Triptolide induced DNA damage in A375.S2 human malignant melanoma cells is mediated via reduction of DNA repair genes

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Abstract. Numerous studies have demonstrated that triptolide induces cell cycle arrest and apoptosis in human cancer cell lines. However, triptolide-induced DNA damage and inhibition of DNA repair gene expression in human skin cancer cells has not previously been reported. We sought the effects of triptolide on DNA damage and associated gene expression in A375.S2 human malignant melanoma cells *in vitro*. Comet assay, DAPI staining and DNA gel electrophoresis were used for examining DNA damage and results indicated that triptolide induced a longer DNA migration smear based on single cell electrophoresis and DNA condensation and damage occurred based on the examination of DAPI straining and DNA gel electrophoresis. The real-time PCR technique was used to examine DNA damage and repair gene expression (mRNA) and results indicated that triptolide led to a decrease in the ataxia telangiectasia mutated (ATM), ataxia-telangiectasia and Rad3-related (ATR), breast cancer 1, early onset (BRCA-1), p53, DNA-dependent serine/threonine protein kinase (DNA-PK) and O^6-methylguanine-DNA methyltransferase (MGMT) mRNA expression. Thus, these observations indicated that triptolide induced DNA damage and inhibited DNA damage and repair-associated gene expression (mRNA) that may be factors for triptolide-mediated inhibition of cell growth *in vitro* in A375.S2 cells.

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Introduction

Of the skin cancers, melanoma is the leading cause of death and the mortality rate is increasing (1-3). Thus, for all ages, melanoma is the primary focus of early detection campaigns. Sun UV has been recognized to cause skin cancer (4-6). UV can cause DNA damage of skin cells (4-6). DNA damage is involved in neurodegeneration in age-related disease, cerebral ischemia, and brain trauma including DNA damage (7-9). It was reported that in anticancer therapy, irradiation and DNA-damaging chemotherapeutic drugs play an important key role based on their ability to induce DNA double-strand breaks leading to cancer cell death (10-12). Thus, if agents can block DNA repair proteins it may lead to increase in the sensitivity of DNA damaging chemotherapeutic agents (13-16).

Triptolide (diterpenoid triepoxide; PG490) extracted from *Tripterygium wilfordii Hook F* (TWHF) has been shown to present anti-fertility function (17), anti-neoplastic activity such as anti-leukemia (18-25), anti-human hepatocellular carcinoma cells (25), colon cancer cells (23,26,27) and cervical cancer cells (28). Furthermore, evidence has been shown that triptolide inhibited the growth and metastasis of various solid tumors and has been suggested capable of acting synergistically with conventional chemotherapeutic drugs (29,30).

Substantial evidence has been demonstrated that triptolide induced cytotoxic effects in many human cancer cell lines but no available information exists to show triptolide-induced DNA damage in human skin cancer cells. Therefore, we investigated the effects of triptolide on DNA damage associated DNA repair

genes expression (mRNA) in A375.S2 human malignant melanoma cells *in vitro*. Our findings demonstrated that triptolide induced DNA damage and also inhibited the expression of DNA repair genes in A375.S2 cells.

Materials and methods

Chemicals and reagents. Triptolide, dimethyl sulfoxide (DMSO), ethidium bromide, propidium iodide (PI), Tris-HCl and Triton X-100 were purchased from Sigma-Aldrich. RPMI-1640 medium, fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin and trypsin-EDTA were purchased from Gibco®/Invitrogen (Grand Island, NY, USA).

Cell culture and chemical treatment. The human malignant melanoma cell line (A375.S2) was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured with minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2 mmol/l of L-glutamine in 75 cm^2 tissue culture flasks and grown in a humidified 5% CO_2 and 95% air at 37˚C (31,32).

Flow cytometric assay for percentage of viable cells. Equal numbers of cells ($2x10^5$ cells/well) were seeded in 12-well plates and allowed to attach overnight. The cells were treated with 0.1% DMSO or triptolide (0, 15, 20, 25 and 30 nM) diluted in MEM with 5% FBS for 24 h. Cells from each treatment were stained with PI (5 μg/ml) and were analyzed for percentage of viable cells by using flow cytometry (Becton-Dickinson, San Jose, CA, USA) and cell viability was calculated as previously described (33,34).

