Differential regulation of survivin by p53 contributes to cell cycle dependent apoptosis

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ABSTRACT

Recent studies indicate that cell-cycle checkpoints are tightly correlated with the regulation of apoptosis, in which p53 plays an important role. Our present works show that the expression of E6/E7 oncogenes of human papillomavirus in HeLa cells is inhibited in the presence of anti-tumor reagent tripchlorolide (TC), which results in the up-regulation of p53 in HeLa cells. Interestingly, under the same TC-treatment, the cells at the early S-phase are more susceptible to apoptosis than those at the middle S-phase although p53 protein is stabilized to the same level in both situations. Significant difference is exhibited between the two specified expression profiles. Further analysis demonstrates that anti-apoptotic gene survivin is up-regulated by p53 in the TC-treated middle-S cells, whereas it is down-regulated by p53 in the TC-treated early-S cells. Taken together, the present study indicates that the differential p53-regulated expression of survivin at different stages of the cell cycle results in different cellular outputs under the same apoptosis-inducer.

Keywords: apoptosis, cell cycle, p53, survivin, HeLa cell.

INTRODUCTION

The balance between cell cycle procession and apoptosis is crucial for normal tissue homeostasis. Any breaks of this balance, which leads to inappropriate cell death and/or abnormal proliferation, can result in diseases even tumorigenesis [1,2]. Numerous works have been done to discover the mechanism of balancing apoptosis and cell cycle progression, and a number of observations have indicated that the regulation of apoptotic process is tightly coupled with the surveillance mechanisms of the cell-cycle checkpoints [3-6].

Recent studies have identified several molecules common to the regulation of the cell cycle and apoptosis. Dual roles of these molecules such as c-Myc, p53, survivin and Bcl-2 provide a rational linkage between cell cycle and apoptosis [3, 6-9]. Among these factors, tumor suppressor p53 performs a pivotal role switching between cell cycle regulation and apoptosis induction [10, 11]. Functional p53 mainly contributes as a transcriptional factor with both capabilities of transactivation and transrepression. Hundreds of p53-responsive genes have been identified [10-12]. It was reported that differential transregulation by p53, which means transactivation or transrepression of different subsets of p53 target genes, was important to determine what downstream event would be elicited [13]. Nonetheless, more details about the differential transregulation of p53 remains to be elucidated.

Many chemotherapeutic agents have been used for tumor therapy by inducing apoptosis of tumor cells. It was reported that cells treated with some antitumor agents had different susceptibility to apoptosis in a cell cycle related manner [14]. This cell cycle stage-dependent susceptibility to apoptosis was also found in cells treated with staurosporine [15] and arsenite [16]. This different susceptibility may be explained by the variety of the lesion severity and the repair ability to the lesion. It is supported by the evidence that p53 can modulate the activity of base excision repair in a cell cycle-specific manner after genotoxic stress induced by γ-irradiation [17]. However, the mechanism controlling the cell-cycle dependent susceptibility to apoptosis is still unknown.

Triptolide is a diterpene triepoxide purified from the Chinese herb *Tripterygium Wilfordii* Hook. It has potent antiproliferative and proapoptotic activities with many types of cancer cells and transformed cell line *in vitro* and *in*

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vivo [18-20]. Recent studies show that triptolide executes anti-neoplastic effect [21-23] and a functional p53 is required for the proapoptotic effect [24]. Tripchlorolide (TC), a derivate of triptolide with similar activity and much lower toxicity [25], has shown its anti-neoplastic activities against leukemic, lung and other tumor cells. Our previous works reported that TC disturbed the cell cycle and induced apoptosis in Chinese hamster ovary (CHO) cells, which resulted from degradation of c-Myc and dysfunctional p53 [26, 27]. In this study, we demonstrated that TC inhibited the expression of viral oncoprotein E6/E7, which resulted in an up-regulation of p53 in HeLa S3 cells. TC induced a p53-mediated apoptosis in Hela S3 cells. Moreover, the susceptibility to apoptosis was cell-cycle specific when the cycling cells were treated with TC. To clarify the molecular foundation, we monitored the expression profiles of TC-treated cells at different cell-cycle stages with microarray assay. Combined the array data with further analysis of survivin, we conclude that the differential expression of survivin regulated by p53 at different cell cycle stages plays an important role in the cell-cycle dependent apoptosis under the TC treatment.

MATERIALS AND METHODS

Cell culture and synchronization

HeLa S3 cells (ATCC) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL) supplemented with 5% fetal bovine serum (FBS, Gibco BRL) at 37ºC in 5% CO_2. The cells were synchronized at G1/S boundary with 2mM hydroxyurea (HU, Calbiochem) for 24 h, and then were released in the HU-free medium for 5 min (the early-S cells) and for 3 h (the middle-S cells), respectively.

Flow cytometric analyses

For the DNA content analysis, the cells were collected and fixed with 70% ethanol. The fixed cells were treated with RNase A and stained with propidium iodide (PI, Sigma). The DNA contents were measured with a flow cytometer (FACScan). For the Annexin V assay, the cells were stained with Annexin V-FITC according to the manufacturer's protocol (PharMingen). For M30-PI double staining, the cells were subjected to the CytoDEATH M30 assay according to the manufacture's instruction (Roche Molecular Biochemicals).

