Chapter

Silencing of Peroxiredoxin-4 in Anticancer Activity of Gamma-Tocotrienol

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Abstract

Peroxiredoxin-4 (PRDX4) is known to have a role in protecting cells from oxidative stress. It has been previously reported to increase in HepG2 liver cancer cells treated with gamma-tocotrienol (GTT). As GTT treatment potentially kills the cancer cell by regulating multiple signaling pathways, this study aims to determine the involvement of PRDX4 in GTT anticancer activity by silencing the *PRDX4* gene. The efficiency of PRDX4 silencing is achieved by optimizing HepG2 cell density, effect of serum presence in transduction media, incubation time of the cells with lentivirus, polybrene concentration, puromycin dose, functional titer, and multiplicity of infection (MOI) of the lentivirus. Silenced HepG2-PRDX4 cells (HepG2-shRNA-PRDX4) were treated with 70 μM of GTT for 48 h. GTT treatment significantly decreased the HepG2-shRNA-PRDX4 cell viability, increased apoptosis rate, and reduced free radical production compared to untreated HepG2-shRNA-PRDX4 cells. These findings are further supported by proteomic analysis, which showed that pro-apoptotic and DNA damage proteins were upregulated, and proteins involved in cell cycle arrest, carcinogenesis, and anti-apoptotic signaling pathways were downregulated in HepG2-shRNA-PRDX4 cells treated with GTT compared to control. In conclusion, PRDX4 plays a role in GTT anticancer activity by increasing free radical production and oxidative damage to induce apoptosis in HepG2 cell.

Keywords: silencing, peroxiredoxin-4, anticancer, gamma-tocotrienol, antioxidant

1. Introduction

Peroxiredoxin-4 (PRDX4) is one of the unique isomers of peroxiredoxin (PRDX) and is located in the endoplasmic reticulum. It has a peroxidase site to oxidize hydrogen peroxide (H_2O_2) and thus functions as an antioxidant, increases cell proliferation, and is involved in regulating multiple signaling transduction pathways [1, 2]. Double cysteine residues at the peroxidase site of PRDX4 oxidize H_2O_2 and form water molecule. Oxidation is one of the processes that control H_2O_2 homeostasis in the cells and thus indirectly reduces the risk of high level of oxidative stress [3]. The increase in reactive oxygen species (ROS) production in the tumor cells has been linked to increased PRDX4 expression, which makes the cell susceptible toward apoptosis [4]. Besides that, PRDX4 is a potential tumor biomarker [5] because of its high expression in most of the tumor tissues as it plays a role in tumor

growth and progression. The pattern of *PRDX4* gene expression is different among cancer cells and depends on its role. *PRDX4* gene may be involved in increasing the immune system of a normal cell to kill the cancer cells or reducing the level of oxidative stress to make the cancer cell more insusceptible toward the anticancer activity. *PRDX4* gene expression is high in hepatocellular, pancreas, colon, and prostate cancer cells, whereas its expression is low in the lung, kidney, and thymus cancer cells [6]. Recent findings have shown that *PRDX4* gene is highly expressed in HepG2 liver cancer cells treated with gamma-tocotrienol (GTT) [7] and reduces the HepG2 viability [8]. However, currently, there are no studies yet that focus on the role of PRDX4 in GTT treatment.

GTT is one of the tocotrienol isomers of vitamin E. It has an unsaturated and short phenyl chain and is differentiated based on the methyl group located on the chromanol ring [9]. This structure enables GTT to pass through the saturated lipid bilayer membrane and to be absorbed by the cells efficiently. GTT and other tocotrienol isomers are renowned as antioxidants [10]. Besides that, GTT has also been proven to have the capability as a signaling molecule to induce anticancer mechanism through activation of multiple pathways [11]. Previous studies have shown that GTT has an effective antitumor activity compared to other tocotrienol isomers [12]. GTT as an anticancer agent is selective on malignant cells, is capable of targeting multiple signaling pathways simultaneously, and has a synergistic effect with chemotherapy [13, 14]. Hence, various studies have been done to identify GTT capabilities and its mechanism in anticancer activities.