Comet assay and DAPI staining for DNA damage. A375.S2 cells at the density of $2x10^5$ cells/well in 12-well plates were incubated with triptolide at final concentrations of 0, 15, 20, 25 and 30 nM, vehicle (1 μl DMSO) and 0.1% of H_2O_2 (positive control) for 24 h or the cells were treated with 20 nM triptolide for 0, 6, 12, 24 and 48 h in MEM medium grown at 37˚C in 5% CO_2 and 95% air. Cells were harvested for the measurement of DNA damage using the Comet assay as described previously (33,35,36). Comet tail length was calculated and quantified by using the TriTek CometScore™ software image analysis system (TriTek Corp., Sumerduck, VA, USA) as described previously (33,35,36). Harvested cells were stained by DAPI then examined and photographed by using fluorescence microscopy as described elsewhere (33,35,37).

DNA gel electrophoresis for DNA damage. A375.S2 cells at the density of $2x10^5$ cells/well in 12-well plates were incubated with triptolide at final concentrations of 0, 15, 20, 25 and 30 nM for 48 h in MEM medium grown in 5% CO_2 and 95% air at 37˚C. Cells in each well were individually isolated by using DNA isolation kit. The isolated DNA (2 μg) from each treatment was examined for DNA damage by using DNA electrophoresis which was carried out in 0.5% agarose gel in Tris/acetate buffer at 15 V for 2 h. At the end of electrophoresis the DNA was stained with ethidium bromide then examined and photographed under a fluorescence microscope as previously described (38-40).

Table I. The DNA sequence was evaluated using the Primer Express software and each assay was run on an Applied Biosystems 7300 Real-time PCR system.

Primer name	Sequences
Human BRCA1	F: CCAGGGAGTTGGTCTGAGTGA
	R: ACTTCCGTAAGGCATCGTAACAC
Human DNA-PK	F: CCAGCTCTCACGCTCTGATATG
	R: CAAACGCATGCCCAAAGTC
Human MGMT	F: CCTGGCTGAATGCCTATTTCC
	R: TGTCTGGTGAACGACTCTTGCT
Human p53	F: GGGTTAGTTTACAATCAGCCACATT
	R: GGGCCTTGAAGTTAGAGAAAATTCA
Human ATM	F: TTTACCTAACTGTGAGCTGTCTCCAT
	R: ACTTCCGTAAGGCATCGTAACAC
Human ATR	F: GGGAATCACGACTCGCTGAA
	R: CTAGTAGCATAGCTCGACCATGGA
Human GAPDH	F: ACACCCACTCCTCCACCTTT
	R: TAGCCAAATTCGTTGTCATACC

Each assay was performed in triplicate to ensure reproducibility. BRCA1, breast cancer gene 1; DNA-PK, DNA-dependent serine/threonine protein kinase; MGMT, O^6-methylguanine-DNA methyltransferase; ATM, ataxia telangiectasia mutated; ATR, ataxia-telangiectasia and Rad3-related; GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

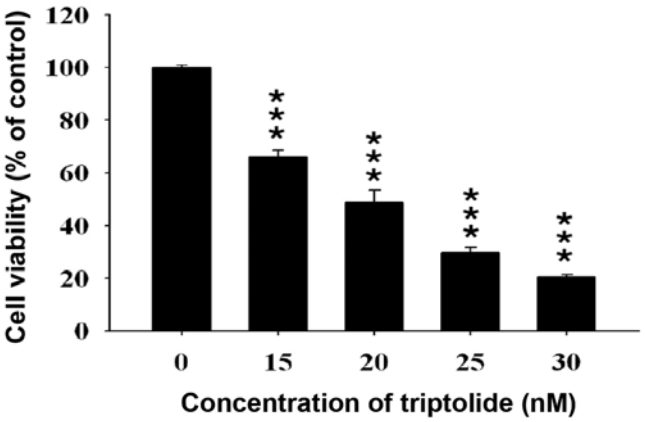

Figure 1. Triptolide decreased the viability of A375.S2 human malignant melanoma cells *in vitro*. A375.S2 cells ($5x10^5$ cells/well) were maintained in 12-well plates and were incubated with 0, 10, 15, 25 and 30 nM of triptolide, vehicle (1 μl DMSO) as control 24 h. Cells were harvested and stained with PI (5 μg/ml) and then were analyzed by flow cytometry as previously described. ***P<0.001.