Isolation of cytosolic and mitochondrial fractions

HeLa S3 cells were treated with a digitonin-buffer (20 mM Hepes-KOH, pH 7.3, 110 mM KAc, 5 mM NaAc, 2 mM $MgAc_2$, 1 mM EGTA and 200 μg/ml digitonin) on ice for 10 min to permeabilize the cell membrane. The cell lysate was then centrifuged at 10,000g at 4ºC for 15 min. The supernatant was collected as a cytosolic fraction, and the pellet was as a mitochondria-fraction.

Western-blotting analysis

HeLa S3 cells were lysed with 1×SDS-loading buffer (50 mM Tris-Cl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol and 0.1% bromophenol blue). In the case of digitonin-permeabilized cells, the cytosolic fraction was mixed with equal volume of 2×SDS-loading buffer, and the mitochondrial fraction was suspended with 1×SDS-loading buffer. Western-blotting was carried out with primary antibodies including anti-p53 and phospho-p53 (Ser15) (Cell Signaling); anti-Bax, anti-Cytochrome *c*, anti-β-actin (Santa Cruz); anti-Bcl-2, Sigma; anti-COX 4 (Molecular Probes); anti-survivin (PharMingen), respectively. The proteins were detected by enhanced chemiluminescence (ECL-plus, Amersham Pharmacia Biotech).

Northern blot analysis

Total RNA of the cells was isolated using the TRIzol (Invitrogen) according to the manufacturer's protocol. Purified total RNA was electrophoresed on 1% formaldehyde denatured agarose gel and transferred to Hybond-N (Amersham Pharmacia Biotech). Hybridization was performed using ExpressHyb Hybridizaton Solution (BD Bioscience) according to the manufacturer's instruction. The probes were labeled with random primer DNA labeling kit (Promega) and the probe templates were generated by RT-PCR amplification from the RNA of HeLa S3 cells with the following primer pair:

p53: f-CCCCTCCTGGCCCCTGTCATCT and r-GGGCGGGG GTGTGGAATCAAC, GAPDH: f-CGCCAGCCGAGCCACATC and r-GCCAGCATCGCCCCACTTGA, E6-E7: f-AGGGAGTAA CCGAAAACG and r-CATAAAACCAGCCGTTAC.

Plasmid construction and transfection

The full-length of Chinese hamster p53 coding sequence was obtained by RT-PCR from CHOC400 cells, which contains a point mutation in its DNA binding domain [28]. This mutated-p53 insert was cloned into SalI/BamHI sites of pEGFP-C1 (CLONTECH) to generate pEGFP-cgTP53 as a p53-dominant negative plasmid. HeLa S3 cells were transfected with pEGFP-cgTP53 by Lipofectamine™ 2000 system (GIBCO BRL).

The 1.1k sequence of the survivin promoter region of HeLa S3 was obtained by PCR-based amplification as described [29]. The plasmid Sur-p was constructed by inserting the survivin promoter on the upstream of the luciferase gene in pGL3-Basic (Promega). An EGFP fused p53 dominant negative construct was co-transfected with the plasmid Sur-p for the experiments on suppression of p53 activities. pEGFP-C1 was also used as a control. HeLa S3 cells were transfected with Sur-p by Nucleofector assay according to the manufacture's instruction (Amaxa biosystems).

RNAi of survivin

The Sur-RNAi plasmid was constructed with pSUPER RNAi System (OligoEngine). A pair of 64-nt oligonucleotides each containing a 19-nt sequence (5'-gatccccTAC ATT CAA GAA CTG GCC CttcaagagaGGG CCA GTT CTT GAA TGT Atttttggaaa-3' and 5'-agcttttccaaaaaTAC ATT CAA GAA CTG GCC CtctcttgaaGGG CCA GTT CTT GAA TGT Aggg-3') derived from the survivin transcript were inserted into the vector pSUPER between BglII and HindIII sites according to the manufacture's instruction. HeLa S3 cells were transfected with Sur-RNAi by Nucleofector assay (Amaxa biosystems).

Microarray assay

Microarray assay was performed with Atlas™ Human Apoptosis Array containing 205 cell cycle-related and apoptotic genes as described in the manufacture's instruction (BD Biosciences). Briefly, the poly-A RNA from HeLa S3 cells was purified, and labeled with

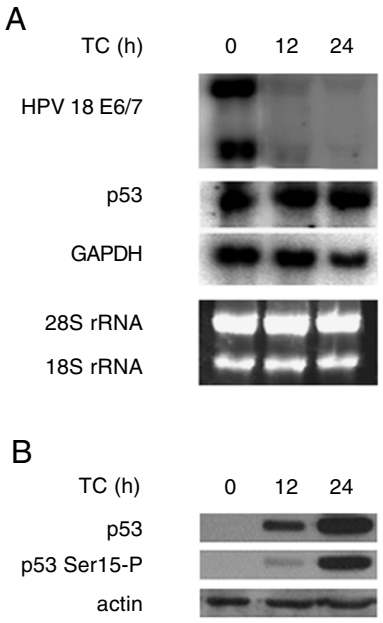


Fig. 1 p53 was restored and activated in HeLa cells under TC treatment. Exponentially growing HeLa S3 cells were treated with 15ng/ml TC for 0, 12 and 24 h, respectively. **(A)** Northern blot of E6/E7 and p53. GAPDH was used as an internal standard of the total mRNA, while the 18S/28S rRNA stained with ethidium bromide was used as loading control of the total RNA. **(B)** Western-blotting of p53 and p53Ser15-P. Actin was used as sample-loading control.

