot displayed the –log10 of p-value on the y-axis versus the fold changes on the x-axis. The red dots are the excluded genes from the total of altered genes with the significant changes at p-value < 0.05.*

Table 1. *The significant entities with p-value < 0.05 from the total 24971 entities.*

Gene Symbol	*p*-value	Fold change	Description
CBX2	0.029	-1.34	Chromobox homolog 2
DUSP13	0.029	-3.83	Dual specificity phosphatase 13
IFI6	0.013	-7.87	Interferon, alpha-inducible pr 6
EB13	0.003	-2.47	Epstein-Bar virus induced 3
OR4S1	0.038	-2.43	Olfactory receptor, f 4, s-f S, m 1
IFIT3	0.037	-5.27	Interferon-I tetratricopeptide
KRT86	0.037	-1.92	Homo sapiens keratin 86
UGT1A6	0.038	-3.74	UDP g-transferase 1, peptide A6
CYP1B1	0.031	-2.33	Cytochrome P450, f 1, s-f B

Table 2. *GO terms satisfying corrected p-value cut-off 0.05 when the entity list chosen from T-test unpaired (T) versus (C).*

Gene Ontology	Corrected *p*-value
Type I interferon-mediated signaling pathway	0
Response to type I interferon	0
Cellular response to type I interferon	0
Cytokine-mediated signaling pathway	0.001
Response to cytokine stimulus	0.001
Cellular response to cytokine stimulus	0.002
Aldo-keto reductase (NADP) ACTIVITY	0.002
Androsterone dehydrogenase activity	0.002
Xenobiotic metabolic process	0.009
Response to xenobiotic stimulus	0.009
Cellular response to xenobiotic stimulus	0.009
Innate immune response	0.018
Oxidoreductase activity	0.021
Response to interferon-gamma	0.041

Table 3. *GO terms satisfying corrected p-value cut-off 0.05 when the entity list chosen from fold change ≥ 2.0.*

Gene Ontology	Corrected *p*-value
Cellular response to type I interferon	0.002
Response to type I interferon	0.002
Type I interferon-mediated signalling pathway	0.002
Cellular response to xenobiotic stimulus	0.022
Response to xenobiotic stimulus	0.022
Xenobiotic dehydrogenese activity	0.022
Androsterone dehydrogenase activity	0.024
Aldo-keto reductase (NADP) activity	0.047

Table 4. *Comparison between MCF7 breast cancer cells and HT-29 colon cancer cells in the most up-regulated genes with cut-off > 2.0 fold from the total 519 up-regulated genes.*

Gene Symbol	Fold changes of up-regulated genes (RT-q PCR)		
	MCF7	HT-29	*p*-value
XLOC00153	Un-d	0.063	-
CARD16	9.02	1.647	0.009 ***
MTR	Un-d	0.913	-

Table 5. *Comparison between MCF7 breast cancer cells and HT-29 colon cancer cells in the most down-regulated genes with cut-off > 2.0 fold from the total 608 down-regulated genes.*

Gene Symbol	Fold changes of up-regulated genes (RT-q PCR)		
	MCF7	**HT-29**	***p*-value**
UGT1A8	0	-1.079	0.0001 ****
SLC7A11	-12.81	-0.823	0.037 *
IFIT1	-0.015	-0.926	0.098 *
IFI6	-0.323	-1.109	0.0001 ****
C17orf64	Un-d	Un-d	-
ALDH3A1	Un-d	-1.079	-
UGT1A6	-4.52	-0.151	0.592 (ns)
IFIT2	-1.32	-0.239	0.613 (ns)
IFIT3	-4.13	-1.366	0.033 *
HMOX1	Un-d	-0.747	-

* Significant at *p*-value < 0.05, *** Significant at *p*-value < 0.001, **** Significant at *p*-value < 0.0001, (ns) Not Significant

4. Discussion

MCF7 breast cancer cell line is a common type of breast cancer arising from epithelial cells and it is known as an estrogen-receptor positive which is under the influence of hormone while colorectal carcinoma is hormone independent. In this study using another cell line with a similar morphology to epithelial breast cancer cell would give an idea about the effect of Thymoquinone on tumor cells arising from epithelial cells apart from the effect of hormone. First, the expression of genes in MCF7 cells treated with Thymoquinone was found through cDNA microarray. Next, expression of the most up and down-regulated genes were compared in HT-29 colorectal carcinoma cell line to find the similar genes involved in the effectiveness of Thymoquinone treatment. The study showed expression of the most up and down-regulated genes with cut-off > 2.0 fold which were about 48% and 52%, respectively.