Real-time PCR assay for examining the expression of DNA repair genes. A375.S2 cells at the density of $1x10^6$ cells/well in 6-well plates were incubated with or without 20 nM of triptolide for 24 h in MEM medium grown at 37˚C in 5% CO_2 and 95% air. The cells from each treatment were collected and total RNA was individually extracted by using the Qiagen RNeasy mini kit (Qiagen, Inc, Valencia, CA, USA) as previously described

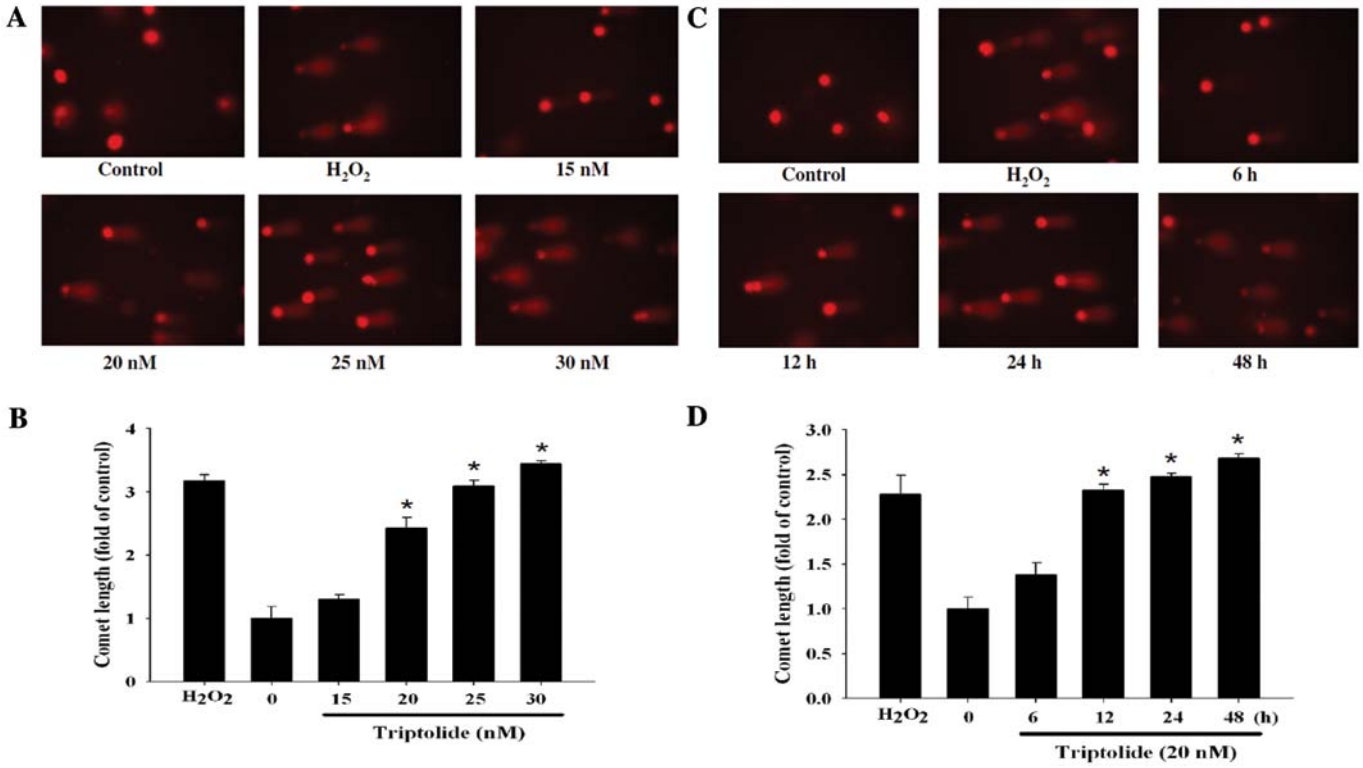

Figure 2. Differential concentration of triptolide-induced DNA damage in A375.S2 cells examined by Comet assay. The A375.S2 cells (5x10^5 cells/well) were maintained in 12-well plates incubated with 0, 15, 20, 25 and 30 nM of triptolide, and 0.1% of H_2O_2 (positive control) for 24 h (A and B) or cells were incubated with 20 nM of triptolide for 0, 6, 12, 24 and 48 h (C and D) and DNA damage was determined by Comet assay as described in Materials and methods. *P<0.05.