To determine the role of PRDX4 in anticancer activity of GTT, *PRDX4* gene in HepG2 cells was silenced using lentivirus particles that carry the RNA sequence of *PRDX4* gene (shRNA-PRDX4). The shRNA-PRDX4 is complementary with the *PRDX4* gene sequence in the HepG2 cell. The *PRDX4* gene and protein expression was reduced and indirectly caused the genotypic and phenotypic changes in the cell [15]. The silenced HepG2 cells were resistant toward puromycin and also encoded green fluorescent protein (GFP). Puromycin resistance enabled the culture of only silenced cell in the media. The presence of GFP as a reporter gene that exhibits bright green fluorescence when exposed to ultraviolet light was used as a visual tag for the expression of other genes. To silence the gene using lentivirus, there are several critical factors to be considered to avoid false positive and to increase silencing efficiency [16]. In this study, the silenced HepG2 cells were treated with GTT for 48 h. Then, cell viability, apoptosis rate, and ROS production were determined. Protein profiling was done to further confirm the proteins involved in the pathways.

2. Gene silencing

Gene silencing is used to study the role of specific genes by introducing antisense RNA to block the translation of messenger RNA (mRNA) and inhibit gene expression or translation. This biological process is known as RNA interference (RNAi). RNA plays a role as a mediator in regulating gene expression. In relation to this, synthetic RNAi is developed to mimic the targeted gene and reduce its expression [17]. The silencing possibly occurs during the transcription or translation phase. When the targeted gene is silenced, its expression is reduced to 70% without eliminating the whole expression [18]. This technique offers one step forward for the therapeutic strategy of specialized medication for patients to undergo treatment for cancer or infectious diseases [19].

There are three types of RNAi which are small interference RNA (siRNA), microRNA (miRNA), and small hairpin RNA (shRNA). siRNA is a double-stranded RNA comprising 20–25 nucleotides. siRNA sequences are coupled with a polymer

and liposome carrier to enter the cell by using exogenous mechanism to silence the gene [20]. This process is known as transfection. shRNA is encoded specific RNA transcription which comprises 19–29 nucleotides. These nucleotides form a bridge for small hairpin of nine nucleotides [21]. shRNA silencing mechanism is known as transduction. The delivery of shRNA to the cell is through vectors such as plasmid, adenovirus, lentivirus, or retrovirus with a U6 promoter to regulate shRNA expression [22]. The vector will ensure that shRNA is expressed to silence the targeted gene. The silenced gene is inherited by the daughter cells [23]. miRNA is single-stranded RNA (ssRNA) which comprises 21–23 nucleotides. This type of RNAi is complementary with mRNA molecule and can be used to silence the gene [24].

In vivo studies have shown that RNAi silences the targeted gene without affecting other cellular activities such as interferon action which may inhibit protein synthesis [25]. siRNA can only be used in actively dividing cells and the silencing effect is temporary. This is because siRNA concentration decreases when the silenced cell divides. siRNA is also limited to cells with low susceptibility toward a foreign molecule [26]. In relation to this, the improvement of transfection method is required by introducing shRNA, which is able to silence the targeted gene in a more specific and effective way. In comparison to siRNA, shRNA and its lentivirus vector have the ability to stably integrate into the host genome. The silencing effect is passed to the daughter cell, resulting in permanent gene silencing. It increases the potential of the targeted gene to be silenced in nondividing cells, and the silencing effect can be delivered to cells that have low susceptibility toward lipid penetration. Thus, it can be applied in cell and animal model [27].

Both siRNA and shRNA have the same silencing mechanism; however, the choice of RNAi method to be used depends on the cell type, the time required to silence the gene, and the duration to silence the gene whether temporarily or permanently. The lentivirus plasmid used in this study has a GFP sequence, which functions as reporter gene, puromycin-resistance sequence for silenced cell selection, and a shRNA sequence that is antisense to the *PRDX4* sequence. Thus, the percentage of transduced cells can be determined by viewing the cells under a fluorescent microscope and culturing the cells with an optimal dose of puromycin in the culture media to select for the silenced cells.