Atlas™ Pure Total RNA Labeling System. The Atlas™ Human Apoptosis Array was hybridized with the ^{32}P-labeled cDNA samples and scanned by PhosphorImager (Molecular Dynamics). The signals were analyzed using the ImageQuant software (Molecular Dynamics). The relative expression of housekeeping genes served to normalize gene expression levels, and two separate experiments were performed.

Luciferase assay

HeLa S3 cells were transfected with Sur-p and other plasmids, respectively. After a 12-h post-transfection, the cells were synchronized with 2mM HU. The cells were harvested after the indicated TC-treatment. The promoter activity was measured with Luciferase Assay System (Promega). β-galactosidase expression plasmid pCH110 (Pharmacia Biotech Inc.) was co-transfected as an internal standard to normalize the luciferase activity (β-Galactosidase Enzyme Assay System, Promega).

RESULTS

p53 restoration and activation of HeLa cells by TC

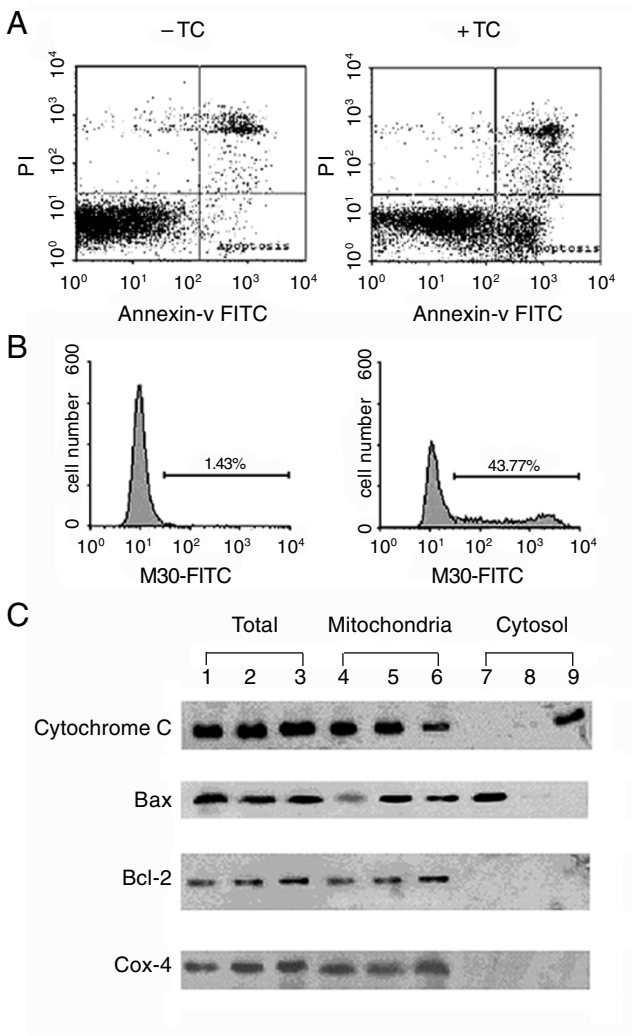
HeLa cell line is well known as a cervical cancer cell line with dormant p53 pathway for its infection of human papillomavirus 18 (HPV18), in which the viral protein E6 accelerates p53 degradation by binding to p53 protein [30-32]. However, when HeLa cells were treated with an potent anti-tumor reagent Tripchlorolide (TC), it was found that the E6/E7 mRNA was diminished significantly after 12-h treatment and was reduced to an undetectable level after 24-h treatment (Fig. 1A), whereas the mRNA level of p53 was increased 1.21- and 2.67-fold after 12- and 24-h treatment (normalized by GAPDH, Fig. 1A). Consistent with the abolition of E6 inhibition, p53 protein was stabilized and elevated significantly in the TC-treated HeLa S3 cells (Fig. 1B). These results indicated that TC-induced restoration of p53 was correlated to the combination of down-regulating the expression of E6/E7 mRNA and up-regulating the expression of p53 mRNA.