(41-43). Isolated RNA samples were individually reverse-transcribed for 30 min at 42˚C with High Capacity cDNA Reverse Transcription kit according to the standard protocol of the supplier (Applied Biosystems, Carlsbad, CA, USA). Quantitative PCR from each sample was conducted as follows: 2 min at 50˚C, 10 min at 95˚C, and 40 cycles of 15 sec at 95˚C, 1 min at 60˚C using 1 μl of the cDNA reverse-transcribed as described above, 2X SYBR Green PCR Master Mix (Applied Biosystems) and 200 nM of forward and reverse primers as shown in Table I, and previously described (41,43,44). Each assay was run on an Applied Biosystems 7300 Real-time PCR system in triplicate. The expression fold-changes were performed by using the comparative CT method.

Statistical analysis. All studies were performed in duplicate. Results are presented as mean ± standard deviation. One-tailed Student's t-test was used to analyze the difference between control and triptolide treated groups. Significance was defined as $p<0.05$.

Results

Effect of triptolide on the percentage of viable A375.S2 cells. A375.S2 cells were incubated with 15, 20, 25 and 30 nM of triptolide for 24 h. At the end of incubation, all samples were collected for determining the percentage of viable cells and the results are presented in Fig. 1, which indicated that triptolide decreased the percentage of viable cells at the concentration of 15-30 nM.

Effects of triptolide on DNA in A375.S2 cells examined by Comet assay and DAPI staining. To confirm whether triptolide can induce DNA damage in A375.S2 cells, after cells were treated with triptolide DNA damage was examined by Comet assay and the results are presented in Fig. 2. Triptolide induced DNA damage in A375.S2 cells and these effects were dose-dependent (Fig. 2B) and time-dependent (Fig. 2D). The higher concentration of triptolide led to a longer DNA migration smear (Comet tail). H_2O_2 is known to be a highly reactive oxygen species, in the present studies, 0.1% H_2O_2 induced Comet tails. Fig. 3 shows DNA damage by DAPI stain and the effects based on the mean fluorescence intensity (Fig. 3A) are dose-dependent (Fig. 3B).

Effects of triptolide on DNA in A375.S2 cells examined by DNA gel electrophoresis. To confirm whether or not triptolide can induced DNA damage in A375.S2 cells, DNA gel electrophoresis was used and results are shown in Fig. 4. The results show that triptolide induced DNA damage and fragments in A375.S2 cells (Fig. 4). The higher dose of triptolide (30 nM) led to more DNA damage and fragments than that of low dose (15 nM) incubation in A375.S2 cells.

Effects of triptolide on DNA damage and of repair gene expression in A375.S2 cells measured by real-time PCR. Figs. 2 and 3 results show that triptolide induced DNA damage and fragments in A375.S2 cells. We investigated whether or not triptolide affects the gene expression of DNA damage and repair in A375.S2 cells. We used DNA