3. Mechanism of gene silencing

The purpose of gene silencing is to regulate gene expression by degrading the targeted gene's product or reducing its mRNA translation through the delivery of RNAi agents into the cytoplasm [28]. Gene silencing mechanism involves both exogenous and endogenous pathways. The silencing mechanism of synthetic RNAi agents such as siRNA, shRNA, and miRNA is through the exogenous pathway, whereas the silencing mechanism for miRNA that exists naturally in the cell is through the endogenous pathway [29]. The mechanism of these three synthetic RNAi agents depends on the RNA-induced silencing complex (RISC) to cleave or degrade the mRNA of the targeted gene.

Further process of siRNA silencing occurs in the cytoplasm, whereas for shRNA and pre-miRNA, DNA integration occurs in the nucleus prior to the changes of pri-miRNA/pri-shRNA to the pre-miRNA/pre-shRNA [30]. The siRNA pathway is activated when dsRNA, together with the carrier complex, penetrates the cell membrane. Then, an endogenous dicer enzyme identifies the dsRNA sequence and splits it into small fragments of siRNA. The RISC complex binds to the siRNA fragments and causes RNA splitting for gene silencing to occur [31]. Plasmid carried by the lentiviral vector encodes the shRNA sequence of the

targeted gene and also shRNA transcripts on the promoter of RNA pol III or pol II. When the lentivirus infects the cells, lentiviral plasmids are transferred to the cytoplasm. An endogenous dicer enzyme identifies the plasmid and splits the shRNA into small fragments of pri-shRNA [32]. The small fragments of pri-shRNA enter the nucleus, are multiplied by reverse transcription, and integrate into the host cell's genome. The integrated genome will enter the shRNA silencing pathway to silence the gene.

Pre-shRNA and microRNA primers (pri-miRNA) are transcribed in the nucleus using a complex of Drosha microprocessors and DGCR8 as intermediators to produce the precursor microRNA (pre-miRNA)/pre-shRNA. Pre-miRNA/pre-shRNA is then exported to the cytoplasm by exportin-5 and splits by a dicer enzyme to dsRNA [33]. The dsRNA is combined with RISC and is resolved by a helicase. The disassembled dsRNA activates the mRNA thread guide to recognize the target gene and Argonaute protein (Ago) in RISC [34]. The RISC complex helps to locate the mRNA thread guide which is complementary to the mRNA molecules of the targeted gene. The targeted gene's mRNA is then degraded by the endonuclease and inactivated [35].

3.1 Critical factors of gene silencing

Each cell has a different level of susceptibility toward lentivirus infection, and this is the biggest challenge to overcome in order to achieve optimal conditions for successful transduction [36]. The efficiency of shRNA silencing on the targeted gene is measured manually by experiment. Therefore, several critical factors must be considered to achieve specificity and efficiency of cell transduction. The factors are cell density, polybrene concentration, serum presence in the transduction media, incubation time, and lentivirus and puromycin dosage [37]. Furthermore, the functional titer and the multiplicity of infection (MOI) should be determined so that the minimum amount of lentivirus needed to transduce the cell is used. The ideal shRNA structure and experimental design should also be taken into account [38].

3.2 Basic conditions of gene silencing

One of the basic conditions that needs to be optimized for successful gene silencing is the cell density. The recommended cell density for transduction is 40–50% of cell confluence [16]. However, it depends on the size and growth rate of the cell. This confluence is to ensure that the cells have enough space to divide during transduction, which takes about 96 h. A previous study showed that cell confluence of more than 50% limits the interaction of lentivirus, cells, and DNA complex [39]. On the other hand, cell confluence of less than 30% will slow down cell growth [40]. Besides that, the cells used for transduction process must be active. This is because active cells take up foreign molecules more efficiently compared to quiescent cells.

The lentivirus membrane and cell wall are negatively charged. This causes difficulty for the lentivirus to infect the cell. Polybrene is a cation polymer that facilitates the lentivirus's infiltration into the cells [41]. Higher concentration of polybrene results in a more effective lentiviral infection. However, if the concentration of polybrene is too high and it is incubated with the cells for a long duration, it may cause toxic effects to the cells [42]. Therefore, a polybrene concentration that does not affect cell viability in long culture periods was chosen as the optimum condition. Studies have reported that the absence of serum in the culture media increases the efficiency of lentivirus DNA uptake by the cell [43]. However,

the absence of serum may affect cell growth. Therefore, the presence of serum in the media during the transduction process has to be determined for efficient transduction.