To identify whether p53 is activated in TC treated HeLa cells, we further assessed the phosphorylation level of p53. Fig. 1B showed the phosphorylation on Ser15 of p53, which an early event in the activation of p53 responding to DNA damage [33, 34]. Furthermore, the transcriptional activation capability of p53 was demonstrated by the array analysis. We utilized the Atlas™ Human Apoptosis Array (BD Biosciences), which contains selected 205 cell-cycle-related and apoptotic genes, to monitor the expression profiles of TC-treated HeLa cells. The results showed that a series of p53 inducing genes such as PIGs (PIG3, PIG7, PIG10, PIG11 and PIG12), p21$^{WAF1/CIP1}$ and GADD45 were up-regulated significantly. Taken together, these results indicated the TC-induced restoration and activation of p53 of HeLa cells.

p53 dependence of TC-induced apoptosis of HeLa cells

To address whether apoptosis of HeLa cells is induced in the presence of TC, we treated exponentially growing HeLa cells with 15ng/ml TC for 24 h. An apoptotic population was identified from the TC-treated cells as measured by Annexin-V [35, 36] or M30 assay [37, 38] (Fig. 2A, 2B). After the drug treatment, the adherent cells as less apoptotic population and the floating ones as completely apoptotic population were collected separately. Western-blotting assays showed that cytochrome *c* release was observed in the floating cells, while it remained in the mitochondria-fractions of the adherent cells and the cells without TC treatment (Fig. 2C, compare lanes 9 to 7 and 8). Furthermore, almost all of the Bax proteins were translocated to mitochondria in the TC treated cells, whereas most remained in the cytosol in the untreated cells (Fig. 2C, compare lanes 5, 6, 8 and 9 to 4 and 7). Collectively, these results indicated that TC induced the apoptosis of HeLa cells through the mitochondrial-regulated apoptotic pathway.

To validate the contribution of p53 in the apoptosis induced with TC, the p53-dominant negative construct



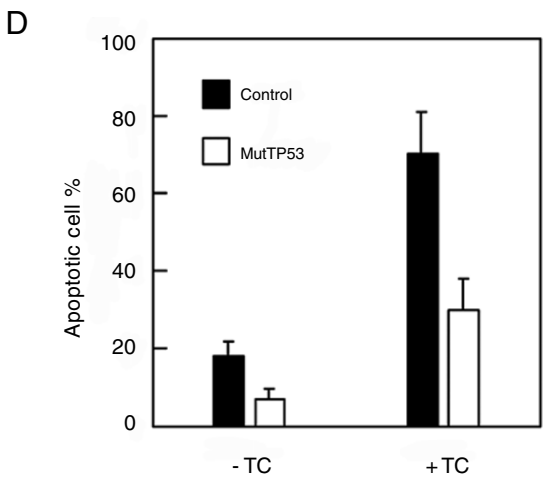


Fig. 2 The TC-induced apoptosis is p53 dependent in HeLa cells. Exponentially growing HeLa S3 cells were treated with 15ng/ml TC for 24 h and analyzed with flow cytometry. **(A)** Apoptotic cells measured by Annexin V assay. The cells were harvested and labeled directly with Annexin V-FITC and PI. **(B)** Apoptotic cells measured by M30 assay. The cells were labeled with M30 CytoDEATH antibody and measured by flow cytometry. **(C)** Western-blotting of Cytochrome *c*, Bax and Bcl-2 of HeLa cells. The untreated cells were collected as a total cell lysate (lane 1) and separated as the mitochondrial and cytosolic fractions (lanes 4, 7); the adherent cells after TC treatment were harvested as a total cell lysate (lane 2) and separated as the mitochondrial and cytosolic fractions (lanes 5, 8); the floating cells after TC treatment were collected as a total cell lysate (lane 3) and separated as the mitochondrial and cytosolic fractions (lanes 6, 9). A mitochondrial protein, Cox 4, was shown as the quality control of the fractionation. **(D)** Inhibition of the TC-induced apoptosis by mutated p53. Exponentially growing HeLa S3 cells were transfected with EGFP-cgTP53 plasmid (mutTP53) and EGFP-C1 vector (control), respectively. The transfected cells were treated with 20 ng/ml TC for 24 h. The condensed apoptotic nuclei stained with DAPI were determined under the fluorescent microscope, of which the percentage of apoptotic cells was counted out of the EGFP-positive cells. Data shown are representative of three separate experiments (mean ± SD).

cgTP53-EGFP was introduced into HeLa cells, which contains a point mutation in its DNA binding domain [28] (see Materials and methods). After the transfection, the cells were treated with 15ng/ml TC for 24 h, and then the condensed nuclei as the representative of apoptotic cells were counted under the fluorescent microscope. The results showed that the number of the apoptotic cells in the population of HeLa cells transfected with cgTP53-EGFP was significant lower than that in the population transfected with EGFP (mock) in the presence of TC (Fig. 2D), indicating that TC-induced apoptosis of HeLa cells is p53-dependent.

Cell-cycle dependence of TC-induced apoptosis

In order to analyze the relationship between the cell cycle regulation and apoptosis, M30-PI double staining assay, which could discriminate apoptotic status as well as the cell-cycle phases of the same cell, was performed to asynchronous HeLa cells treated with TC for the indicated times. The flow cytometric analyses showed that the apoptotic cells in S phase were significantly more than that in non-S phase including G_1 and G_2/M phases (Fig. 3A), suggesting that the TC-treated cells within S phase very likely initiate the apoptosis.