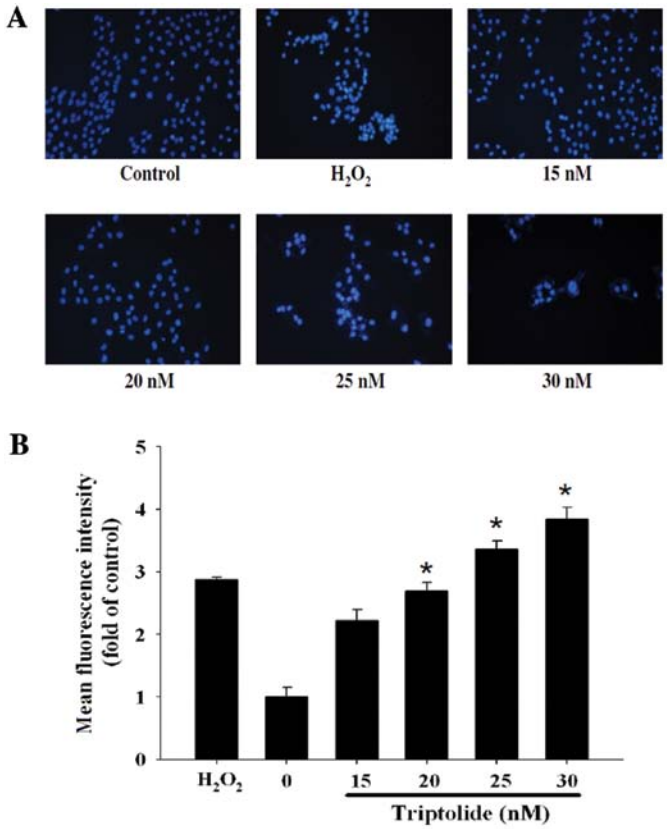

Figure 3. Triptolide-induced DNA damage in A375.S2 cells examined by DAPI staining. The A375.S2 cells ($5x10^5$ cells/well) were maintained in 12-well plates and incubated with 0, 15, 20, 25 and 30 nM of triptolide, and 0.1% of H_2O_2 (positive control) for 24 h and DNA damage was determined by DAPI staining as described in Materials and methods. *P<0.05.

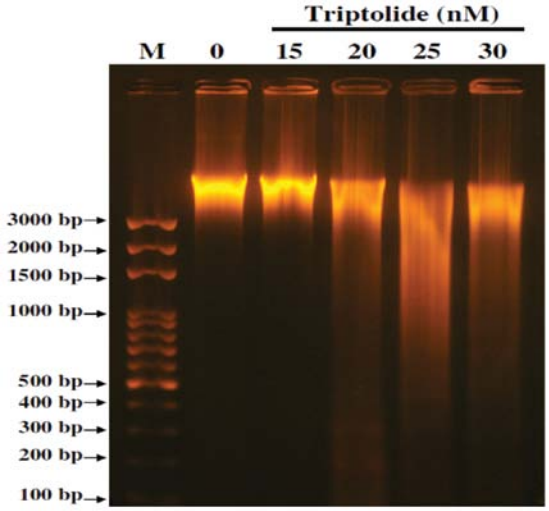

Figure 4. Triptolide-induced DNA damage in A375.S2 cells examined by DNA gel electrophoresis. The A375.S2 cells ($1x10^6$ cells/well) were placed in 12-well plates were incubated with 0, 15, 20, 25 and 30 nM of triptolide for 48 h. Cells were collected and DNA were isolated for DNA gel electrophoresis as described in Materials and methods.

agarose gel electrophoresis for examining the products from real-time PCR and results are shown in Fig. 5. The results indicated that all examined gene expression including ATM,

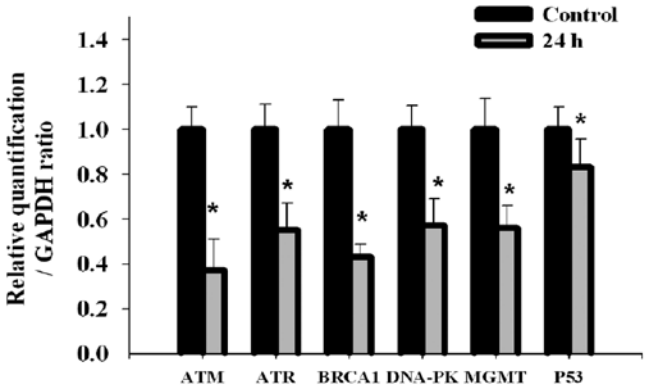

Figure 5. Triptolide-inhibited DNA damage and expression of repair genes in A375.S2 cells determined by real-time PCR. The total RNA was extracted from the A375.S2 cells after incubation with 0 and 20 nM triptolide for 24 h, RNA samples were reverse-transcribed, and cDNA used for real-time PCR as described in Materials and methods. The ratios of ATM, ATR, BRCA-1, p53, DNA-PK and MGMT mRNA/GAPDH are presented. Data represents mean ± SD of three experiments. *P<0.05.

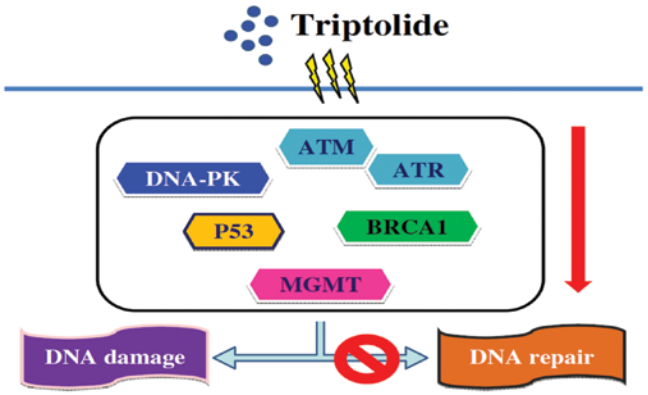

Figure 6. Proposed flow chart for triptolide-induced DNA damage and inhibition of gene expression, DNA damage and repair in A375.S2 human malignant melanoma cells.