Stable silenced cells are selected through puromycin resistance. Non-transduced cells will die as puromycin inhibits protein synthesis [44]. Cells are cultured in media containing different concentrations of puromycin for 7 days. Then, a kill curve is constructed. The optimal dosage of puromycin to be used is the lowest dosage of puromycin that kills the cell significantly from the third to the seventh day. Cell viability is calculated starting from the third day because the cells only respond to the antibiotic exposure after 48 h.

Each cell type has different susceptibilities to lentivirus infection. Therefore, the functional titer needs to be determined in order to know the minimum lentivirus concentration required to infect the cell. Functional titer is the smallest transducing unit required for lentivirus to infect the cell. The transducing unit needs to be parallel to the ratio between the lentivirus and cell in culture, which is known as MOI. Functional titer and MOI are important to ensure optimum condition for shRNA expression for transduction to occur. Technically, only a small concentration of functional titer is required to transduce cells at a consistent MOI ratio [45]. MOI is the ratio of lentivirus that can infect the cell at one time. As the MOI increases, the lentivirus volume required in a fixed amount of functional titer also increases. The minimum MOI should be determined so that the optimum concentration of shRNA that is sufficient to infect the cells at the recommended confluence is used [46].

3.3 Small hairpin RNA (shRNA) design

The design of shRNA vector is important to control the optimum expression of shRNA. The optimum expression of shRNA is determined by the lentiviral vector, promoters, and shRNA structures [47]. A study reported that human cytomegalovirus (hCMV) promoter is able to infect 95% of HepG2 cells and stably integrate into the host cell genome [48]. Lentivirus infection has low toxicity and does not affect the phenotype of the cell [49]. An efficient combination of promoter and vector will have an influence on the functional titer. Thus, shRNA can be expressed optimally to specifically silence a target gene.

3.4 Experimental design

A good experimental design is essential in gene silencing so that the silencing effect can be validated as specific silencing without any false positive. The recommended experimental design for transduction method comprises a positive control, a negative control, and unsilenced cell [50]. The positive control is a sequence of normalized genes that are highly expressed in cells to identify whether the silencing have effects on the cell cycle or any pathways that may affect the growth of the silenced cells [51]. The presence of nontargeted lentivirus negative control (NTC) is to validate whether the plasmid of the lentivirus itself has an effect on the targeted gene expression. NTC is consists of shRNA sequence that is not complementary to any mammalian gene by having three or less nucleotide equivalents. It also contains GFP and also puromycin-resistance sequences. This control is important to avoid any false positive.

The NTC control should have no effect on the targeted gene and the transduced cell [52]. The non-transduced cell controls are standardized to be compared with NTC controls. If the NTC and non-transduced cell controls show no difference in mRNA and protein expression of targeted proteins, this proves that

the plasmid carried by the lentivirus has no effect on the cell. Although shRNA functions are at the mRNA level, only changes in protein expression may contribute to phenotypic changes. Therefore, validation of the mRNA and protein expression of the targeted proteins should be carried out to ensure that the gene expression is suppressed [53]. In addition, it is essential to validate the silencing effect by using more than two sequences of targeted gene shRNA. Every segment of gene sequence has a different effect in silencing the gene. The best sequence is selected based on the higher silencing rates shown through reduction of mRNA and protein expression of targeted gene.

4. Peroxiredoxin-4

4.1 Peroxiredoxin-4 and its function

H_2O_2 is produced during the disulfide bond formation in protein synthesis and from a side effect of cell biology mechanism located in the ER lumen and extracellular matrix [54]. PRDX4 is localized in the endoplasmic reticulum and highly expressed in the pancreas, liver, and heart but is low in blood and brain leukocytes [5]. PRDX4 plays a role as an antioxidant by scavenging H_2O_2 and is a chaperone molecule to activate ER stress pathway [55]. PRDX4 is important for cell protection by reducing H_2O_2 to water in order to reduce oxidative stress.