To further address this question, HeLa cells synchronized at different stages of S phase were explored. HeLa cells synchronized at G_1/S border by the treatment of hydroxyurea would progress into early-S phase directly after the releasing (as early-S cells) and into middle-S phase after 3-h releasing (as middle-S cells) (Fig. 4A). The synchronized early-S cells and middle-S cells were treated

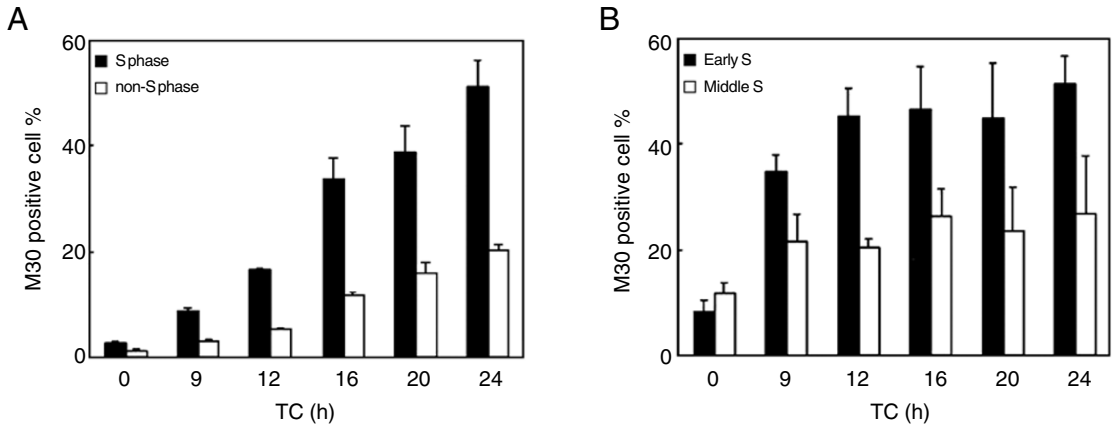


Fig. 3 Apoptosis induced by TC is cell-cycle dependent. **(A)** the apoptotic cells in the particular stages of cell cycle were measured. The exponentially growing HeLa S3 cells treated with 15ng/ml TC were harvested at indicated time points, and then double stained with M30 CytoDEATH antibody and PI. The populations of "S-phase" and "None S- phase" including G1 and G2/M phase were determined according to DNA content analysis through the flow cytometer. **(B)** the number of TC-induced apoptotic cells from the early-S or middle-S population was measured. The early S-phase and middle S-phase cells were prepared as described in Materials and Methods. The synchronized cells were treated with 15ng/ml TC, and then harvested at indicated time points. Flow cytometric analyses were done with M30 CytoDEATH and PI double staining. Data shown are representative of three separate experiments (mean ± SD).

with TC, respectively. It was showed that the TC-treated early-S cells slowed down its progression of S phase (Fig. 4A) and underwent a high level of apoptosis (Fig. 3B), whereas the middle S-phase cells treated with TC could progress to the next G_1 phase within 12 h (Fig. 4A) and went a lower apoptotic degree (Fig. 3B). Taken together, these results suggest that TC-induced apoptosis of HeLa cells is cell-cycle dependent.

Profiling of genes differentially expressed between early-S and middle-S cells under TC treatment

To understand the molecular behaviors that result in the different susceptibility in TC-treated HeLa cells at different stages of S phase, we monitored the gene expression profiles with Atlas™ Human Apoptosis Array. The synchronized HeLa cells in early and middle S phase were treated with 15ng/ml TC for 13 h, respectively. The array analyses showed that the expression profile in TC-treated early-S cells presented a tendency of down-regulation, among which the ratio of TC-treated sample/control of total 59 genes was below 0.5. In contrast, the expression profile in TC-treated middle-S cells showed a tendency of up-regulation, among which the ratio of TC-treated sample/control of total 52 genes was over 1.5 (Fig. 4B). These results suggest that RNA transcription machine in the early-S cells is more sensitive to TC treatment than that of the middle-S cells.

Further analyses showed that few of the genes between the TC-treated early-S cells and middle-S cells had the same expression tendency (Fig. 4B). Interestingly, many pro-apoptotic genes were up-regulated in the early-S cells, whereas they were down-regulated in the middle-S cells. For example, the genes promoting apoptosis such as caspases 6 and 10 were up-regulated to 1.62- and 1.61-fold, respectively, in TC-treated early-S cells, but down-regulated to 0.55- and 0.31- fold, respectively, in TC-treated middle-S cells. In contrast, the microarray data showed that survivin, an inhibitor of apoptosis (IAP) protein [39], was down-regulated in the TC-treated early-S cells but up-regulated in the TC-treated middle-S cells. These results suggest that the output of the gene-expression network in the TC-treated early-S cells promotes apoptosis, whereas no such case was in the TC-treated middle-S cells.

Survivin differentially expressed between early-S and middle-S cells under TC treatment

Survivin is a member of IAP family involving in both the apoptotic regulation and the cell-cycle control [39, 40]. In the present study, the expression patterns of survivin within the stages of the HeLa cell cycle were first analyzed. Consistent with the results of Altieri DC's lab [3], survivin was highly expressed in the G2/M phase and lowered in the G1 phase (Fig. 5A).