The first selected up-regulated gene was *XLOC001537* listed as lincRNAs. Transcripts from the non-coding parts of genome which do not present any function in the form of RNA are referred to the non-coding RNAs [8]. LincRNAs is known as the key regulators of various cellular responses. To date, understanding the individual function of each lincRNA is stated as a challenge. It is stated that lincRNAs can be co-expressed with their nearby genes and their expression is similar to nearby protein-coding genes. It is reported that a large number of long non-coding genes (IncRNAs) can be transcribed from mammalian genomes. There is an idea of probability connection between IncRNAs and the regulation of physiological functions whose distraction can be led to different diseases [9]. The second up-regulated gene was *CARD16* which is introduced as one of the adaptor molecules regulated caspases cascade. It enhances the activation of CASP1 which lead to apoptosis process [10]. The small EST profile of this gene in normal mammary gland is reported to be 6 bp and in breast and colon cancers is 10 and 8 bp, respectively. The microarray results showed that the fold changes of *CARD16* is 5.25 up-regulated after treatment of breast cancer cells with Thymoquinone. The over expression of *CARD16* was validated with RT-qPCR method which was 9.02 up-fold change in breast cancer cell but showed only 1.64 up-fold in colon cancer (Table 5). Previous study on different type of colon cancer reported that Thymoquinone 40 µM concentration cannot lead the cell significantly in the apoptosis process after 24 hours especially in HT-29 cell line [11]. The expression of *MTR* gene in breast cancer was found to be up-regulated in microarray result by 3.91. The EST profile of *MTR* gene in breast cancer is 21 bp. The expression of *MTR* gene was undetermined in MCF7 breast cancer cells by RT-qPCR assay and it was about 0.913 up-fold in HT-29 colon cancer cells. The EST profile for *MOBKL2B* gene is 4 bp in breast cancer. It was up-regulated in microarray results by 3.67. The expression of *MOBKL2B* gene in colon cancer was found to be undetermined through the RT-qPCR results. The EST profile indicates very low expression of *MOBKL2B* gene in breast and colon cancers.

The length of EST is about 300-500 bp named as the short sequences of expressed tags. In addition, these small sequences of mRNA or cDNA which presented a snapshot of genes involved in the specific tissue came from coding or even non-coding tags which might not be able to present any biological function [12]. There were not any expressed tags (EST profile) in the up-regulated genes *C2CD4B*, *UBQLNL*, *TMEM100*, *RNF17*, *STON1GTF2* and *OR52J3* in breast cancer, whereas in this present study the microarray results showed there is changes in the expression of these genes due to the treatment with Thymoquinone. There was a similarity between both cell lines in terms of expression of top 10 up-regulated genes which showed only *CARD16*, *MTR* and *MOBKL2B* are expressing in both breast and colon cancers. Generally, down-regulated genes presented differences in the EST profile of mammary gland and breast cancer. Although the EST profile of *UGT1A8* gene showed no expression in both mammary gland and breast cancer tissue, the microarray results revealed -16.26 fold changes.