In addition, PRDX4 has a role in regulating the activation of NF-κB transcription factor and TNF-related apoptosis-inducing ligand (TRAIL) [56]. TRAIL is a transmembrane protein involved in apoptosis extrinsic pathway by binding to receptors DR4 or DR5. A reduction in PRDX4 expression leads to TRAIL activation and apoptosis induction, whereas an increase in PRDX4 expression inhibits the TRAIL activation and leads to cell survival [57]. The expression of PRDX4 in the cytosol increases NF-κB activity through phosphorylation of IκB-α [58]. Meanwhile, the expression of PRDX4 in the endoplasmic reticulum reduces NF-κB activity. NF-κB plays a role in cell proliferation and survival. A study has shown that PRDX4 is bound to the endothelium and is secreted when there is a redox change in the extracellular matrix [59]. This study is supported with the presence of high PRDX4 in the serum that indicates membrane leakage due to tissue destruction and cell apoptosis [60].

4.2 Mechanism of peroxiredoxin-4 as an antioxidant

PRDX4 triggers H_2O_2 signaling and protects cells from oxidative stress by oxidizing thioredoxin (Trx) proteins. Trx is a group of co-factor proteins with chaperone activity to control cell homeostasis and inhibit H_2O_2 signaling pathway [61]. PRDX4 requires Trx peroxidase activity as an electron donor. The oxidized Trx is recycled by Trx reductase and NADPH as the primary electron donor. The main target of H_2O_2 molecules is two cysteine residues on PRDX4 to form catalytic peroxide mechanisms [62]. PRDX4 has two cysteine residues known as peroxidatic cysteine residue (CysP-) and resolution cysteine residue (CysR-). These two cysteine subunits have peroxidatic properties to oxidize H_2O_2.

When the oxidative stress level is high, CysP- acts on peroxide to form sulfenic acid (CysP-SOH). CysP- is one of the cysteine units located near the N-terminal of PRXD4. CysP-SOH then reacts with another systemic subunit known as CysR- to form a stable disulfide bond, and water molecule is produced. Trx enzyme reduces the disulfide bond and completes the PRDX4 catalytic cycle by detoxifying the peroxide and producing an active thiol. If the oxidative stress keeps increasing, –SOH is

oxidized to $–SO_2H$ [63]. PRDX4 in $-SO_2H$ is reduced and re-oxidized by sulfiredoxin (Srx) through the reduction of ATP to ADP. This process is reversible [1]. However, in high oxidation state, $–SO_2H$ is continuously oxidized to $–SO_3H$, and PRDX4 will undergo hyperoxidation. This will cause inhibition of oxidation, and PRDX4 will activate other pathways to counteract the oxidation state. In conclusion, PRDX4 antioxidant activity is inhibited in high oxidation state and indirectly activates the ER-pressure pathway [64].

Studies have shown that the ER produces higher level of H_2O_2 than the mitochondria [65]. The main source of H_2O_2 in the ER lumen is the reaction of oxidase-1 in the ER (Ero1) with disulfide isomerase protein (PDI) for disulfide bond formations during protein synthesis. The disulfide bonds are important to prevent the protein from degrading after it is excreted from the ER lumen. Disulfide bonds formation involves high oxidative processes, and the unfolded bonds on damaged proteins may cause unfolded protein response (UPR). Unfolded proteins are degraded by dissolution of disulfide bonds before the protein is bound to the cytosol membrane [66]. UPR is the action of cells to balance the increasing oxidative stress and repair the function of or degrade the unfolded protein. The UPR will activate chaperone proteins of multiple pathways to balance the oxidative stress [67]. High oxidative stress in the ER will induce UPR and activate Ero1 to oxidize PDI catalytic enzymes by using oxygen molecules as the electron receiver [68]. The UPR action will form three intermediate sensors which are ATF6, IRE1α, and PERK and at the same time increase Ero1 and PRDX4 expressions as homeostatic chaperones [69].