In order to investigate the expression of survivin of different cell cycle stages under TC treatment, early-S and middle-S cells were treated with 15ng/ml TC for 13h,

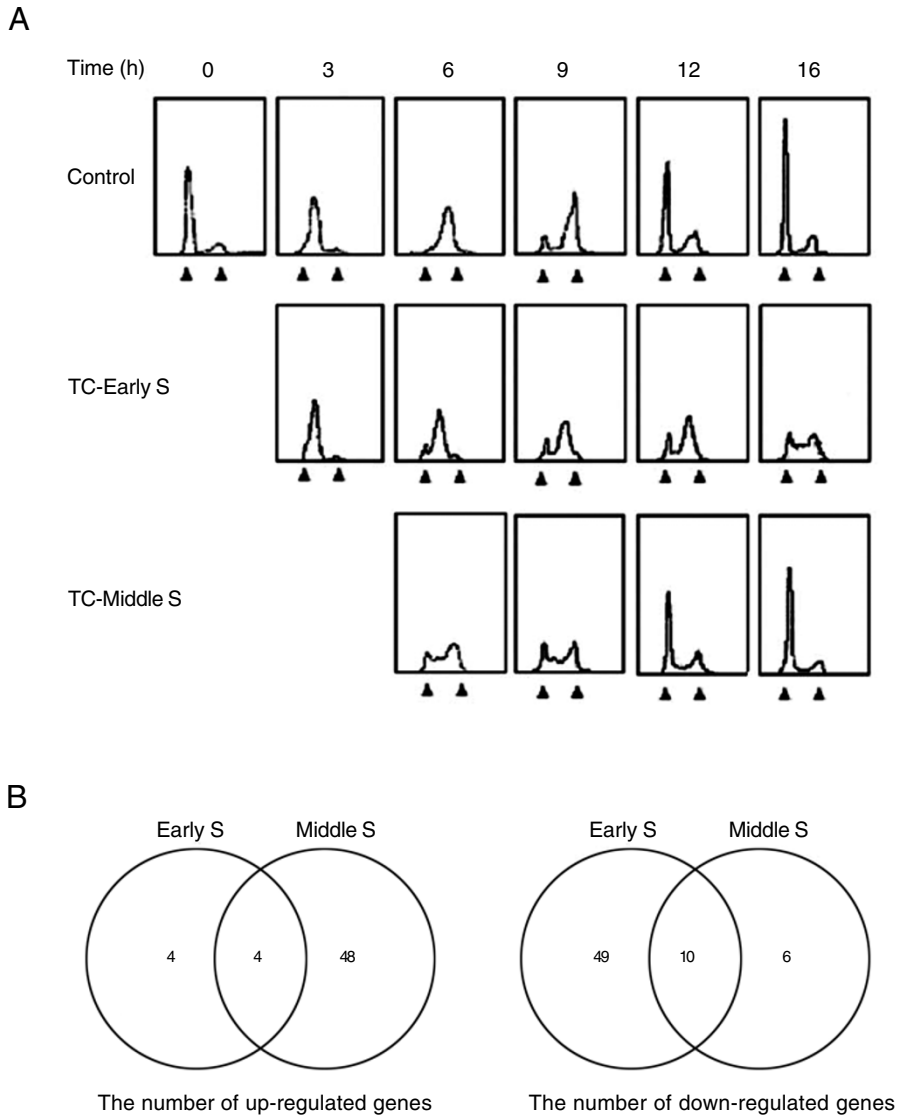


Fig. 4 Different responses between TC-treated early-S cells and TC-treated middle-S cells. **(A)** TC induced different cell-cycle arrests at different stages of S phase. The early S-phase HeLa cells (released from G_1/S border for 5 min) and middle S-phase (released for 3 h) HeLa cells were treated with 15ng/ml TC respectively. The released cells with no TC treatment were used as controls. Cells were harvested at the indicated times and analyzed with flow cytometry. **(B)** profiling of genes differentially expressed between TC-treated early-S cells and TC-treated middle-S cells. The early S-phase and middle S-phase HeLa cells were prepared and treated with 15ng/ml TC for 13 h, respectively. The untreated early S-phase and middle S-phase cells were used as controls. The microarray assays were performed as described in Materials and Methods. The up-regulated genes (the ratio of TC-treated cells/control is over 1.5) and down-regulated genes (the ratio of TC-treated cells/control is below 0.5) from the averages of two duplicate experiments were accounted.

respectively. The results showed that the mRNA level of survivin was down-regulated in the TC-treated early-S cells but up-regulated in the TC-treated middle-S cells (Fig. 5B). This change was further confirmed at protein level by Western-blotting analysis (Fig. 5C). In contrast, Western-blotting analysis showed the level of the p53 proteins induced after TC treatment was increased to the same extent in both the early-S and middle-S cells (Fig. 5C). The uncovered result of differential expression of survivin suggests that survivin plays an important role in the regulation of the cell-cycle dependent apoptosis.