The UGTs enzymes are membrane-bound and implicate in the biotransformation of important endogenous compounds as well as drugs and xenobiotics [13]. UGT-glucuronosyl-transferase 1A8 is known as an enzyme in human body which is encoded by the *UGT1A8* gene and belongs to the UGTs and express in extra-hepatic tissues. Normally, every xenobiotic compound which enters inside body has to be metabolized and removed from the body via detoxification processes. During the second phase of detoxification, xenobiotic compounds are transformed into a water-soluble compound through conjugation reaction and exerted via bile or urine. In fact, glucuronidation is known as one of the important types of conjugation reactions. Besides, the *UGT1A8* and *UGT1A6* genes are known as the enzymes involved in glucuronidation [14,15], and serve in the xenobiotic metabolism in the detoxification system [13]. Furthermore, in the current study the expression of *UGT1A6* gene was also found in the microarray experiment (-5.68 fold). The EST profile showed its low expression in breast cancer. The *UGT1A6* gene is known to transform steroids, bilirubin, hormones and drugs

into water-soluble metabolites and led them to the process of detoxification [16]. Therefore, the expression alteration of *UGT1A8* and *UGT1A6* genes which was seen in the microarray results could be because of the indispensable biological function. On the other hand, the biological changes cannot be detected through the RT-qPCR assay [17]. The expression of *UGT1A8* and *UGT1A6* genes in normal human colon tissue is reported through microarray analysis which is about 6 and 7, respectively [18]. The EST profile of *UGT1A8* and *UGT1A6* genes is 0 and 21 in normal human colon tissue, respectively. The RT-qPCR assay showed 1.079 and 0.151 down-fold in the expression of these genes due to Thymoquinone treatment in colon cancer, respectively. The number of EST profile of *SLC7A11* gene in breast and colon cancer is 42 bp and 35 bp indicates short sequence of this gene [12]. Down-regulation of *SLC7A11* gene by -0.823 fold was found in HT-29 colon cancer cells with RT-qPCR method. Both gene expression methods showed similar fold changes after treatment with Thymoquinone by -12.99 and -12.81 down-fold, respectively. The *SLC7A11* gene is known as one of the genes which can control the level of intracellular glutathione (GSH) [19]. Moreover, the role of antioxidant GSH in the regulation of reactive oxygen intermediates and consequently its effect on the lymphoid functions in the immune system to trigger immune defense. The *SLC7A11* gene encodes the X_c^- cystine/glutamate anti-porter [20]. Previous researches showed that the resistance of cancer cells to therapy can be associated with the higher amount of GSH within tumor cells [19]. The anti-oxidant GSH is known as a compound that promoted drug-resistance via removal of free radicals [19]. Success in cancer treatment based on the regulation of GSH level can be related to the control of its level inside the cancer cell. The down-regulation of *SLC7A11* in this study suggested the ability of Thymoquinone in the reduction of the GSH level and it would show its ability to reduce resistance of MCF7 cells to chemotherapy. Besides, previous studies categorized Thymoquinone between groups of ROS inducer plant-derived anti-cancer compounds [21]. It was also reported in p53-mutated Jurkat cells [22] in prostate cancer cell [23], in lymphoma cell line [24], in acute lymphocyte leukemia cell line [25], in pancreatic cancer cells [2] and in colorectal cancer cells [11].

The EST profiles of *IFIT1*, *IFIT2*, *IFIT3* and *IFI6* genes showed small numbers of expressed tags (EST profile) indicated the sequences less than 500 bp [12]. The RT-qPCR assay results in MCF7 cell line showed the down-fold alteration of *IFIT1*, *IFIT2*, *IFIT3* and *IFI6* genes which were 0.015, 1.32, 4.13 and 0.323, respectively. Besides, the RT-qPCR assay results in colon cancer cell line showed 0.926, 0.239, 1.366 and 1.109 down-fold changes, correspondingly. The results suggested alteration of these genes after Thymoquinone treatment.

In conclusion, anti-cancer effect of Thymoquinone has been reported on different types of tumors which showed alteration of some genes. This study revealed the breast cancer cellular response through the expression of significant genes, the important genes involved in biological process, the up-fold and down-fold genes and their biological functions after Thymoquinone treatment. The most up- and down-fold genes in breast cancer cell were expressed in colorectal cancer cell although there were significant differences between the expressions of some of the genes but there were not significant differences between two types of cancer cells due to the Thymoquinone treatment.

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