5. The effect of viability, apoptosis rate, and reactive oxygen species production in HepG2 cell silenced with peroxiredoxin-4 and treated with GTT

Gamma-tocotrienol has been shown to have high antitumor activity [12] that exhibits cell cycle arrest and apoptosis in alveolar adenocarcinoma epithelial cells, A549 [70], and colorectal cancer cell HT-29 [71]. Its action is specific and causes the cancer cells to be more susceptible and sensitive to treatment [72]. A previous study by Sazli et al. [7] reported that PRDX4 was upregulated when HepG2 cells were treated with GTT. In this study, we silenced *PRDX4* gene and treated with 70 μM of GTT to determine the role of PRDX4 in anticancer activity of GTT. Finding has shown that GTT treatment reduces HepG2-shRNA-PRDX4 cell viability significantly ($p < 0.01$) compared to its control (HepG2-shRNA-PRDX4) and also compared to HepG2 treated with GTT ($p < 0.05$) (**Table 1**). This viability assay is the first line of observation to show that GTT treatment does have an effect toward the silenced *PRDX4* gene.

To investigate further the cause of reduction in cell viability, an apoptosis assay was done. The findings showed that the apoptosis rate was significantly increased ($p < 0.05$) in HepG2 treated with GTT compared to HepG2 control. Besides that, apoptosis rate increased in HepG2-shRNA-PRDX4-GTT ($p < 0.05$) compared to HepG2-shRNA-PRDX4 and HepG2-GTT ($p < 0.05$). GTT is capable to induce intrinsic and extrinsic apoptotic pathways in cancer cells such are breast cancer cell line, MCF-7, and MDA-MB-231 by increasing biomarker stress in the endoplasmic reticulum [73]. It is suggested that the main reason for the apoptosis in GTT-treated group is high level of ROS production. GTT has triggered ROS production through PRDX4 activity. Studies reported that one of the anticancer activities of GTT is apoptosis induction through increases of ROS production [74]. We have postulated that GTT might trigger the ROS production through PRDX4 activity.

Cell groups	Cell viability (%)	Apoptosis rate (%)	Free radical production (arbitrary unit)
HepG2	100 ± 0.08	13.26 ± 1.97	1.00 ± 0.01
HepG2-shRNA-PRDX4	98 ± 12.33	18.91 ± 2.90	3.59 ± 0.33[a]
HepG2 with 70 μM GTT	90 ± 8.98	76.09 ± 4.89[a]	2.32 ± 0.09[a]
HepG2-shRNA-PRDX4 with 70 μM GTT	63 ± 10.38[b,c]	92.65 ± 6.58[b,c]	1.61 ± 0.01[b]

The data is presented as the mean ± standard deviation. Each group consists of technical triplicate and three biological replicates. GTT, gamma-tocotrienol; PRDX4, peroxiredoxin-4; HepG2-shRNA-PRDX4, HepG2 cell with PRDX4 gene silenced.
[a]A significant difference compared to HepG2 group ($p < 0.05$).
[b]A significant difference compared to HepG2-shRNA-PRDX4 group ($p < 0.01$).
[c]A significant difference compared to HepG2 GTT-treated group ($p < 0.05$).

Table 1.
The effect of GTT treatment on HepG2 and HepG2-shRNA-PRDX4 cell viability, apoptosis rate, and free radical production.

Hence, the level of ROS in each group was measured. The result showed that GTT increased ROS production ($p < 0.05$) in HepG2 group compared to the control but reduced ROS production in HepG2-shRNA-PRDX4 group. In HepG2-shRNA-PRDX4, ROS production was even higher compared to other groups but causes no effect on the cell viability. This proves that the function of PRDX4 is to reduce ROS level and oxidative stress. GTT either increases ROS production [75] to stimulate apoptosis pathway in HepG2 group or directly activate the apoptosis pathway in HepG2-shRNA-PRDX4.