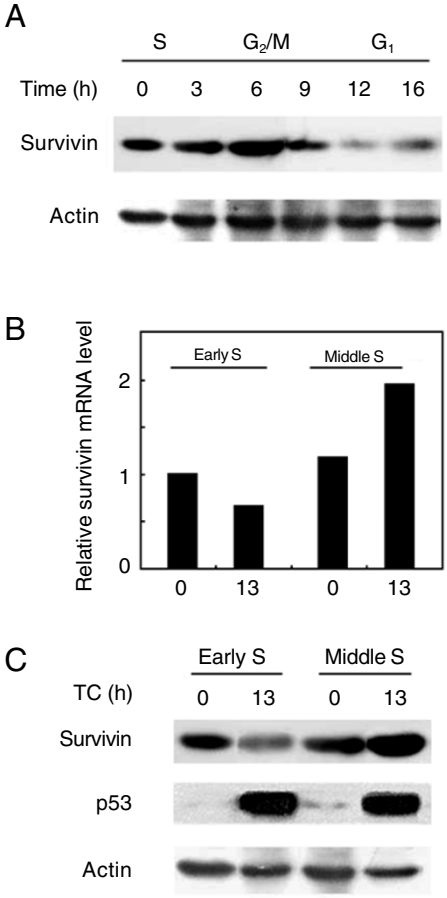


Fig. 5 Survivin differentially expressed between TC-treated early-S cells and TC-treated middle-S cells. **(A)** expression of survivin in synchronized cells. The cells synchronized at G_1/S border were released and maintained in fresh medium. The expression of survivin was detected by western blot at the indicated times. **(B)** relative survivin mRNA level of early-S and middle-S cells in the presence of TC. The early S-phase and middle S-phase HeLa cells were prepared and treated with 15ng/ml TC for 13 h, respectively. The relative expression fold of survivin mRNA was got from array data. Result was confirmed with RT-PCR. **(C)** protein level of survivin and p53 in TC-treated early-S cells and the TC-treated middle-S cells. The early S-phase and middle S-phase HeLa cells were prepared and treated with 15ng/ml TC for 13 h, respectively. Actin was used as sample-loading control.

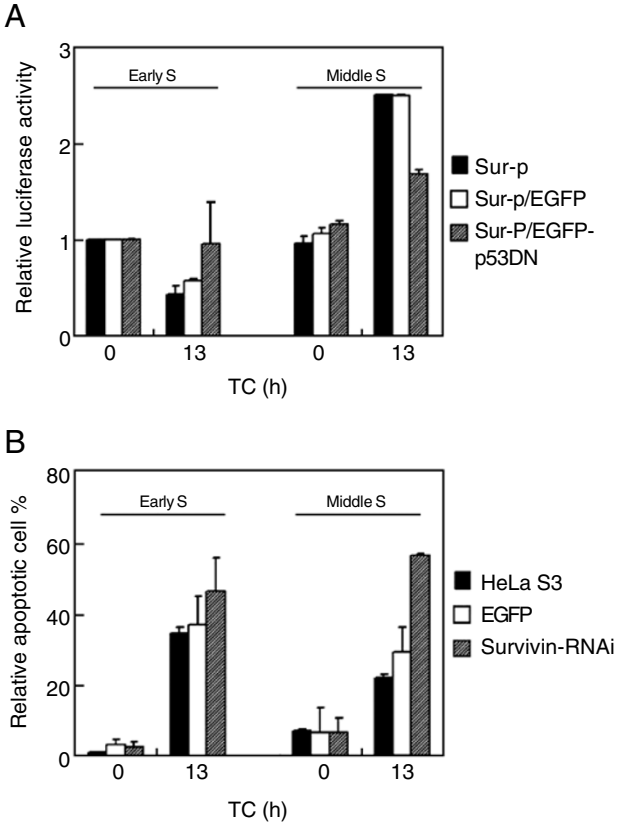


Fig. 6 The expression of survivin differentially regulated by p53 involves in the cell-cycle dependent apoptosis. **(A)** p53 differentially regulates the survivin expression in the TC-treated S-phase cells. HeLa S3 cells were transfected with indicated plasmids, respectively, and then synchronized with HU. The cells were treated with 15ng/ml TC for 13 h and subjected to luciferase assay. **(B)** RNAi of survivin elevates the apoptosis of the TC-treated middle-S cells. HeLa S3 cells were transfected with indicated plasmids, respectively, and then synchronized with HU. The cells were treated with 15ng/ml TC for 13 h, and the apoptotic ratio was counted as described in Fig. 3. Data shown are representative of three separate experiments (mean ± SD).

Differential regulation of survivin by p53 in different stages of the cell cycle in the presence of TC

It has been well documented that the expression of survivin is down-regulated by p53, and the repression of survivin is tightly related to p53-dependent apoptosis [29, 41]. Since our results showed that p53 level of the TC-treated early-S cells was activated to the same extent as the TC-treated middle-S cells (Fig. 5C), it is possible that p53 can differentially regulate the survivin expression under the different cellular context. To address this possibility, a plasmid containing luciferase driven by the promoter of survivin was transfected into HeLa cells and its expression activity was analyzed by luciferase assay. The results showed that the survivin-promoter related expression was down-regulated in the TC-treated early-S cells ($P<0.01$), and this suppression was abolished by co-transfection of the dominant negative p53 (Fig. 6A). On the other hand, the survivin-promoter related expression was increased in the TC-treated middle-S cells, while the co-transfection of dominant negative p53 inhibited this up-regulated expression (Fig. 6A). These results suggest that the effect

of p53 on the transcription of survivin in the TC-treated early-S cells is opposite to that in the TC-treated middle-S cells (see Discussion).