ATR, BRCA-1, DNA-PK, MGMT and p53 mRNA were decreased in 24 h treatment with triptolide. ATM and BRCA-1 gene were more sensitive than the other genes (ATR, DNA-PK, MGMT and p53). P53 was the least sensitive compared to the other genes.

Discussion

Numerous experiments have shown that triptolide induces cell death via induction of apoptosis in human cancer cell lines (26,45,46), but no available information exists to demonstrate triptolide induced DNA damage and affected DNA repair gene expression in human skin cancer cells. We found that A375.S2 cells treated with various concentrations of triptolide led to decreased percentage of viable cells (Fig. 1) and it also induced DNA damage (Figs. 2 and 3) and inhibited gene expression of DNA repair genes (Fig. 5) in A375.S2 cells. These findings are based on the observations from i) flow cytometric assay showing the decrease of percentage of viable cells (Fig. 1); ii) Comet assay and DAPI staining,

the longer comet tail means higher DNA damage (Fig. 2); the light of fluorescence means higher DNA condensation (Fig. 3); iii) DNA fragments in DNA gel electrophoresis indicate high dose of triptolide treatment led to high DNA damage and fragments (Fig. 4) and iv) RT-PCR showed that triptolide inhibited the gene expression (mRNA) of DNA associated repair genes (Fig. 5).

It is well documented that Comet assay is a highly sensitive technique for DNA damage examination (47,48) and trend-break formation during the process of excision repair of DNA in cells (49,50). Herein, our results showed triptolide-induced DNA damage, which was examined by Comet assay and DAPI staining. The DNA damage of A375.S2 cells from triptolide treatment was also confirmed by DNA gel electophoresis (Fig. 4).

It was reported that agent-induced DNA damage can be reduced in cells via the DNA repair system through eliminating DNA lesions (49,50). Thus, we further investigated whether or not triptolide can affect the DNA repair gene expression in A375.S2 cells and results indicated that triptolide inhibit the expression of mRNA such as ataxia telangiectasia mutated (ATM), ataxia-telangiectasia (ATR), breast cancer gene 1 (BRCA-1), p53, DNA-dependent protein kinase (DNA-PK) and O^6-methylguanine DNA methyltransferase (MGMT) in the examined A375.S2 cells. The results in Fig. 5 indicate that p53 gene has the lowest sensitivity to triptolide when compared to the other examined genes.

It was reported that DNA damage responses of cells could lead to p53 activation and activated p53 regulates the cell cycle arrest, DNA repair and apoptosis (51,52). The role of p53 in skin cancer cell response to triptolide-induced DNA damage and repair is unclear. Our results show that triptolide inhibited p53 gene expression in A375.S2 cells. In response to DNA damage, DNA damage checkpoints associate with cell cycle for maintaining genomic integrity (53-55). It was reported that both ATM and ATR are master checkpoint kinases which can be activated by double-stranded DNA breaks (52,56). Our results also show that triptolide inhibited the ATM and ATR gene expression in A375.S2 cells.

DNA-PK plays an important role in DNA damage repair (52) and the deficiency in DNA-PK activity of human glioblastoma cells can lead to a slow, error prone repair process causing increased formation of chromosome aberrations (52). BRCA1 plays and important roles in DNA damage and repair response, homologous recombination, cell cycle regulation, protein ubiquitination and apoptosis (57,58) and loss of BRCA1 causes a defective DNA repair response and G_2/M cell cycle checkpoint in breast cancer cells (57,59). MGMT reduces cytotoxicity of therapeutic or environmental alkylating agents (60,61). Our results showed that triptolide inhibited the gene expression (mRNA) of DNA-PK, MGMT and BRCA-1.

In conclusion, A375.S2 cells were exposed to various concentrations of triptolide and DNA damage occurred. Moreover, the proposed flow chart for triptolide effect on DNA in A375.S2 human malignant melanoma cells is summarized in Fig. 6. Triptolide induces DNA damage in a dose response followed by inhibition of DNA repair-associated gene expression including ATM, ATR, BRCA-1, p53, DNA-PK and MGMT, then leading to DNA damage (Fig. 6).

Acknowledgements

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