To investigate further, protein profiling was done using LCMS machine to detect proteins expressed in HepG2-shRNA-PRDX4 group treated with GTT compare with HepG2-shRNA-PRDX4 as its control group. Protein profiling has shown a total of 3413 proteins expressed in HepG2-shRNA-PRDX4 and 3659 proteins expressed in HepG2-shRNA-PRDX4 treated with GTT. There are 2121 similar proteins expressed in both HepG2-shRNA-PRDX4 control group and HepG2-shRNA-PRDX4 treated with GTT. Statistical analysis has been done to differentiate protein which significantly expressed HepG2-shRNA-PRDX4 treated with GTT compared to HepG2-shRNA-PRDX4 control group using Fisher Exact test. The proteins are significantly expressed if the p value is less than 0.00398. Then, the significantly expressed protein is filtered and selected based on their functional processes that are involved in anticancer activity using UniProt and Reactome database.

There are 6 upregulated protein expressions that are CLU, NDRG1, NUDT2, PRDX5, RALB, and SLC25A6 (**Table 2**) and 14 downregulated proteins expression (**Table 3**) in HepG2-shRNA-PRDX4 group treated with GTT compared to HepG2-shRNA-PRDX4 control group. The downregulated protein expressions are EEF1A1, DHX9, PRDX1, RPS27, HIST2H2AA3, UBA52, UTP20, GSTP1, HSPB1 NPM1, PRDX2, PRDX6, PRKDC, and TXN—the significant different expressed proteins involved in multiple anticancer mechanism targeted pathway. Most of the upregulated proteins are involved in apoptotic pathway and DNA damage, whereas for the 14 downregulated proteins are involved in carcinogenesis pathway, anti-apoptotic, and cell cycle arrest. NDRG1, NUDT2, and PRDX5 expressions resulted from cellular response on ROS production causes on the downstream action. Those proteins trigger cell cycle arrest due to DNA damage and apoptosis [76]. This situation worsens the cell conditions; thus, GTT has increased pro-apoptotic proteins to induce apoptosis. The apoptotic pathway is regulated by the expression of RALB, SLC25A6, and CLU which mediate the releases of cytochrome c from mitochondria [77].

Functional cluster/ protein name	Accession name (Swiss Prot)	*P*-value	Fold change	Functional process	Mechanism
Clusterin	CLU	0.0013	1.0	Release of cytochrome c from mitochondria	Pro-apoptosis
Protein NDRG1	NDRG1	0.0001	3.4	DNA damage response, signal transduction by p53 class mediator, cellular response to hypoxia	DNA damage
Bis(5′-nucleosil)-tetraphosphatase	NUDT2	0.00056	8.5	Apoptotic process, cellular response to oxidative stress	Pro-apoptosis
Peroxiredoxin-5, mitochondrial	PRDX5	0.0023	1.6	Apoptotic process, cell redox homeostasis, cellular response to reactive oxygen species	Pro-apoptosis
Ras-related protein Ral-B	RALB	0.0020	4.8	Apoptotic process, cell cycle	Pro-apoptosis
ADP/ATP translocase 3	SLC25A6	0.0003	1.4	Apoptotic process	Pro-apoptosis

Table 2.
List of upregulated protein expression in HepG2-shRNA-PRDX4 treated with GTT compared to HepG2-shRNA-PRDX4 control group.

The treatment of GTT on HepG2-shRNA-PRDX4 causes downregulated protein expression that is involved in cell cycle arrest, carcinogenesis pathway, proteins resulted in the ER stress, and anti-apoptotic. The level of ROS production was reduced in HepG2-shRNA-PRDX4 group treated with GTT, while the apoptosis activity was induced. EEF1A1, PRDX 1, PRDX2, PRDX6, and TXN are the proteins that function to reduce ROS level and thus become negative regulator for cell apoptosis [78]. Thus, GTT has shown to reduce the ROS accumulation in HepG2-shRNA-PRDX4. GTT also suppressed the HIST2H2AA3, UBA52, and UTP20 protein expressions that are involved in cell cycle arrest. The reduction of ROS level promotes cell proliferation [79], and this justifies the downregulation of protein expression for cell cycle arrest. The expression of DHX9 is downregulated, and it plays a role in regulating DNA repair. GTT in silenced cells reduces the ROS level and stimulates an apoptotic pathway to cause cell death.