The further experiments indicated that the lower percentage of apoptosis in the TC-treated middle-S cells, at least partially, resulted from the up-regulation of survivin expression. HeLa cells transfected with a plasmid containing survivin-RNAi were synchronized to the early and middle S phase, respectively, and then treated with TC for indicated times. The silencing of survivin enhanced significantly the apoptotic response to TC treatment in the middle-S cells, whereas the apoptotic cells were only slightly increased in the TC-treated early-S population (Fig. 6B). The results support the argument that the up-regulation of survivin contributes to the survival of the middle-S cell in the presence of TC.

DISCUSSION

The regulation of cell cycle is tightly correlated with apoptotic control when the cells are under external or internal stresses. Our studies indicated that TC-treated HeLa cells in the S phase, especially in the early S phase, are highly susceptible to initiation of apoptosis (Fig. 3). This observation was very similar to the results revealed by Schlegel lab [42]. They found that apoptosis was induced in S-phase-arrested HeLa cells by staurosporine, caffeine, 6-dimethylaminopurine or okadaic acid, whereas little amount of apoptosis took place in asynchronously growing cells or in the cells arrested in G_1 phase under the same treatment [42].

Since the CDK2 activation is a key factor for the transition from G_1 to S phase, it is possible that activated CDK2 links the S phase and apoptosis. A previous report showed that CDK2 activation during thymocyte apoptosis was regulated by p53, Bax and Bcl-2 [9]. In addition, the inactivation of CDKs by its dominant negative mutants suppressed the drug-induced apoptosis of HeLa cells [43]. Our microarray analyses here suggest another possibility that the extensive down-regulation of most genes in TC-treated early-S cells results in their susceptibility to apoptosis (Fig. 4B). It will be worth knowing that which factor(s) is responsible for this extensive down-regulation pattern of the early-S cells in the presence of TC.

Survivin, a member of the inhibitor of apoptosis protein (IAP) family, has been revealed to participate the control of apoptosis and G_2/M checkpoint [3, 44, 45]. It was reported that the expression of survivin was at low level in G_1 and S phase but was elevated in G_2/M phase, and this G_2/M specific expression has been related to mitotic spindle checkpoint function [3]. The present studies indicate that survivin also plays an important role in the regulation of apoptosis in S-phase HeLa cell treated with TC. The results showed that the survivin was up-regulated in the TC-treated middle-S cells (Fig. 5B, 5C), and the suppression of the survivin up-regulation by the RNA interference caused the increase of apoptotic cells (Fig. 6B).

Survivin has been reported as a p53-regulated gene. A number of data show that the transcription of survivin is repressed by p53 [29, 41]. In the present studies, the suppression of survivin-promoter by p53 was identified in the TC-treated early-S cells (Fig. 6A). To our surprise, it was showed that the expression of survivin-promoter was up-regulated in the TC-treated middle-S cells, and the up-regulation was diminished by introduction of the p53 dominant negative construct. This result implies that the p53-regulating survivin is much more complicated than the simple repression-diagram. In fact, a previous observation already showed that a correlation between accumulated p53 and survivin expression existed in gastric carcinomas [46].

Since TC treatment caused the same extent of p53 activation in both early-S cells and middle-S cells (Fig. 5C), this result provides a new argument that the particular p53-mediated networks rather than p53 molecule alone in the different stages of the cell cycle determine the functional performance of p53 responded to genotoxic stresses. Recently, Dobbelstein's lab argued that the p53-mediated repression of survivin expression depended mainly on the induction of $p21^{WAF1/CIP1}$ gene [47]. Moreover, another experiment suggested that the modification of chromatin within the survivin promoter rather than p53-binding sites in the survivin promoter could be for the p53-regulated suppression of survivin transcription [41].

Bernard et al. showed that the p53 activity might involve in the apoptotic susceptibility of HPV infected cells when they compared the HPV-positive cells containing wild type p53 with the HPV-negative cells containing mutated p53 [15]. Our results here demonstrated that the cell-cycle dependent apoptosis was related with the differential transregulation of p53 on the basis of the silencing of E6 and restoration of functional p53. It is well documented the oncoprotein E6 expressed by human papillomavirus (HPV) neutralizes the functions of tumor suppressor p53 by promoting p53 degradation, which represents a key step in cervical carcinogenesis [32, 48, 49]. Therefore, one of therapeutic strategies for cervical tumors is to repress the expression of HPV oncogenes and then re-activate the tumor-suppress genes. Recently, Lane's lab reported that the anti-tumor compounds, leptomycin B and actinomycin D, reduced the expression of HPV E6/E7 mRNA in HeLa cells, which is a cervical cancer-derived cell line, and resulted in the activation of p53 [30]. The experiments presented here demonstrated that TC also significantly decreased the expression of E6/E7 mRNA and increased the p53 expression (Fig. 3). Our results suggest that TC

can be used as a potential anti-tumor reagent in the treatment of cervical cancer.

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