GTT also suppressed PRDX1 and RPS27 expression that are involved in carcinogenesis. PRDX1 is a positive regulator of stress-activated MAPK cascade, whereas protein RPS27 is involved in JNK cascade and Wnt signaling pathway. The activation of ER stress response leads to the expression of protein that involves the MAPK pathway through the activation of JNK as mediator [80]. Most of the downregulated proteins are involved in direct negative regulator of apoptosis or negative regulator of oxidative stress-induced proteins. The proteins are GSTP1, HSPB1, NPM1, PRDX2, PRDX6, PRKDC, TXN, and UBA52. Protein profiling of HepG2-shRNA-PRDX4 showed that GTT induces apoptosis by reducing oxidative stress in the endoplasmic reticulum and upregulated pro-apoptotic protein expression.

Functional cluster/protein name	Accession name (Swiss Prot)	*P*-value	Fold change	Functional process	Mechanism
ATP-dependent RNA helicase A	DHX9	0.0015	0.80	Positive regulation of DNA repair	Carcinogenesis
Cluster of elongation factor 1-alpha 1	EEF1A1	0.0001	0.80	Response to endoplasmic reticulum stress	ER stress
Glutathione S-transferase P	GSTP1	0.0001	0.60	Negative regulation of extrinsic apoptotic signaling pathway	Anti-apoptosis
Cluster of histone H2A type 2-A	HIST2H2AA3	0.0001	0.70	Negative regulation of cell proliferation	Cell cycle arrest
Heat shock protein beta-1	HSPB1	0.0001	0.70	Negative regulation of oxidative stress-induced intrinsic apoptotic signaling pathway	Anti-apoptosis
Nucleophosmin	NPM1	0.0018	0.80	Negative regulation of apoptotic process	Anti-apoptosis
Peroxiredoxin-1	PRDX1	0.0001	0.60	Regulation of stress-activated MAPK cascade, response to oxidative stress	Carcinogenesis
Peroxiredoxin-2	PRDX2	0.0001	0.70	Negative regulation of apoptotic process, response to oxidative stress	Anti-apoptosis
Peroxiredoxin-6	PRDX6	0.0001	0.60	Negative apoptosis regulation by regulating reactive oxygen species	Anti-apoptosis
DNA-dependent protein kinase catalytic subunit	PRKDC	0.0001	0.80	Negative regulation of apoptotic process	Anti-apoptosis
Cluster of 40S ribosomal protein S27	RPS27	0.0025	0.30	JNK cascade, Wnt signaling pathway	Carcinogenesis

Functional cluster/protein name	Accession name (Swiss Prot)	*P*-value	Fold change	Functional process	Mechanism
Thioredoxin	TXN	0.0001	0.01	Negative regulation of hydrogen peroxide-induced cell death	Anti-apoptosis
Ubiquitin-60S ribosomal protein L40	UBA52	0.0011	0.70	Signal transduction by p53 class mediator resulting in cell cycle arrest, negative regulation of apoptotic process	Cell cycle arrest and Anti-apoptosis
Small subunit processome component 20 homolog	UTP20	0.00010	0.20	Negative regulation of cell proliferation	Cell cycle arrest

Table 3.
List of downregulated protein expression in HepG2-shRNA-PRDX4 treated with GTT compared to HepG2-shRNA-PRDX4 control group.

6. Conclusion

Gene silencing is a technique to prevent the expression of certain genes. This technique is very useful to study biochemical pathway or produce therapeutics to treat cancer and diseases. Optimizing on the basic criteria for gene silencing is very important to achieve efficient silencing. GTT treatment reduces cell viability and causes apoptosis in both silenced HepG2-shRNA-PRDX4 and non-silenced HepG2 groups, but ROS production was increased non-silenced cells. The silencing of *PRDX4* gene in HepG2 cells caused ROS accumulation but did not cause cell death. Proteomic technique showed that GTT caused HepG2 cell death through activation of multiple pathways. It either triggers the apoptosis pathway directly in silenced cell or increases ROS production through PRDX4 activity, thus increasing pro-apoptotic and reducing anti-apoptotic protein expressions.

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Conflict of interest

The authors declare no conflict of interest.

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